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Design and Production of a Recombinant FliC-Antigen Co-Expression Platform for Increased Vaccine Efficacy

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DESIGN AND PRODUCTION OF A RECOMBINANT FliC-ANTIGEN CO-EXPRESSION

PLATFORM FOR INCREASED VACCINE EFFICACY

by

SARAH C. BOYD

Under the Direction of Sidney A. Crow, Jr.

ABSTRACT

The protein monomer of bacterial flagella, FliC, is known to stimulate human innate immunity through activation of Toll-like receptor five. Linking native *Salmonella* FliC with various antigens has demonstrated an increased immune response as compared to single antigen presentation. To drastically reduce production time and allow for a more cost effective recombinant vaccine adjuvant, a synthetic construct was created that enables genetic linkage of FliC to other known antigens. The construct contains the necessary components for immune

system stimulation while the non-essential regions were replaced with commonly used restriction enzyme recognition sites to aid in ligation with other antigens and cloning into various expression vectors and hosts. After synthesis in the inducible expression vector pJ404, the construct was transformed into competent BL21 *E. coli* and expression was confirmed through SDS-PAGE, Western blot, and MALDI MS/MS. The cells were adapted to fermentation media and re-screened for expression, and upon confirmation a 20-liter fermentation was conducted. The resulting samples were analyzed for expression within the insoluble and soluble cellular fractions to further optimize fermentation conditions. Once purified, this synthetic FliC will serve as a platform technology for the standardized co-expression of the TRL5 activator with a variety of antigens in both prokaryotic and eukaryotic systems.

INDEX WORDS: FliC, TLR5, Vaccine, Adjuvant

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by

SARAH C. BOYD

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of

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SARAH C. BOYD

Committee Chair: Sidney A. Crow

Committee: George Pierce

Eric Gilbert

Electronic Version Approved:

Office of Graduate Studies

College of Arts and Sciences

Georgia State University

August 2014

DEDICATION

For my daughter, Olivia.

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I would like to thank my mentors, Dr. Crow and Dr. Pierce. They took a chance on me a few years ago and welcomed me into the lab, rescuing me from the dangerous world of coffee. To my fellow lab mates, thank you for making this journey bearable. Specifically I would like to recognize Brandi Campbell, Chris Cornelison, Katie Segars, Courtney Barlament, Amber Keller, and Sup. These individuals will remain my respected colleagues for life. I would also like to thank my mom for her daily encouragement and willingness to *try* to understand my project. Lastly, I'd like to thank my husband, Erick, for driving me to and from school in the early years, making the money, and being an unwavering source of love and support.

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The global use of safe and effective vaccines is needed to ward against potentially harmful, infectious diseases (Curtiss, 2002). According to the World Health Organization (WHO), 1.5 million children under the age of 5 died from vaccine-preventable diseases (VPDs) in 2012 (Global Immunization Data, 2014). Closer to home, the September 11, 2001 attacks on the United States have increased governmental awareness and funding for defense programs against bioterrorism involving both troops and civilians (Cohen and Marshall, 2001). With such foci, diseases like measles, tetanus, yellow fever, and pertussis can all currently be avoided by following proper vaccination protocols (CDC, 2006). The American Academy of Microbiology (2005) has identified a variety of agents that pose a significant health threat for which there are currently no licensed vaccines (Table 1). To significantly reduce the number of individuals affected by preventable diseases and work toward disease eradication and terrorism protection, vaccine development must be at the forefront of research and funding endeavors. To this end, microbial expression systems can be designed to produce high yields of immunogenic vaccine components in a short amount of time, reducing the overall process time and cost.

Vaccines simulate initial pathogen exposure through the delivery of known antigenic cellular components, often with the addition of adjuvants to help surmount an immune response. Current adjuvants include aluminum salts, oil-in-water emulsions, virosomes, and phospholipids and serve to enhance the efficacy of weak antigens or to decrease the dosage required to activate the immune system (Reed *et al*., 2013). Upon antigen recognition, the body begins a complex signaling cascade to rid the bacteria, virus, toxins, or other non-self molecule through the innate

and adaptive immune systems. The specialized adaptive immune system consists primarily of B and T cells that, upon antigen-recognition and subsequent activation, recruit the production of specific antibodies to clear the invader in a matter of days.

Table 1: Infectious agents that pose significant human health problems with no licensed vaccines available (Vaccine Development: Current Status and Future Needs. A report from the American Academy of Microbiology, 2005)

Sexually Transmitted Agents	Human Immunodeficiency Virus (HIV) Human Papilloma Virus (HPV) Herpes Simplex Virus (HSV) *Chlamydia trachomatis* *Neisseria gonorrhoeae* *Treponema pallidum*
Respiratory Agents	Respiratory Syncytial Virus (RSV) Parainfluenza Virus Human Metapneumovirus (HMPV) Group A Streptococci *Chlamydia pneumoniae* Meningococcus B
Enteric Agents	*Salmonella* species *Shigella* species *Escherichia coli* (ETEC, EHEC, EPEC) *Helicobacter pylori* Noroviruses
Vectorborne Agents	*Plasmodium falciparum* Dengue Fever Virus Hantaviruses *Borrelia burgdorferi* *Schistosoma* species *Leishmania* species Hookworm
Nosocomial Agents	*Staphylococcus aureus* *Pseudomonas* species *Enterococcus* species Gram negative enteric bacteria *Candida* species
Other Agents	Hepatitis C, E viruses Cytomegalovirus Group B Streptococcus

The innate immune system detects non-self molecules through the activation of Toll-like receptors (TLRs) within hours after initial exposure, leading to an inflammatory response and

activation of adaptive immune cells. TLRs consist of a leucine-rich extracellular domain and a

highly conserved intra-cytoplasmic domain (Takeda *et al*., 2003) and are expressed on a variety

of lymphocytes, including dendritic and natural killer (NK) cells (Figure 1, Kaisho and Akira,

2002). Dendritic cells at the site of infection utilize TLRs to recognize non-self molecules,

causing these cells to migrate to nearby lymph nodes where they play a critical role in antigen

presentation and T-cell activation. Concurrently, activation of TLRs on NK cells leads to the

release of inflammatory cytokines and antimicrobial molecules. Through this interworking, the

innate immune system not only provides the first line of defense against invading

microorganisms, but it also recruits a more long-lasting, effective immunity.
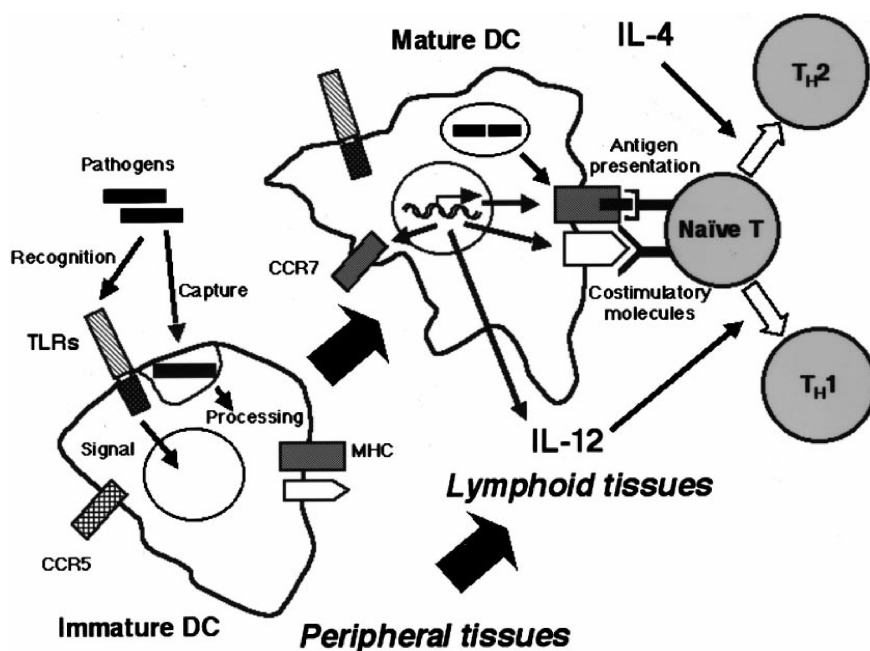
Figure 1: Role of TLRs in Innate Immunity
Toll-like receptors are found on dendritic cells and play a crucial role in
antigen presentation and T-cell activation.

Mammals have ten highly conserved TLRs, each recognizing a specific pathogen-

associated molecular pattern (PAMP) (Janeway, 1989). These PAMPs are evolutionarily stable

and critical to the organism's survival. For example, TLR3 recognizes double stranded RNA,

like that of retroviruses, while TLR4 recognizes the lipopolysaccharide of Gram-negative bacteria (McInturff *et al.*, 2006). This research, however, specifically focuses on the activation of TLR5 by FliC, the major structural component of *Salmonella* flagellin (Hayashi *et al.*, 2001).

1.2   FliC Structure and TLR5 Recognition

Bacterial flagella aid in locomotion and attachment and are therefore critical virulence factors (Mobley *et al.*, 1996). Attached to a molecular motor, each flagellar filament is made of eleven protofilaments, comprised of flagellin monomers termed FliC, that have four domains (D_0, D_1, D_2, and D_3) (Figure 2, Yonekura *et al.*, 2003). The crystallized hairpin structure of each monomer reveals these domains to be discontinuous (Namba *et al.*, 1989), so that the linear structure is: N terminus D_0- D_1- D_2 - D_3- D_2 - D_1- D_0 - C terminus.

Comprising the D_1 domain, the amino- and carboxyl- terminal ends of FliC are highly conserved among *Salmonella* and other Gram-negative bacteria (Namba *et al.,* 1989 and Mimori-Kiyosue *et al.*, 1998) and are known to interact with TLR5 (Eaves-Pyles *et al.*, 2001; Donnelly and Steiner, 2002; Murthy *et al.*, 2004) through three lateral helices (Figure 3, Yoon *et al.*, 2012). The D_3 domain is neither conserved nor required for TLR5 activation, and is therefore referred to the hypervariable region (Lino, 1977).

1.3   Flagellin as a Biological Adjuvant

Because FliC activates innate immunity through TLR5, it can serve as a biological vaccine adjuvant when genetically linked to other antigens. Presentation of such heterologous proteins could surmount a greater immune response than the singular counterpart. Ruth Arnon and colleagues were the first to use flagellin as a biological adjuvant and others have since demonstrated its capacity in this role (McEwen *et al.*, 1992; Levi and Arnon, 1996; Ben-Yedidia and Arnon, 1998; Ben-Yedidia *et al.*, 1998; 1999a; 1999b). Since then, many other researchers

have linked the TLR5 activator to known antigens (Table 2), establishing it as an effective

vaccine adjuvant.
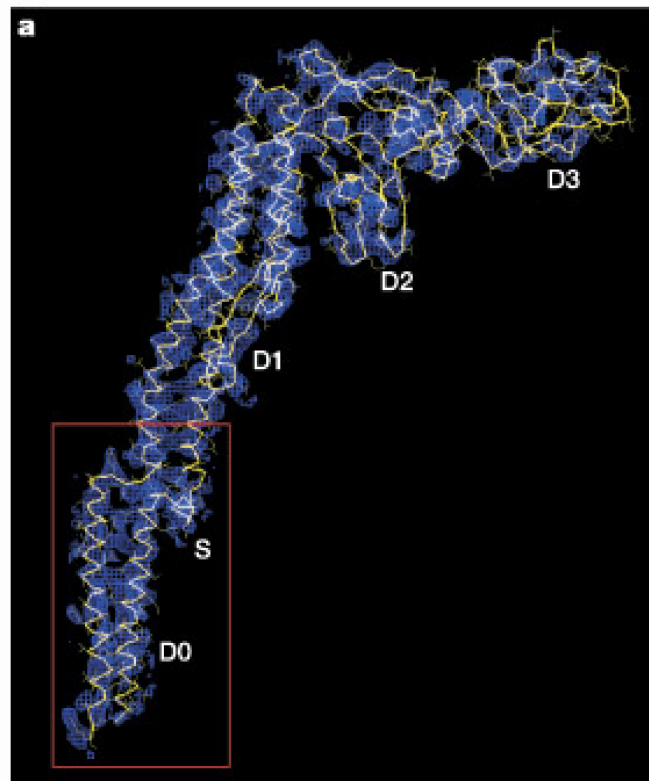
Figure 2: Full Length Flagellin Protein Structure
Flagellin is comprised of four structural domains that aid in protein folding and TLR5 recognition.
Kaisho, T. and S. Akira. **2002**. Toll-like receptors as adjuvant receptors. Biochim. Biophys. Acta. **1589**: 1-13.
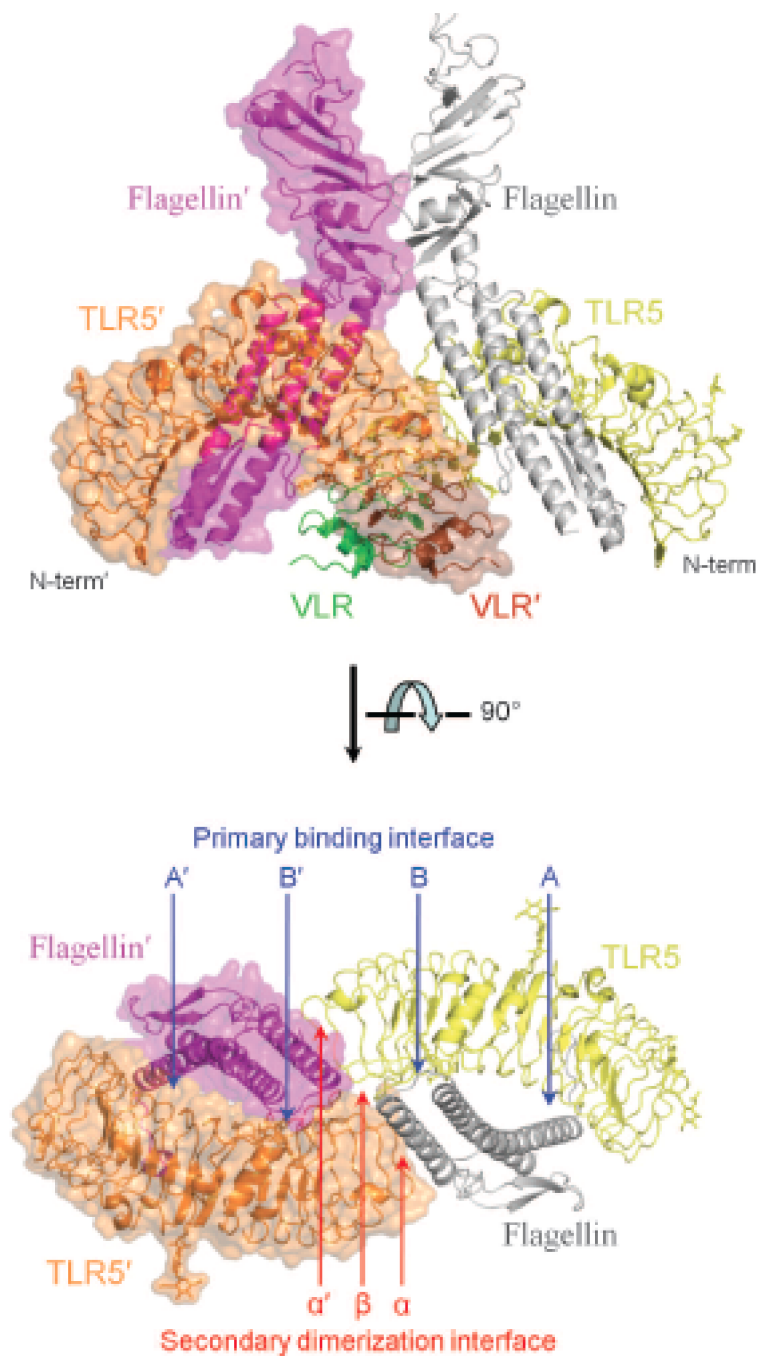
Figure 3: FliC interacts with TLR5
The D_1 domain contains three highly conserved helices required for TLR5 activation.
Yoon, S., O. Kurnasov, V. Natarajan, M. Hong, A.V. Gudkov, A.L. Osterman, and A.I.Wilson. **2012**.
Structural basis of TLR5-flagellin recognition and signaling. Science **335**: 859-864.

Table 2: Recombinant Flagellin Vaccines

Recombinant Flagellin Vaccine	Reference
Flagellin – Influenza hemagglutinin epitopes	McEwen *et al*., 1992 Levi and Arnon, 1996 Ben-Yedidia and Arnon, 1998 Ben-Yedidia *et al.,* 1998 Ben-Yedidia *et al*., 1999a Jeon *et al*., 2002 Adar *et al*., 2009
Flagellin – Influenza hemagglutinin globular head domain	Song *et al*., 2009
Flagellin – Influenza virus M2e ectodomain	Huleatt *et al*., 2008
Flagellin – *Schistosoma mansoni* epitope	Ben-Yedidia *et al*., 1999b
Flagellin – *Campylobacter coli* maltose-binding protein	Lee *et al*., 1999
Flagellin – *Escherichia coli* colonization factor I epitope	das Graças Luna *et al*., 2000 McSorley *et al*., 2000 Strindelius *et al*., 2004
Flagellin – *Escherichia coli* heat-stable toxin	Pereira *et al*., 2001
Flagellin – Tetanus toxoid	Lee *et al*., 2006
Flagellin – West Nile virus envelope protein	McDonald *et al*., 2007
Flagellin – *Plasmodium* antigen	Bargieri *et al*., 2008 Bargieri *et al*., 2010 Leal *et al*., 2013
Flagellin – *Yersinia pestis* F1 antigen	Honko *et al*., 2006
Flagellin – Vaccinia virus L1R and B5R antigens	Delaney *et al*., 2010
Flagellin – *Helicobacter pylori* antigen	Mori *et al*., 2012
Flagellin – *Pseudomonas aeruginosa* OprF epitope	Weimer *et al*., 2009a Weimer *et al.,* 2009b

1.4   First Generation FliC:Antigen Fusion Protein

Previous research within this lab linked native *Salmonella* FliC (Figure 4, Genbank

Acquisition: D13689) with antigens like those for Marberg and influenza.

```
GTTATCGGCAATCTGGAGGCAAAGTTTAATGATAATTTTGCAAAAATAATGCGCGGAATAATGATGCATA
AAGCGGCTATTTCGCCGCCTAAGAAAAAGATCGGGGGAAGTGAAAAATTTTCTAAAGTTCGAAATTCAGG
TGCCGATACAAGGGTTACGGTGAGAAACCGTGGGGAACAGCCCAATAACATCAAGTTGTAATTGATAAGG
AAAAGATCATGGCACAAGTCATTAATACAAACAGCCTGTCGCTGTTGACCCAGAATAACCTGAACAAATC
CCAGTCCGCTCTGGGCACCGCTATCGAGCGTCTGTCTTCCGGTCTGCGTATCAACAGCGCGAAAGACGAT
GCGGCAGGTCAGGCGATTGCTAACCGTTTTACCGCGAACATCAAAGGTCTGACTCAGGCTTCCCGTAACG
CTAACGACGGTATCTCCATTGCGCAGACCACTGAAGGCGCGCTGAACGAAATCAACAACAACCTGCAGCG
TGTGCGTGAACTGGCGGTTCAGTCTGCTAACAGCACCAACTCCCAGTCTGACCTCGACTCCATCCAGGCT
GAAATCACCCAGCGCCTGAACGAAATCGACCGTGTATCCGGCCAGACTCAGTTCAACGGCGTGAAAGTCC
TGGCGCAGGACAACACCCTGACCATCCAGGTTGGTGCCAACGACGGTGAAACTATCGATATCGATCTGAA
GCAGATCAACTCTCAGACCCTGGGTCTGGATACGCTGAATGTGCAACAAAAATATAAGGTCAGCGATACG
GCTGCAACTGTTACAGGATATGCCGATACTACGATTGCTTTAGACAATAGTACTTTTAAAGCCTCGGCTA
CTGGTCTTGGTGGTACTGACCAGAAAATTGATGGCGATTTAAAATTTGATGATACGACTGGAAAATATTA
CGCCAAAGTTACCGTTACGGGGGGAACTGGTAAAGATGGCTATTATGAAGTTTCCGTTGATAAGACGAAC
GGTGAGGTGACTCTTGCTGGCGGTGCGACTTCCCCGCTTACAGGTGGACTACCTGCGACAGCAACTGAGG
ATGTGAAAAATGTACAAGTTGCAAATGCTGATTTGACAGAGGCTAAAGCCGCATTGACAGCAGCAGGTGT
TACCGGCACAGCATCTGTTGTTAAGATGTCTTATACTGATAATAACGGTAAAACTATTGATGGTGGTTTA
GCAGTTAAGGTAGGCGATGATTACTATTCTGCAACTCAAAATAAAGATGGTTCCATAAGTATTAATACTA
CGAAATACACTGCAGATGACGGTACATCCAAAACTGCACTAAACAAACTGGGTGGCGCAGACGGCAAAAC
CGAAGTTGTTTCTATTGGTGGTAAAACTTACGCTGCAAGTAAAGCCGAAGGTCACAACTTTAAAGCACAG
CCTGATCTGGCGGAAGCGGCTGCTACAACCACCGAAAACCCGCTGCAGAAAATTGATGCTGCTTTGGCAC
AGGTTGACACGTTACGTTCTGACCTGGGTGCGGTACAGAACCGTTTCAACTCCGCTATTACCAACCTGGG
CAACACCGTAAACAACCTGACTTCTGCCCGTAGCCGTATCGAAGATTCCGACTACGCGACCGAAGTTTCC
AACATGTCTCGCGCGCAGATTCTGCAGCAGGCCGGTACCTCCGTTCTGGCGCAGGCGAACCAGGTTCCGC
AAAACGTCCTCTCTTTACTGCGTTAATGCGTTAATCCGGCGATTGATTCACCGACACGTGGTACACAATC
AATGGCAGCGAAAGCTGCCTTTTTTAACCGCGCACGCCCTATGTAATGAAAGAAATCACCGTACCTGAAC
CTGCCT
```

Figure 4: Full length flagellin 5' - 3' DNA sequence
5' – 3' DNA coding sequence of *Salmonella* flagellin. GenBank Acquisition: D13689

This approach utilized naturally occurring, blunt-ended restriction enzyme recognition sites throughout the *fliC* sequence but had several pitfalls that required unnecessarily complicated steps during the cloning and expression processes. First, the DNA sequences of both *flic* and the proposed antigens must be extensively analyzed for the presence of unwanted restriction enzyme recognition sites. If the antigen were to be linked to FliC using the FatI enzyme, that site should only be located once in both sequences to avoid an unwanted digest. If the site exists multiple times, PCR must be utilized to construct a sequence without the site while maintaining the same amino acid sequence. Redundancy of the genetic code (Figure 5) allows single nucleotide substitutions to yield the same protein sequence as that containing an unwanted restriction enzyme recognition site.

	U	C	A	G	
U	UUU ⌐ Phe UUC ⌐ UUA ⌐ Leu UUG ⌐	UCU ⌐ UCC ⌐ Ser UCA UCG ⌐	UAU ⌐ Tyr UAC UAA* Stop UAG* Stop	UGU ⌐ Cys UGC UGA* Stop UGG Trp	U C A G
C	CUU ⌐ CUC ⌐ Leu CUA CUG ⌐	CCU ⌐ CCC ⌐ Pro CCA CCG ⌐	CAU ⌐ His CAC CAA ⌐ Gln CAG ⌐	CGU ⌐ CGC ⌐ Arg CGA CGG ⌐	U C A G
A	AUU ⌐ AUC ⌐ Ile AUA ⌐ AUG** Met	ACU ⌐ ACC ⌐ Thr ACA ACG ⌐	AAU ⌐ Asn AAC ⌐ AAA ⌐ Lys AAG ⌐	AGU ⌐ Ser AGC ⌐ AGA ⌐ Arg AGG ⌐	U C A G
G	GUU ⌐ GUC ⌐ Val GUA GUG** ⌐	GCU ⌐ GCC ⌐ Ala GCA GCG ⌐	GAU ⌐ Asp GAC ⌐ GAA ⌐ Glu GAG ⌐	GGU ⌐ GGC ⌐ Gly GGA GGG ⌐	U C A G

Figure 5: The Genetic Code
There are 64 codons that code for only 20 amino acids.
Image Source: http://patentimages.storage.googleapis.com/WO2005021588A1/imgf000009_0001.png

For example, the DNA sequence 5' ACG-AAT-TCC 3' codes for the peptide sequence Threonine-Asparagine-Serine but also contains the EcoRI restriction recognition site, 5' GAATTC 3'. To remove this unwanted site, one base must be substituted that would maintain the aforementioned protein sequence. The corrected DNA sequence 5' ACA-AAT-TCC 3' maintains the polypeptide but lacks the EcoRI site. While this oversimplified example is easily corrected, removing an unwanted restriction enzyme recognition site is very time and labor intensive, especially if the unwanted site is located within the middle portion of a gene, requiring the use of overlapping PCR (Figure 6).
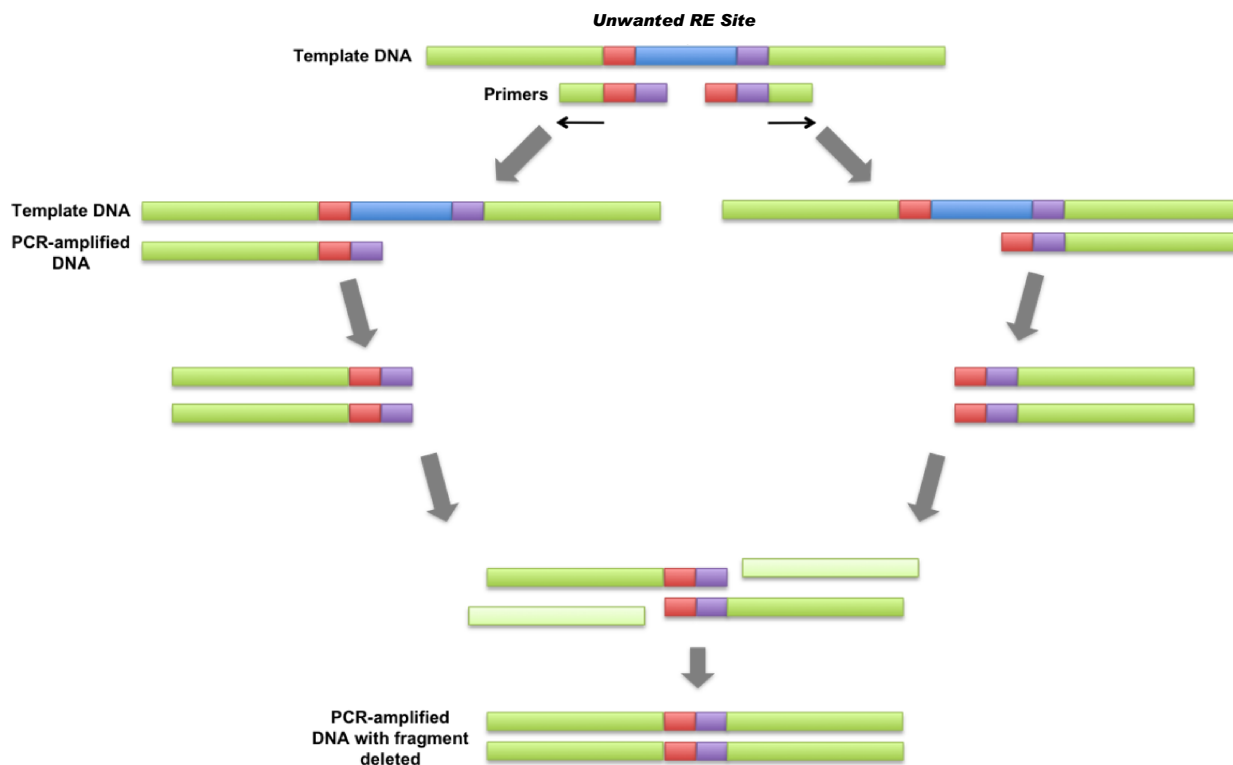
Figure 6: Overlapping PCR
Overlapping PCR can be used to remove unwanted restriction enzyme recognition sites. Primers are used to
introduce a needed mutation and to create a final fragment without the site.
Image adapted from: http://upload.wikimedia.org/wikipedia/commons/8/8b/Overlap_Extension_PCR.png

Secondly, it is commonly known that the use of blunt-ended restriction enzymes leads to low ligation efficiency and transformation yield. It would be optimal to utilize enzymes that leave "sticky ends," ensuring greater recombination success with proper orientation. Lastly, utilizing the native *fliC* sequence requires separate cloning strategies for each antigen of interest, increasing overall time and the variety of needed enzymes. For these reasons, a more robust and customizable FliC was desired to serve as reusable platform for the integration and co-expression of many different antigenic proteins.

2   MATERIALS AND METHODS

2.1   *In silico* Construct Design

The native DNA sequence of *Salmonella typhimurium* phase 1 flagellin (Figure 4) was

divided into each of its 7 subdomains by manually counting nucleotides (Figure 7). The

subdomains were then translated using the ExPASy online translator tool found at

http://web.expasy.org/translate/ (Figure 8) and BLASTED

(http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi?PAGE=Proteins) against the native sequence

for confirmation.

Figure 7: 5' 3' Native *Salmonella* FliC Domains - Color Coded

DOMAIN	AMINO ACID SEQUENCE
D0 (N)	**Met** A Q V I N T N S L S L L T Q N N L N K S Q S A L G T A I E R L S S G L R I N S A K D D
D1 (N)	A A G Q A I A N R F T A N I K G L T Q A S R N A N D G I S I A Q T T E G A L N E I N N N L Q R V R E L A V Q S A N S T N S Q S D L D S I Q A E I T Q R L N E I D R V S G Q T Q F N G V K V L A Q D N T L T I Q V G A N D G E T I D I D L K Q I N S Q T L G L D T L N V Q Q
D2 (N)	K Y K V S D T A A T V T G
D3	Y A D T T I A L D N S T F K A S A T G L G G T D Q K I D G D L K F D D T T G K Y Y A K V T V T G G T G K D G Y Y E V S V D K T N G E V T L A G G A T S P L T G G L P A T A T E D V K N V Q V
D2 (C)	A N A D L T E A K A A L T A A G V T G T A S V V K **Met** S Y T D N N G K T I D G G L A V K V G D D Y Y S A T Q N K D G S I S I N T T K Y T A D D G T S K T A L N K L G G A D G K T E V V S I G G K T Y A A S K A E G H N F K A Q P D L A E A A A T T T E
D1 (C)	N P L Q K I D A A L A Q V D T L R S D L G A V Q N R F N S A I T N L G N T V N N L T S A R S R I E
D0 (C)	D S D Y A T E V S N **Met** S R A Q I L Q Q A G T S V L A Q A N Q V P Q N V L S L L R **Stop**

Figure 8: Amino Acid Sequences of Each FliC Domain

The D_1 and D_2 domains on both termini, responsible for immune system recognition, were screened for the absence of all commercially-available restriction enzyme sites by using the New England BioLabs Cutter located at http://tools.neb.com/NEBcutter2/index.php and choosing the "0 cutters" option. These resulting restriction enzymes were manually screened to remove those that leave blunt ends, resulting in a list of restriction enzymes that do not cut within the sequence but will leave sticky ends after cleavage. This list was further shortened to include only those restriction enzymes that recognize multiples of 3 DNA nucleotides, aiding in frame maintenance for future cloning (Figure 9).

AatII (G_ACGT^C)
Acc65I (G^GTAC_C)
AccI (GT^MK_AC)
AclI (AA^CG_TT)
AflIII (C^TTAA_G)
AgeI (A^CCGG_T)
ApaI (G_GGCC^C)
ApaLI (G^TGCA_C)
ApoI (R^AATT_Y)
AvrII (C^CTAG_G)
BamHI (G^GATC_C)
BanII (G_RGCY^C)
BclI (T^GATC_A)
BglII (A^GATC_T)
BmtI (G_CTAG^C)
BsaHI (GR^CG_YC)
BsaWI (W^CCGG_W)
BsiHKAI (G_WGCW^C)
BsiWI (C^GTAC_G)
BsoBI (C^YCGR_G)
Bsp1286I (G_DGCH^C)
BspEI (T^CCGG_A)
BspHI (T^CATG_A)
BsrGI (T^GTAC_A)
BssSI (C^ACGA_G)
BstBI (TT^CG_AA)
BstYI (R^GATC_Y)
BtgI (C^CRYG_G)
EagI (C^GGCC_G)
EcoRI (G^AATT_C)
HindIII (A^AGCT_T)
KasI (G^GCGC_C)

KpnI (G_GTAC^C)
MfeI (C^AATT_G)
MluI (A^CGCG_T)
NarI (GG^CG_CC)
NcoI (C^CATG_G)
NdeI (CA^TA_TG)
NgoMIV (G^CCGG_C)
NheI (G^CTAG_C)
NotI (GC^GGCC_GC)
NsiI (A_TGCA^T)
NspI (R_CATG^Y)
PaeR7I (C^TCGA_G)
PciI (A^CATG_T)
PflFI (GACN^N_NGTC)
PluTI (G_GCGC^C)
PpuMI (RG^GWC_CY)
PspOMI (G^GGCC_C)
PvuI (CG_AT^CG)
SacI (G_AGCT^C)
SacII (CC_GC^GG)
SalI (G^TCGA_C)
SmlI (C^TYRA_G)
SpeI (A^CTAG_T)
SphI (G_CATG^C)
StyI (C^CWWG_G)
TatI (W^GTAC_W)
TspMI (C^CCGG_G)
Tth111I (GACN^N_NGTC)
XbaI (T^CTAG_A)
XhoI (C^TCGA_G)
XmaI (C^CCGG_G)

Figure 9: Possible Restriction Enzymes to be Used in the FliC Construct

To aid in ligation into commercially available expression vectors, the most commonly used restriction enzymes were incorporated on the 5' and 3' ends, flanking the D_2 domains. The length of D_3 domain/hypervariable region was maintained, although the DNA sequence was altered so that one of the restriction enzyme recognition sites (Figure 9) was incorporated between every 15 native nucleotides (Figure 18).

2.2 Construct Synthesis

The following 5'-3' sequence of the Synthetic FliC Expression Platform (Figure 10) was

optimized for expression in *E. coli* (Figure 11) and synthesized by DNA2.0 (Menlo Park, CA) in

the pJ404 expression vector for a total recombinant length of 4140 base pairs:

```
AAGCGATCAGTACGATGGACTGACGTACCATGGGAATTCACTAGTTCTAGAGCGGCAGGTCAGGCGAT
TGCTAACCGTTTTACCGCGAACATCAAAGGTCTGACTCAGGCTTCCCGTAACGCTAACGACGGTATCTC
CATTGCGCAGACCACTGAAGGCGCGCTGAACGAAATCAACAACAACCTGCAGCGTGTGCGTGAACTG
GCGGTTCAGTCTGCTAACAGCACCAACTCCCAGTCTGACCTCGACTCCATCCAGGCTGAAATCACCCA
GCGCCTGAACGAAATCGACCGTGTATCCGGCCAGACTCAGTTCAACGGCGTGAAAGTCCTGGCGCAG
GACAACACCCTGACCATCCAGGTTGGTGCCAACGACGGTGAAACTATCGATATCGATCTGAAGCAGAT
CAACTCTCAGACCCTGGGTCTGGATACGCTGAATGTGCAACAAAAATATAAGGTCAGCGATACGGCTG
CAACTGTTACAGGAGACGTCGATACTACGATTGCTGGGCCCAATAGTACTTTTAAAGCTAGCGCTACTG
GTCTTGGTGAGCTCGACCAGAAAATTGATCATATGTTAAAATTTGATGATCAATTGGGAAAATATTACGC
CCCGCGGACCGTTACGGGGGGACGATCGAAAGATGGCTATTATACCGGTTCCGTTGATAAGACGGCATG
CGAGGTGACTCTTGCTGTGCACGCGACTTCCCCGCTTACGCGTGGACTACCTGCGACAAGATCTGAGG
ATGTGAAAAATCCTAGGGTTGCAAATGCTGATTTGACAGAGGCTAAAGCCGCATTGACAGCAGCAGGT
GTTACCGGCACAGCATCTGTTGTTAAGATGTCTTATACTGATAATAACGGTAAAACTATTGATGGTGGTT
TAGCAGTTAAGGTAGGCGATGATTACTATTCTGCAACTCAAAATAAAGATGGTTCCATAAGTATTAATAC
TACGAAATACACTGCAGATGACGGTACATCCAAAACTGCACTAAACAAACTGGGTGGCGCAGACGGC
AAAACCGAAGTTGTTTCTATTGGTGGTAAAACTTACGCTGCAAGTAAAGCCGAAGGTCACAACTTTAA
AGCACAGCCTGATCTGGCGGAAGCGGCTGCTACAACCACCGAAAACCCGCTGCAGAAAATTGATGCT
GCTTTGGCACAGGTTGACACGTTACGTTCTGACCTGGGTGCGGTACAGAACCGTTTCAACTCCGCTATT
ACCAACCTGGGCAACACCGTAAACAACCTGACTTCTGCCCGTAGCCGTATCGAATGAGGATCCAAGCT
TCTCGAGGCGGCCGCAAGCGATCAGTACGATGGACTGACGTA
```

Figure 10: Original 5'-3' DNA sequence of FliC Expression Platform
Original 5' – 3' DNA sequence submitted to DNA2.0, before optimization for expression in *E. coli*

```
AGGAGGTAAAACACCATGGGAATTCGGTCTAGAACTAGTGCAGCCGGCCAGGCGATTGCGAA
TCGTTTTACGGCGAACATCAAGGGTCTGACCCAAGCCTCTCGTAATGCAAATGATGGTATTAGC
ATCGCACAAACCACCGAAGGCGCGCTGAACGAGATCAACAATAACTTGCAACGTGTCCGTGA
GCTGGCAGTTCAGAGCGCGAACAGCACGAATAGCCAGTCCGATTTGGACAGCATCCAGGCGG
AGATTACGCAACGTTTGAACGAAATCGACCGCGTCAGCGGTCAAACGCAGTTTAATGGTGTGA
AAGTTCTGGCCCAGGATAACACCCTGACGATTCAGGTTGGCGCGAACGACGGTGAAACGATT
GATATTGACCTGAAGCAGATCAACAGCCAAACCCTGGGTCTGGACACCCTGAACGTGCAACA
AAAGTATAAGGTGTCGGACACGGCCGCTACCGTGACCGGCGACGTCGATACTACGATTGCTGG
GCCCAATAGTACTTTTAAAGCTAGCGCTACTGGTCTTGGTGAGCTCGACCAGAAAATTGATCAT
ATGTTAAAATTTGATGATCAATTGGGAAAATATTACGCCCCGCGGACCGTTACGGGGGGACGAT
CGAAAGATGGCTATTATACCGGTTCCGTTGATAAGACGGCATGCGAGGTGACTCTTGCTGTGCA
CGCGACTTCCCCGCTTACGCGTGGACTACCTGCGACAAGATCTGAGGATGTGAAAAATCCTAG
GGTTGCCAACGCTGACCTGACCGAGGCGAAAGCGGCGCTGACCGCAGCGGGCGTTACTGGTA
CCGCAAGCGTTGTGAAAATGAGCTACACCGACAATAATGGTAAAACTATCGATGGCGGTCTGG
CGGTCAAAGTCGGCGACGACTACTATTCCGACCACCCAGAACAAAGACGGCAGCATCAGCATTA
ACACTACGAAATACACCGCAGATGACGGCACGAGCAAAACGGCACTGAATAAGCTGGGCGGT
GCGGATGGTAAGACCGAAGTTGTTAGCATTGGTGGTAAAACCTATGCCGCGTCCAAGGCAGAG
GGTCACAATTTCAAGGCGCAGCCGGATCTGGCGGAAGCTGCAGCGACCACGACCGAGAATCC
GTTGCAGAAGATTGATGCGGCGCTGGCACAGGTCGATACGCTGCGCTCTGACCTGGGTGCCGT
ACAAAACCGTTTCAATAGCGCGATCACCAATCTGGGCAACACCGTGAACAATCTGACCTCTGC
TCGCAGCCGTATTGAGGGATCCAAGCTTCTCGAGTAGGCGGCCGCAAGC
```

Figure 11: Final 5'-3' DNA Sequence of FliC Expression Platform
5'-3' DNA sequence after codon optimization for expression in *E. coli*

2.3   Transformation of BL21 *E. coli* Expression Strain

One tube of BL21 competent *E. coli* cells (New England Biolabs, C2530) was thawed on ice for 10 minutes and mixed with 100ng of reconstituted pJ404 plasmid DNA containing the FliC construct by gentle pipetting. The mixture was placed on ice for 10 minutes, transferred to a 42°C water bath for exactly 10 seconds, and placed back on ice for 5 minutes. Cells were allowed to recover for 60 minutes in 950μL Super Optimal broth with Catabolite repression (SOC) medium (2% w/v Tryptone, 0.5% w/v Yeast Extract, 10mM NaCl, 2.5mM KCl, 10mM MgCl$_2$, 20mM Glucose) shaking at 200rpm at 37°C. Meanwhile, Luria Bertani (LB) Agar selection plates containing 100μg/mL ampicillin were warmed to 37°C. 100μL of transformed cells and 100μL of each of three serial dilutions (10^{-1}, 10^{-2}, 10^{-3}) were used to inoculate 4 separate warmed plates. The plates were allowed to sit upright on the bench top for one hour and then incubated upside down at 37°C overnight.

2.4   Culture Maintenance

2.4.1   LB Glycerol Stocks

One resulting colony was picked from the undiluted sample and transferred to a 50mL falcon tube containing 20mL LB broth with 100μg/mL ampicillin and allowed to grow at 37°C, shaking at 200rpm overnight. 0.5mL of the overnight culture was added to 0.5mL filter-sterilized 60% glycerol, to achieve a 30% glycerol stock, and stored at -80°C.

2.4.2   Adaptation to Fermentation Media

100mL of *E. coli* Adaptation Media [(ECAM) per liter: 7.8g KH$_2$PO$_4$, 1.0g Citric Acid, 2.33g (NH$_4$)$_2$SO$_4$, 1mL Trace Metal Solution, 1mL Thiamine HCl Solution, 40mL Glucose Stock Solution, 13mL MgSO$_4$ Solution, 0.5mL CaCl$_2$ Solution; Trace Metal Solution: 900mL warm

ddH_2O, 5g EDTA, 10g $FeSO_4.7H2O$, 2g $ZnSO_4.7H_2O$, 2g $MnSO_4.H_2O$, 0.2g $CoCl_2.6H_2O$, 0.1g

$CuSO_4.5H_2O$, 0.2g $NaMoO_4.2H_2O$, 0.1g H_3BO_3, dissolved and brought up to 1L with ddH_2O,

autoclaved at 121°C for 30 minutes; Thiamine HCl Stock: 950mL ddH_2O, 10g Thiamine HCl,

dissolved and made up to 1L with ddH_2O and filter sterilized; Glucose Stock Solution: 700mL

ddH_2O, 250g glucose, dissolved and made up to 1L with ddH_2O and filter sterilized; $MgSO_4$

Stock Solution: 900mL ddH_2O, 78g $MgSO_4$, dissolved and made up to 1L with ddH_2O and filter

sterilized; $CaCl_2$ Stock: 950mL ddH_2O, 80g $CaCl_2$, dissolved and made up to 1L with ddH_2O and

filter sterilized] containing 100µg/mL ampicillin was warmed to 37°C and inoculated with a

1mL glycerol stock of the BL21 *E. coli* containing the recombinant pJ404 plasmid in a sterile

250mL flask. The culture was allowed to grow overnight at 37°C, shaking at 200rpm. The

following day, the OD_{600} was measured and the volume needed to yield an OD_{600} of 0.05 was

used to inoculate a 250mL flask containing 100mL ECAM with 100µg/mL ampicillin. Once an

OD_{600} of 0.8-1.2 was achieved, 0.5mL of the culture was added to 0.5mL filter-sterilized 60%

glycerol to achieve a 30% glycerol stock and stored at -80°C as ECAM-adapted cells.

2.5   Induction Analysis

2.5.1   Cytotoxicity Assay

A 1.0mL 30% glycerol stock of either BL21 housing recombinant pJ404 or BL21 with no

plasmid was used to inoculate two 50 mL falcon tubes containing 20mL LB broth with and

without 100µg/mL ampicillin, respectively. The cultures were incubated overnight at 37°C

shaking at 200rpm. A volume of these cultures was used to inoculate the following experimental

samples to an initial OD_{600} of 0.1 in 10mL LB broth plus antibiotics where appropriate in 15mL

falcon tubes: BL21 with no plasmid, Uninduced BL21 with recombinant pJ404, and Induced

BL21 with recombinant pJ404. 1mM IPTG was added to the Induced culture once the OD_{600} reached 0.2. The cultures were allowed to grow for 300 minutes, shaking at 200RPM at 37°C, and triplicate samples were taken every 30 minutes to measure the OD_{600}.

2.5.2   Flask work

20mL of LB, 100mL of ECAM, and 100mL of Partially Defined *E. coli* Adaptation Media [(PECAM) per liter: 10g Yeast Extract, 20g Tryptone, 3g KH_2PO_4, 6g K_2HPO_4, 2g $(NH_4)_2SO_4$, 1mL Trace Metal Solution, 0.02g Thiamine HCl, 20g Glucose, 1g $MgSO_4.7H20$, 0.5mL $CaCl_2$ Solution] each containing 100μg/mL ampicillin, were warmed to 37°C and inoculated with a 1mL glycerol stock of the adapted BL21 *E. coli* containing the recombinant pJ404 plasmid. The culture was allowed to grow overnight at 37°C, shaking at 200rpm. The following day, the OD_{600} was measured and the volume needed to yield an OD_{600} of 0.05 was used to inoculate 2, 250mL flasks with 100mL of each media containing 100μg/mL ampicillin. Once an OD_{600} of 0.8-1.2 was achieved, an 80mM IPTG stock solution was added to one culture to yield a final concentration of 1mM IPTG and the other culture served as the uninduced control. After three hours of growth at 37°C, shaking at 200rpm, 10mL samples were taken from each culture and placed in 15mL falcon tubes and stored for up to 8 hours at 4°C.

2.6   Fermentation with ECAM and PECAM

2.6.1   Inoculum preparation: ECAM

A 1mL adapted glycerol stock stored at -80°C was used to inoculate 250mL ECAM with 100μg/mL ampicillin in a sterile1L flask. The culture was incubated at 37°C while shaking at 200rpms for 14.5 hours. After, 2.8mL of the culture was transferred to 250mL ECAM with

100μg/mL in a 1L flask to yield a starting OD_{600} of 0.1. After 6 hours the OD_{600} was 1.5 and 80mL of this culture was used to inoculate the vessel to a starting OD_{600} of 0.012.

2.6.2 Inoculum preparation: PECAM

250μL of a 1mL adapted glycerol stock stored at -80°C was used to inoculate 250mL ECAM with 100μg/mL ampicillin in a sterile1L flask. The culture was incubated at 37°C while shaking at 200rpms for 16 hours. After, 2.5mL of the culture was transferred to 250mL PECAM with 100μg/mL in a 1L flask to yield a starting OD_{600} of 0.1. After 5 hours the OD_{600} was 5.0 and 20mL of this culture was used to inoculate the vessel to a starting OD_{600} of 0.012.

2.6.3 Vessel Set Up/ Fermentation Run: ECAM

The Biostat C 20L vessel was autoclaved with 2.5L of ddH_2O and 1.5L of 10X EBAT (750μL Antifoam, 22g KH_2PO_4, 10g Citric Acid, 45g $(NH_4)_2SO_4$,10mL Trace Metal Solution, 10mL Thiamine HCl Solution) and 2L of 10X EBAT II (875mL Glucose Stock Solution, 130mL $MgSO_4$ Solution, 5mL $CaCl_2$ Solution, 100mg/L ampicillin, dissolved and brought up to 2L with ddH_2O and filter sterilized) was added. After inoculation, a 10mL sample was taken and a loopful was transferred to both a Nutrient Agar (NA) plate and a LB agar plate with 100μg/mL ampicillin and incubated at 37°C to check for contamination and insure plasmid integrity, respectively. The glucose concentration was 16.8g/L and the ampicillin concentration was 100mg/L.

The temperature was set at 30°C and the pH was maintained at 7.0. Dissolved oxygen was maintained at 30% by cascade control with agitation, with a minimum value of 150rpm and a maximum of 450rpm, and gas mix. Airflow was maintained 10 LPM.

2.6.4 Vessel Set Up/ Fermentation Run: PECAM

The Biostat C 20L vessel was autoclaved with 8.5L of double deionized water and 1.5L

of 10X PEBAT (10X PECAM with 750μL antifoam) was added. After inoculation, a 10mL

sample was taken and a loopful was transferred to both a Nutrient Agar (NA) plate and a LB agar

plate with 100μg/mL ampicillin and incubated at 37°C to check for contamination and insure

plasmid integrity, respectively. The glucose concentration was 19.6g/L and the ampicillin

concentration was 100mg/L.

The temperature was set at 30°C and the pH was maintained at 7.0. Dissolved oxygen

was maintained at 30% by cascade control with agitation, with a minimum value of 150rpm and

a maximum of 450rpm, and gas mix. Airflow was maintained 10 LPM.

2.6.5 Induction with 1mM IPTG

Upon glucose depletion as determined by the YSI, the feed rate was increased to confirm

0% net glucose accumulation. A 10mL, uninduced sample was taken and a filter-sterilized IPTG

solution was injected into the bioreactor to achieve a final concentration of 1mM. During the

ECAM fermentation, 10mL samples were taken at 1, 2, 3, 4,5, and 22 hours post induction for

analysis. During the PECAM fermentation, samples were taken at 1, 2, and 3 hours post

induction for analysis.

2.7 Preparation of Soluble, Insoluble, and Conditioned Media of Uninduced and Induced
Samples

3mL of each flask sample and 1mL of each fermentation sample were centrifuged at

14krpm for 5 minutes. The supernatant was removed as "Conditioned Media" and the pellet was

resuspended in 850μL Phosphate Buffered Saline (137mM NaCl, 12mM Phosphate, 2.7mM

KCl, pH 7.4) and sonicated on ice 3 times for 10 seconds (1 second on / 0.5 second off). The

sonicated samples were spun at 14krpm for 15 minutes. The resulting supernatant was removed

as "Soluble Protein" and the pellet was resuspended in 850μL 50mM Tris, 8M Urea, pH 8.0 as

"Insoluble Protein."

2.8   Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis (SDS-PAGE)

10μL of 4x NuPAGE loading buffer (Novex) was added to 30μL of each sample,

vortexed, and heated at 80°C for 5 minutes. The samples were centrifuged at 14krpm for 5

minutes. 10μL of each sample was loaded into each well of a NuPAGE 4-12% Bis-Tris Gel

(Novex). The gel was run at 200 volts for 60 minutes in 1X MOPS SDS running buffer (Novex)

stained overnight in Coommassie Brilliant Blue, and destained in ddH$_2$0. The gel was imaged

using a Typhoon 9400 scanner (Amersham Biosciences) and Image Quant TL software (General

Electric).

2.9   Western Blot

The proteins separated by SDS-PAGE were transferred onto a nitrocellulose membrane

(Invitrogen, LC2000) for 1 hour at 30 volts. Gel-to-membrane contact was maintained

throughout by pressure from blotting pads and filter paper soaked in 1X Transfer Buffer [50mL

20X NuPAGE Transfer Buffer (Novex NP0006), 100mL methanol, 850mL ddiH$_2$0], sandwiched

within the Blot Module filled with 1X Transfer Buffer.

After, non-specific binding sites were blocked by incubating the membrane in 5% nonfat

milk in 1X TBST [100mL 10X TBS (0.2M Trizma-HCl, 1.5M NaCl, pH 7.6), 900mL ddH$_2$0,

1mL Tween-80], overnight shaking at 60rpm at 4°C. The membrane was washed 3 times for 5

minutes with 1X TBST shaking at 100rpm at 4°C, followed by an hour incubation shaking at

100rpm at 4°C with the Mouse Anti-Flagellin Primary Antibody (InvivoGen) diluted to 1:1000

in 1X TBST. The membrane was then washed once with 1X TBST for 15 minutes, shaking at

100rpm at 4°C, followed by four more washes for 5 minutes, shaking at 100rpm at 4°C. The

membrane was then incubated for one hour, shaking at 100rpm at 4°C, with the Goat Anti-

Mouse Antibody (Invitrogen, 62-6720), diluted to 1:10,000 in 1X TBST. The membrane was

then washed once with 1X TBST for 15 minutes, shaking at 100rpm at 4°C, followed by four

more washes for 5 minutes, shaking at 100rpm at 4°C.

The membrane was incubated with Chemiluminescence Reagent (PerkinElmer,

NEL103001EA) according to the manufacturer's protocol and imaged using the

ImageQuant4000 (General Electric).

2.10 Protein Analysis via MALDI MS/MS

Bands of interest were excised from the stained SDS-PAGE gel and placed in 200μL

ddH$_2$0 and submitting to the protein sequencing facility in the Georgia State University Core

Facility for Matrix Assisted Laser Deabsorption Ionization (MALDI) mass spectrophotometry/

mass spectrophotometry (MS/MS) analysis.

3 RESULTS

3.1 Cytotoxicity Assay

Presence of the recombinant pJ404 plasmid in BL21 *E. coli* cells showed no negative

impact on growth as compared to that of cells without the plasmid (Figure 12). Also, the addition

of 1mM IPTG for protein induction at approximately 30 minutes post-inoculation also did not

negatively impact cell growth. The optical densities of the induced sample decreased after the

7th time point (210 minutes post-inoculation), however this slight decline was attributed to

variability within the shaker.

3.2 **Flask work: Induction in LB**

SDS-PAGE and Western blot analyses of recombinant BL21 *E. coli* cells grown in 20mL

LB broth and induced with 1mM IPTG demonstrated target protein expression primarily in the

insoluble fractions (IP) after both 3 and 24 hours of induction (Figure 13). The major band

located in the insoluble fraction after 24 hours of induction was sequenced in the GSU Core

Facility using MALDI MS/MS. The resulting sequence matched that of native FliC, correlating

to 53 and 59 peptides on the N and C termini, respectively. The lower major band (~42kDa) was

also sequenced and demonstrated homology with OmpF, an outer membrane protein that is seen

in most of the *E. coli* protein work conducted in this laboratory. The third major band (~38kDa)

could be the result of degradation or truncation, cleavage, or an aggregation of proteins that

migrate more rapidly than their fully denatured counterparts. The faint band seen around 55kDa

could be attributed to dimerization of the synthetic FliC protein.
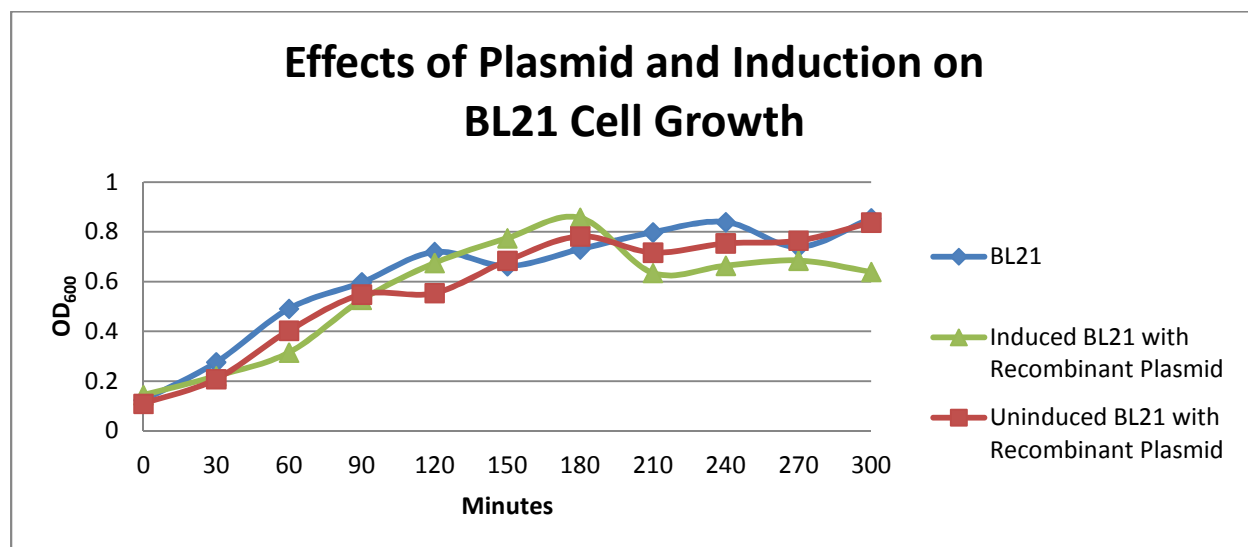
Figure 12: Effects of Plasmid and Induction on BL21 Cell Growth.
Cells were grown in LB Broth supplemented with 100μg/mL ampicillin at 37°C shaking at 200rpm for 300 minutes. Triplicate samples were taken every 30 minutes and the OD_{600} values were measured and averaged.

Figure 13: Induction in LB: Flask work.
SDS-PAGE and Western Blot of protein samples extracted from BL21 with the recombinant
plasmid grown in LB broth at 37°C shaking at 200rpm, uninduced and induced with 1mM IPTG for 3 and 24 hours.
CM: Conditioned Media, SP: Soluble Protein, IP: Insoluble Protein

3.3   Flask work: Induction in ECAM

After growth and induction in 100mL ECAM, SDS-PAGE analysis demonstrated a major

band in the insoluble fraction, a minor band in the soluble fraction, and a faint band in the
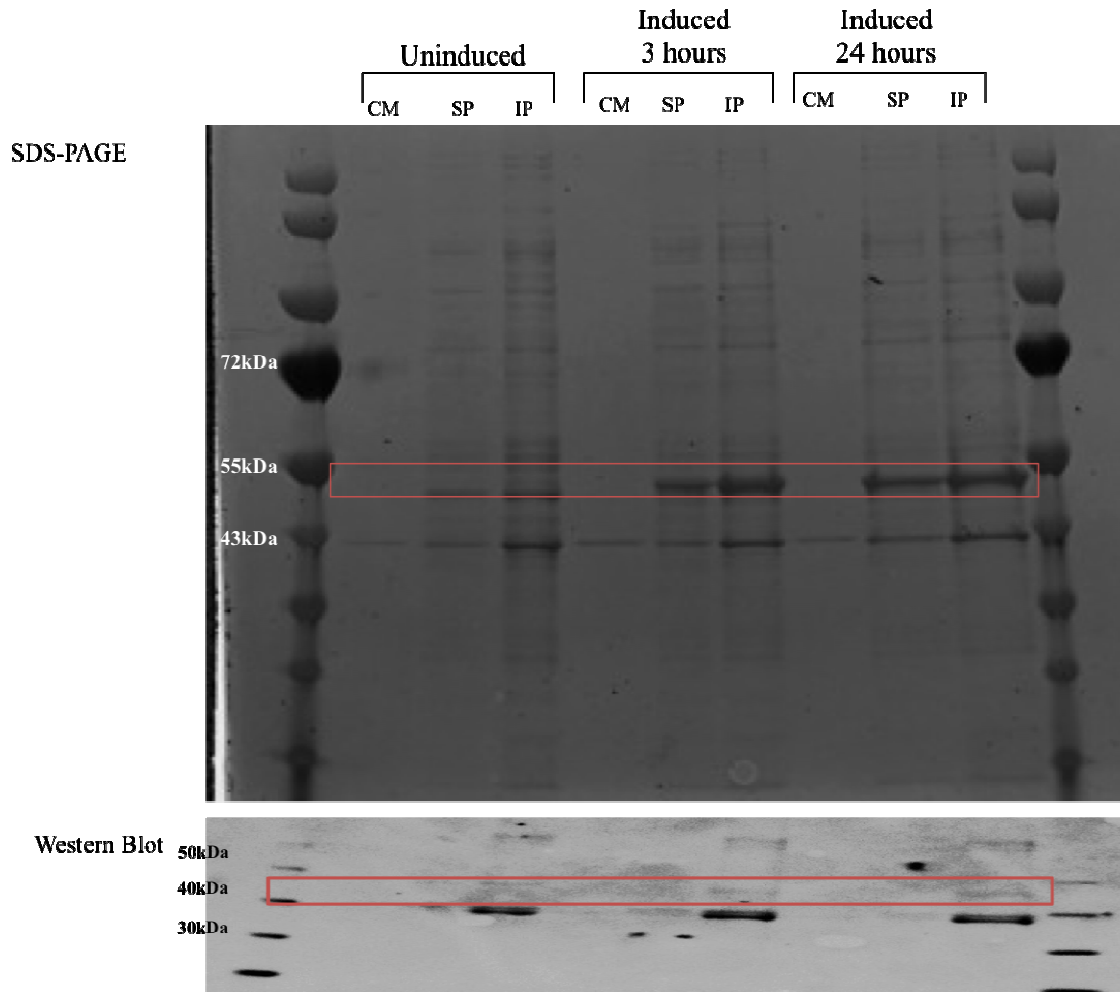
conditioned media (Figure 14). Western blot analysis confirmed strong expression within the

insoluble fraction after only 3 hours of induction with 1mM IPTG. The small, 38kDa protein

produced after growth in ECAM (Figure 14) and in LB (Figure 13) could be the product of

protein degradation, cleavage, or aggregation

3.4   Flask work: Induction in PECAM

Growth and induction in PECAM followed by SDS-PAGE and Western blot analyses of

cellular fragments resulted in a major protein band at approximately 45kDA, primarily in the

induced insoluble and soluble fractions, indicating strong induction of the FliC construct (Figure

15). As seen in Figures 13 and 14, growth in PECAM also produced a 38kDa protein, indicating

possible protein degradation or clumping.

3.5   Fermentation in ECAM

After a 20-liter fermentation in ECAM involving 22 hours of induction, the expected

45kDa protein was only present in the conditioned media after SDS-PAGE and Western blot

analysis (Figure 16). Full-length product was seen neither in the soluble nor insoluble fractions,

even after 22 hours of induction. However, a smaller band around 28kDa was confirmed in the

Western blot to be part of FliC and displayed a strong banding pattern in the insoluble fractions.

This sized fragment was not created in the LB (Figure 13), ECAM (Figure 14), or PECAM

(Figure 15) flask work. Also of note, the band commonly seen at 38kDA was only present in the

conditioned media.

3.6   Fermentation in PECAM

A 20-liter fermentation in PECAM demonstrated target protein expression after 1,2, and

3 hours of induction with 1mM IPTG compared to the uninduced control, as seen in the SDS-

PAGE and Western blot in Figure 17. The ~38kDA protein present in the ECAM (Figure 14) and

PECAM (Figure 15) flask work but absent in the soluble and insoluble fractions of the ECAM

fermentation samples (Figure 16) was primarily expressed in the insoluble fractions after

fermentation using PECAM (Figure 17). To add, the 28kDA protein in the soluble and insoluble

fractions of the ECAM fermentation samples (Figure 16) was absent from the PECAM

fermentation samples (Figure 17).

Figure 14: Induction in ECAM: Flask Work.
SDS-PAGE and Western Blot of protein samples extracted from BL21 with the
recombinant plasmid grown in ECAM at 37°C shaking at 200rpm, uninduced and induced with 1mM IPTG for 3 hours.
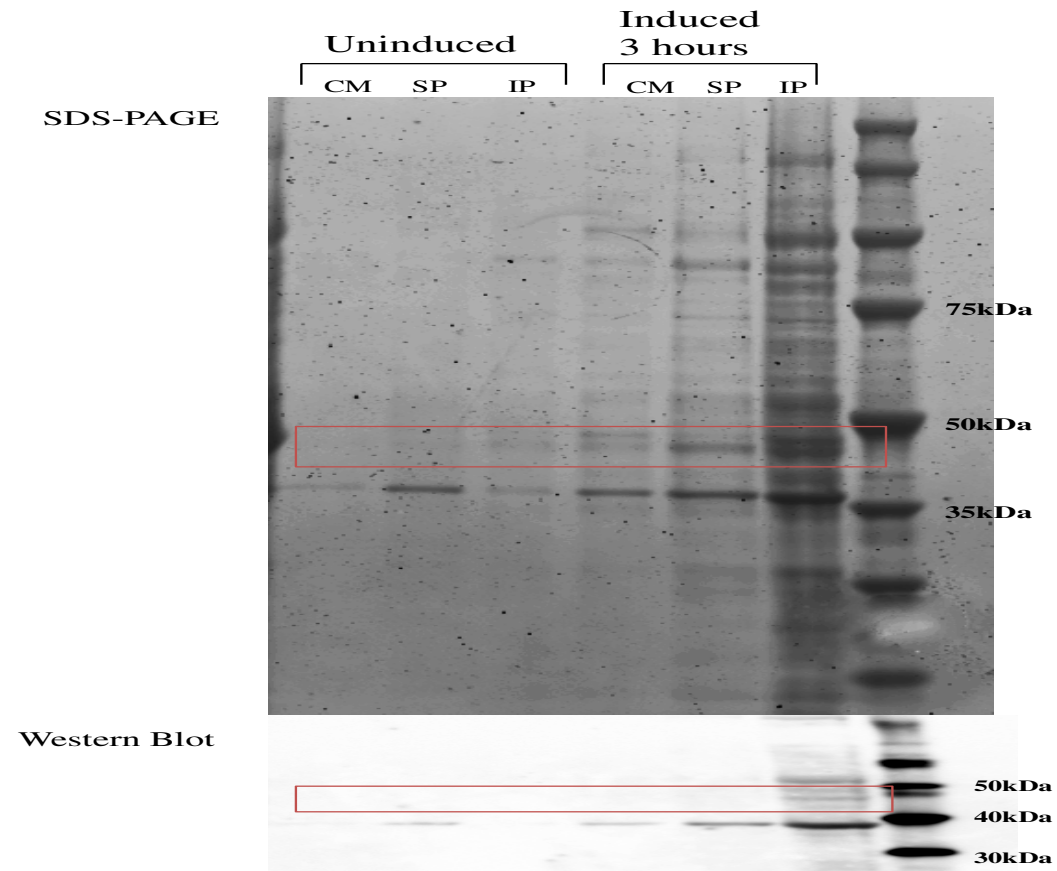CM: Conditioned Media, SP: Soluble Protein, IP: Insoluble Protein

Figure 15: Induction in PECAM: Flask Work
SDS-PAGE and Western Blot of protein samples extracted from BL21 with the
recombinant plasmid grown in PECAM at 37°C shaking at 200rpm, uninduced and induced with 1mM IPTG for 3 hours.
CM: Conditioned Media, SP: Soluble Protein, IP: Insoluble Protein

Figure 16: ECAM Fermentation.
SDS-PAGE and Western Blot of protein samples extracted from BL21 with the
recombinant plasmid grown in 20 Liter ECAM Fermentation at 30°C and induced with 1mM IPTG
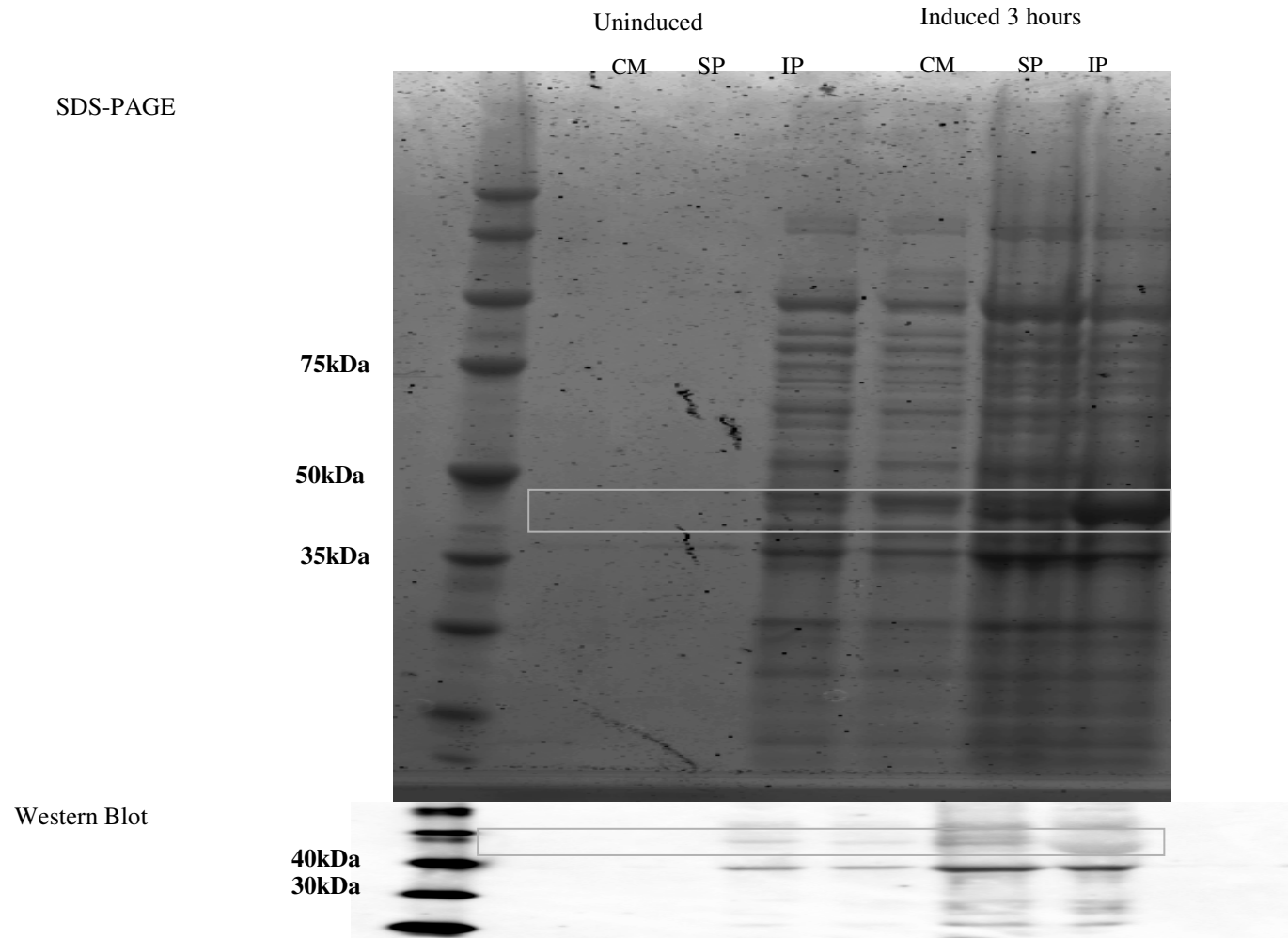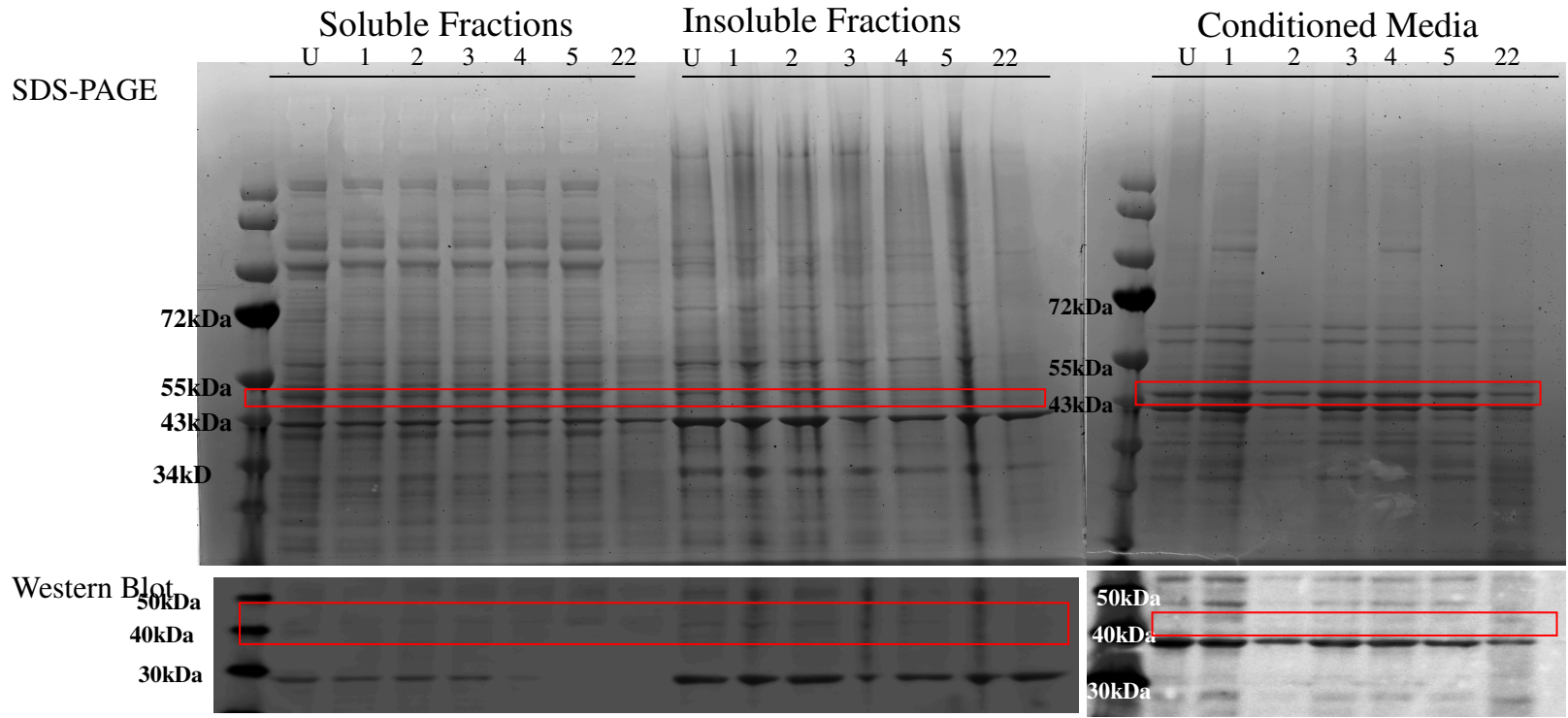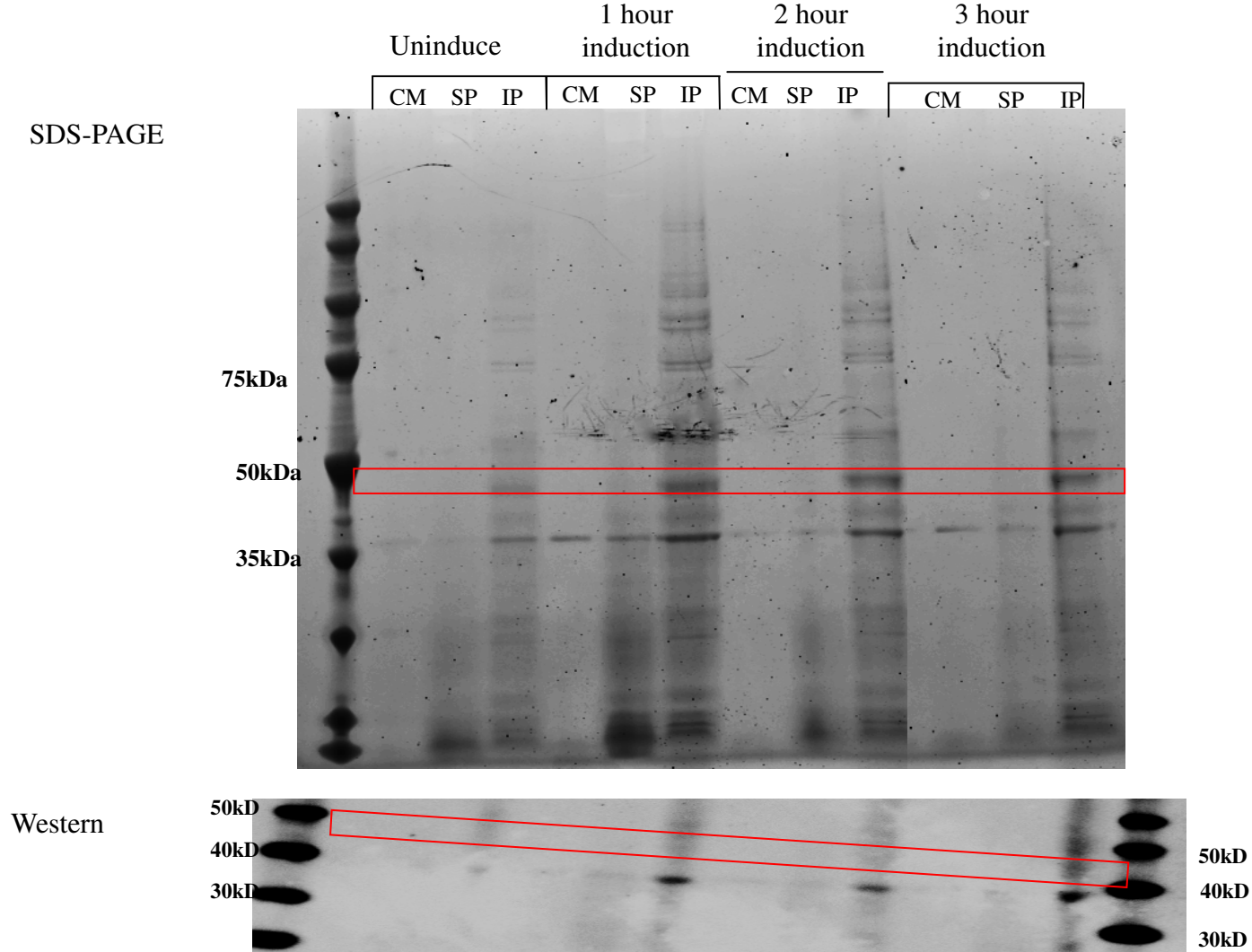U: Uninduced 1,2,3,4,5,22 – Hours post induction

Figure 17: PECAM FERMENTATION.
SDS-PAGE and Western Blot of protein samples extracted from BL21 with the
recombinant plasmid grown in 20 Liter PECAM Fermentation at 30°C and induced with 1mM IPTG
CM: Conditioned Media SP: Soluble Protein IP: Insoluble Protein

4 DISCUSSION

4.1 Second Generation FliC-Antigen Co-Expression Platform

To effectively use FliC as a platform technology for the production of biological vaccine adjuvants, a construct was created that allows antigenic proteins to be placed at the N-terminus, C-terminus, and within the hypervariable region (Figure 18). The N-terminal and C-terminal D_0 domains were replaced with commonly used restriction enzyme recognition sequences, as these native sequences have not been demonstrated necessary for TLR5 activation, and the hypervariable region was replaced with a synthetic sequence of restriction enzyme recognition sites between every fifteen native nucleotides.

4.1.1 Rationale For N and C Terminal Sequences

Eaves-Pyles and colleagues (2001) were the first to study the effects of FliC mutations on immune response and begin pinpointing the sequences necessary for inflammation. They created a series of FliC protein-fraction fusions and tested for bioactivity, determining that the most conserved amino and carboxyl termini are required for an immune response. In 2002, Donnelly and Steiner worked to further isolate the FliC amino acids required for an inflammatory response by creating a series of deletion and insertion mutants and measuring subsequent Interleukin (IL)-8 release. They concluded that the last 121 residues of the N-terminal D_1 and the first 12 residues in D_2 are required for inflammation. One year later Smith *et al*. also constructed a variety of FliC mutants and determined that a deletion of the first 100 amino acids in the N-terminal D_1 domain resulted in complete loss of TLR5 activation and removing amino acids 416-444 in the C terminus caused the same effect. In studying the mechanism by which some flagellated bacteria circumvent the immune system, Andersen-Neissen and colleagues (2005) discovered that amino

acids 89-96 within the N terminus are required for TLR5 activation. In 2012, Yoon *et al*. continued this research by resolving the crystal structure of Zebrafish TLR5 complexed with *Salmonella* FliC. It was determined that only the D_1 domain interacts with the receptor. Combining these data, removing the D_0 domain should have no effect on TLR5 activation as long as the D_1 and D_2 domains remain intact, and therefore the second generation FliC-Antigen Co-expression platform contains popular restriction enzyme recognition sites at this location.

4.1.2   Rationale For Hypervariable Region Sequence

Eaves-Pyles *et al*. (2001) created a flagellin mutant containing the native N and C terminal conserved domains and replaced the hypervariable region with an unrelated *E. coli* DNA sequence and found this mutant to stimulate innate immunity like wild type flagellin. They then speculated that the D_3 domain allows the D_1 and D_2 domains to interact and activate TLR5. Donnelly and Steiner (2002) confirmed that a large deletion within this region had no effect on inflammation but an insertion led to decreased inflammation. This insertion might have a consequence on the spatial relationship between the D_1 and D_2 domains, causing diminished TLR5 activation. Liu *et al*. (2010) set out to definitively identify the role of the hypervariable domain in innate immunity and adjuvancy by creating three distinct deletion mutants: Δ190-278, Δ220-320, Δ180-400, all of which lost their antigenicity. Based on these results combined, the length of the D_3 domain is required for proper protein folding and subsequent TLR5 activation but the sequence is irrelevant. Therefore, the hypervariable domain of the synthetic FliC construct is the same length as that of native *flic* but contains a 6 base pair restriction enzyme recognition sequence every 15 nucleotides, allowing antigens of a variety of lengths to be inserted into this region.
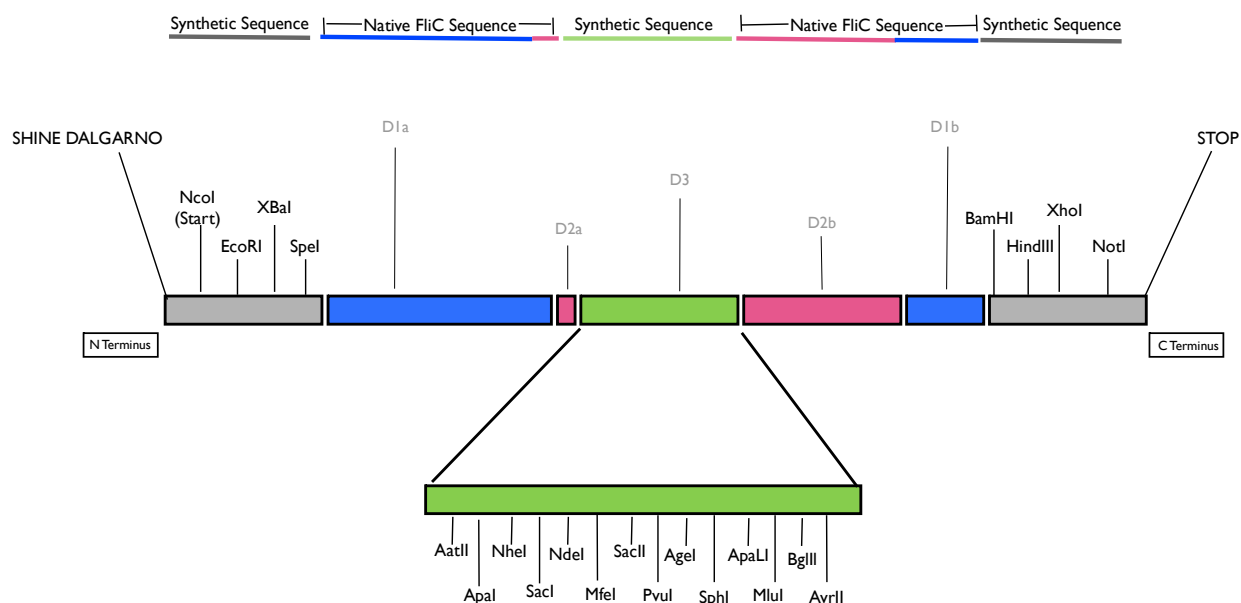
Figure 18: Synthetic FliC Expression Platform
D$_0$ and D$_3$ domains were replaced with commonly used restriction enzyme recognition sites while D$_1$ and D$_2$ remain for proper TLR5 activation.

4.2   Production in BL21 *E. coli* via pJ404

4.2.1   Synthesis in pJ404

The FliC-Antigen co-expression platform is capable of stable integration within a variety of expression vectors, given that the most commonly used restriction enzymes are located on the 5' and 3' ends of the platform DNA sequence. For initial synthesis, DNA2.0 provided an inducible expression vector, pJ404, which utilizes an IP-free T5 promoter. This conscious choice to move away from the traditionally used T7 promoter safeguards the future patentability of the construct, should the Brookhaven National Laboratory, under contract with the U.S. Department of Energy, decide to patent and require licensure for the production of transcripts utilizing the T7 promoter (Studier, 2005). The T5 promoter system was first

described in 1985 by Gentz and Bujard, and has since been used and modified by many companies. DNA2.0 has not patented their changes to the T5 promoter system and insures their gene constructs are IP-free. The recombinant pJ404 was transformed into chemically competent BL21 *E. coli* cells via heat shock. The BL21 strain is suitable for the expression of all non-T7 transcripts and is deficient of both Lon and OmpT proteases. Such strains are very useful for the expression of recombinant proteins because the otherwise present proteases would cleave misfolded and exogenous proteins, respectively.

4.2.2   Induction in LB

When BL21 cells transformed with pJ404 containing the synthetic FliC construct were grown in LB broth and induced with 1mM IPTG, expression of the construct was seen primarily in the insoluble fractions (Figure 13). This protein sequence was confirmed through MALDI MS/MS and was used as an indicator band for all future induction studies. Although the amount of protein loaded in each lane of the gel in Figure 13 is not exactly the same, each processed pellet was resuspended in the same volume of buffer and therefore expression levels can be compared across samples with some level of certainty. In this way, there was no apparent increase in synthetic FliC production after 24 hours of induction, as compared to 3 hours of induction. Also, there was no major shift in protein expression between the soluble to insoluble fractions over time, as is often the case when proteins begin to form inclusion bodies and aggregate.

To add, the synthetic FliC construct was either cleaved or formed fragments, as seen at the 38kDA band on the SDS-PAGE and confirmed by interaction with the anti-FliC primary antibody in the Western blot (Figure 13). This smaller version of the construct was present in all three protein fractions but was most apparent in the insoluble fractions of both uninduced and

induced samples. Although it is unclear why the cell would process the protein in this way, it is plausible that the *E. coli* is truncating the monomer in an effort to form a functional flagellum.

4.2.3   Induction in ECAM

Because traditional media like LB is insufficient at maintaining high cell densities common to large-scale production, a defined media containing ample amounts of glucose and transcriptional cofactors was used for cell adaptation and fermentation. After two passes in ECAM, the cells were glycerol stocked and used as inoculum for flask work and 20 liter fermentations.

In the flask, recombinant cells grown in ECAM and induced with 1mM IPTG express the synthetic FliC protein primarily in the insoluble fraction, but it is also seen in the conditioned media and soluble fractions after 3 hours of induction (Figure 14). Interestingly, growth in ECAM also promotes the formation of the 38kDA FliC fraction, similar to that produced in LB. In ECAM, however, there is difference in expression levels between the uninduced and induced samples that was not noted in the LB samples.

Using ECAM for a 20 liter fermentation, BL21 cells induced to express synthetic FliC demonstrated production only in the conditioned media and this was confirmed through Western blot (Figure 16). The 38kDA fragment noted in both LB (Figure 13) and ECAM (Figure 14) flask work also only appeared in the conditioned media. To add, a new fragment around 28kDa was produced primarily in the insoluble fractions but was also seen in the soluble samples. The presence of a smaller protein could indicate that the cell is cleaving the FliC or that processing conditions did not inactivate all proteases.

4.2.4   Induction in PECAM

To promote the induction of full-length flagellin, the ECAM media was supplemented with defined and scalable amounts of tryptone and yeast extract, according to Tabandeh *et al*. (2004). The additional tryptone serves as a source of amino acids, promoting expression of recombinant proteins. As seen in Figure 15, the 45kDa protein of interest was induced and expressed in all three fractions after only 3 hours of induction in the flask, as compared to the uninduced control. The 38kDa truncated FliC was also present in all three fractions but was primarily produced in the soluble and insoluble fractions after induction.

Using PECAM in a 20 liter fermentation, full length synthetic FliC was produced in the insoluble fractions after 1, 2 and 3 hours of induction (Figure 17). To add, the 38kDa, truncated version was also present in the insoluble fractions but the smaller 28kDa fragment produced during the ECAM fermentation was not. To promote the production of full length synthetic FliC, and inhibit any truncated forms, PECAM serves as a viable candidate for future fermentation experiments to optimize the concentration of IPTG, temperature, and oxygen levels.

4.3   Utilizing Other Expression Vectors and Hosts

4.3.1   pET Blue Vectors

If expression in a different vector or host is required for future applications, the synthetic FliC construct can readily be cloned into the pETBlue-2 plasmid for expression in other strains of *E. coli* (Novagen, Figure 19). Utilizing the 5' restriction enzyme site NcoI and the 3' site NotI, the construct can be ligated into the multiple cloning site of pETBlue-2. The NcoI site is unique in that it also contains the start codon ATG, initiating transcription and translation of any downstream DNA sequence.

pETBlue-2 sequence landmarks

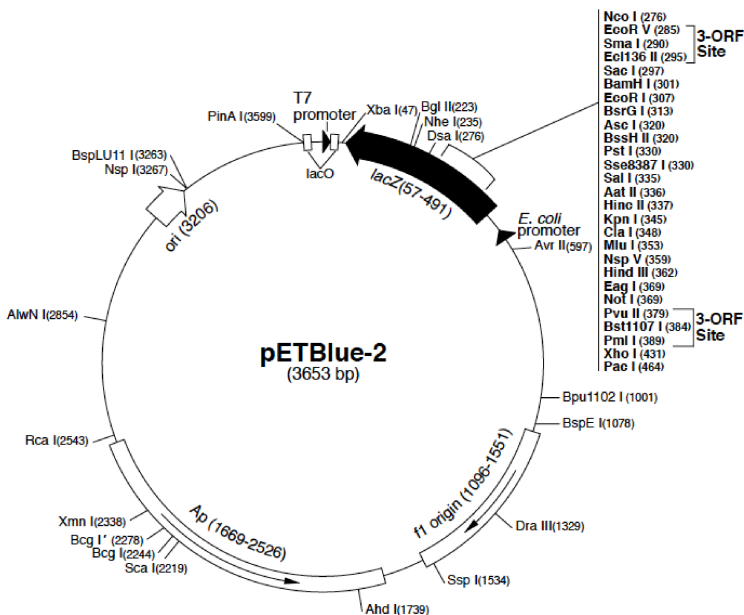|	
lac operator	3606–3625
T7 promoter	1–17
lac operator	22–42
T7 transcription start	18
multiple cloning region (*Nco* I–*Pac* I)	276–467
His•Tag® coding sequence	437–454
HSV•Tag® coding sequence	395–430
lacZ start codon	491
lacZ α-peptide ORF	57–491
E. coli promoter	541–569
f1 origin	1096–1551
bla coding sequence	1669–2526
pUC origin	3206

Figure 19: pETBlue-2
pETBlue-2 vector for expression in *E. coli* (Novagen)

This system utilizes two cell types: one for cloning and one for expression. The NovaBlue cloning host provides a quick blue/white screen for insertion within the multiple cloning site. This cell type contains a lacZ ω-fragment that is complemented by the plasmid's lacZ α-fragment, producing a functional β-galactosidase that can degrade X-gal to produce a blue colony. When the multiple cloning site is disrupted, the α-fragment is not produced and the cells are rendered white. This quick screen circumvents the need for colony PCR, removing an extra day from the total process time. After successful digest and ligation, white clones are further confirmed through PCR and sequencing, and the pETBlue-2 plasmid is extracted and then transformed into the DE3(tuner)pLacI *E. coli* expression host. Basal levels of protein expression are kept to a minimum in this cell type through the use of a chromosomal T7 RNA Polymerase that is under control of the lacUV5 promoter and subsequently repressed by the addition of 1%

glucose in the media. IPTG can then be used to induce expression of genes placed within the multiple cloning site.

4.3.2   *Production in a Eukaryotic Host*

In addition to *E. coli,* this platform can also be successfully produced and secreted by *Pichia pastoris* through the use of the pGAPZα-B plasmid (Invitrogen, Figure19). This B-type plasmid is one within a set of three (A, B, and C) for cloning in frame with an N-terminal α-secretion factor from *Saccharomyces cerevisiae* that is cleaved by the expression host.

This vector is industrially optimized for the constitutive expression of recombinant proteins through stable integration within the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter. Because this gene is a critical part of the glycolysis pathway, it is always transcribed and translated. Therefore, transgenic proteins located within the GAPDH promoter will also be constitutively expressed. The pGAPZα-B plasmid contains a region of homology for crossover and subsequent expression of the gene of interest, Zeocin antibiotic resistance, and GAPDH. Using this system, initial cloning occurs in *E. coli* through standard electroporation or heat shock and proper ligation is confirmed using PCR. The recombinant plasmid is then linearized and transformed into competent *Pichia* via homologous recombination.

4.4   Real World Applications/Relevance of Project

Production of a novel, recombinant FliC-Antigen co-expression platform will lead to a variety of applications. First, this synthetic FliC can be used to increase the efficacy of currently available vaccines or to increase the immune response of antigens previously found to not be efficacious. Additionally, this construct can be used to simultaneously express several antigens, incorporating them into a multivalent vaccine while cutting down on production costs and time. For instance, the Diphtheria, Tetanus and Pertussis vaccine (DTaP) is currently given as one

injection but is produced in three different hosts. Using the FliC platform, each antigen can be

expressed in the same host within the same protein structure to yield a more efficacious vaccine.

Lastly, using recombinant DNA technologies and producing antigenic proteins in microbial

systems drastically reduces the time and costs associated with the current egg-based vaccine

production which could be completely wiped out in the event of pandemic bird flu.
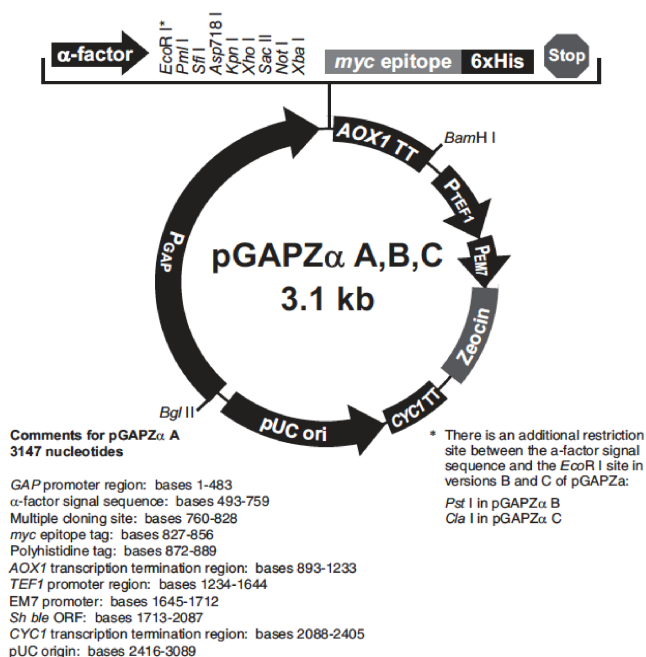

Figure 20:pGAPZ
pGAPZ vector for expression in *Pichia pastoris* (Invitrogen)

Work has already begun to incorporate a variety of antigens within this expression

platform. Of note, cloning strategies for incorporating antigenic StxB from *Shigella*

dysenteriae SD197, Stx2B from *E. coli* O157:H7, and HagA from *Porphyromonas*

gingivalis have been determined.

5 CONCLUSION

5.1 Experimental Conclusions

The recombinant FliC-antigen co-expression platform has been successfully produced in the bioreactor using both ECAM and PECAM. The former yields target protein expression in the media, potentially reducing the overall purification processing time, while the latter demonstrates expression in the insoluble cellular fraction. Under both conditions, truncated versions of the protein were produced that interacted with the anti-FliC antibodies. These smaller fragments could be a result of early translation termination or a byproduct of over-induction, and the growth variables must be optimized for full-length protein production.

5.2 Future Work

The novel, recombinant FliC-antigen co-expression platform will be produced in both *E. coli* and *P. pastoris*. The conditions for optimal fermentation and expression induction will be determined and the protein will be produced in large quantities. From these preliminary studies, it is clear that IPTG concentrations, media components, temperature, induction OD, and oxygen levels must be optimized. These parameters will also need to be adjusted for each subsequent antigen co-expressing clone. The resulting cell paste will then be subjected to Akta purification using column chromatography to isolate only the FliC protein or Flic-Antigen chimera. This purified product will be tested using the TLR5 cell assay to determine its immune response efficiency. If the protein stimulates the immune response at the same level or greater than native FliC, work will continue to incorporate other antigenic proteins within the sequence. In addition to serving as a vaccine adjuvant, the synthetic FliC protein could potentially be sold as a research chemical, once sufficiently purified and proven to active TLR5.

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