UC Berkeley
UC Berkeley Electronic Theses and Dissertations

Title
Engineering heterologous protein secretion for improved production

Permalink
https://escholarship.org/uc/item/70f4r5n9

Author
Metcalf, Kevin James

Publication Date
2016

Peer reviewed|Thesis/dissertation

Engineering heterologous protein secretion for improved production

By

Kevin James Metcalf

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Chemical Engineering

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor Danielle Tullman-Ercek, Chair
Professor Dave Schaffer
Professor Wenjun Zhang
Professor J. Chris Anderson

Spring 2016

Abstract

Engineering heterologous protein secretion for improved production

by

Kevin James Metcalf

Doctor of Philosophy in Chemical Engineering

University of California, Berkeley

Professor Danielle Tullman-Ercek, Chair

Heterologous protein production in bacteria is often a batch process, where the cells are lysed and the protein of interest is purified from the cellular milieu. A frequent approach is to accumulate the protein of interest in the cytoplasm of the cell, requiring extensive purification to separate the protein of interest from other cellular constituents. Secretion of heterologous protein produced with gram-negative bacteria holds many advantages that have not yet been realized due to low yields, and success has been protein-specific. The extracellular space is largely void of proteins, resulting in simplified protein purification and enabling continuous processing for production of a protein of interest. The type III secretion system is an ideal target for engineering generalizable protein secretion at high titer because it is not essential and is proven to secrete heterologous proteins. This allows direct engineering of the secretion system, in contrast to previous efforts that used essential secretion systems.

In this dissertation, I describe approaches taken to characterize and improve the process of protein production using the type III secretion system. In Chapter 2, I describe methods for quantification of secreted protein titer. In Chapters 3 and 4, I describe two complementary approaches to increase product titer. In Chapter 3, I describe a genetic approach to engineer control of the expression of the ~40 genes that comprise the *Salmonella* pathogenicity island 1 (SPI-1) type III secretion system. The positive transcriptional regulator HilA serves as a node in the regulatory network and is required for expression of the SPI-1 genes. Controlling the expression of *hilA* allows for control of the many downstream genes required for secretion. This modification increases secreted protein titer by over ten-fold and the effect is generalized for all proteins tested. Importantly, the timing and level of SPI-1 expression is synthetically controlled and are no longer restricted to growth conditions that endogenously induce expression of these genes. In Chapter 4, I describe a protein engineering strategy on the genome to mutate the gene *prgI*, which codes for a major structural component of the SPI-1 type III secretion system. The structure, termed the secretion apparatus, is thought to be dynamically regulated. I identify amino acid substitutions that result in greater secreted protein titer. The effect of the *prgI* mutation on secreted protein titer was general for two different model proteins.

In Chapter 5, I characterize product quality by probing the folded state of several different test proteins. Proteins are unfolded during secretion. Secreted proteins are then ejected into the extracellular space in an unfolded state, where refolding takes place in a dilute, aqueous environment. I used protein function as a proxy for protein folding, and demonstrate function in

the extracellular space, indicating that secreted proteins indeed refold after secretion. Genetic and chemical methods are used to probe the folded state of the model enzymes beta-lactamase and alkaline phosphatase and a single-chain variable fragment of an antibody to confirm that these proteins are spontaneously adopting a functional conformation. Further, the folding efficiency is a function of the chemical composition of the media, suggesting that a process using secretion to produce proteins must consider media composition to control protein folding.

Dedication Page

To my parents, Loretta and Bruce.

ACKNOWLEDGEMENTS

To Danielle, you have been a role model for me. You are patient with your students; you gave me the space and time I needed to grow as a researcher. I grew immensely as a scientist and a person while in your lab.

To my committee, thank you for giving me your time. From our meetings with you all in the last several years, I have learned many things on how to be a successful researcher and group leader.

To my friends in the duck pond, thank you for being great scientists and friends. I have enjoyed working with and alongside all of you. I have learned how to be a careful and creative scientist by sharing lab space every day with you all.

To the students who worked with me, you have made great contributions to my work. You can see your fingerprints all over my dissertation. I hope that you all continue to grow as scientists, even if you do not stay in science.

To my friends in Berkeley, you have been a great inspiration. Wow! We traveled to a lot of places and saw a lot of things! You all shared passions for science and for adventure. Although we are moving on to different places, I hope to see you all often.

To my family, you have always been there for me. You supported me and were interested in my work, no matter how obtuse the subject. You always had love for me and kept me grounded when I was on the other side of the country and far away from you.

To Lam-Kiu, thank you for supporting me and helping me be a better person. May our train take us on an incredible journey. I love you.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

1.1 Biotechnology and protein production

Recombinant DNA technology has transformed molecular biology and made significant contributions to medicine, chemicals, food, and other fields. Entailed by this transformative technology is the ability to take a gene from any source, introduce the gene to a transgenic organism, such that the host organism expresses this gene and the protein is produced. Thus, therapeutic proteins (e.g., human insulin) (Williams et al. 1982) and enzymatic proteins for food production (e.g., chymosin) (Marston et al. 1984) can be produced by the bacterium *Escherichia coli* and other host organisms. Further, production of many other proteins that perform diverse functions is achieved in a heterologous host, such as strong, elastic materials made from spider silk (Hinman, Jones, and Lewis 2000; Widmaier et al. 2009). Protein products constitute an industry greater than $250 \cdot 10^9$ USD/year (Dewan 2014) and represent the dominant product of the biotechnology sector.

Heterologous protein production, the production of foreign proteins in a non-native host organism, makes use of the central dogma of molecular biology. All known forms on life on planet earth encode and replicate a genetic program that is a nucleic acid polymer called deoxyribonucleic acid (DNA). By the central dogma, cellular mechanisms transcribe DNA into another polynucleotide, ribonucleic acid (RNA) molecule, which is then translated into a protein, a poly(amino acid). The transcription and translation of DNA into protein is defined by the genetic code, with many organisms following the standard genetic code, though some variants exist. Thus, this standard genetic code allows a gene encoded in the DNA of one organism to be transferred to another organism and make the same protein molecule. Biotechnologists make use of this fact. In theory, any protein can be produced by any organism, provided that the DNA coding sequence is known. Thus, a protein of interest (POI) can be produced in a host organism that is optimized for industrial protein production, though the origin of the POI is from an organism that is not feasible to cultivate industrially.

Beyond commercial protein products, the aforementioned advances in biotechnology enables production of heterologous proteins on the lab scale. This strategy enables production of a protein in sufficient quantity to execute many experiments to understand the structure and/or function of the protein. This was previously not feasible, as the protein had to be produced and purified from the natural source, which is often difficult.

Thus, an indispensable component of many diverse fields in science and engineering is the production of proteins in a heterologous host organism using genetic engineering. However, the process is not quite so simple. In this chapter, I describe protein production strategies and engineering solutions to the problem of production of a given POI. It must be acknowledged that this field is so large that one cannot comprehensively cover the field in just one document; I point the reader to primary literature that covers these subjects in excellent depth that I omit here.

Hosts organisms from any domain of life can be used, though bacteria and eukaryotes are most often employed. The focus of this dissertation is on bacterial hosts. However, eukaryotic hosts will be discussed where clear application of progress in these organisms informs bacterial host expression.

1.2 Heterologous protein production strategies

Production of proteins in a heterologous host is a mature process with well-defined standards. First, an organism and a strain must be selected for production. Next, the DNA that codes for the POI must be cloned and transformed into the selected strain. The strain carrying the gene coding for the POI is then grown in conditions that produce this protein, and purification strategies are performed to isolate the POI. However, the ability to isolate these proteins from the heterologous host presents many challenges. Specific challenges presented include: unstable expression of the gene, poor stability of the full-length protein product, difficult purification of the protein from the other cellular constituents, and poor folding of the protein target (Baneyx 1999; Baneyx and Mujacic 2004; Schein 1989).

Heterologous proteins can be isolated from a culture in two primary forms: as soluble protein molecules and as insoluble particles, known as inclusion bodies (Baneyx 1999; Georgiou and Valax 1996; Guise, West, and Chaudhuri 1996; Schein 1989; Vallejo and Rinas 2004). Each strategy has unique advantages. Typically, the goal is to recover large quantities of correctly folded protein, which soluble recovery obviously achieves. However, purification of proteins by this strategy is difficult, as all other soluble cellular proteins may co-purify as contaminants. Further, soluble production often achieves a lower titer of protein, compared with inclusion body production. Purification using inclusion bodies is much more facile; indeed the majority of an inclusion body can be the POI, up to 95% (Ramón, Señorale-Pose, and Marín 2014). This serves as an excellent first purification step, as isolation of the inclusion body removes many of the cellular proteins that are not desired. However, inclusion bodies present a unique challenge, in that these particles must be first solubilized to allow for refolding of the POIs, a low-yield and laborious process (Guise, West, and Chaudhuri 1996; Singh et al. 2015; Vallejo and Rinas 2004).

Several strategies have attempted to mitigate problems encountered during heterologous protein production. These strategies can be grouped into three categories: engineering the heterologous host, engineering of the protein, and process engineering. This dissertation is concerned with engineering the heterologous host for selective secretion of the protein of interest (POI) into the extracellular space, which will be addressed in detail. The other strategies are presented in abridged form here to provide context to the approach taken.

1.2.1 Host engineering
By engineering the heterologous host for improved heterologous protein production, an optimized microbial cell factory is created. First, care must be taken to select the appropriate host. For any heterologous protein production process, the requirements of the protein must be first enumerated. Researchers must ask the titer, purity, post-translational modification state, tolerance of contaminants, etc. of the POI. Many examples of existing hosts across different domains of life exist to address different product requirements. After the host is selected, specific molecular information helps to determine the engineering approach to take. Common strategies include: optimization of protein secretion, co-expression of chaperones, and evolution of strains for

improved protein production. However, modification of the host for increased protein titer can be problematic, as the engineered host may not be as fit or as stable.

Several disparate rational engineering strategies have been used to increase heterologous protein titer. To achieve increased protein titer efforts include overexpression of genes coding for chaperones or other functions (Joly, Leung, and Swartz 1998; Makino et al. 2011; Shusta et al. 1998; Wentz and Shusta 2007; Wülfing and Plückthun 1994), deletion of genes coding for endogenous proteases (Grodberg and Dunn 1988), optimization of genetic context (Baneyx 1999; Baneyx and Mujacic 2004; Terpe 2006), and secretion into the periplasm or extracellular space (Georgiou and Segatori 2005; Majander et al. 2005; Stader and Silhavy 1990). Overexpression of chaperones is thought to help prevent misfolding and aggregation, which can result product losses and cellular toxicity (Baneyx and Mujacic 2004). Deletion of genes coding for proteases helps to decrease proteolysis of the POI. Improved control of expression can increase protein titer by optimizing the expression conditions and context. The promoter, gene copy number (Terpe 2006), locus (Mairhofer et al. 2013), translation initiation signal (Salis, Mirsky, and Voigt 2009), and codon usage (Welch et al. 2009) are just a short list of the many variables that can be controlled when optimizing a strain for protein production. Several reviews cover this topic is excellent detail (Baneyx 1999; Georgiou and Valax 1996; Terpe 2006). Finally, secretion of protein is used to localize a protein to a more optimal folding environment, limit toxicity due to overexpression, and decrease the steps of purification steps required (Georgiou and Segatori 2005; Stader and Silhavy 1990).

Secretion of the protein product is advantageous from a process perspective. Purification of the POI from the extracellular space is much easier, as only a subset of all cellular proteins are present in this culture fraction. These contaminating cellular proteins, along with the POI, are released when cells are lysed, which greatly increases the difficulty of protein purification. Additionally, proteins are produced in batch, as cell lysis is required for intracellular protein accumulation. A continuous process is enabled by secretion of protein to the extracellular space (Reed and Chen 2013; Stader and Silhavy 1990). Finally, protein-specific reasons for secretion exist. For toxic POIs, accumulation of the POI in the extracellular space mitigates host toxicity. Additionally, some heterologous proteins have a desired extracellular activity, as is the case with cellulase enzymes in consolidated bioprocessing. Again, secretion is able to correctly localize the POI to improve culture performance (Ni and Chen 2009).

Proteins produced heterologously in eukaryotes is most often achieved by secretion to the extracellular space. Secretion of protein into the extracellular space in eukaryotes occurs primarily by one mechanism: proteins are secreted by the general secretory pathway. Although there is not the same diversity in secretory pathways as found in bacteria, there are still diverse approaches taken to increase the titer of secreted protein titer. The first reported secreted heterologous protein in *Saccharomyces cerevisiae* was the bovine protein prochymosin (Smith, Duncan, and Moir 1985). Progress in heterologous protein secretion in eukaryotes is covered expertly in several recent reviews (Idiris et al. 2010; Kim, Kim, and Lee 2011; Jayapal 2007). It is notable that the majority of heterologous protein production processes employ protein secretion, as the advantages of this type of process work well with the native secretory capacity of eukaryotes. It is worth noting here that bacteria can grow much faster than eukaryotes, in general, and the volumetric titer of a heterologous protein over a defined time period is both dependent on the titer per batch and the batch length. This causes many processes to still prefer bacteria, as the process allows.

1.2.2 Protein of interest engineering

Approaches to engineer the POI itself also has increased the yield of a protein production process. Mutations are made to the gene that change a property of the gene or the gene product such that the titer of the product is increased. Mutations known to confer improved physical properties are made. A rationally-designed mutant is created if the protein's mutational landscape is known. This work often proceeds from a crystal structure. Mutations are then made to improve a known physical property, such as minimization of exposed hydrophobic residues (Boock et al. 2015). However, a rational approach in many cases may in fact be irrational. Many mutations will be made that do not, in fact, confer the desired physical property to the protein. Additionally, the understanding of both the protein titer as a function of a physical property, and the physical property as a function of primary amino acid sequence is not complete. As a result, many rational mutations must be screened with the hope that one of these rational mutations is actually beneficial. When a rational approach is not possible or not successful, directed evolution is a strategy to achieve greater protein titer via identification of a mutant gene allele. Directed evolution strategies create libraries of genes, derived from the parent gene, that create many mutations is a pseudo-random manner. Next, the gene(s) in the library that give rise to the desired phenotype, in this case highest protein titer, are isolated through the use of a selection or screen. Remarkably, this strategy often identifies mutations that often cannot be rationally identified *a priori*. Some examples include the production of a human-derived single chain variable fragment fusion from an antibody (scFv) in *E. coli* (Fisher and DeLisa 2009; Makino et al. 2011), neuron growth factors in *S. cerevisiae* (Burns et al. 2014), and the fungal cellulase Cel5A in *E. coli* (Boock et al. 2015).

This approach must be attempted with some trepidation. POI engineering should be done in a way that maintains the function of the parent protein, a property often at odds with stability and titer (Tokuriki et al. 2008). However, these tests are not able to be done comprehensively and are often done in idealized conditions *in vitro*. Proper caution must be taken, as optimization of a protein for expression (or any other property) may also change another property in unintended and detrimental ways. This is protein-specific, as a mutation that increases expression, but also immunogenicity, may be detrimental for some proteins (e.g., insulin), but advantageous for other proteins (e.g., antigens for vaccines). Analysis of the engineered POI must be prudent and ensure that the new protein variant does not have any deleterious properties. In light of this challenge, the wild-type POI can be engineered via protein elements *in cis* that are proteolytically removed. Addition of domains that are cut off by proteolytic events can help to increase the protein titer without changing the protein properties, as the sequence has not changed. This is achieved by modification of secretion signals (Burns et al. 2015), addition of pro leader sequences (Baker, Silen, and Agard 1992), and folding enhancers (i.e., maltose binding protein, glutathione S-transferase, etc.) with a protease site (Terpe 2003).

1.2.3 Process engineering

Process engineering to increase the yield of heterologous protein product is nearly ubiquitous in the field and is an essential step for process design. Parameters such as temperature, culture length, and medium are all optimized for a given process. Process requirements depend on the requirements of the POI. Culturing conditions are controlled for optimal expression, folding, and other process considerations. These variables are difficult to generalize the effect on product quantity and quantity. Several general rules do exist and are covered in great detail in several publications (Lee 1996; Terpe 2006). Additionally, purification and refolding are optimized, as

necessary. This is beyond the scope of this dissertation and is covered expertly elsewhere (Lange and Rudolph 2005).

1.3 Requirements for a heterologous protein product

Essential to the development of a heterologous protein production process is the requirements of the POI. The quantity and quality of the protein product must be carefully considered when designing the process.

The requirement for sufficient quantity of product at an economical cost is the main driver of a process. Different host organisms have different volumetric productivity capabilities. Bacteria are often preferred, as their fast growth and inexpensive growth media makes for an ideal process (Georgiou and Valax 1996; Terpe 2006). Bacterial culture can reach extremely high cell densities, such that over 30% of the fermentor volume is cellular volume (Wurm 2004) and dry cell mass is 10% of the total mass in a fermentor (Choi, Keum, and Lee 2006). This enables accumulation of a POI on the order of 10^1-10^2 g/L. However, not all of this protein is recovered; the purification and refolding steps represent significant losses in product.

Additionally, the quality of the product must be considered. Many proteins products require correct protein sequence, structure, and post-translational modification. The product's tolerance of contaminants, both non-target POI isoforms and other proteins and molecules, must also be considered. Size constraints require that I acknowledge the omission of the plethora of great work in protein expression in eukaryotes and bacterial species that are not *E. coli* or *Salmonella enterica*. This work has been covered expertly in many reviews (Andersen and Krummen 2002; Demain and Vaishnav 2009; Oka and Rupp 1990; Schmidt 2004; Wurm 2004; Binnie, Cossar, and Stewart 1997; Terpe 2006). Henceforth I will cover only protein expression in *E. coli* and *S. enterica* for heterologous protein production.

1.4 Previous protein secretion approaches in bacteria

Protein secretion systems in bacteria are diverse in origin, function, and requirements. There exists nine known classes of protein secretion systems in bacteria (Costa et al. 2015). Additionally, there are two classes of proteins translocation systems, which translocate proteins across the inner membrane into the periplasmic space in double-membraned bacteria. All these secretion systems have one thing in common: they provide a means by which a protein is moved across the cellular membrane.

It is important here to develop clear and consistent nomenclature regarding protein secretion. For the entirety of this manuscript, "translocation" will refer to the movement of a protein across a membrane, while "secretion" will refer to protein translocation into the extracellular space, a formal naming convention (Desvaux et al. 2006) made necessary by different secretion mechanisms.

Another important and related distinction is that bacteria fall into two morphologically distinct groups. Bacteria can have one or two membranes that comprise the cellular envelope. In addition, polymeric cellular compartments (e.g., cell wall, lipopolysaccharide) different across genera, species, strains, and even growth conditions, and thus must be considered in a process-

dependent fashion. This requires consideration of cellular morphology when selecting a secretion strategy.

1.4.1 Translocation to the periplasm

Proteins are translocated into the periplasm by the Sec and Tat pathways in bacteria (Driessen, Fekkes, and van der Wolk 1998; Palmer and Berks 2012). Here, it is useful to clarify that there is a periplasm in both single and double membraned bacteria. The space between the inner and outer membrane is the periplasm. Additionally, the cellular compartment between the cell membrane and the thick peptidoglycan cell wall in single membrane bacteria is also a periplasm. Thus, both single and double membraned bacteria possess mechanisms for translocation of proteins across a membrane in the periplasm. Secretion to this cellular compartment has several uses: disulfide bonds can form in the oxidizing periplasm, fewer proteins are in the periplasm for simpler purification, and function of the protein may be ideal in the periplasm (i.e., enzymes with periplasmic substrates) (Ni and Chen 2009; Reed and Chen 2013)

Several commonalities exist between the Sec and Tat pathways, although there are many substantial differences, as well. An N-terminal signal sequence is translationally fused to the POI and this N-terminal signal is necessary for translocation. In addition, a membrane-embedded protein structure, the secretion machinery, spans the membrane and is required for translocation. The secretion machinery includes a peptidase that proteolytically cleaves the N-terminal signal sequence during translocation. Proteins are translocated N-terminus first. Secretion titers are depend on many factors and are protein-dependent. Notably, use of the Sec pathway can yield over 1 g/L of translocated heterologous protein, while use of the Tat pathway typically produces lower product titers. It is worth noting that the Tat pathway translocates only folded protein, while proteins are unfolded during translocation by the Sec pathway (Lee, Tullman-Ercek, and Georgiou 2006). Recovery from the periplasm requires compromisation of the outer membrane and removal of other periplasmic proteins. Secretion to the extracellular space addresses these issues.

1.4.2 Secretion to the extracellular space

The extracellular space as a final destination for a secreted protein can be a difficult endeavor. Proteins must pass across at least one membrane and get through one or more polymeric layers, such as the peptidoglycan and lipopolysaccharide. Generally, there are two strategies to achieve secretion to the extracellular space: specific secretion to the extracellular space, and non-specific leakage.

Specific mechanisms exist in nature to secrete proteins into the extracellular space. The type I secretion system is one example and is found in double-membraned bacteria. This secretion system is often associated with a pathogenic lifestyle. An example is the α-hemolysin transporter. This system is composed of three protein trimers, HlyB, HlyD, and TolC, that forms a structure that spans the inner and outer membrane. Secretion is ATP-driven and the secretion signal is encoded in the C-terminus of HlyA, the natively secretion protein of this system. The native α-hemolysin transporter has been used to secrete a diverse set of heterologous proteins into the extracellular space, with a secreted protein titer of up to 500 mg/L (Blight and Holland 1994; Gentschev, Dietrich, and Goebel 2002). During secretion, it is thought that the protein is unfolded in order to pass through the pore formed by the transporter structure (Reed and Chen 2013). However, an scFv is able to fold after secretion, even forming disulfide bonds (Fernández and De Lorenzo 2001), indicating that this protein may fold and form disulfide bonds in the extracellular

space. The secreted titer of cutinase, from the bacterium *Thermobifida fusca*, was increased over 2-fold to 1.5 g/L by overexpression of the structural proteins HlyB and HlyD (Su et al. 2012). The effect could be explained as overexpression of type I secretion system transporters allows increases the number of transporters and increases protein secretion, though their data does not preclude increased cellular lysis by decreased membrane integrity. Increased secreted titer of subtilisin E and an scFv is conferred by mutation to the secretion system structural proteins HlyB and HlyD (Sugamata and Shiba 2005).

Other specific secretion systems exist to secrete protein into the extracellular space. There exists in total nine known secretion systems in double membraned bacteria that achieve protein secretion to the extracellular space (Costa et al. 2015), though only a handful have been investigated for the secretion of heterologous proteins as a protein production platform. The type II and type V secretion systems secrete proteins in two steps: first a protein is translocated to the periplasm, that is then secreted to the extracellular space (Costa et al. 2015). Several approaches to secrete heterologous proteins using these secretion systems have been successful and are covered elsewhere (Ni and Chen 2009; Reed and Chen 2013). Type III secretion systems are covered in greater detail below. Secretion of heterologous proteins by the type IV, VI, and VII secretion systems occurs as a one-step secretion from the cytoplasm directly to the extracellular space (Costa et al. 2015). To date, we are not aware of a report of secretion of heterologous proteins using the type IV, VI, or VII secretion systems (Reed and Chen 2013).

An interesting approach is to fuse a POI to the *E. coli* proteins OsmY, OmpF, or YebF (Jeong and Lee 2002; Qian et al. 2008; Zhang, Brokx, and Weiner 2006). The secretion mechanism for these fusions is not well-understood, but the secretion of a diverse array of proteins is achieved via fusion to these sequences in *E. coli* (Bokinsky et al. 2011; Cheng et al. 2014). It is likely that these proteins are secreted in a two-step process (type II or type V), but the mechanism is not well-characterized.

Non-specific secretion mechanisms also exist. For example, proteins can reach the extracellular space in double membraned bacteria via compromisation of the outer membrane. Chemical or genetic methods are used to create large holes or pores in the outer membrane, which allows for non-specific leakage of periplasmic proteins into the extracellular space. This strategy requires destruction of cellular structures and releases many proteins that are not of interest (Georgiou and Segatori 2005).

1.5 Type III secretion biology

Proteins are secreted by the type III secretion system (T3SS) across the two bacterial membranes in a concerted, one-step process (Cornelis 2006; Galán and Collmer 1999). T3SS fall into two classes: flagellar and injectisome. It is thought that both secretion systems evolved from the same origin, and the flagellar T3SS evolved first (Diepold and Armitage 2015). Divergent evolution allowed for specialization; the injectisome T3SS is used for pathogenic functions, while the flagella is used for motility. Indeed, the canonical function of injectisome T3SS is to secrete proteins, termed effectors, into the cytosol of a host eukaryotic cell, crossing two bacterial membranes and a eukaryotic membrane in one step (Carleton et al. 2013; Cornelis 2006; Galán et al. 2014). Interestingly, it is thought that the genes that code for the injectisome are transferred

between different species horizontally (Coombes 2009; Cornelis 2006; Pallen, Beatson, and Bailey 2005).

The injectisome T3SS secretes proteins carrying a proteinaceous N-terminal secretion signal, though some evidence supports the secretion signal may be, at least in part, encoded in the 5' untranslated region (UTR) of the messenger RNA (mRNA) of the secretion protein (Niemann et al. 2013; Blaylock, Sorg, and Schneewind 2008). The N-terminus of natively secreted proteins also has a chaperone binding sequence, such that specific protein chaperones interact with the secreted protein *in trans* to assist with protein secretion. It is thought that this function is largely to maintain the protein in an unfolded state (Stebbins and Galán 2001). Injectisome T3SSs have only been found in Gram-negative bacteria and are often encoded on plasmids or genomic island. Morphologically, the injectisome spans both the inner and outer membrane of cells and projects 50-600 nm into the extracellular space. Notably, two injectisome T3SS exist in *S. enterica*. The *Salmonella* pathogenicity island one (SPI-1) is expressed first during infection (Cornelis 2006) and 10-100 secretion strutctures exist per cell (Kubori et al. 1998). The *Salmonella* pathogenicity island two (SPI-2) is expressed after SPI-1, and only ~1 structure exists per cell (Chakravortty et al. 2005).

Flagellar T3SSs differ in several ways from injectisome T3SSs. First, the secretion signal is not as well-understood. Conflict in the literature as to the secretion signal exists; both N-terminal protein and 5' UTR signals are thought to signal for secretion (Diepold and Armitage 2015; Majander et al. 2005; Singer, Erhardt, and Hughes 2014). For heterologous proteins, conclusive evidence is given for a signal coded in the 5' UTR, suggesting that secretion may be co-translational (Majander et al. 2005). Flagellar T3SSs are found in both Gram-negative and Gram-positive bacteria and are encoded in the genome and show more evidence of co-evolution with the genome. Flagellar T3SS structures can span the cellular membrane (in the case of single-membraned bacteria) or both the inner and outer membranes (in the case of double-membraned bacteria). The structure extends 1-10 μm into the extracellular space, at least an order of magnitude further than the injectisome T3SS (Diepold and Armitage 2015).

1.6 *Salmonella* pathogenicity island one biology

The SPI-1 T3SS is encoded in a genomic island, as evidenced by the name. The apparatus coded by SPI-1 secretes proteins into the cytosol of gut epithelial cells. These proteins remodel the actin cytoskeleton of the host such that *S. enterica* internalizes into the *Salmonella*-containing vacuole. This niche affords evasion of the host immune response (Galán and Collmer 1999) and is thought to contribute to *S. enterica* proliferation in the gut (Faber and Bäumler 2014).

1.6.1 Transcriptional regulation
The SPI-1 gene cluster is contains over four operons and 35 coding genes (Figure 1.1). Several transcription factors are encoded in this gene cluster and control gene expression from this locus. Environmental factors, such as dissolved oxygen concentration, media osmolarity, pH, and others, contribute to SPI-1 gene expression (Lostroh and Lee 2001; Tartera and Metcalf 1993). These conditions ultimately induce expression of the *hilA* gene, which codes for the positive transcriptional regulator HilA (Bajaj, Hwang, and Lee 1995; Lee, Jones, and Falkow 1992). HilA serves as a regulatory node in the transcriptional network and is required for SPI-1 gene expression. The essential role of HilA is supported by the observation that all environmental conditions that

regulate the expression of HilA-dependent genes also regulate *hilA* expression (Lostroh and Lee 2001), further supporting HilA function as a master regulator.
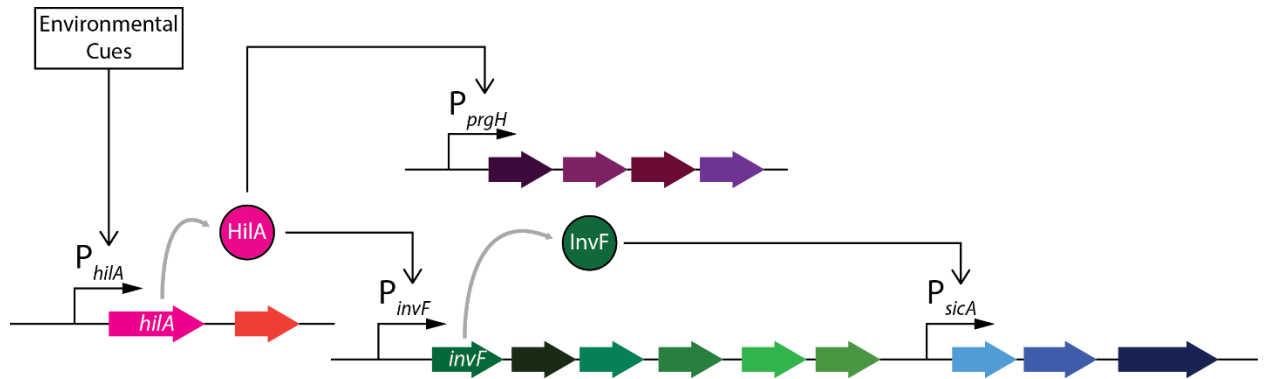
Figure 1.1 Transcriptional network of SPI-1 gene expression. Figure modified from (Lostroh and Lee 2001).

The HilA transcription factor directly controls the expression of three promoters in SPI-1: *hilA*, *prgH*, and *invF* (De Keersmaecker et al. 2005; Lostroh, Bajaj, and Lee 2000). Additionally, the transcription factor InvF controls the *sicA* promoter, when in complex with the secretion chaperone SicA (Eichelberg and Galán 1999; Darwin and Miller 1999; Lostroh and Lee 2001). Together, the HilA and InvF transcription factors likely control all that is necessary for SPI-1 dependent protein secretion.

However, on a population basis, only a portion of cells express SPI-1 genes (Sturm et al. 2011). Cells that do express SPI-1 genes appear to have 10-100 apparatus per cell (Kubori et al. 1998), and it has been suggested that not all apparatus are in the same secretion state (Schlumberger et al. 2005). In other words, not all cells are physiologically able to secrete proteins, as they have no apparatus. And not all apparatus secrete at the same rate, as it appears that the apparatus is activated for secretion. Thus, it is reasonable to propose that *S. enterica* cells exist in at least three states: 1) no secretion, absence of secretion apparatus; 2) low secretion, presence of secretion apparatus; and 3) high secretion, presence of secretion apparatus.

1.6.2 Structure
The proteins that form of the structure of the secretion apparatus are presented in Table 1.1. It is important to emphasize that this protein secretion structure is a large, multiprotein structure composed of more than 100 protein chains that spans the inner and outer membrane and projects outward from the cell. Each structure is ~3.5 MDa (Radics, Königsmaier, and Marlovits 2013). A two-dimensional cartoon version of a structure determined by cryo-electron microscropy is adapted from Schraidt and Marlovits 2011 and presented in Figure 1.2.

Table 1.1 List of proteins that constitute the structural component of the SPI-1 T3SS.

Protein	Structural component	Localization*	Reference
SipB	translocon (needle tip), hydrophobic pore formers	extracellular	Cornelis 2006
SipC	translocon (needle tip), hydrophobic pore formers	extracellular	Cornelis 2006
PrgI	needle filament	extracellular	Cornelis 2006; Galán 2001
SipD	needle tip, scaffold for pore formation	extracellular	Cornelis 2006; Galán 2001; Zhou and Galán 2001
InvG	secretin/outer ring	OM	Cornelis 2006
InvH	assists in secretin insertion into OM, lipoprotein	OM	Cornelis 2006
PrgJ	inner rod protein	periplasm	Cornelis 2006; Galán 2001
InvA	export apparatus, basal structure	IM	Cornelis 2006
PrgH	larger inner ring protein, MS ring	IM	Cornelis 2006
PrgK	smaller inner ring protein, MS ring, lipoprotein	IM	Cornelis 2006; Kubori et al. 2000
SpaP	export apparatus, basal structure	IM	Cornelis 2006
SpaQ	export apparatus, basal-structure	IM	Cornelis 2006
SpaR	export apparatus, basal structure	IM	Cornelis 2006
SpaS	export apparatus, basal structure, involved in substrate specificity switching	IM	Cornelis 2006
InvC	ATPase	cytoplasm	Cornelis 2006

*IM = inner membrane; OM = outer membrane
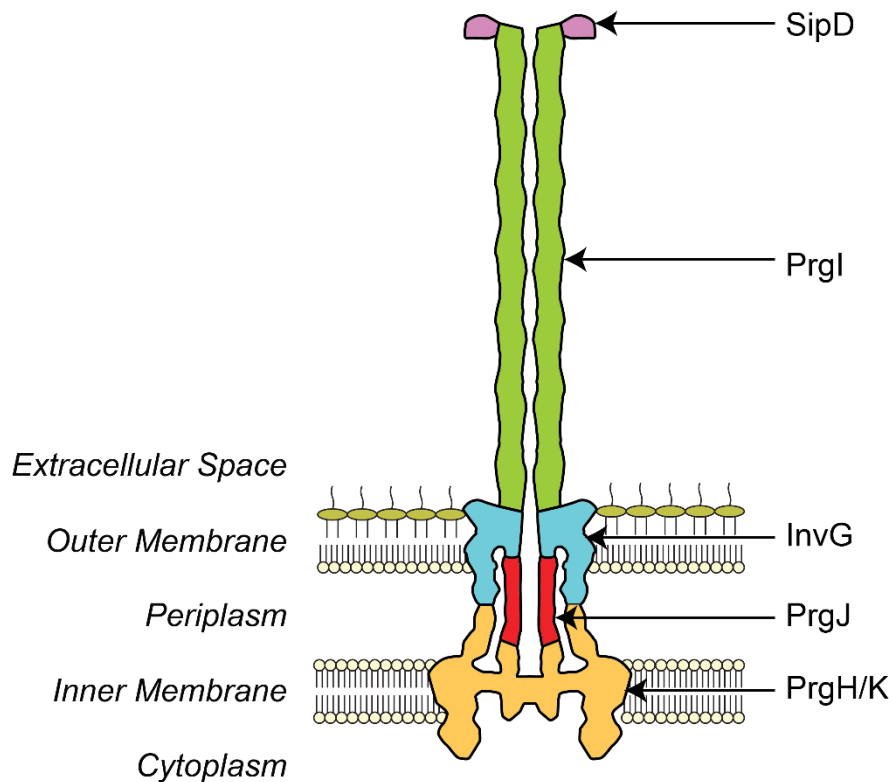
Figure 1.2 Cartoon of full apparatus structure. Figure is adapted from the electron density map presented by Schraidt and Marlovits 2011.

The needle length is controlled by the protein InvJ in the native system and measures ~50 nm (Galán et al. 2014; Radics, Königsmaier, and Marlovits 2013). It is likely that the proteins in Table 1.1 that are localized extracellularly are secreted by the T3SS to build the apparatus to the correct length.

1.6.3 Secreted proteins

Natively secreted proteins by the SPI-1 T3SS are characterized by a non-cleaved N-terminal secretion signal. In sum, 20 proteins are known to be secreted by the SPI-1 T3SS, which included proteins outside of the SPI-1 locus (Cornelis 2006; Galán 2001; Lostroh and Lee 2001; McGhie et al. 2009; Zhou and Galán 2001). Some secreted proteins are maintained in the bacterial cytosol bound to a cognate chaperone. It is thought that the chaperone helps to prevent aggregation of the secreted protein inside of the cell and maintain the protein unfolded in a secretion-competent state (Stebbins and Galán 2001). Fusion of the signal sequence to heterologous proteins affords secretion of these fusion proteins into the extracellular space (Stebbins and Galán 2001; Widmaier and Voigt 2010; Widmaier et al. 2009). Of the 20 secreted proteins, only nine signal sequences are known (Bronstein, Miao, and Miller 2000; Ehrbar et al. 2003; Fu and Galán 1998; Higashide and Zhou 2006; Hong and Miller 1998; Karavolos et al. 2005; Knodler et al. 2006; Lee and Galán 2003; Lee and Galán 2004; Rüssmann et al. 2002; Tucker and Galán 2000; Wood et al. 2004; Zhang et al. 2002). The SptP signal sequence gives the highest secreted protein titer for most proteins, though this is not true for all proteins (Widmaier et al. 2009).

It is not well understood the characteristics of a fusion protein that can be secreted. Observations from a limited number of studies have indicated that folded protein stability, length, and formal charge may contribute to protein secretion, though more thorough studies are needed (Radics, Königsmaier, and Marlovits 2013; Widmaier and Voigt 2010). Notably, the green fluorescent protein (GFP) is not secreted by the SPI-1 T3SS (Radics, Königsmaier, and Marlovits 2013), though GFP is secreted by other T3SSs (Derouazi et al. 2008; Epaulard et al. 2008). Further, the effect of fusion protein properties on secreted protein titer has not been investigated.

Proteins must be unfolded as the proteins pass through the secretion pore, approximately 20 Å in diameter (Radics, Königsmaier, and Marlovits 2013). The protein InvC, localized on the inner leaflet of the inner membrane at the base of the basal body, has ATPase unfoldase activity, which is thought to aid in the secretion of proteins (Eichelberg, Ginocchio, and Galán 1994; Galán et al. 2014). However, deletion of *fliL*, an *invC* homolog in the *S. enterica* flagellar T3SS, was shown to not be required for secretion (Erhardt et al. 2014). Further, the loss of secretion caused by a catalytically inactive InvC mutant was rescued by deletion of *atp*, indicating that the proton motive force may be an important energy source for powering secretion (Erhardt et al. 2014; Galán et al. 2014). Additionally, these data show that InvC is not necessary for secretion in certain conditions. Together, these data indicate the poor understanding the field has of the energy source that powers protein secretion.

While the regulation of the action of secretion is not known, it is thought that protein secretion is activated by host cell contact, potentially by a signal transduction event (Galán et al. 2014; Zierler and Galán 1995). Proteins are expressed and held in the cytosol as latent pools, which are rapidly depleted by protein secretion upon activation of the secretion system (Schlumberger et al. 2005). After activation, proteins are rapidly secreted at a rate of 10^0-10^1 proteins/s/apparatus (Schlumberger et al. 2005; Singer et al. 2012). Several hypotheses exist that describe the activation of an apparatus for secretion. In one model, an allosteric regulatory role is attributed to the needle structure (Figure 1.2) that extends from the outer membrane into the extracellular space (Galán et al. 2014).

1.7 Previous engineering of Type III secretion for protein production

Several different types and host-origin T3SS have been explored for heterologous protein secretion. A table of organisms, T3SS types, POIs, and yields is presented in Table 1.2.

Table 1.2 List of reported heterologous protein production using a T3SS.

Organism	T3SS type	POI(s)	Yield (mg/L)‡	Reference
E. coli	flagellar	GFP, domains and full-length proteins of bacterial origin	15	Majander et al. 2005
S. typhimurium	injectisome	spider silks	15	Widmaier et al. 2009
S. typhimurium	flagellar	neuroactive peptides	NR*	Singer et al. 2012
Pseudomonas aeruginosa	injectisome	GFP, ovalbumin, catechol dehydrogenase	NR*	Derouazi et al. 2008; Epaulard et al. 2008
S. typhimurium	injectisome	tropoelastin- and resilin-domains, and spider silks	20	Azam et al. 2015

*NR means value not reported by the authors

‡Yields are either directly reported or calculated from the information provided by the authors

It is worth noting that it is difficult to directly compare these reported uses of the T3SS for heterologous protein production due to the differences in culture duration, cell densities, and POIs. However, the general problem is two-fold: not enough protein is secreted, and we do not have control over secretion. In this case, the ability to control the later, as outlined in Section 1.6, will address the former.

We use rough estimates (Flamholz, Phillips, and Milo 2014; Milo 2013) to approximate the secreted protein titer that we can expect from a given culture performance. From previously reported values (Widmaier and Voigt 2010; Widmaier et al. 2009), we estimate that 1 OD of cells ($\sim 10^9$ cells/mL) (Moran, Phillips, and Milo 2010) secrete a 50 kDa protein for 8 hours, and 30% of the cells have 10^1-10^2 apparatus (Kubori et al. 1998), while the other cells are not secretion-active (Sturm et al. 2011). We would then expect a secreted protein titer of 0.01-1 g/L, if we assume that the secretion rate is bounded between 10^3 and 10^4 amino acids/s/apparatus (Schlumberger et al. 2005; Singer et al. 2012). Note that the value reported in Table 1.2 is within this expected domain, lending support to the accuracy of this crude analysis.

Notably, proteins are secreted at a rate of 10^3-10^4 amino acids/s/apparatus (Schlumberger et al. 2005; Singer et al. 2012). These data suggest three conclusions: 1) proteins are secreted at an exceptionally fast rate; 2) secretion rate is on the same order as translation rate; and 3) a secretion apparatus is secretion-active for a short time.

The first conclusion is a comment that applies a human value to a physical property, but is nonetheless worth highlighting. This rate is much faster than translation (Moran, Phillips, and Milo 2010). Note that in the secretion of a protein, the peptide travels from the inner leaflet of the inner

membrane to the extracellular space, a distance of more than 50 nm. This process occurs on the order of 0.1 to 1 seconds.

Conclusion two is a comparison of rates, so I further detail my claim. If we assume that a *S. enterica* cell has 10^4-10^5 ribosomes, and that they all are actively translating at 10^1 amino acids/s (Moran, Phillips, and Milo 2010), then we are producing a 50 kDa protein at a maximal rate of 10^3 proteins/s/cell. If we assume 10^1-10^2 apparatus/cell (Kubori et al. 1998), then we are secreting protein at a maximal rate of 10^3 proteins/s/cell. Thus we conclude that it is physically possible to match the translation and secretion rates. At steady-state, one could maintain a low intracellular concentration of POI while enabling continuous protein production. Matching the expression and secretion rates is thought to increase secreted protein titer. Indeed, titer of several human growth factors was maximized by RBS engineering in *E. coli*, using the Sec system (Simmons and Yansura 1996). Further, increased secreted protein titer of GFP in *S. cerevisiae* is achieved by matching expression and secretion rates (Huang, Gore, and Shusta 2008).

However, to address the third conclusion, we acknowledge that the cell does not perform as we describe. Cells cannot devote protein synthesis exclusively to POI production, as cells produce many different proteins from the same pool of ribosomes, some of which are essential for proper cellular function. Additionally, the secretion apparatus is thought to shut off after a given time, as accumulation of secreted protein stops midway through the culture (Widmaier and Voigt 2010; Widmaier et al. 2009). Finally, secreted protein pools in the cell are rapidly depleted after secretion is initiated, as evidenced by real-time time-lapse microscopy has shown that (Schlumberger et al. 2005). Thus, we expect that the secretion rate is likely quite low in the native system, although the pseudo-zeroth order rate constant is quite high. This is supported by comparing our order-of-magnitude analysis on the previous page with the reported values in Table 1.2. The reported literature values are on the low end of the range of expected secreted protein titer, supporting the hypothesis that cells are secretion-active for a short time, and not the whole length of the culture.

1.8 Outlook and objectives

The SPI-1 T3SS of *S. enterica* is an excellent target for engineering protein secretion, because it has been shown to secrete heterologous proteins (Widmaier et al. 2009), and it is not essential to cell viability (Galán and Collmer 1999; Cornelis 2006), in contrast to most other secretion pathways in bacteria (Simonen and Palva 1993; Choi and Lee 2004). Engineering the T3SS is expected to improve production of proteins that are not currently produced well using traditional intracellular production methods, such as toxic or difficult-to-fold proteins (Schein 1989).

We further the crude analysis from Section 1.7 to evaluate parameters that are engineering targets. First, if we assume that all cells are secretion-active, then we would expect a secreted protein titer of 0.05-5 g/L, an over three-fold increase. Next, if add in the assumption that the culture density is increased to 10 OD, a density that is a reasonable target, the expected titer increases to 0.5-5 g/L, a titer that is very competitive with current state-of-the-art. Additionally, if we could increase the time that the cells are actively secreting to 24 hours, then we would expect a secreted protein titer of 1-100 g/L. Clearly, an increase of any of these parameters would give a

commensurate increase in expected secreted protein titer. From this analysis, it becomes clear that a large effect to increase secreted protein titer would be achieved by optimizing:

1. the number of cells that are secretion-active
2. the culture density
3. the number of apparatus per cell
4. the secretion rate
5. the culture time with which proteins are secreted

One would expect that some of these parameters are easier to modify that others. However, it is not readily apparent which parameters these are; indeed the secretion system seems to be closely tied to growth phase (Lostroh and Lee 2001; Tartera and Metcalf 1993). Thus, the work described within this document begins to search through these five listed parameters and considers the potential for optimization of each.

In this document, I describe several approaches taken to characterize secretion performance and implement engineering solutions to increase secreted protein titer. First, I outline the development of quantitative approaches to calculate the secreted protein titer and purity in the extracellular space, a dilute complex mixture (Chapter 2). I then describe two strategies that I took to increase secreted protein titer: controlling expression of the SPI-1 T3SS (Chapter 3) and engineering the structure (Chapter 4). Additionally, I characterized the folding of the secreted protein product (Chapter 5), an important process consideration for the production of heterologous proteins.

1.9 References

Andersen, Dana C., and Lynne Krummen. 2002. "Recombinant Protein Expression for Therapeutic Applications." *Current Opinion in Biotechnology* 13 (2): 117–23. doi:10.1016/S0958-1669(02)00300-2.

Azam, Anum, Cheng Li, Kevin J. Metcalf, and Danielle Tullman-Ercek. 2015. "Type III Secretion as a Generalizable Strategy for the Production of Full-Length Biopolymer-Forming Proteins." *Biotechnology and Bioengineering*, June. doi:10.1002/bit.25656.

Bajaj, Vivek, Clara Hwang, and Catherine A. Lee. 1995. "*hilA* Is a Novel *ompR/toxR* Family Member That Activates the Expression of *Salmonella* Typhimurium Invasion Genes." *Molecular Microbiology* 18 (4): 715–27. doi:10.1111/j.1365-2958.1995.mmi_18040715.x.

Baker, David, Joy L. Silen, and David A. Agard. 1992. "Protease Pro Region Required for Folding is a Potent Inhibitor of the Mature Enzyme." *Proteins* 12 (4): 339–44. doi:10.1002/prot.340120406.

Baneyx, François. 1999. "Recombinant Protein Expression in *Escherichia coli*." *Current Opinion in Biotechnology* 10 (5): 411–21. doi:10.1016/S0958-1669(99)00003-8.

Baneyx, François, and Mirna Mujacic. 2004. "Recombinant Protein Folding and Misfolding in *Escherichia coli*." *Nature Biotechnology* 22 (11): 1399–1408. doi:10.1038/nbt1029.

Binnie, Craig, J. Douglas Cossar, and Donald I. H. Stewart. 1997. "Heterologous Biopharmaceutical Protein Expression in *Streptomyces*." *Trends in Biotechnology* 15 (8): 315–20. doi:10.1016/S0167-7799(97)01062-7.

Blaylock, Bill, Joseph A. Sorg, and Olaf Schneewind. 2008. "*Yersinia enterocolitica* Type III
    Secretion of YopR Requires a Structure in its mRNA." *Molecular Microbiology* 70 (5):
    1210–22. doi:10.1111/j.1365-2958.2008.06474.x.

Blight, Mark A., and I.Barry Holland. 1994. "Heterologous Protein Secretion and the Versatile
    Escherichia coli Haemolysin Translocator." *Trends in Biotechnology* 12 (11): 450–55.
    doi:10.1016/0167-7799(94)90020-5.

Bokinsky, Gregory, Pamela P. Peralta-Yahya, Anthe George, Bradley M. Holmes, Eric J. Steen,
    Jeffrey Dietrich, Taek Soon Lee, Danielle Tullman-Ercek, Christopher A. Voigt, Blake
    A. Simmons, and Jay D. Keasling. 2011. "Synthesis of Three Advanced Biofuels from
    Ionic Liquid-Pretreated Switchgrass Using Engineered *Escherichia coli*." *Proceedings of
    the National Academy of Sciences*, November. doi:10.1073/pnas.1106958108.

Boock, Jason T., Brian C. King, May N. Taw, Robert J. Conrado, Ka-Hei Siu, Jessica C. Stark,
    Larry P. Walker, Donna M. Gibson, and Matthew P. DeLisa. 2015. "Repurposing a
    Bacterial Quality Control Mechanism to Enhance Enzyme Production in Living Cells."
    Journal of Molecular Biology 427 (6, Part B): 1451–63. doi:10.1016/j.jmb.2015.01.003.

Bronstein, Philip A., Edward A. Miao, and Samuel I. Miller. 2000. "InvB Is a Type III Secretion
    Chaperone Specific for SspA." *Journal of Bacteriology* 182 (23): 6638–44.
    doi:10.1128/JB.182.23.6638-6644.2000.

Burns, Michael L., Thomas M. Malott, Kevin J. Metcalf, Benjamin J. Hackel, Jonah R. Chan,
    and Eric V. Shusta. 2014. "Directed Evolution of Brain-Derived Neurotrophic Factor for
    Improved Folding and Expression in *Saccharomyces cerevisiae*." *Applied and
    Environmental Microbiology* 80 (18): 5732–42. doi:10.1128/AEM.01466-14.

Burns, Michael L., Thomas M. Malott, Kevin J. Metcalf, Arthya Puguh, Jonah R. Chan, and Eric
    V. Shusta. 2016. "Pro-Region Engineering for Improved Yeast Display and Secretion of
    Brain Derived Neurotrophic Factor." *Biotechnology Journal* 11(3):425-36. doi:
    10.1002/biot.201500360.

Carleton, Heather A., María Lara-Tejero, Xiaoyun Liu, and Jorge E. Galán. 2013. "Engineering
    the Type III Secretion System in Non-Replicating Bacterial Minicells for Antigen
    Delivery." *Nature Communications* 4 (March): 1590. doi:10.1038/ncomms2594.

Chakravortty, Dipshikha, Manfred Rohde, Lorenz Jäger, Jörg Deiwick, and Michael Hensel.
    2005. "Formation of a Novel Surface Structure Encoded by *Salmonella* Pathogenicity
    Island 2." *The EMBO Journal* 24 (11): 2043–52. doi:10.1038/sj.emboj.7600676.

Cheng, Chiu-Min, Shey-Cherng Tzou, Ya-Han Zhuang, Chien-Chiao Huang, Chien-Han Kao,
    Kuang-Wen Liao, Ta-Chun Cheng, Chih-Hung Chuang, Yuan-Chin Hsieh, Ming-Hong
    Tai, Tian-Lu Cheng. 2014. "Functional Production of a Soluble and Secreted Single-
    Chain Antibody by a Bacterial Secretion System." *PLoS ONE* 9 (5): e97367.
    doi:10.1371/journal.pone.0097367.

Choi, J. H., and S. Y. Lee. 2004. "Secretory and Extracellular Production of Recombinant
    Proteins Using *Escherichia coli*." *Applied Microbiology and Biotechnology* 64 (5): 625–
    35. doi:10.1007/s00253-004-1559-9.

Choi, Jong Hyun, Ki Chang Keum, and Sang Yup Lee. 2006. "Production of Recombinant
    Proteins by High Cell Density Culture of *Escherichia coli*." *Chemical Engineering
    Science* 61 (3): 876–85. doi:10.1016/j.ces.2005.03.031.

Coombes, Brian K. 2009. "Type III Secretion Systems in Symbiotic Adaptation of Pathogenic
    and Non-Pathogenic Bacteria." *Trends in Microbiology* 17 (3): 89–94.
    doi:10.1016/j.tim.2008.11.006.

Cornelis, Guy R. 2006. "The Type III Secretion Injectisome." *Nature Reviews Microbiology* 4 (11): 811–25. doi:10.1038/nrmicro1526.

Costa, Tiago R. D., Catarina Felisberto-Rodrigues, Amit Meir, Marie S. Prevost, Adam Redzej, Martina Trokter, and Gabriel Waksman. 2015. "Secretion Systems in Gram-Negative Bacteria: Structural and Mechanistic Insights." *Nature Reviews Microbiology* 13 (6): 343–59. doi:10.1038/nrmicro3456.

Darwin, K. Heran, and Virginia L. Miller. 1999. "InvF Is Required for Expression of Genes Encoding Proteins Secreted by the SPI1 Type III Secretion Apparatus in *Salmonella* Typhimurium." *Journal of Bacteriology* 181 (16): 4949–54.

De Keersmaecker, Sigrid C. J., Kathleen Marchal, Tine L. A. Verhoeven, Kristof Engelen, Jos Vanderleyden, and Corrella S. Detweiler. 2005. "Microarray Analysis and Motif Detection Reveal New Targets of the *Salmonella enterica* Serovar Typhimurium HilA Regulatory Protein, Including *hilA* Itself." *Journal of Bacteriology* 187 (13): 4381–91. doi:10.1128/JB.187.13.4381-4391.2005.

Demain, Arnold L., and Preeti Vaishnav. 2009. "Production of Recombinant Proteins by Microbes and Higher Organisms." *Biotechnology Advances* 27 (3): 297–306. doi:10.1016/j.biotechadv.2009.01.008.

Derouazi, M., B. Toussaint, L. Quénée, O. Epaulard, M. Guillaume, R. Marlu, and B. Polack. 2008. "High-Yield Production of Secreted Active Proteins by the *Pseudomonas aeruginosa* Type III Secretion System." *Applied and Environmental Microbiology* 74 (11): 3601–4. doi:10.1128/AEM.02576-07.

Desvaux, Mickaël, Michel Hébraud, Ian R. Henderson, and Mark J. Pallen. 2006. "Type III Secretion: What's in a Name?" *Trends in Microbiology* 14 (4): 157–60. doi:10.1016/j.tim.2006.02.009.

Dewan, Shalini Shahani. 2014. "Global Markets for Enzymes in Industrial Applications." *BCC Research Market Forecasting* BIO030H (June).

Diepold, Andreas, and Judith P. Armitage. 2015. "Type III Secretion Systems: The Bacterial Flagellum and the Injectisome." *Phil. Trans. R. Soc. B* 370 (1679): 20150020. doi:10.1098/rstb.2015.0020.

Driessen, Arnold J. M., Peter Fekkes, and Jeroen P. W. van der Wolk. 1998. "The Sec System." *Current Opinion in Microbiology* 1 (2): 216–22. doi:10.1016/S1369-5274(98)80014-3.

Ehrbar, Kristin, Andrea Friebel, Samuel I. Miller, and Wolf-Dietrich Hardt. 2003. "Role of the *Salmonella* Pathogenicity Island 1 (SPI-1) Protein InvB in Type III Secretion of SopE and SopE2, Two *Salmonella* Effector Proteins Encoded Outside of SPI-1." *Journal of Bacteriology* 185 (23): 6950–67. doi:10.1128/JB.185.23.6950-6967.2003.

Eichelberg, Katrin, and Jorge E. Galán. 1999. "Differential Regulation of *Salmonella* Typhimurium Type III Secreted Proteins by Pathogenicity Island 1 (SPI-1)-Encoded Transcriptional Activators InvF and HilA." *Infection and Immunity* 67 (8): 4099–4105.

Eichelberg, Katrin, Christine C. Ginocchio, and Jorge E. Galán. 1994. "Molecular and Functional Characterization of the *Salmonella* Typhimurium Invasion Genes *invB* and *invC*: Homology of InvC to the F_0F_1 ATPase Family of Proteins." *Journal of Bacteriology* 176 (15): 4501–10.

Epaulard, Olivier, Madiha Derouazi, Carole Margerit, Raphaël Marlu, Didier Filopon, Benoît Polack, and Bertrand Toussaint. 2008. "Optimization of a Type III Secretion System-Based *Pseudomonas aeruginosa* Live Vector for Antigen Delivery." *Clinical and Vaccine Immunology* 15 (2): 308–13. doi:10.1128/CVI.00278-07.

Erhardt, Marc, Max E. Mertens, Florian D. Fabiani, and Kelly T. Hughes. 2014. "ATPase-Independent Type-III Protein Secretion in *Salmonella enterica*." *PLoS Genetics* 10 (11): e1004800. doi:10.1371/journal.pgen.1004800.

Faber, Franziska, and Andreas J. Bäumler. 2014. "The Impact of Intestinal Inflammation on the Nutritional Environment of the Gut Microbiota." *Immunology Letters* 162 (2 Pt A): 48–53. doi:10.1016/j.imlet.2014.04.014.

Fernández, Luis A., and Víctor De Lorenzo. 2001. "Formation of Disulphide Bonds during Secretion of Proteins through the Periplasmic-Independent Type I Pathway." *Molecular Microbiology* 40 (2): 332–46. doi:10.1046/j.1365-2958.2001.02410.x.

Fisher, Adam C., and Matthew P. DeLisa. 2009. "Efficient Isolation of Soluble Intracellular Single-Chain Antibodies Using the Twin-Arginine Translocation Machinery." *Journal of Molecular Biology* 385 (1): 299–311. doi:10.1016/j.jmb.2008.10.051.

Flamholz, Avi, Rob Phillips, and Ron Milo. 2014. "The Quantified Cell." *Molecular Biology of the Cell* 25 (22): 3497–3500. doi:10.1091/mbc.E14-09-1347.

Fu, Yixin, and Jorge E. Galán. 1998. "Identification of a Specific Chaperone for SptP, a Substrate of the Centisome 63 Type III Secretion System of *Salmonella* Typhimurium." *Journal of Bacteriology* 180 (13): 3393–99.

Galán, Jorge E. 2001. "*Salmonella* Interactions with Host Cells: Type III Secretion at Work." *Annual Review of Cell and Developmental Biology* 17: 53–86. doi:10.1146/annurev.cellbio.17.1.53.

Galán, Jorge E, and Alan Collmer. 1999. "Type III Secretion Machines: Bacterial Devices for Protein Delivery into Host Cells." *Science* 284 (5418): 1322–28. doi:10.1126/science.284.5418.1322.

Galán, Jorge E., Maria Lara-Tejero, Thomas C. Marlovits, and Samuel Wagner. 2014. "Bacterial Type III Secretion Systems: Specialized Nanomachines for Protein Delivery into Target Cells." *Annual Review of Microbiology* 68 (1): 415–38. doi:10.1146/annurev-micro-092412-155725.

Gentschev, Ivaylo, Guido Dietrich, and Werner Goebel. 2002. "The *E. coli* α-Hemolysin Secretion System and Its Use in Vaccine Development." *Trends in Microbiology* 10 (1): 39–45. doi:10.1016/S0966-842X(01)02259-4.

Georgiou, George, and Laura Segatori. 2005. "Preparative Expression of Secreted Proteins in Bacteria: Status Report and Future Prospects." *Current Opinion in Biotechnology* 16 (5): 538–45. doi:10.1016/j.copbio.2005.07.008.

Georgiou, George, and Pascal Valax. 1996. "Expression of Correctly Folded Proteins in *Escherichia coli*." *Current Opinion in Biotechnology* 7 (2): 190–97. doi:10.1016/S0958-1669(96)80012-7.

Grodberg, Jennifer, and John J. Dunn. 1988. "*ompT* Encodes the *Escherichia coli* Outer Membrane Protease That Cleaves T7 RNA Polymerase during Purification." *Journal of Bacteriology* 170 (3): 1245–53.

Guise, Andrew, Shuna West, and Julian Chaudhuri. 1996. "Protein Folding in Vivo and Renaturation of Recombinant Proteins from Inclusion Bodies." *Molecular Biotechnology* 6 (1): 53–64. doi:10.1007/BF02762323.

Higashide, Wendy, and Daoguo Zhou. 2006. "The First 45 Amino Acids of SopA Are Necessary for InvB Binding and SPI-1 Secretion." *Journal of Bacteriology* 188 (7): 2411–20. doi:10.1128/JB.188.7.2411-2420.2006.

Hinman, Michael B., Justin A. Jones, and Randolph V. Lewis. 2000. "Synthetic Spider Silk: A Modular Fiber." *Trends in Biotechnology* 18 (9): 374–79. doi:10.1016/S0167-7799(00)01481-5.

Hong, K. Heran, and Virginia L. Miller. 1998. "Identification of a Novel *Salmonella* Invasion Locus Homologous to *Shigella ipgDE*." *Journal of Bacteriology* 180 (7): 1793–1802.

Huang, Dagang, Patrick R. Gore, and Eric V. Shusta. 2008. "Increasing Yeast Secretion of Heterologous Proteins by Regulating Expression Rates and Post-Secretory Loss." *Biotechnology and Bioengineering* 101 (6): 1264–75. doi:10.1002/bit.22019.

Idiris, Alimjan, Hideki Tohda, Hiromichi Kumagai, and Kaoru Takegawa. 2010. "Engineering of Protein Secretion in Yeast: Strategies and Impact on Protein Production." *Applied Microbiology and Biotechnology* 86 (2): 403–17. doi:10.1007/s00253-010-2447-0.

Jayapal, Karthik P., Katie. F. Wlaschin, Wei-Shou Hu, and Miranda G. S. Yap. 2007. "Recombinant Protein Therapeutics from CHO Cells - 20 Years and Counting." *Chemical Engineering Progress* 103 (10): 40–47.

Jeong, Ki Jun, and Sang Yup Lee. 2002. "Excretion of Human β-Endorphin into Culture Medium by Using Outer Membrane Protein F as a Fusion Partner in Recombinant *Escherichia coli*." *Applied and Environmental Microbiology* 68 (10): 4979–85. doi:10.1128/AEM.68.10.4979-4985.2002.

Joly, John C., W. S. Leung, and James R. Swartz. 1998. "Overexpression of *Escherichia coli* Oxidoreductases Increases Recombinant Insulin-like Growth Factor-I Accumulation." *Proceedings of the National Academy of Sciences* 95 (6): 2773–77.

Karavolos, M. H., M. Wilson, J. Henderson, J. J. Lee, and C. M. A. Khan. 2005. "Type III Secretion of the *Salmonella* Effector Protein SopE Is Mediated via an N-Terminal Amino Acid Signal and Not an mRNA Sequence." *Journal of Bacteriology* 187 (5): 1559–67. doi:10.1128/JB.187.5.1559-1567.2005.

Kim, Jee Yon, Yeon-Gu Kim, and Gyun Min Lee. 2011. "CHO Cells in Biotechnology for Production of Recombinant Proteins: Current State and Further Potential." *Applied Microbiology and Biotechnology* 93 (3): 917–30. doi:10.1007/s00253-011-3758-5.

Knodler, Leigh A., Michela Bertero, Calvin Yip, Natalie C. J. Strynadka, and Olivia Steele-Mortimer. 2006. "Structure-Based Mutagenesis of SigE Verifies the Importance of Hydrophobic and Electrostatic Residues in Type III Chaperone Function." *Molecular Microbiology* 62 (4): 928–40. doi:10.1111/j.1365-2958.2006.05418.x.

Kubori, Tomoko, Yukiyasu Matsushima, Dai Nakamura, Jaimol Uralil, Maria Lara-Tejero, Anand Sukhan, Jorge E. Galán, and Shin-Ichi Aizawa. 1998. "Supramolecular Structure of the *Salmonella* Typhimurium Type III Protein Secretion System." *Science* 280 (5363): 602–5. doi:10.1126/science.280.5363.602.

Kubori, Tomoko, Anand Sukhan, Shin-Ichi Aizawa, and Jorge E. Galán. 2000. "Molecular Characterization and Assembly of the Needle Complex of the *Salmonella* Typhimurium Type III Protein Secretion System." *Proceedings of the National Academy of Sciences* 97 (18): 10225–30. doi:10.1073/pnas.170128997.

Lange, Christian, and Rainer Rudolph. 2005. "Production of recombinant proteins for therapy, diagnostics and industrial research by in vitro folding." In: Kiefhaber, T., Buchner, J. (Eds.), Protein Folding Handbook. Wiley-VCH, Weinheim, 1245–1280. doi: 10.1002/9783527619498.ch71.

Lee, Catherine A., Bradley D. Jones, and Stanley Falkow. 1992. "Identification of a *Salmonella* Typhimurium Invasion Locus by Selection for Hyperinvasive Mutants." *Proceedings of the National Academy of Sciences* 89 (5): 1847–51.

Lee, Philip A., Danielle Tullman-Ercek, and George Georgiou. 2006. "The Bacterial Twin-Arginine Translocation Pathway." *Annual Review of Microbiology* 60: 373–95. doi:10.1146/annurev.micro.60.080805.142212.

Lee, Sang Ho, and Jorge E. Galán. 2003. "InvB Is a Type III Secretion-Associated Chaperone for the *Salmonella enterica* Effector Protein SopE." *Journal of Bacteriology* 185 (24): 7279–84. doi:10.1128/JB.185.24.7279-7284.2003.

Lee, Sang Ho, and Jorge E Galán. 2004. "Salmonella Type III Secretion-Associated Chaperones Confer Secretion-Pathway Specificity." *Molecular Microbiology* 51 (2): 483–95. doi:10.1046/j.1365-2958.2003.03840.x.

Lee, Sang Yup. 1996. "High Cell-Density Culture of *Escherichia coli*." *Trends in Biotechnology* 14 (3): 98–105. doi:10.1016/0167-7799(96)80930-9.

Lostroh, C. Phoebe, Vivek Bajaj, and Catherine A. Lee. 2000. "The Cis Requirements for Transcriptional Activation by HilA, a Virulence Determinant Encoded on SPI-1." *Molecular Microbiology* 37 (2): 300–315. doi: 10.1046/j.1365-2958.2000.01991.x.

Lostroh, C. Phoebe, and Catherine A. Lee. 2001. "The *Salmonella* Pathogenicity Island-1 Type III Secretion System." *Microbes and Infection* 3 (14–15): 1281–91. doi:10.1016/S1286-4579(01)01488-5.

Mairhofer, Juergen, Theresa Scharl, Karoline Marisch, Monika Cserjan-Puschmann, and Gerald Striedner. 2013. "Comparative Transcription Profiling and In-Depth Characterization of Plasmid-Based and Plasmid-Free *Escherichia coli* Expression Systems under Production Conditions." *Applied and Environmental Microbiology* 79 (12): 3802–12. doi:10.1128/AEM.00365-13.

Majander, Katariina, Lena Anton, Jenni Antikainen, Hannu Lång, Mirko Brummer, Timo K. Korhonen, and Benita Westerlund-Wikström. 2005. "Extracellular Secretion of Polypeptides Using a Modified *Escherichia coli* Flagellar Secretion Apparatus." *Nature Biotechnology* 23 (4): 475–81. doi:10.1038/nbt1077.

Makino, Tomohiro, Georgios Skretas, Tae-Hyun Kang, and George Georgiou. 2011. "Comprehensive Engineering of *Escherichia coli* for Enhanced Expression of IgG Antibodies." *Metabolic Engineering* 13 (2): 241–51. doi:10.1016/j.ymben.2010.11.002.

Marston, Fiona A. O., Peter A. Lowe, Michael T. Doel, Joyce M. Schoemaker, Susan White, and Sarojani Angal. 1984. "Purification of Calf Prochymosin (Prorennin) Synthesized in *Escherichia coli*." *Nature Biotechnology* 2 (9): 800–804. doi:10.1038/nbt0984-800.

McGhie, Emma J., Lyndsey C. Brawn, Peter J. Hume, Daniel Humphreys, and Vassilis Koronakis. 2009. "*Salmonella* Takes Control: Effector-Driven Manipulation of the Host." *Current Opinion in Microbiology* 12 (1): 117–24. doi:10.1016/j.mib.2008.12.001.

Milo, Ron. 2013. "What Is the Total Number of Protein Molecules per Cell Volume? A Call to Rethink Some Published Values." *BioEssays* 35 (12): 1050–55. doi:10.1002/bies.201300066.

Moran, Uri, Rob Phillips, and Ron Milo. 2010. "SnapShot: Key Numbers in Biology." *Cell* 141 (7): 1262–1262.e1. doi:10.1016/j.cell.2010.06.019.

Niemann, George S., Roslyn N. Brown, Ivy T. Mushamiri, Nhu T. Nguyen, Rukayat Taiwo, Afke Stufkens, Richard D. Smith, Joshua N. Adkins, Jason E. McDermott, and Fred

Heffron. 2013. "RNA Type III Secretion Signals That Require Hfq." *Journal of Bacteriology*, February. doi:10.1128/JB.00024-13.

Ni, Ye, and Rachel Chen. 2009. "Extracellular Recombinant Protein Production from *Escherichia coli*." *Biotechnology Letters* 31 (11): 1661–70. doi:10.1007/s10529-009-0077-3.

Oka, Melvin S., and Randall G. Rupp. 1990. "Large-Scale Animal Cell Culture: A Biological Perspective." In: Anthony S. Lubiniecki (Ed.), Large-scale mammalian cell culture technology. Marcel Dekker, Inc., New York, 71–92.

Pallen, Mark J., Scott A. Beatson, and Christopher M. Bailey. 2005. "Bioinformatics, Genomics and Evolution of Non-Flagellar Type-III Secretion Systems: A Darwinian Perpective." *FEMS Microbiology Reviews*, Bacterial Genomics, 29 (2): 201–29. doi:10.1016/j.femsre.2005.01.001.

Palmer, Tracy, and Ben C. Berks. 2012. "The Twin-Arginine Translocation (Tat) Protein Export Pathway." *Nature Reviews Microbiology* 10 (7): 483–96. doi:10.1038/nrmicro2814.

Qian, Zhi-Gang, Xiao-Xia Xia, Jong Hyun Choi, and Sang Yup Lee. 2008. "Proteome-Based Identification of Fusion Partner for High-Level Extracellular Production of Recombinant Proteins in *Escherichia coli*." *Biotechnology and Bioengineering* 101 (3): 587–601. doi:10.1002/bit.21898.

Radics, Julia, Lisa Königsmaier, and Thomas C. Marlovits. 2013. "Structure of a Pathogenic Type 3 Secretion System in Action." *Nature Structural & Molecular Biology* 21 (December): 82–87. doi:10.1038/nsmb.2722.

Ramón, Ana, Mario Señorale-Pose, and Mónica Marín. 2014. "Inclusion Bodies: Not That Bad…." *Frontiers in Microbiology* 5 (February). doi:10.3389/fmicb.2014.00056.

Reed, Ben, and Rachel Chen. 2013. "Biotechnological Applications of Bacterial Protein Secretion: From Therapeutics to Biofuel Production." *Research in Microbiology* 164 (6): 675–82. doi:10.1016/j.resmic.2013.03.006.

Rüssmann, Holger, Tomoko Kubori, Jeannette Sauer, and Jorge E. Galán. 2002. "Molecular and Functional Analysis of the Type III Secretion Signal of the *Salmonella enterica* InvJ Protein." *Molecular Microbiology* 46 (3): 769–79. doi:10.1046/j.1365-2958.2002.03196.x.

Salis, Howard M., Ethan A. Mirsky, and Christopher A. Voigt. 2009. "Automated Design of Synthetic Ribosome Binding Sites to Control Protein Expression." *Nature Biotechnology* 27 (10): 946–50. doi:10.1038/nbt.1568.

Schein, Catherine H. 1989. "Production of Soluble Recombinant Proteins in Bacteria." *Nature Biotechnology* 7 (11): 1141–49. doi:10.1038/nbt1189-1141.

Schlumberger, Markus C., Andreas J. Müller, Kristin Ehrbar, Brit Winnen, Iwan Duss, Bärbel Stecher, and Wolf-Dietrich Hardt. 2005. "Real-Time Imaging of Type III Secretion: *Salmonella* SipA Injection into Host Cells." *Proceedings of the National Academy of Sciences of the United States of America* 102 (35): 12548–53. doi:10.1073/pnas.0503407102.

Schmidt, F. R. 2004. "Recombinant Expression Systems in the Pharmaceutical Industry." *Applied Microbiology and Biotechnology* 65 (4): 363–72. doi:10.1007/s00253-004-1656-9.

Schraidt, Oliver, and Thomas C. Marlovits. 2011. "Three-Dimensional Model of *Salmonella*'s Needle Complex at Subnanometer Resolution." *Science* 331 (6021): 1192–95. doi:10.1126/science.1199358.

Shusta, Eric V., Ronald T. Raines, Andreas Plückthun, and K. Dane Wittrup. 1998. "Increasing the Secretory Capacity of *Saccharomyces cerevisiae* for Production of Single-Chain Antibody Fragments." *Nature Biotechnology* 16 (8): 773–77. doi:10.1038/nbt0898-773.

Simmons, Laura C., and Daniel G. Yansura. 1996. "Translational Level Is a Critical Factor for the Secretion of Heterologous Proteins in *Escherichia coli*." *Nature Biotechnology* 14 (5): 629–34. doi:10.1038/nbt0596-629.

Simonen, Marjo, and Ilkka Palva. 1993. "Protein Secretion in *Bacillus* Species." *Microbiological Reviews* 57 (1): 109–37.

Singer, Hanna M., Marc Erhardt, and Kelly T. Hughes. 2014. "Comparative Analysis of the Secretion Capability of Early and Late Flagellar Type III Secretion Substrates." *Molecular Microbiology* 93: 505-20. doi:10.1111/mmi.12675.

Singer, Hanna M, Marc Erhardt, Andrew M Steiner, Min-Min Zhang, Doju Yoshikami, Grzegorz Bulaj, Baldomero M Olivera, and Kelly T Hughes. 2012. "Selective Purification of Recombinant Neuroactive Peptides Using the Flagellar Type III Secretion System." *mBio* 3 (3). doi:10.1128/mBio.00115-12.

Singh, Anupam, Vaibhav Upadhyay, Arun K. Upadhyay, Surinder M. Singh, and Amulya K. Panda. 2015. "Protein Recovery from Inclusion Bodies of *Escherichia coli* Using Mild Solubilization Process." *Microbial Cell Factories* 14 (1): 41. doi:10.1186/s12934-015-0222-8.

Smith, Robert A., Margaret J. Duncan, and Donald T. Moir. 1985. "Heterologous Protein Secretion from Yeast." *Science* 229 (4719): 1219–24. doi:10.2307/1696025.

Stader, Joan A., and Thomas J. Silhavy. 1990. "Engineering *Escherichia coli* to Secrete Heterologous Gene Products." *Methods in Enzymology* 185:166–87. doi:10.1016/0076-6879(90)85017-I.

Stebbins, C. Erec, and Jorge E. Galán. 2001. "Maintenance of an Unfolded Polypeptide by a Cognate Chaperone in Bacterial Type III Secretion." *Nature* 414 (6859): 77–81. doi:10.1038/35102073.

Sturm, Alexander, Matthias Heinemann, Markus Arnoldini, Arndt Benecke, Martin Ackermann, Matthias Benz, Jasmine Dormann, and Wolf-Dietrich Hardt. 2011. "The Cost of Virulence: Retarded Growth of *Salmonella* Typhimurium Cells Expressing Type III Secretion System 1." *PLoS Pathog* 7 (7): e1002143. doi:10.1371/journal.ppat.1002143.

Sugamata, Yasuhiro, and Toshikazu Shiba. 2005. "Improved Secretory Production of Recombinant Proteins by Random Mutagenesis of *hlyB*, an Alpha-Hemolysin Transporter from *Escherichia coli*." *Applied and Environmental Microbiology* 71 (2): 656–62. doi:10.1128/AEM.71.2.656-662.2005.

Su, Lingqia, Sheng Chen, Li Yi, Ronald W. Woodard, Jian Chen, and Jing Wu. 2012. "Extracellular Overexpression of Recombinant *Thermobifida fusca* Cutinase by Alpha-Hemolysin Secretion System in *E. coli* BL21(DE3)." *Microbial Cell Factories* 11: 8. doi:10.1186/1475-2859-11-8.

Tartera, Carmen, and Eleanor S. Metcalf. 1993. "Osmolarity and Growth Phase Overlap in Regulation of *Salmonella* Typhi Adherence to and Invasion of Human Intestinal Cells." *Infection and Immunity* 61 (7): 3084–89.

Terpe, Kay. 2003. "Overview of Tag Protein Fusions: From Molecular and Biochemical Fundamentals to Commercial Systems." *Applied Microbiology and Biotechnology* 60 (5): 523–33. doi:10.1007/s00253-002-1158-6.

Terpe, Kay. 2006. "Overview of Bacterial Expression Systems for Heterologous Protein Production: From Molecular and Biochemical Fundamentals to Commercial Systems." *Applied Microbiology and Biotechnology* 72 (2): 211–22. doi:10.1007/s00253-006-0465-8.

Tokuriki, Nobuhiko, Francois Stricher, Luis Serrano, and Dan S. Tawfik. 2008. "How Protein Stability and New Functions Trade Off." *PLoS Comput Biol* 4 (2): e1000002. doi:10.1371/journal.pcbi.1000002.

Tucker, Stephanie C., and Jorge E. Galán. 2000. "Complex Function for SicA, a *Salmonella enterica* Serovar Typhimurium Type III Secretion-Associated Chaperone." *Journal of Bacteriology* 182 (8): 2262–68. doi:10.1128/JB.182.8.2262-2268.2000.

Vallejo, Luis F., and Ursula Rinas. 2004. "Strategies for the Recovery of Active Proteins through Refolding of Bacterial Inclusion Body Proteins." *Microbial Cell Factories* 3 (1): 11. doi:10.1186/1475-2859-3-11.

Welch, Mark, Sridhar Govindarajan, Jon E. Ness, Alan Villalobos, Austin Gurney, Jeremy Minshull, and Claes Gustafsson. 2009. "Design Parameters to Control Synthetic Gene Expression in *Escherichia coli*." *PLoS ONE* 4 (9): e7002. doi:10.1371/journal.pone.0007002.

Wentz, Alane E., and Eric V. Shusta. 2007. "A Novel High-Throughput Screen Reveals Yeast Genes That Increase Secretion of Heterologous Proteins." *Applied and Environmental Microbiology* 73 (4): 1189–98. doi:10.1128/AEM.02427-06.

Widmaier, Daniel M., Danielle Tullman-Ercek, Ethan A. Mirsky, Rena Hill, Sridhar Govindarajan, Jeremy Minshull, and Christopher A. Voigt. 2009. "Engineering the *Salmonella* Type III Secretion System to Export Spider Silk Monomers." *Molecular Systems Biology* 5 (1): 309. doi:10.1038/msb.2009.62.

Widmaier, Daniel M., and Christopher A. Voigt. 2010. "Quantification of the Physiochemical Constraints on the Export of Spider Silk Proteins by *Salmonella* Type III Secretion." *Microbial Cell Factories* 9 (1): 78. doi:10.1186/1475-2859-9-78.

Williams, Daniel C., Richard M. Van Frank, William L. Muth, and J. Paul Burnett. 1982. "Cytoplasmic Inclusion Bodies in *Escherichia coli* Producing Biosynthetic Human Insulin Proteins." *Science* 215 (4533): 687–89. doi:10.1126/science.7036343.

Wood, Michael W., Christopher Williams, Abhishek Upadhyay, Andrew C. Gill, Didier L. Philippe, Edouard E. Galyov, Jean M. H. van den Elsen, and Stefan Bagby. 2004. "Structural Analysis of *Salmonella enterica* Effector Protein SopD." *Biochimica Et Biophysica Acta* 1698 (2): 219–26. doi:10.1016/j.bbapap.2003.12.003.

Wülfing, Christoph, and Andreas Plückthun. 1994. "Correctly Folded T-Cell Receptor Fragments in the Periplasm of *Escherichia coli*: Influence of Folding Catalysts." *Journal of Molecular Biology* 242 (5): 655–69. doi:10.1006/jmbi.1994.1615.

Wurm, Florian M. 2004. "Production of Recombinant Protein Therapeutics in Cultivated Mammalian Cells." *Nature Biotechnology* 22 (11): 1393–98. doi:10.1038/nbt1026.

Zhang, Guijin, Stephen Brokx, and Joel H. Weiner. 2006. "Extracellular Accumulation of Recombinant Proteins Fused to the Carrier Protein YebF in *Escherichia coli*." *Nature Biotechnology* 24 (1): 100–104. doi:10.1038/nbt1174.

Zhang, Shuping, Renato L. Santos, Renee M. Tsolis, Silke Stender, Wolf-Dietrich Hardt, Andreas J. Bäumler, and L. Garry Adams. 2002. "The *Salmonella enterica* Serotype Typhimurium Effector Proteins SipA, SopA, SopB, SopD, and SopE2 Act in Concert to

Induce Diarrhea in Calves." *Infection and Immunity* 70 (7): 3843–55. doi:10.1128/IAI.70.7.3843-3855.2002.

Zhou, Daoguo, and Jorge Galán. 2001. "*Salmonella* Entry into Host Cells: The Work in Concert of Type III Secreted Effector Proteins." *Microbes and Infection* 3 (14–15): 1293–98. doi:10.1016/S1286-4579(01)01489-7.

Zierler, Michael K., and Jorge E. Galán. 1995. "Contact with Cultured Epithelial Cells Stimulates Secretion of *Salmonella* Typhimurium Invasion Protein InvJ." *Infection and Immunity* 63 (10): 4024–28.

CHAPTER 2

METHODS FOR QUANTIFICATION

2.1 Introduction

Accurate and precise quantification of secreted protein titer is essential to describe the effect size of the different modifications described in Chapters 3 and 4. It is important here to specify that this quantification is an absolute concentration of POI, as many existing techniques rely on quantification in arbitrary units. Arbitrary units do not capture the relevant effect size accurately, and this dissertation eschews use of arbitrary units, when possible. Western blotting and dot blotting were investigated for accuracy, precision, and throughput. Ideally, this assay is accurate and precise. The reason for this is obvious, but I will consider each aspect explicitly. First, a method must be accurate. This requires quantification to be robust to the different conditions tested.

Notably, the protein sample in the extracellular space is very dilute, on the order of 10^0-10^1 mg/L of the protein of interest and 10^2 mg/L total protein content. This concentration is below the detection limit for several of the techniques described below and requires that the sample be concentrated quantitatively. However, different concentrating techniques may introduce a bias based on molecular interactions that are protein- or sample-dependent. Given the low concentrations of sample, precision is also important, as a small change in concentration will give a relatively large effect size. To prepare samples for quantification, the concentration of POI in the solution must be within the sensitivity of the detection method. Here it is useful to consider the detection limit of different assays, even in this case different protocols/reagents for the same assay. In order to achieve accurate and precise quantification, the concentration of sample must be above the detection limit of the assay. Given that proteins are secreted into the culture supernatant, I consider first the chemical environment of this culture fraction. If a 50 kDa protein is secreted at a titer of 1 mg/L, then it is at a concentration of 20 nM, which is qualitatively relatively dilute. This is a reasonable order of magnitude to consider, given the reported literature values in Table 1.2. In addition, many other proteins are secreted into the extracellular space. Let us assume that the POI is at 30% purity. This means that there is ~3 mg/L of total protein in the culture supernatant. Finally, the culture supernatant is in a solution of lysogeny broth (LB), a complex mixture that includes peptides of undefined composition and concentration. Given the undefined composition and concentration of samples, it is important to validate the quantification method within the context of experiments presented in subsequent chapters.

A high-throughput assay increases the number of conditions that can be tested. Using directed approaches, a small number of variables can be manipulated, requiring the ability to measure 10^2 samples. However, to apply random mutation techniques to create a large library of conditions, a much larger throughput is required. In this chapter, I highlight throughput of each technique in a qualitative manner.

2.2 Methods

2.2.1 Strains and growth conditions

All strains were grown from colonies from fresh transformations or fresh streaks from frozen stock in lysogeny broth (LB-Lennox, LB-L) (10 g/L tryptone, 5 g/L yeast extract, and 5 g/L NaCl) (VWR #EM1.00547.5007) with appropriate antibiotics for 12–16 hours at 37 °C and 225 rpm in an orbital shaker. Cells were cultured in 24-well blocks (Axygen). Cells were subcultured 1:100 into fresh LB-Lennox with appropriate antibiotics and IPTG, as indicated, and grown in an orbital shaker for 8 hr at 37 °C and 225 rpm. A *S. enterica subsp. enterica* serovar Typhimurium str. SL1344 derived strain was used for all secretion experiments (Hoiseth and Stocker 1981) and was transformed with plasmids using electroporation. For strains that carried an upregulation vector (P_{lacUV5} *hilA*) and 100 µM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the growth media at the time of subculture, unless indicated otherwise.

2.2.2 DNA manipulations

PCR was performed with *Pfu* or *Phusion* DNA polymerase. Restriction enzymes and ligase (NEB) were used according to the manufacturer's instructions. For all cloning, *E. coli* DH10B cells were used. All plasmids used in this study are presented in Table 2.1. P_{trc99a} *gfp^{mut2}* is from the Tullman-Ercek lab plasmid collection. The upregulation vector (P_{lacUV5} *hilA*, pKJM035) was derived from the BglBrick plasmid collection (Anderson et al. 2010) and is described in full detail in Section 3.2.2. The export vectors are derived from a modified pPROTet.133 backbone vector (BD Clontech) under control of the *sicA* promoter, as in Widmaier et al. 2009. Export vectors using P_{tet} are derived from the BglBrick plasmid collection (Anderson et al. 2010). The *nfsA* and *nfsB* genes were amplified from the *E. coli* DH10B genome. The *sptP* gene was amplified from the *S. enterica* SL1344 genome. The *DH* gene was amplified from pCASP *sicP sptP-DH* (Widmaier et al. 2009). The primers used for each PCR amplification is given in Table 2.2. The *ADF3* and *ADF4* genes were excised from plasmids (Widmaier et al. 2009) using the *notI* and *hindIII* sites and cloned into the same sites in the P_{sicA} *DH* vector.

Table 2.1 List of all plasmids used in this chapter.

Plasmid name	ORFs under inducible control	ORI	ab^R	Plasmid ID	Reference
P_{lacUV5} *hilA*	*hilA*	p15a	kan	pKJM035	Metcalf et al. 2014
P_{trc99A} *gfp^{mut2}*	*gfp^{mut2}*	colE1	carb	pKJM006	DTE lab stock
P_{sicA} *nfsA*	*sicP; sptP-nfsA-2xF-6xH*	colE1	cam	pKJM084	This study
P_{tet} *nfsB*	*sicP; sptP-nfsB-2xF-6xH*	colE1	cam	pKJM083	This study
P_{sicA} *DH*	*sicP; sptP-DH-2xF-6xH*	colE1	cam	pKJM026	Metcalf et al. 2014
P_{tet} *DH*	*sicP; sptP-DH-2xF-6xH*	colE1	cam	pKJM026	Metcalf et al. 2014
P_{sicA} *ADF3*	*sicP; sptP-ADF3-2xF-6xH*	colE1	cam	pKJM026	Metcalf et al. 2014
P_{sicA} *ADF4*	*sicP; sptP-ADF4-2xF-6xH*	colE1	cam	pKJM026	Metcalf et al. 2014
P_{tet} *ADF4*	*sicP; sptP-ADF4-2xF-6xH*	colE1	cam	pKJM026	This study
P_{sicA} *sptP*	*sicP; sptP-3xF*	colE1	cam	pKJM072	This study

Table 2.2 List of all primers used in this chapter.

Sequence	Used for the construction of:
FWD: ATTAAGATCTCTGTAAGAGAATACACTATTATCATGCC REV: ATTActcgagtttggatccTTACCGTAATTTAATCAAGCGGG	P_{lacUV5} *hilA*
FWD: ttaaAAGCTTACGCCAACCATTGAACTTATTTGTG REV: aattGCGGCCGCTGCGCGTCGCCCAAC	P_{sicA} *nfsA*
FWD: ttaaAAGCTTGATATCATTTCTGTCGCCTTAAAGCG REV: aattGCGGCCGCTCACTTCGGTTAAGGTGATGTTTTG	P_{tet} *nfsB*
FWD: ATTAAGATCTACAGATAACAGGAGTAAGTAATGCAAGC REV: ATTActcgagtttggatccTTAGTGGTGATGGTGATGATGC	P_{tet} *DH*
FWD: ttaaAAGCTTCCTTTACTCGATATCGCGCTAAAG REV: aattGCGGCCGCTGCTTGCCGTCGTCATAAGC	P_{sicA} *sptP*

2.2.3 Sample preparation

Culture supernatant samples were harvested from the cell culture by two sequential centrifugation steps of 2,272*g* for 10 minutes. Samples were precipitated in 20% trichloroacetic acid (TCA) overnight at 4 °C, washed twice with cold acetone and dried by heating. Samples were resuspended in buffer to solubilize precipitated protein films. Unless noted, the solubilization buffer for dot blot samples was Buffer A (20 mM Tris, 150 mM NaCl, 8 M urea, pH 7). For SDS-PAGE separation, samples were then mixed with Laemmli buffer.

2.2.4 Western blot

Samples were separated by SDS-PAGE. The FLAG-BAP protein (Sigma) or Multiple Tag protein (GenScript) was used to create known dilutions to create a standard curve, as indicated. Proteins were transferred using the TransBlot SD unit (Bio-Rad) to a nitrocellulose (Whatman) membrane for fluorescence detection or to a PVDF (Millipore) membrane for chemiluminescence detection. Membranes were interrogated with anti-FLAG or anti-GroEL antibodies per manufacturer's instructions (Sigma). For chemiluminescence detection, a secondary labeling step was carried out with horse-radish-peroxidase-conjugated anti-Mouse IgG or anti-Rabbit IgG antibodies, as appropriate, per manufacturer's instructions (Thermo). Bands were visualized with west-pico chemiluminescent substrate (Thermo) and imaged with a ChemiDoc XRS+ unit (Bio-Rad). For fluorescence detection, a secondary labeling step was carried out with Cy5-conjugated anti-Mouse IgG antibodies, per manufacturer's instructions (GE) and imaged with a Typhoon 9410 imager (GE).

2.2.5 Dot blot

Dot blot samples were precipitated with TCA and resuspended in the appropriate amount of resuspension buffer (between 10–100X concentrated). A 2 µL aliquot of each sample was spotted onto a nitrocellulose membrane and allowed to absorb for at least 1 minute. The FLAG-BAP protein (Sigma) or Multiple Tag protein (GenScript) was used to create known dilutions to create a standard curve, as indicated. The membrane was then incubated in 5 w/w% milk, 0.05 v/v% Tween-20, Tris-buffered saline, pH 7.5 for 1 hour at room temperature with shaking. The membrane was then probed with primary and secondary antibody, as in Section 2.2.4.

2.2.6 Protein purification

Proteins were purified from culture lysate as described in Section 3.2.4.

2.3 Results

2.3.1 Sample preparation

Detection limit by western blot depends on antigen presentation, protein adsorption to the membrane, band size, antibody binding efficiency, and other factors. As such, it is often necessary to increase the concentration of a sample to be able to detect the protein of interest. Protein-containing supernatant samples were concentrated with spin concentrators and with TCA precipitation. I evaluate each technique on three metrics: 1) protein retention; 2) throughput; and 3) introduced bias.

Both techniques require many manual steps. Both techniques are also limited by the number of samples that can be centrifuged. Further, samples must be handled after each spin for both techniques. Spin concentrated samples are spun in a centrifuge until a desired amount of volume has passed through the membrane. The retentate is then removed, as this fraction contains the protein of interest. Note that the flow rate across the membrane is not uniform for multiple samples and this step can add significant error. Further, proteins can adsorb to the membrane, making sample removal from the unit dependent on the sample composition and operation of the unit. Samples that are precipitated chemically are also subject similar variances in sample handling that are described for spin concentrated samples. First, protein precipitation is highly dependent on many chemical factors in the solution, such as protein chemistry and protein concentration (Rajalingam et al. 2009; Sivaraman et al. 1997). Also, the precipitant is easily lost during sample handling.

To compare the two techniques, two supernatant samples were taken from several different culture conditions (Figure 2.1) and two levels of analysis were performed. First, the change in the signal of a protein expected to be invariant across samples, GroEL, was determined. GroEL is a cytoplasmic chaperone that is released to the extracellular space by cell lysis. This protein is expected be present in the culture supernatant at a low concentration that is expected to be only a function of cell density in the conditions tested. Note that all samples grown in the same "T3SS" condition reach the similar OD_{600} and GroEL signal can be compared. The densitometry signals from each band is plotted in Figure 2.2. The anti-GroEL signals from the spin concentrator sample is too small to accurately assign peaks. Also, comparing the signal of the anti-FLAG or anti-GroEL western blot shows that the signal from the sample prepared by TCA precipitation is greater than that prepared by spin concentration. This likely is due to greater yield of protein using the TCA method. Note that this method may introduce extra variability, as the signal in lanes 9–13 (Figure 2.2) is expected to be similar for these samples. Next, the signal from the anti-FLAG blot was used to compare the signal produced by the protein of interest. This signal was quantified on a relative basis and the plotted in Figure 2.3. The dotted line is included to guide the eye and to describe the case of identical signal between the two techniques. The signals between the two techniques are correlated. For all samples tested, a higher signal is seen in the TCA-precipitated sample, relative to the spin concentrated sample. Greater signal is observed for all samples after TCA precipitation, giving this method greater sensitivity due to increased protein yield after concentration steps. Moving forward, all samples will be prepared with TCA precipitation for increasing the protein concentration, as needed.
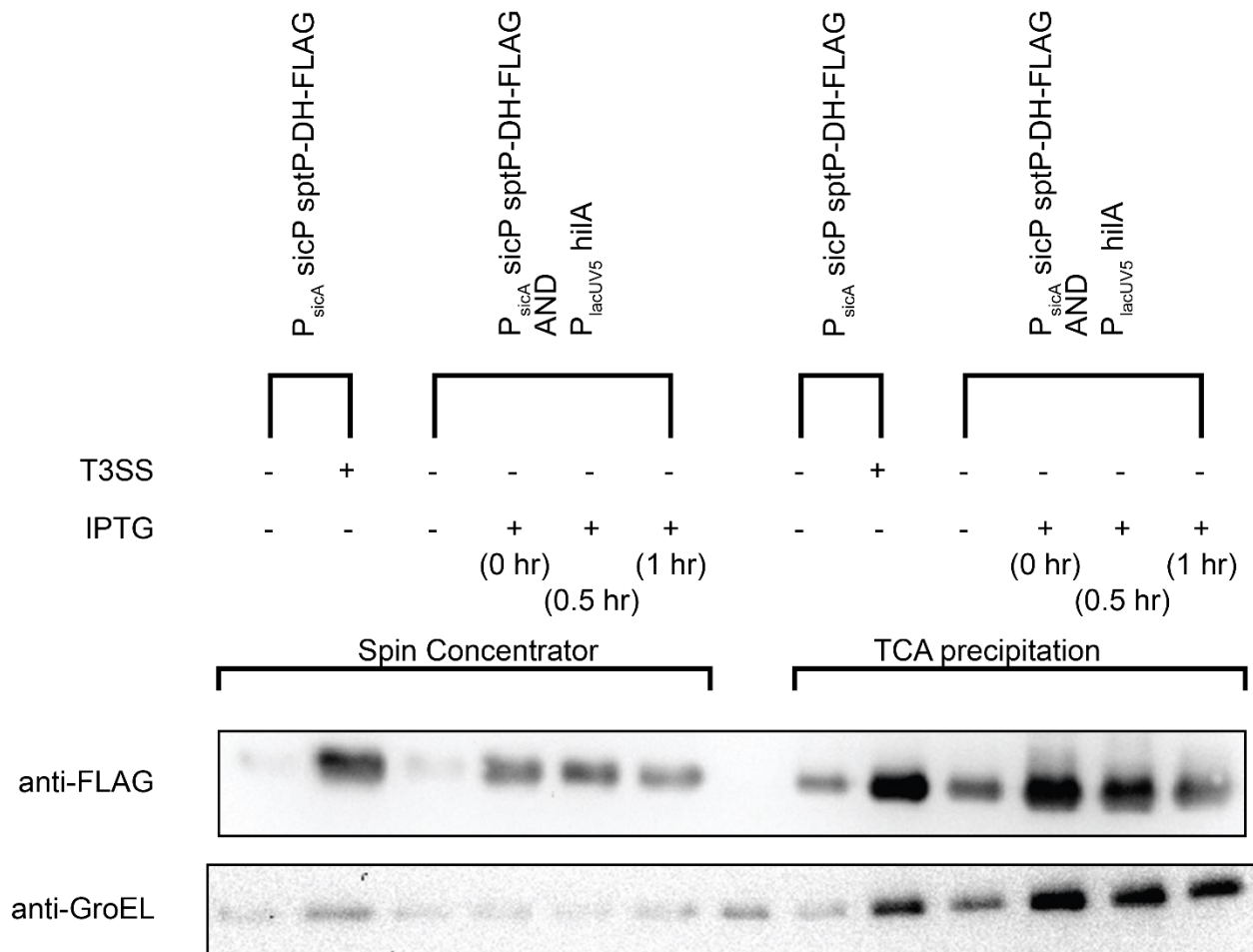
Figure 2.1 Western blot of identical samples preparred by spin concentrators and TCA precipitation. Samples were loaded corresponding to equal OD and probed for fusion protein and GroEL by SDS-PAGE followed by a western blot. Samples that are uninduced were grown in a shaker that was experiencing temperature control problems and the culturing temperature ranged from 30-37°C for these samples.
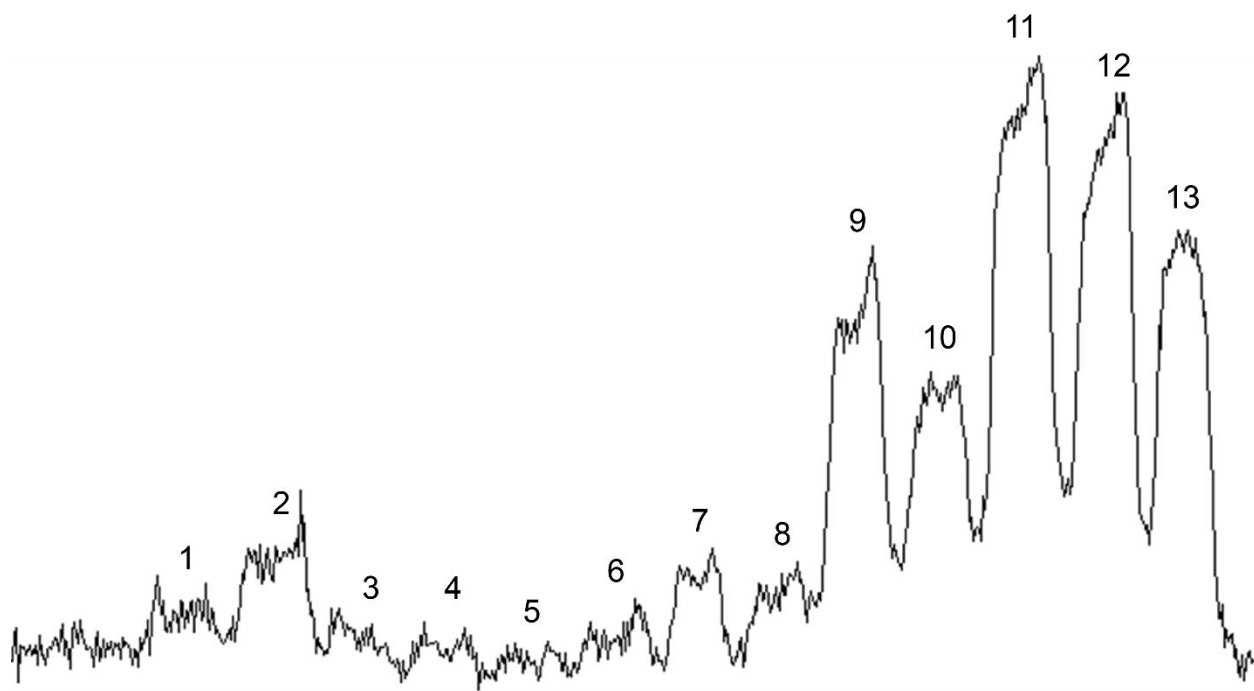
Figure 2.2 Densitometry plot of signal from anti-GroEL blot presented in Figure 2.1. Lane numbering is left to right with respect to Figure 2.1.
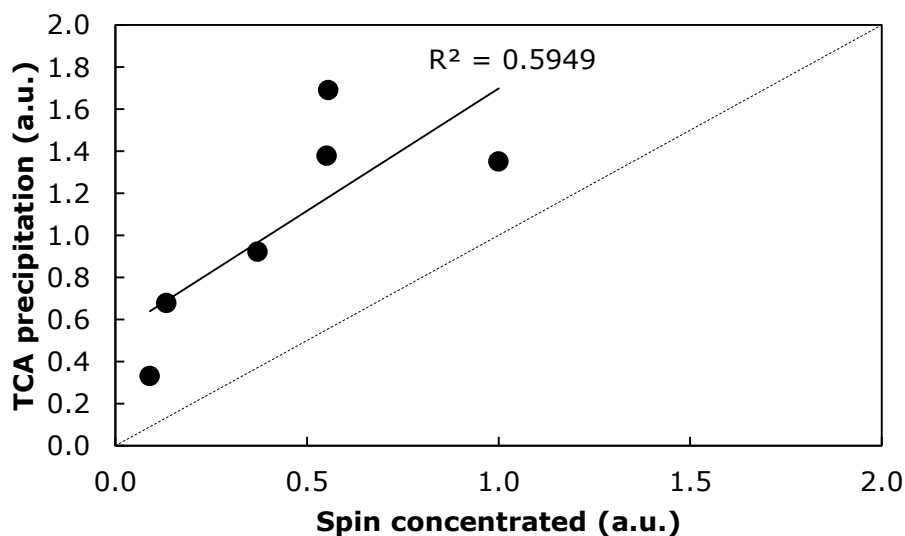
Figure 2.3 Quantification of signal from anti-FLAG blot presented in Figure 2.1. Samples from the western blot in Figure 2.1 were quantified by densitometry relative to the sample in lane 2, the positive control that was concentrated using a spin concentrator. The solid line is a linear best fit, and the dotted line shows the 1:1 relationship.

Resuspension of the precipitated protein films was also tested as a function of resuspension buffer composition. This was to improve the adsorption of protein samples on nitrocellulose membranes using a dot blot. Sodium dodecyl sulfate, the surfactant molecule present in Laemmli

buffer, can inhibit binding of proteins to nitrocellulose membranes. Several different buffers were tested for the ability to solubilize precipitated culture supernatant sample (Figure 2.4). The same sample was precipitated by TCA, and resuspended in the same volume of eight different buffer formulations. The buffer that gave the highest signal, potentially due to increased solubilization, the sample in spot 4. This buffer, termed Buffer A (20 mM Tris, 100 mM NaCl, 8 M urea, pH 7), was used for all dot blot experiments described in Section 2.3.3.
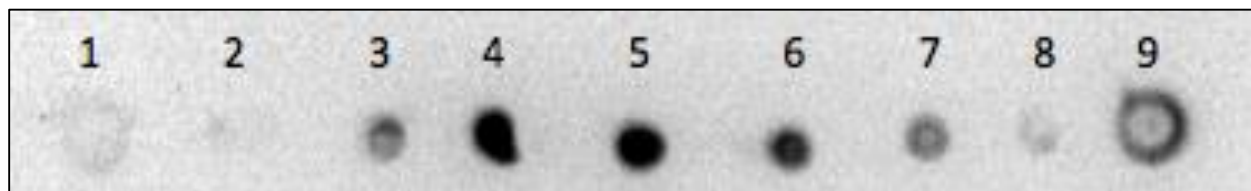
Figure 2.4 Dot blot of identical samples precipitated with TCA and resuspended in different buffers. Samples were precipitated with TCA and resuspended in different buffers. Two µL of each sample was spotted onto a membrane. The membrane was interrogated with the appropriate antibodies and bands were visualized with SuperSignal West Pico chemiluminesce substrate (Thermo). Buffers are: 1) Laemmli buffer; 2) phosphate-buffer saline; 3) 0.05 v/v% Tween-20, Tris-buffered saline, pH 7.5; 4) 20 mM Tris, 100 mM NaCl, 8 M urea; 5) 20 mM Tris, 100 mM NaCl, 4 M guanidinium thiocyanate; 6) 20 mM HEPES; 7) 20 mM MOPS, pH 6.8; 8) ddH$_2$O; and 9) Laemmli buffer.

2.3.2 Quantitative western blot

The data presented here was used to calculate the titers presented in Figure 3.11. The Multiple Tag (GenScript) was used as a standard protein for all blots. To correlate the signal from the Multiple Tag protein with the secreted proteins (with format: SptP-POI-2xFLAG-6xHIS, where POI is the protein of interest), the signal from known concentrations of Multiple Tag were compared with the signal from known concentrations of purified SptP-DH-2xFLAG-6xHIS (Figure 2.5 and Figure 2.6). The purified SptP-DH-2xFLAG-6xHIS samples were subjected to the TCA precipitation protocol before separation by SDS-PAGE and western blotting (Figure 2.5) to mimic sample yields after TCA precipitation for secreted samples.
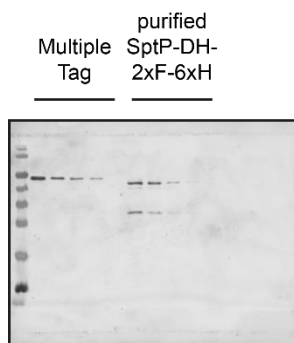
Figure 2.5 Western blot of Multiple Tag and purified SptP-DH-2xFLAG-6xHIS. Lower band is likely a truncated form of the SptP-DH-2xF-6xH protein that copurifies. The secondary antibody used was the anti-Mouse cy5-conjugated antibody.
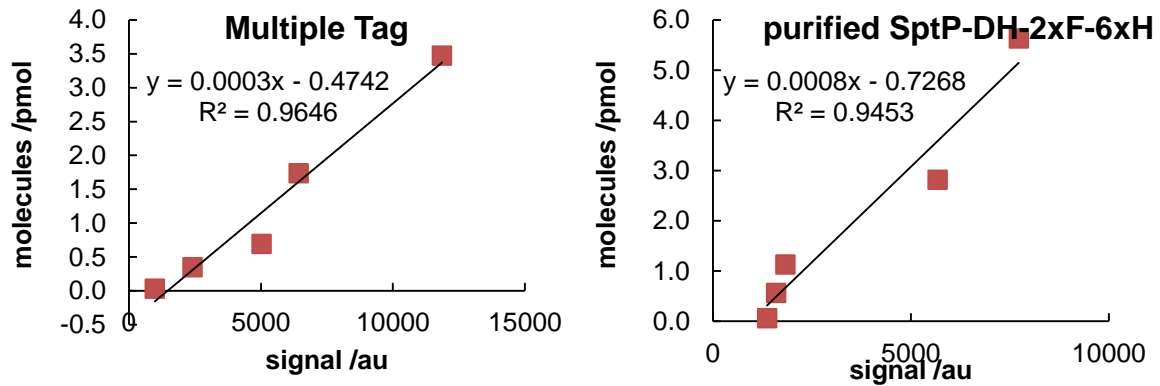
Figure 2.6 Correlation of densitometry signal from western blot presented in Figure 2.5. Note that only the higher molecular weight species of the purified SptP-DH-2xF-6xH sample was used for calculation. The signals measured for samples in Figure 2.7 were used to calculate the quantity of Multiple Tag protein using the standard curve generated for each blot (Figure 2.8). This value was then used to calculate effective Multiple Tag quantity using the Multiple Tag standard curve above to transform the value to the exposure of the blot in Figure 2.5. This signal value was then used to calculate protein quantity using the standard curve for the purified SptP-DH-2xF-6xH. Finally, the concentration of secreted protein was calculated using this quantity and the dilution factor presented in Table 2.3.

Secreted protein samples were then quantified using a western blot. Culture supernatant samples were concentrated by the TCA protocol and then resuspended in Laemmli buffer. The volume used to resuspend the sample was varied, according to the expected titer value to ensure that all bands in the western blot had similar intensities and would be within the domain of the standard curve (Table 2.3). The raw western blots are presented in Figure 2.7. The standard curves generated for each western blot are presented in Figure 2.8.

Table 2.3 Concentration factors for secreted samples analyzed by quantitative western blot in Figure 2.7.

Blot number	genotype	T3SS growth condition	IPTG	Volume resuspended (uL)	Concentration factor
1	P$_{sicA}$ *sptP-DH-2xF-6xH*	+	-	20	50.0
1	P$_{sicA}$ *sptP-DH-2xF-6xH*	-	+	500	2.0
1	P$_{sicA}$ *sptP-DH-2xF-6xH*	+	+	200	5.0
2	P$_{tet}$ *sptP-DH-2xF-6xH*	+	-	20	50.0
2	P$_{tet}$ *sptP-DH-2xF-6xH*	-	+	300	3.3
2	P$_{tet}$ *sptP-DH-2xF-6xH*	+	+	200	5.0
3	P$_{tet}$ *sptP-nfsB-2xF-6xH*	+	-	20	50.0
3	P$_{tet}$ *sptP-nfsB-2xF-6xH*	-	+	20	50.0
3	P$_{tet}$ *sptP-nfsB-2xF-6xH*	+	+	20	50.0
4	P$_{sicA}$ *sptP-ADF4-2xF-6xH*	+	-	20	50.0
4	P$_{sicA}$ *sptP-ADF4-2xF-6xH*	-	+	20	50.0
4	P$_{sicA}$ *sptP-ADF4-2xF-6xH*	+	+	20	50.0
6	P$_{tet}$ *sptP-ADF4-2xF-6xH*	+	-	20	50.0
6	P$_{tet}$ *sptP-ADF4-2xF-6xH*	-	+	40	25.0
6	P$_{tet}$ *sptP-ADF4-2xF-6xH*	+	+	20	50.0
7	P$_{sicA}$ *sptP-ADF3-2xF-6xH*	+	-	20	50.0
7	P$_{sicA}$ *sptP-ADF3-2xF-6xH*	-	+	40	25.0
7	P$_{sicA}$ *sptP-ADF3-2xF-6xH*	+	+	20	50.0
8	P$_{sicA}$ *sptP-bla-2xF-6xH*	+	-	20	50.0
8	P$_{sicA}$ *sptP-bla-2xF-6xH*	-	+	20	50.0
8	P$_{sicA}$ *sptP-bla-2xF-6xH*	+	+	20	50.0
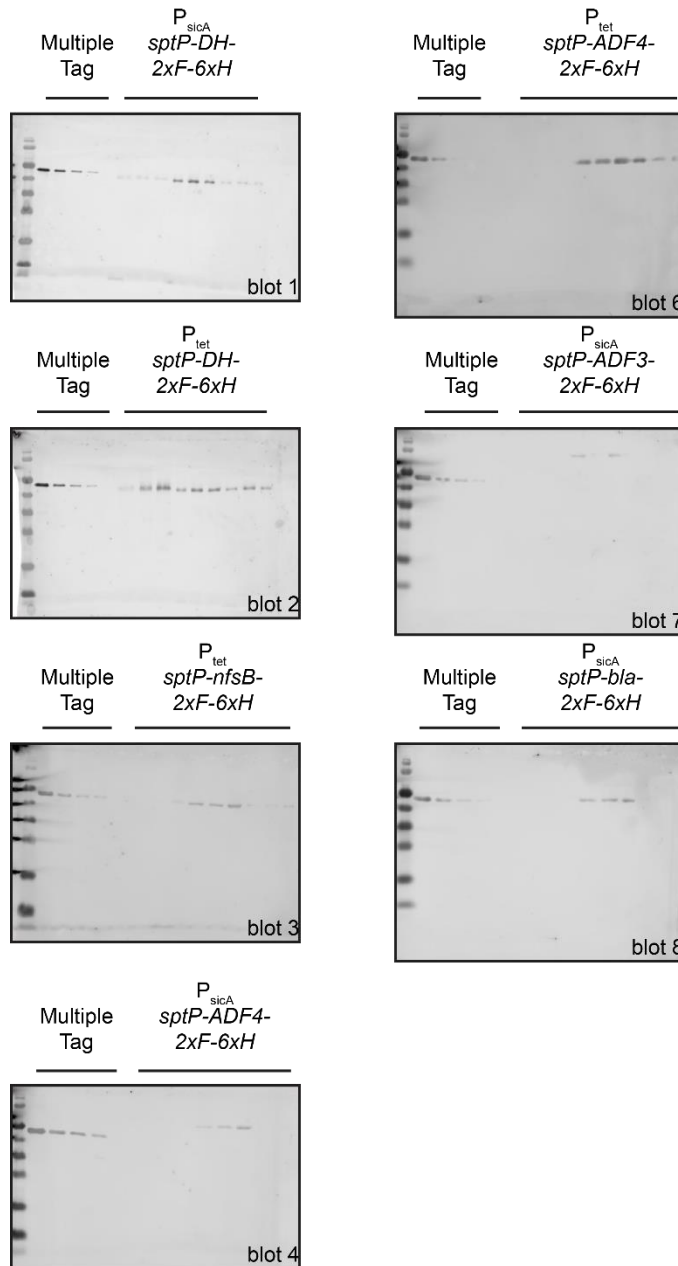
Figure 2.7 Western blot of secreted culture supernatant samples. The secreted samples are loaded in the same pattern for each blot. The first three secreted samples are biological triplicate samples of cultures grown in the "+T3SS –IPTG" condition, where cultures did not carry the upregulation vector. The next three secreted samples are biological triplicate samples of cultures grown in the "-T3SS +IPTG" condition. The last three secreted samples are biological triplicate samples of cultures grown in the "+T3SS +IPTG" condition. The Multiple Tag samples are loaded with the same quantity in each blot and are: 14.2, 7.1, 2.8, 1.4, and 0.1 picomoles. Note that samples were concentrated as indicated in Table 2.3. The secondary antibody used was the anti-Mouse cy5-conjugated antibody.

blot 1
y = 762.92x + 221.26
R² = 0.9976

blot 6
y = 171.26x + 370.76
R² = 0.986

blot 2
y = 713.91x + 110.79
R² = 0.9853

blot 7
y = 594.34x - 816.06
R² = 0.9508

blot 3
y = 704.33x + 638.37
R² = 0.9706

blot 8
y = 252.75x + 34.691
R² = 0.9864

blot 4
y = 542.79x + 910.95
R² = 0.9632
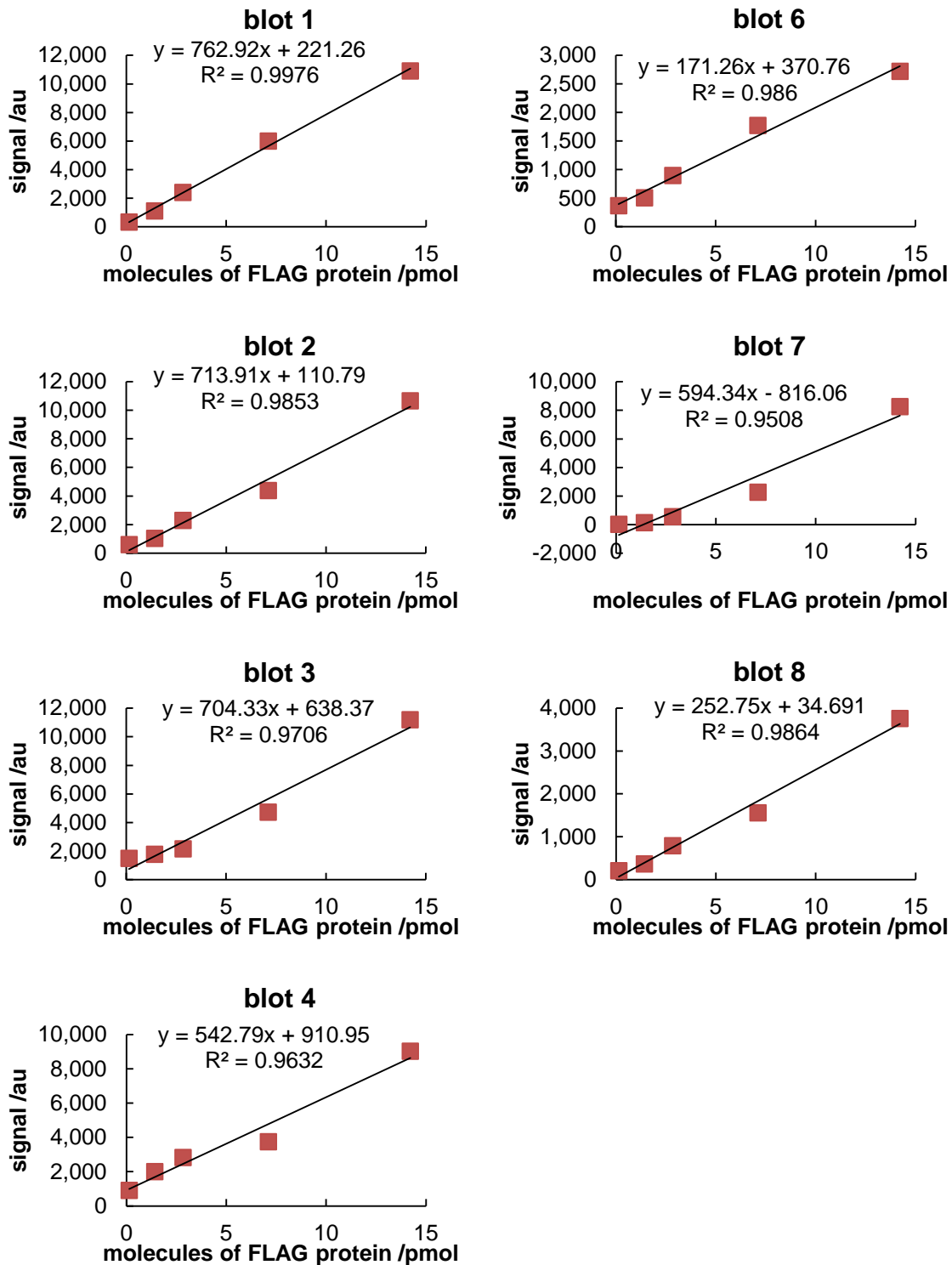
Figure 2.8 Standard curve of Multiple Tag protein standard for each blot presented in Figure 2.7. Linear least-squares regression was used to determine the signal as a function of the quantity of Multiple Tag protein standard.

Note that the standard curve shows two orders of magnitude linear signal (Figure 2.8), which means that the western blot could be accurate over a wide range of sample quantities. A limitation of this is analysis is the inability to appropriately address assay variability. The coefficient of variance in the signal by densitometry for each of the samples was between 16 and 63%, but this variance captures both assay variability and biological variance. Quantification of the secreted protein titer for the samples presented in the raw images in Figure 2.7 is presented in Figures 3.2B, 3.6A, and 3.11.

2.3.3 Quantitative dot blot

A dot blot was performed for three different samples at different concentrations (Figure 2.9). Five different quantities of a standard protein, FLAG-BAP (Sigma), was spotted on the membrane on the top row of the blot. Experimental samples were precipitated with TCA and resuspended with different volumes of Buffer A, relative to the original sample volume. The signal for the 50x and 100x concentrations is higher than the standard for samples 5 and 7. This high signal requires extrapolation of the standard curve and may not be a valid analysis due to potential nonlinearity between the signal and protein quantity at high masses.

Three different methods to calculate the concentration of each samples was used: 1) ChemiDoc software; 2) ImageJ with peak height; and 3) ImageJ with peak integration. ChemiDoc software was investigated but is not preferred because the algorithm is not fully manipulatable. A standard curve using the FLAG-BAP standard (Sigma) is presented in Figure 2.10. Note that both peak height and peak integration are qualitatively similar. The samples in Figure 2.9 were then quantified by the three methods and the results are presented in Table 2.4.
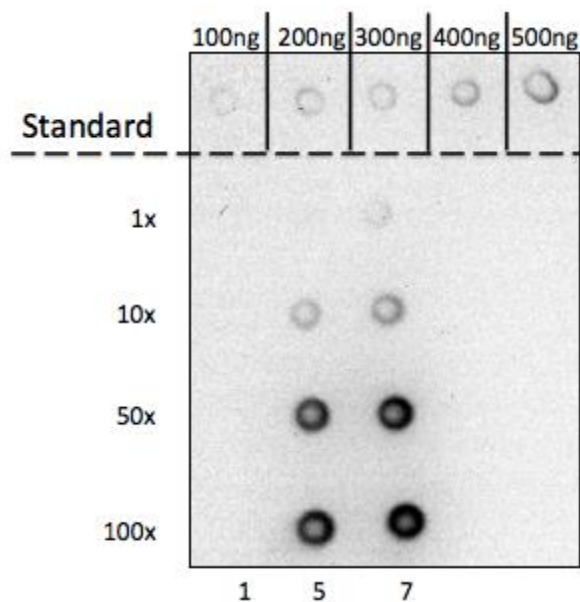
Figure 2.9 Dot blot of culture supernatant samples from three different growth conditions. Signal was collected using chemiluminescence. Standard protein is FLAG-BAP (Sigma). The culture supernatant samples were precipitated with TCA and were resuspended and concentrated to between 1 and 100 times the original volume using Buffer A.
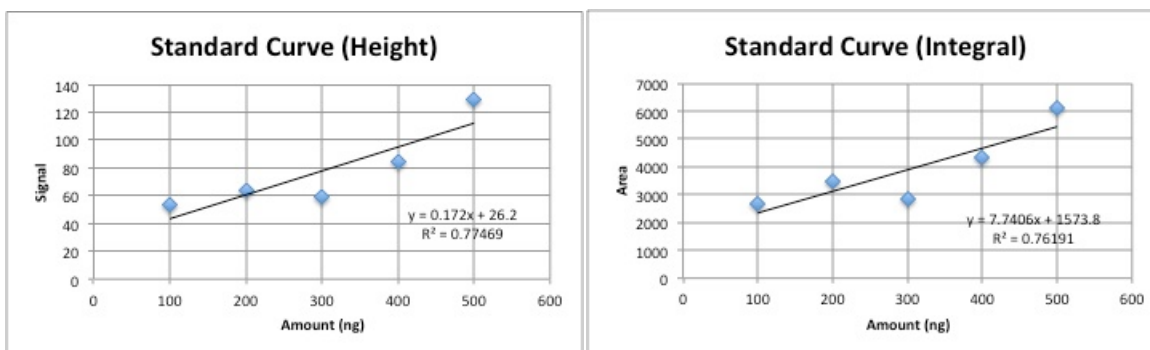
Figure 2.10 Standard curve of FLAG-BAP samples used for quantification of dot blot in Figure 2.9. Analysis was performed using ImageJ (NIH).

Table 2.4 Quantification of samples from Figure 2.9. Samples were quantified using the ChemiDoc software and ImageJ (NIH).

	ChemiDoc (Integral)	ImageJ (Height)	ImageJ (Integral)
Sample 1 (1x)	NSD	NSD	NSD
Sample 1 (10x)	NSD	NSD	NSD
Sample 1 (50x)	NSD	NSD	NSD
Sample 1 (100x)	NSD	NSD	NSD
Sample 5 (1x)	155.4ng (NSD)	NSD	NSD
Sample 5 (10x)	342.1ng	327.72ng	404.44ng
Sample 5 (50x)	1,260.7ng	1,053.07ng	1378.59ng
Sample 5 (100x)	1,703.8ng	1,287.28ng	1792.98ng
Sample 7 (1x)	202.7ng	174.79ng	154.73ng
Sample 7 (10x)	532.7ng	494.57ng	597.68ng
Sample 7 (50x)	1,598.8ng	1305.30ng	1689.71ng
Sample 7 (100x)	1,841.7ng	1413.40ng	1964.03ng

*NSD – No Signal Detected

The calculated values of protein titer is similar across the different techniques. However, the values that are calculated are not as predicted for each of the different sample concentrations. A sample that is 100x concentrated should contain 10 times more sample than a 10x concentrated sample. None of the predicted relationships between the different concentrations are held. This may be due to extrapolation of the standard curve or differential resuspension of the pellet after precipitation.

The domain of sample concentration that yield a linear response in detection was determined by measuring the signal from different dilutions of the same sample. Culture supernatant samples were precipitated with TCA and resuspended in Buffer A. Samples concentrated to various dilutions and spotted onto a membrane. Raw images of the membranes probed with labeling antibodies is given in Figure 2.11. The linear range of detection is at least one order of magnitude (200 - 2000 ng), as seen in Figure 2.12.
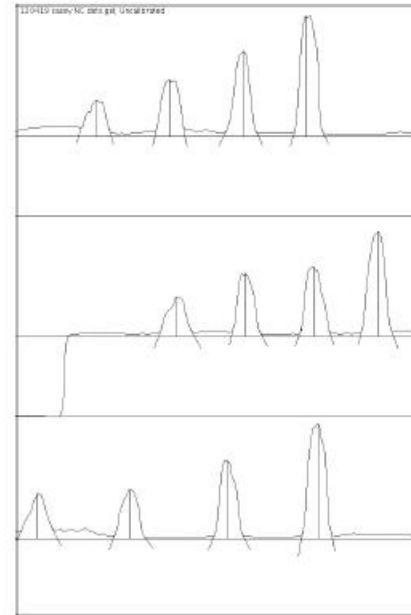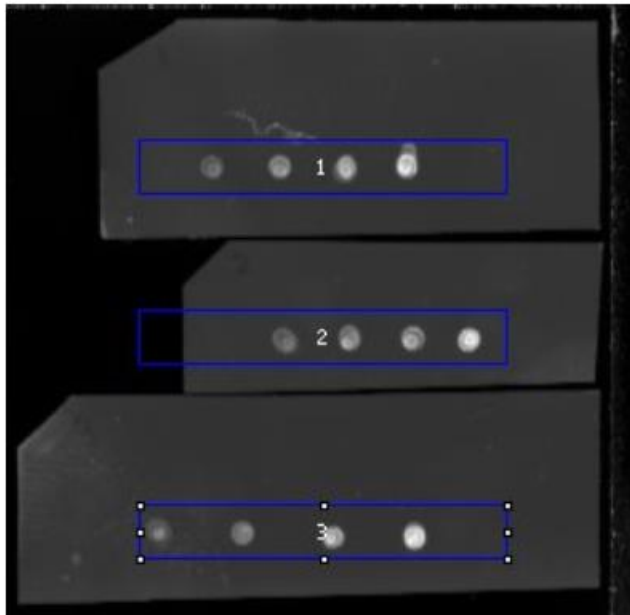
Figure 2.11 Raw dot blot of secreted samples. Secreted DH protein was concentrated to different dilutions using TCA precipitation and quantified using a dot blot with fluorescently-labeled antibody.

DB1: Integral Method

y = 41.617x + 1735.6
R² = 0.98628

DB2: Integral Method

y = 31.609x + 1940.6
R² = 0.98198

DB3: Integral Method

y = 38.233x + 1791.8
R² = 0.97666
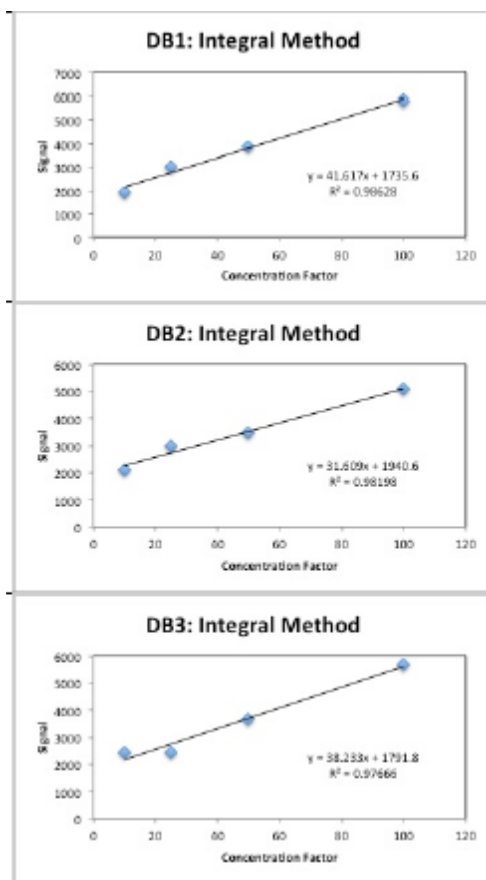
Figure 2.12 Plot of dot blot signal as a function of sample concentration. Quantification of samples from Figure 2.11 was performed using ImageJ and the integral method. Plots show the signal measured as a function of the dilution of sample.

Next, the ability to scale-up the dot blot procedure was tested. The dot blot throughput must be greater than ~10 samples to be a viable alternative to the western blot. Culture supernatant samples were precipitated with TCA and resuspended in an appropriate amount of Buffer A that corresponded to the expected concentration of sample such that the final concentration of the protein of interest was within the linear detection range. Seventy-eight samples were spotted on a nitrocellulose membrane, in addition to five different concentrations of a standard protein (FLAG-BAP). The raw image of this membrane after antibody probing and fluorescence visualization is given in Figure 2.13. For reference, the number of samples analyzed on one dot blot is equivalent to 9 mini-sized gels or 4 midi-sized gels. The ability to analyze a large number of samples on one membrane may greatly increase the number of samples that can be analyzed.

Comparing the raw images in Figure 2.11 and Figure 2.13, it is obvious that there is a big difference in the signal/background signal in the samples. The larger blot has a much higher background signal. Also, the signal from the samples is not as regular in shape in the blot with many samples, compared with the blot with fewer samples. These differences likely arose from the manual application of samples to the membrane. Application of samples took over 10 minutes. Samples thus had a very long time to interact with the dry membrane before hydration in the blocking buffer. Note, however, that the poor quality of the image in Figure 2.13, relative to that in Figure 2.11, did not result in poor quantitation. As seen in the standard curve presented in Figure

39

2.14, the relationship between number of molecules and signal is linear between 2 and 80 picomoles (100 – 4000 ng).

Maximal secreted protein titer of several different test proteins was calculated and is presented in Figure 2.15. The proteins DH, NfsA, and NfsB were measured to be 72 ± 25, 5 ± 4, and 12 ± 3 mg/L in the conditions tested, respectively. The dot blotting procedure gives relatively consistent results. It must be noted that the standard protein in this experiment is FLAG-BAP (Sigma), which contains a C-terminal 1xFLAG tag. The experimental test proteins all contained a C-terminal 2xFLAG-6xHIS tag. Given the differences in sample handling, epitope number and sequence, these calculated titers cannot be taken as absolute, due to the potential differences in the relationship between signal and number of molecules in the standard and test proteins. A more thorough consideration of this problem was covered in Section 2.3.2.
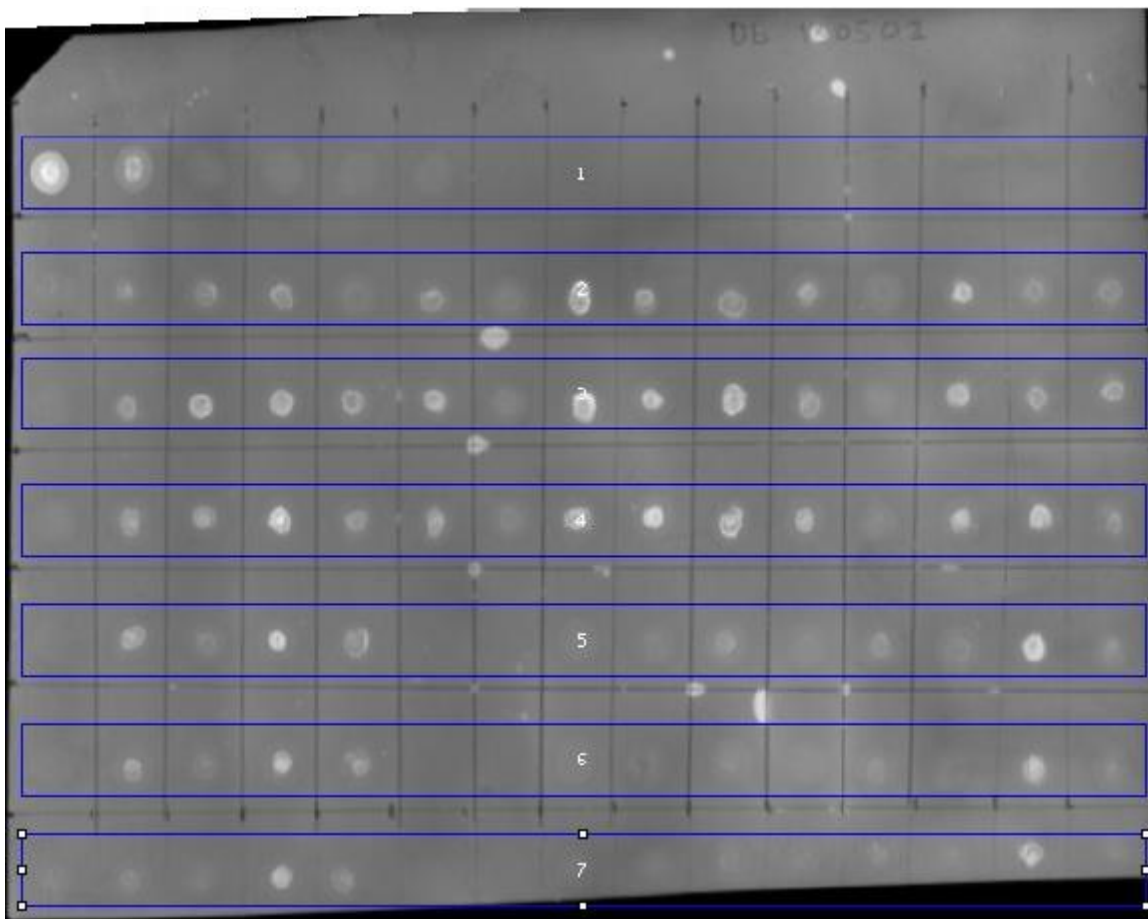
Figure 2.13 Raw image of dot blot. Samples were precipitated with TCA, resuspended in Buffer A, and spotted on a nitrocellulose membrane. Samples were given an expected concentration value, and were resuspended to 10-, 50-, or 100-fold in Buffer A, such that the expected concentration was within the linear range.
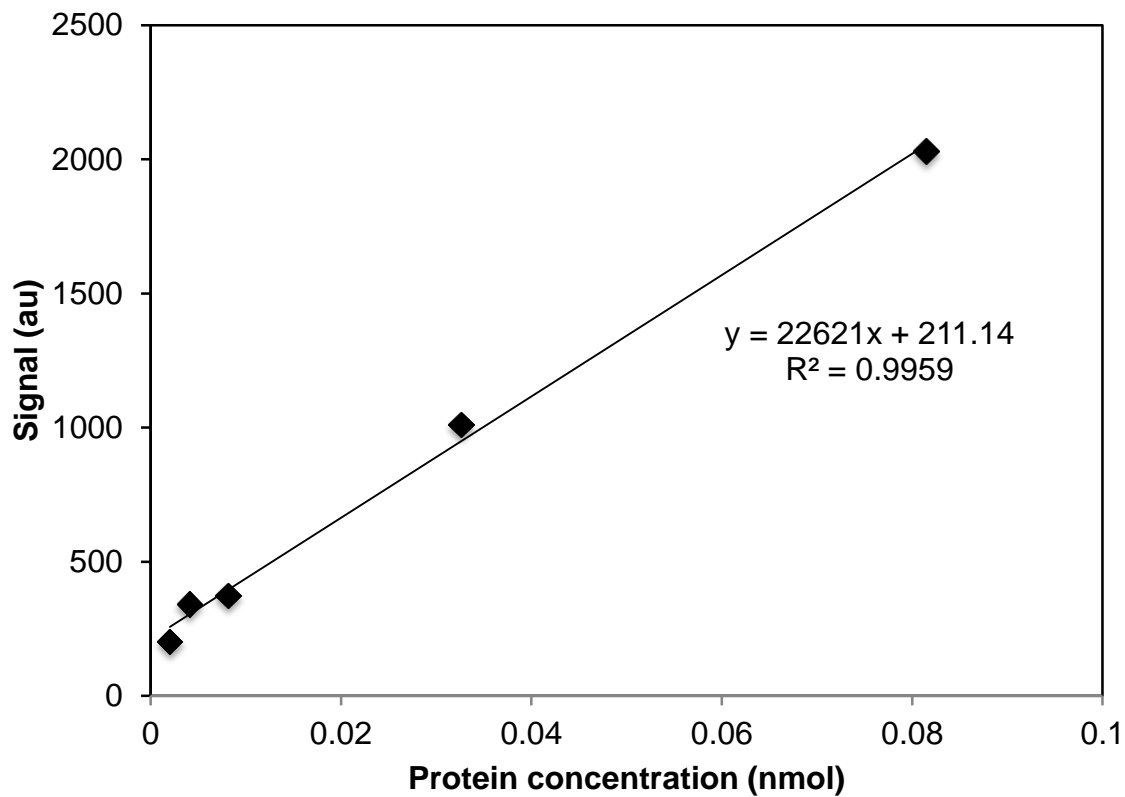
Figure 2.14 Standard curve for FLAG-BAP (Sigma) standard protein dilutions applied to membrane in dot blot given in Figure 2.13. The integral method was used for quantitation.
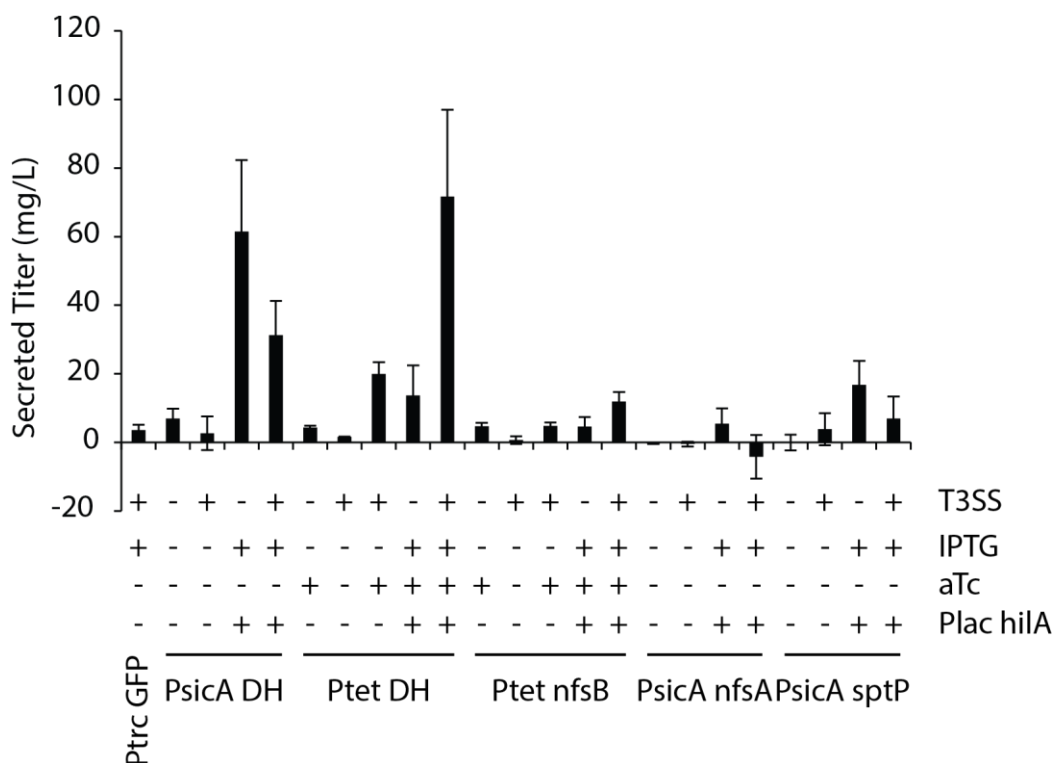
Figure 2.15 Calculated titers of secreted proteins from samples presented in Figure 2.13. P_{tet} *DH*, P_{tet} *nfsB*, P_{sic} *nfsA*, and P_{sic} *sptP* samples were performed three times in biological replicate. P_{trc99A} *gfp* and P_{sic} *DH* samples were performed six times in biological replicate. The mean is plotted and error bars represent one standard deviation.

2.4 Discussion

In this chapter, I describe several different approaches to evaluate the secreted protein titer. The protocols tested were evaluated on the accuracy, precision, throughput, and experimental ease. The standard method for calculating secreted protein titer before work on this dissertation began was based on a western blot and is described in Widmaier et al. 2009. Other methods were analyzed in order to improve upon the quantification protocol.

A higher throughput assay would enable future experiments. For example, analysis of many mutants or culture conditions would be facilitated by an accurate, precise, and high throughput quantitative method. A rational design protein engineering approach to increase the secreted protein titer is described in Chapter 4. To extend the results of that chapter, a library-based protein engineering approach could be taken where many mutants are created and analyzed. As described in Section 2.3.3, a dot blot approach greatly increases the number of samples that can be analyzed, as the number of lanes in an SDS-PAGE gel limits the number of samples that can be analyzed using a western blot. However, at the time of the work presented here, the dot blot was not sensitive enough to detect signal from raw culture supernatant samples. Using more sensitive detection methods, such as improved conjugated reporter molecule or substrate, could increase the signal to allow for the dot blot method to be more feasible. Further, as evidenced by the variability introduced in sample processing steps, efforts to improve the detection limit to decrease sample handing should greatly decrease sample variability.

Given the results at the time, the quantitative western blot was used for all quantification in the following chapters. However, I use this section to suggest that future work to improve the quantitative assay could greatly increase accuracy, precision, and throughput. Chiefly, minimizing sample processing is a key issue for improving the assay. Given the relatively dilute concentration of the protein of interest, the trade-off between sensitivity and sample processing must be considered during assay development. Further, the dot blot could be improved by integration of robotics to perform sample application to the nitrocellulose membrane, which could minimize measurement errors and increase speed and throughput.

2.5 Acknowledgements

I would like to acknowledge the great work that Casey Finnerty performed in this section. Development and validation of the different blotting techniques described in this chapter featured many of his contributions.

2.6 References

Anderson, J. Christopher, John E. Dueber, Mariana Leguia, Gabriel C. Wu, Jonathan A. Goler, Adam P. Arkin, and Jay D. Keasling. 2010. "BglBricks: A Flexible Standard for Biological Part Assembly." *Journal of Biological Engineering* 4 (1): 1–12. doi:10.1186/1754-1611-4-1.

Hoiseth, Susan K., and B. A. D. Stocker. 1981. "Aromatic-Dependent *Salmonella* Typhimurium Are Non-Virulent and Effective as Live Vaccines." *Nature* 291 (5812): 238–39. doi:10.1038/291238a0.

Metcalf, Kevin J., Casey Finnerty, Anum Azam, Elias Valdivia, and Danielle Tullman-Ercek. 2014. "Using Transcriptional Control To Increase Titers of Secreted Heterologous Proteins by the Type III Secretion System." *Applied and Environmental Microbiology* 80 (19): 5927–34. doi:10.1128/AEM.01330-14.

Rajalingam, Dakshinamurthy, Charles Loftis, Jiashou J Xu, and Thallapuranam Krishnaswamy S Kumar. 2009. "Trichloroacetic Acid-Induced Protein Precipitation Involves the Reversible Association of a Stable Partially Structured Intermediate." *Protein Science : A Publication of the Protein Society* 18 (5): 980–93. doi:10.1002/pro.108.

Sivaraman, T., T. K. S. Kumar, G. Jayaraman, and C. Yu. 1997. "The Mechanism of 2,2,2-Trichloroacetic Acid-Induced Protein Precipitation." *Journal of Protein Chemistry* 16 (4): 291–97. doi:10.1023/A:1026357009886.

Widmaier, Daniel M., Danielle Tullman-Ercek, Ethan A. Mirsky, Rena Hill, Sridhar Govindarajan, Jeremy Minshull, and Christopher A. Voigt. 2009. "Engineering the *Salmonella* Type III Secretion System to Export Spider Silk Monomers." *Molecular Systems Biology* 5 (1): 309. doi:10.1038/msb.2009.62.

CHAPTER 3

TRANSCRIPTIONAL CONTROL OF THE T3SS FOR INCREASED SECRETED PROTEIN TITER

3.1 Introduction

Bacterial heterologous protein production is a bedrock of modern molecular biology. A gene that codes for a protein of interest can be inserted into a bacterial cell, often *Escherichia coli*, and the cell is able to produce the desired protein. Bacteria are the preferred organisms for many heterologous expression experiments due to the ease with which they can be genetically manipulated, high protein yield, fast growth, and growth to high cell density (Baneyx, 1999; Terpe, 2006). However, not all genes can be expressed at high levels in a heterologous host, for reasons such as toxicity of the desired protein (Miroux and Walker, 1996). Also, natively folded proteins can be difficult to purify from the milieu of biomolecules present in a culture. Even after recovery of the protein, many processes require resolubilization of inclusion bodies and proper refolding of the protein of interest, which causes significant loss of product (Swartz, 2001). Secretion of heterologous proteins into the extracellular fluid addresses these limitations (Choi and Lee, 2004; Georgiou and Segatori, 2005).

There are seven known classes of secretion systems present in bacteria that transport proteins to the extracellular space (Hochkoeppler, 2013). The type III secretion system (T3SS) is of particular interest because it secretes proteins in one step from the cytoplasm out of the cell. The T3SS is not essential for growth in standard laboratory culturing conditions (Cornelis, 2006; Galán and Collmer, 1999), which makes it more amenable to engineering efforts and enables its use solely for heterologous protein cargo. The T3SS is a transmembrane heteroprotein structure that spans both the inner and outer plasma membrane (Cornelis, 2006; Galán and Collmer, 1999) and secretes between 1,000 and 10,000 amino acid residues per second (Schlumberger et al., 2005; Singer et al., 2012). Two classes of T3SS are well characterized, and are known as the injectisome and flagella (Desvaux et al., 2006). Heterologous proteins secreted by the T3SS include spider silk (Widmaier et al., 2009), fibronectin-binding protein (Majander et al., 2005), neuroactive peptides

(Singer et al., 2012), resilin (Azam et al., 2015), and tropoelastin (Azam et al., 2015). Despite the numerous successes secreting proteins by the type III mechanism, application of the wild-type T3SS for the export of heterologous proteins suffers from low yields and poor secretion efficiency (Widmaier and Voigt, 2010; Widmaier et al., 2009). Moreover, T3SS-induction conditions limit cell growth and stationary phase density, which is not desirable for large-scale protein production.

Using the well-characterized *Salmonella* Pathogenicity Island 1 (SPI-1) T3SS as a model secretion system, we hypothesized that controlling the expression of the T3SS genes would be essential to increasing the amount of protein that is secreted. SPI-1 gene products give rise to an injectisome-type T3SS that secretes enzymes, termed effectors (Cornelis, 2006; Galán and Collmer, 1999). In the native system, external environmental cues are necessary to induce expression of T3SS genes (Lostroh and Lee, 2001a; Tartera and Metcalf, 1993). HilA is a positive regulator of the *invF* (regulatory gene products) and the *prgH* (structural gene products) operons in SPI-1, and overexpression of *hilA* increases SPI-1 gene expression (Sturm et al., 2011), the number of secretion needle complexes (Carleton et al., 2013), and cell invasion (Lee et al., 1992). Given these data, we hypothesized that secreted protein titer could be greatly increased by overexpression of SPI-1 genes via *hilA* overexpression. In this work, the external environmental cues are decoupled from T3SS gene expression and T3SS gene expression is modulated through the addition of a small molecule that induces HilA production. The controlled expression of *hilA* results in increased titers of secreted heterologous proteins. Moreover, expression of SPI-1 genes is increased on both a per cell and population basis by engineering *hilA* expression. Finally, the enzyme beta-lactamase is secreted and adopts an active conformation upon reaching the culture media, enabling its application in future secretion titer assays.

3.2 Materials and Methods

3.2.1 Strains and growth conditions

All *S. enterica* experiments used derivatives of the SL1344 strain (Hoiseth and Stocker, 1981). All strains were grown from colonies from fresh transformations in LB-Lennox (LB-L) (10 g/L tryptone, 5 g/L yeast extract, and 5 g/L NaCl) (VWR #EM1.00547.5007) overnight at 37 °C and 225 rpm in an orbital shaker. Cells were cultured in 24-well blocks (Axygen). Samples were subcultured 1:100 into fresh media, LB-L for "-T3SS" samples and LB-IM (10 g/L tryptone, 5 g/L yeast extract, and 17 g/L NaCl) for "+T3SS" samples. Samples designated "-T3SS" were grown at 37 °C and 225 rpm in an orbital shaker while samples designated "+T3SS" were grown at 37 °C and 120 rpm in an orbital shaker, inducing SPI-1 gene expression and protein secretion using native regulation (Temme et al., 2008). Cultures carrying the *hilA* overexpression plasmid were induced with 100 µM IPTG unless otherwise noted. For the experiments measuring secreted titer, cultures were grown for eight hours. For all other experiments, cultures were grown for six hours. Supernatant samples were harvested from the cell culture by two sequential centrifugation steps of 2,272 x *g* for 10 minutes. Samples were precipitated in 20% trichloroacetic acid (TCA) overnight at 4 °C, washed twice with cold acetone and dried by heating. Samples from the extracellular media were resuspended in Laemmli buffer normalized to culture OD_{600} for SDS-PAGE analysis and denoted "S". Cell pellet samples were resuspended in BPERII (Thermo) normalized to culture OD_{600} and centrifuged at 13,000 x *g* for 5 minutes. The supernatant from the lysed cell pellets was considered the soluble cell pellet fraction, and denoted "C".

3.2.2 DNA manipulations

PCR was performed with *Pfu* DNA polymerase. Restriction enzymes and ligase (NEB) were used according to the manufacturer's instructions. For all cloning, *E. coli* DH10B cells were used. The SPI-1 promoters P_{prgH} (Lostroh and Lee, 2001b; Temme et al., 2008), P_{invF} (Lim et al., 2012; Lostroh et al., 2000), and P_{sicA} (Darwin and Miller, 2001; Temme et al., 2008) were identified from literature and cloned from *S. enterica* subsp. *enterica* serovar Typhimurium strain SL1344 into a modified pPROTet.133 backbone (BD Clontech) to control the *gfp mut2* gene (Cormack et al., 1996) using the *xhoI* and *xbaI* restriction sites. Genes of proteins of interest (POIs) to be secreted were cloned into the same modified pPROTet.133 backbone vector under control of the *sicA* promoter, as in Widmaier *et al.* (Widmaier et al., 2009) and illustrated in Figure 3.1. POIs expressed under control of the *tet* promoter were cloned into a "BglBrick" vector (Anderson et al., 2010) via the *bglII* and *xhoI* sites with a colE1 origin and chloramphenicol resistance cassette. All secretion vectors expressed the SptP chaperone, *sicP,* and the *sptP* signal sequence (nucleotides 1-477) (Widmaier et al., 2009). All POIs were fused to the C-terminus of the SptP secretion signal at the genetic level using *hindIII* and *notI* restriction sites, and a 2xFLAG-6xHis C-terminal tag was also genetically incorporated into all POIs. The *hilA* gene from SL1344, including the first 23 nucleotides 5' of the start codon, was cloned into a P_{lacUV5} "BglBrick" expression vector with a p15a origin and neomycin resistance cassette using the *bglII* and *xhoI* restriction sites. Deletion of *prgI* from the SL1344 wild-type strain was performed by the methods of Datsenko and Wanner (Datsenko and Wanner, 2000). All DNA sequences were verified by Sanger sequencing (Quintara). A table of strains and plasmids is given in Table 3.1. A table of primers used in this study is given in Table 3.2.

```
CTCGAGCCACAAGAAAACGAGGTACGGCATTGAGCCGCGTAAGGCAGTAGCGATGTATTCATTGGGCGTTTTTTGAA
TGTTCACTAACCACCGTCGGGGTTTAATAACTGCATCAGATAAACGCAGTCGTTAAGTTCTACAAAGTCGGTGACAG
ATAACAGGAGTAAGTAATGCAAGCACACCAGGATATTATCGCTAATATTGGTGAGAAATTGGGTTTACCGCTCACTT
TTGACGACAACAATCAGTGCTTATTATTACTCGATAGCGATATTTTTACGTCTATTGAAGCTAAAGATGATATCTGG
TTATTGAACGGTATGATTATACCGTTATCGCCTGTTTGTGGCGATTCTATCTGGCGGCAGATTATGGTGATTAATGG
TGAACTGGCTGCGAATAATGAAGGTACGTTAGCGTATATTGATGCCGCAGAGACGTTGTTGCTTATACATGCAATTA
CCGATCTGACAAATACTTACCATATTATATCGCAGCTTGAGTCATTTGTGAATCAGCAGGAAGCGCTCAAAAACATA
CTGCAGGAATATGCTAAAGTATGAGGAGAGAAAATTGAATAATTTAACGTTGTCTTCGTTTTCAAAAGTTGGTGTGT
CGAATGATGCCCGACTTTATATTGCTAAGGAAAATACTGATAAGGCATATGTTGCGCCTGAAAAATTTTCGTCAAAA
GTATTAACCTGGCTTGGAAAAATGCCGTTATTTAAAAACACTGAAGTGGTGCAAAAACATACGGAAAATATCAGAGT
ACAGGACCAAAAGATTTTACAGACATTTCTCCATGCACTAACGGAAAAATATGGGGAAACAGCGGTTAATGACGCAC
TGTTAATGTCCCGTATAAATATGAACAAACCCCTTACCCAACGTTTAGCAGTGCAGATCACGGAGTGTGTAAAAGCT
GCTGACGAAGGGTTTATAAACCTTATTAAGAGCAAGGATAATGTTGGTGTCAGGAATGCCGCTTTAGTCATAAAAGG
CGGCGATACAAAAGTGGCAGAAAAAAATAACGATGTTGGAGCAGAAAGTAAGCTT...goi...AGCGGCCGCGATT
ATAAAGATGACGATGACAAGGATTATAAAGATGACGATGACAAGCATCATCACCATCACCACTAATCTAGA
```

Figure 3.1 Generalized DNA sequence for the export vector. The sequence coding for the protein to be secreted (gene of interest, *goi*) was cloned into the *hindIII* and *notI* restriction sites. The export vector is derived from the pPROTet.133 vector (BD Clontech), with the *sicA* promoter, *sicP* chaperone, *sptP* signal sequence, and C-terminal FLAG and HIS epitopes between the *xhoI* and *xbaI* restriction sites.

Table 3.1 List of strains and plasmids used in this study.

Strain name	Comment	strain ID	Reference
Wild-type	SL1344-derived lab strain	sKJM002	Widmaier et al., 2009
SL1344 *prgI*	Deletion of *prgI*$^{1-231}$ with pKD13 and FLP-out	sKJM085	This study

Plasmid name	ORFs under inducible control	ORI	ab^R	plasmid ID	Reference
P$_{sicA}$ *DH*	*sicP; sptP-DH-2xF-6xH*	colE1	cam	pKJM026	This study
P$_{sicA}$ *ADF3*	*sicP; sptP-ADF3-2xF-6xH*	colE1	cam	pKJM108	This study
P$_{sicA}$ *ADF4*	*sicP; sptP-ADF4-2xF-6xH*	colE1	cam	pKJM092	This study
P$_{sicA}$ *bla*	*sicP; sptP-bla-2xF-6xH*	colE1	cam	pKJM112	This study
P$_{tet}$ *DH*	*sicP; sptP-DH-2xF-6xH*	colE1	cam	pKJM046	This study
P$_{invF}$ *gfp^{mut2}*	*gfp^{mut2}*	colE1	cam	pKJM057	This study
P$_{prgH}$ *gfp^{mut2}*	*gfp^{mut2}*	colE1	cam	pKJM002	Temme et al., 2008
P$_{sicA}$ *gfp^{mut2}*	*gfp^{mut2}*	colE1	cam	pKJM001	Temme et al., 2008
P$_{lacUV5}$ *hilA*	*hilA*	p15a	kan	pKJM035	This study
pKD46				-	Datsenko et al., 2000
pCP20				pKJM014	Datsenko et al., 2000

Table 3.2 Primers used in this study.

Sequence	Used for the construction of:
FWD: CCCAAGCCCACTTTAATTTAACGTAAATAAGGAAGTCATTGTGTAGGCTGGAGCTGCTTC REV: CAATCGACATAATCCACCTTATAACTGATTAACGGAAGTTATTCCGGGGATCCGTCGACC	SL1344 *prgI* deletion strain
FWD: ATTAAGATCTCTGTAAGAGAATACACTATTATCATGCC REV: ATTActcgagtttggatccTTACCGTAATTTAATCAAGCGGG	P$_{lacUV5}$ *hilA* plasmid
FWD: ataaTCTAGAATGCAGCTTTTGCGCG REV: attaCTCGAGAGCCAACGGTGATATGGC	P$_{invF}$ *gfp* plasmid
FWD: ttaaAAGCTTGATATCATTTCTGTCGCCTTAAAGCG REV: aattGCGGCCGCTCACTTCGGTTAAGGTGATGTTTTG	P$_{sicA}$ *sicP sptP-nfsB-2xF-6xH* plasmid
FWD: ttaaAAGCTTCACCCAGAAACGCTGGTG REV: aattGCGGCCGCTCCAATGCTTAATCAGTGAGGC	P$_{sicA}$ *sicP sptP-bla-2xF-6xH* plasmid
FWD: ATTAggtctcTCTAGAGATTATAAAGATGACGATGACAAGGATTATAAAGATG REV: ATTAggtctcTCTAGAAGCGGCCGCAGCGGCCGCGATTATAAAG	P$_{sicA}$ *sicP sptP-ADF3-2xF-6xH* plasmid
FWD: ATTAggtctcTaattcGATTATAAAGATGACGATGACAAGGATTATAAAGATG REV: ATTAggtctcTaattcAGCGGCCGCAGCGGCCGCGATTATAAAG	P$_{sicA}$ *sicP sptP-ADF4-2xF-6xH* plasmid
FWD: ATTAAGATCTACAGATAACAGGAGTAAGTAATGCAAGC REV: ATTActcgagtttggatccTTAGTGGTGATGGTGATGATGC	P$_{tet}$ *sicP sptP-DH-2xF-6xH* plasmid

3.2.3 Protein separation and western blotting

Samples were separated by SDS-PAGE by the methods of Laemmli (Laemmli, 1970). Proteins were transferred to a PVDF (Millipore) membrane for chemiluminescence detection, using the TransBlot SD unit (Bio-Rad). Membranes were interrogated with anti-FLAG or anti-GroEL antibodies per manufacturer's instructions (Sigma). A secondary labeling step was carried out with anti-Mouse IgG or anti-Rabbit IgG antibodies, as appropriate, per manufacturer's instructions (Thermo). Bands were visualized with west-pico chemiluminescent substrate (Thermo) and imaged with a ChemiDoc XRS+ unit (Bio-Rad).

3.2.4 Protein purification

The SptP-DH-2xF-6xH protein was purified from bacterial culture. The *E. coli* strain DH10B was used to express the protein and the culture was homogenized by sonication. Culture homogenate was purified using a His GraviTrap column (GE Healthcare # 11-0033-99). Eluted protein sample was separated by SDS-PAGE, stained with Coomassie G-250, and quantified using densitometry relative to a bovine serum albumin standard (Thermo).

3.2.5 Secreted protein quantification

Supernatant samples were harvested from the cell culture as described earlier. Dried supernatant samples were resuspended in an appropriate volume of Laemmli buffer, separated by SDS-PAGE, and transferred to a nitrocellulose membrane (Whatman). The membrane was interrogated with anti-FLAG (Sigma # F3165) and anti-Mouse IgG-Cy5 (GE # PA45010) antibodies. Membranes were imaged with a Typhoon 9410 imager (GE). The Multiple Tag protein was used to generate a standard curve. The purified SptP-DH-2xF-6xH protein was used to generate a correction to the standard protein standard curve to adjust for the FLAG epitope differences. The purified SptP-DH-2xF-6xH protein was diluted in PBS to 10 mg/L and then precipitated with TCA, as mentioned previously. Then, a quantitative western blot was run with the standard protein to compare the signal at different concentrations, and a linear best fit was used to correct the calculated value for the specific precipitation protocol and epitope differences. ImageJ (Schneider et al., 2012) was used to quantify the signal by densitometry. Each peak was manually bounded and the peak area was used to calculate the signal, without background correction (Gassmann et al., 2009). The experiment was performed on different days in biological triplicate. Error bars represent one standard deviation.

3.2.6 Flow cytometry

Samples were grown overnight in LB-L media, subcultured 1:100 in LB-L media, grown for two hours to dilute overnight expression, and then subcultured 1:10 into fresh LB media with the appropriate salt concentration, inducer, and antibiotic, as required. Culture samples were taken every hour, with samples diluted into phosphate-buffered saline (PBS) with 2 mg/mL kanamycin to 0.01-0.1 OD_{600} and stored at 4 °C for analysis. After the induction was complete, all samples were diluted to 0.001 OD_{600} in PBS and analyzed by flow cytometry. For each sample, 10,000 events within a gated population determined to be cells were collected on a Guava easyCyte 8HT flow cytometer (Millipore). Data analysis was performed in FlowJo 7.6.4 (Tree Star, Inc.). To determine the fraction of cells from the population that are induced for SPI-1 gene expression, cells were gated by green fluorescence above cellular autofluorescence. The experiment was performed on different days in biological triplicate. Error bars represent one standard deviation.

3.2.7 Beta-lactamase activity assay

Samples were grown overnight in LB-L media, then subcultured 1:100 in LB-L media and grown for eight hours with "-T3SS" conditions. The cultures were pelleted by one centrifugation step of 2,272 x g for 10 minutes and the supernatant was passed through a 0.45 μm filter. Samples were then subjected to a nitrocefin hydrolysis assay, per the substrate vendor (Sigma). 100 μL of reaction buffer (0.1 M phosphate, 1 mM EDTA, 50g/mL nitrocefin (EMD Millipore), 0.5% DMSO, pH 7) was mixed with 10 μL culture supernatant and the absorbance at 486 nm was observed over time. The slope of the linear region was calculated and the initial reaction velocity was calculated for $\xi = 20,500$ M^{-1} cm^{-1}. The experiment was performed on different days in biological triplicate. Error bars represent one standard deviation.

3.3 Results

3.3.1 *hilA* overexpression increases secreted protein titer

Typically, induction of secretion has been achieved by "+T3SS" growth conditions that mimic the microanaerobic and high osmolarity conditions of the intestinal lumen (Tartera and Metcalf, 1993; Widmaier et al., 2009). HilA is known to be an upstream positive regulator of the SPI-1 T3SS (Bajaj et al., 1995; Lee et al., 1992), so we reasoned that controlled expression of this transcription factor could induce secretion in the absence of the "+T3SS" condition. To this end, an "upregulation vector" was generated, in which *hilA* is under control of the isopropyl β-D-1-thiogalactopyranoside- (IPTG) inducible P$_{lacUV5}$ promoter on a low-copy vector. Since a cryptic secretion-targeting sequence from the N-terminal sequence of native secreted proteins is required for secretion (Widmaier and Voigt, 2010; Widmaier et al., 2009), an "export vector" was created by fusing the N-terminal signal sequence from the native secreted protein SptP (Stebbins and Galán, 2001; Widmaier et al., 2009) to the gene encoding a model protein of interest (POI). The SptP signal sequence was previously shown to be required to direct the secretion of heterologous proteins (Widmaier et al., 2009). The soluble catalytic DH domain from the human protein intersectin-1L is the POI for the initial experiments described here (Ahmad and Lim, 2010; Yeh et al., 2007). This fusion was placed under the control of the native SPI-1 effector promoter P$_{sicA}$. The two vectors were then co-transformed into a derivative of *S. enterica* subsp. *enterica* serovar Typhimurium str. SL1344. Under the appropriate inducing conditions, the SptP-DH-2xF-6xH fusion protein is secreted by the T3SS in the presence or absence of the upregulation vector, and controlled expression of *hilA* from the upregulation vector increased the yield of protein recovered in the culture supernatant (Figure 3.2A). The secreted titer of the SptP-DH-2xF-6xH protein was quantified to determine the improvement from *hilA* overexpression. Remarkably, *hilA* overexpression increases the secreted titer by over 10-fold, and the highest secreted titer observed was 28 ± 9 mg/L when grown in the "-T3SS" condition (Figure 3.2B).

To confirm that proteins recovered in the culture supernatant resulted from secretion by the SPI-1 T3SS and not increased cell lysis, a probe was introduced to test for the presence of GroEL, a soluble cytoplasmic chaperone, in the culture supernatant (Majander et al., 2005; Widmaier et al., 2009). No significant accumulation of GroEL in the culture supernatant was observed, indicating that *hilA* overexpression did not increase cell lysis (Figure 3.2A). Also, the titer of secreted protein is greater with *hilA* overexpression in the "-T3SS" induction conditions, as compared to the "+T3SS" conditions with and without *hilA* overexpression (Figure 3.2B). The increased protein secretion observed with *hilA* overexpression requires a full T3SS. Protein secretion is not observed in a strain with a genomic deletion of *prgI* (Figure 3.2C), which codes

for a structural protein required for functional SPI-1-based secretion (Kimbrough and Miller, 2000).

Overexpression of *hilA* results in secretion in the absence of "+T3SS" environmental cues, allowing for higher density cultures (Figure 3.2D) in addition to greater recovery of secreted protein on a per-OD_{600} basis (Figure 3.2A). Interestingly, overexpression of *hilA* does not retard growth (Figure 3.2D), while growth in "+T3SS" conditions does suppress growth rate (Sturm et al., 2011) and results in lower culture densities at stationary phase. Thus, increased volumetric secreted protein titer from *hilA* overexpression (Figure 3.2B) can be attributed in part to increased culture density in the "-T3SS" condition and in part to increased secretion on a per-OD_{600} basis.
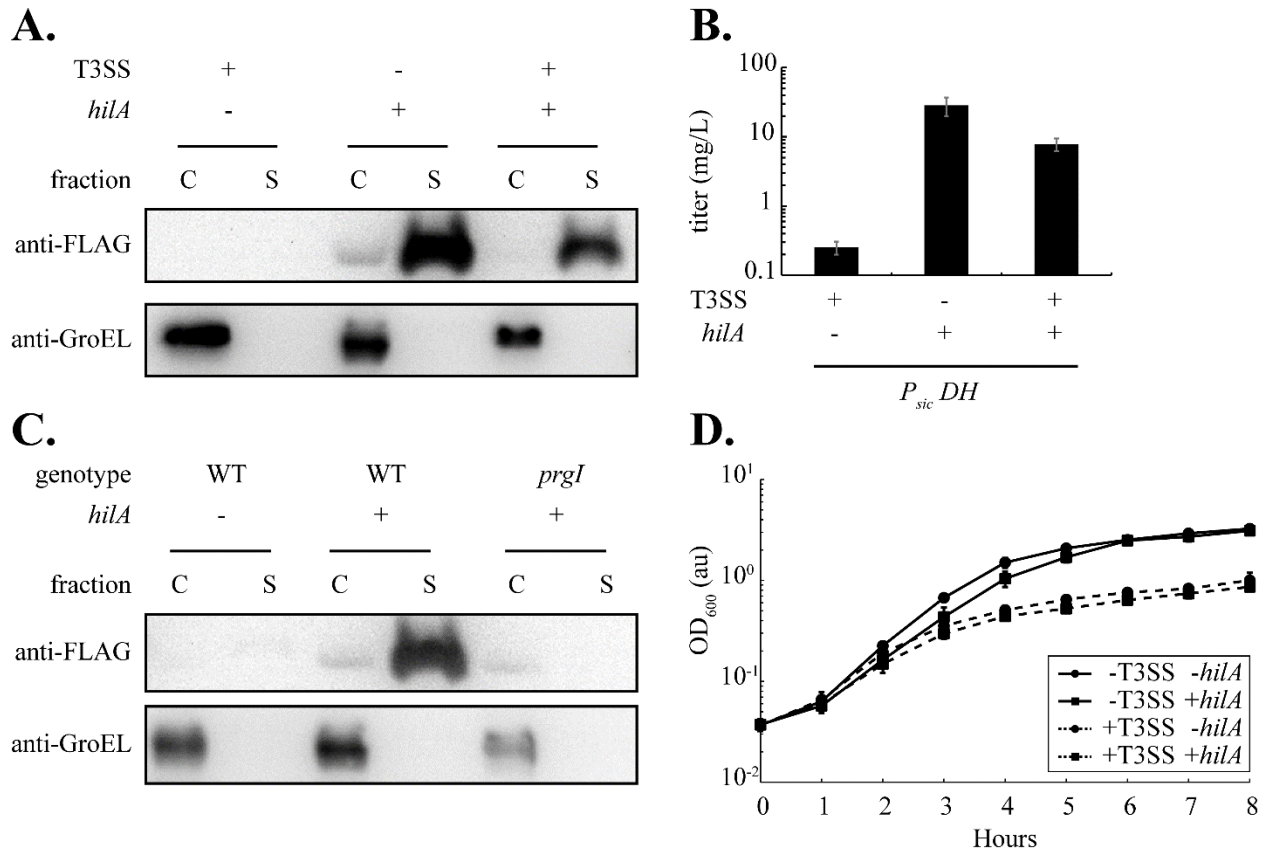
Figure 3.2 Effect of *hilA* overexpression on secretion and cell growth. Cultures carrying the upregulation vector were induced with 100 μM IPTG at the time of subculture and denoted "+*hilA*". Cultures that did not carry the upregulation vector are denoted "-*hilA*". **A.** Western blot of soluble cell fraction ("C") and supernatant ("S") samples, with samples loaded equal OD_{600}. **B.** Quantification of SptP-DH-2xF-6xH secreted protein titer using the P_{sicA} *DH* export plasmid with varying growth conditions and *hilA* overexpression. **C.** Western blot of soluble cell fraction and supernatant samples of wild-type and *prgI* deletion strains, loaded equal OD_{600}, from cultures grown in the "-T3SS" condition. **D.** Growth of *S. enterica*. Cultures grown in the "-T3SS" and "+T3SS" condition are denoted with a solid and dashed black line, respectively. Cultures without and with *hilA* overexpression are marked with a circle and rectangle, respectively. For all quantitative results, experiments were performed on different days in biological triplicate. Error bars represent one standard deviation.

3.3.2 *hilA* overexpression increases SPI-1 locus gene expression

hilA overexpression is known to increase SPI-1 gene expression (Bajaj et al., 1995, 1996; Eichelberg and Galán, 1999; Sturm et al., 2011) and the number of secretion apparatus per cell (Carleton et al., 2013). To confirm that the increased secreted protein titer correlates with *hilA* overexpression, we quantified the activity of SPI-1 promoters in the context of our experimental conditions. HilA directly activates *invF* and *prgH* operon expression and indirectly activates *sicA* operon expression, which code for regulatory, structural, and secreted proteins, respectively (Lostroh and Lee, 2001b). Therefore, a transcriptional reporter using green fluorescent protein (GFP) was used to track the expression from these three promoters and cells were analyzed by flow cytometry.

The expression of both the *invF* and *prgH* promoters increases with *hilA* overexpression (Figure 3.3), and these promoters show a graded response, in agreement with reported results (Temme et al., 2008). Growth of the cells in the "+T3SS" condition increased GFP signal from the *sicA* promoter, relative to the "-T3SS" condition (Figure 3.4), but the fraction of cells induced was similar (Figure 3.5). Additionally, the *sicA* promoter increased expression (Figure 3.4) as well as the fraction of the population that is induced (Figure 3.5) upon *hilA* overexpression. Interestingly, with *hilA* overexpression, nearly all of the cells are induced for P_{sicA} expression in the "-T3SS" condition. For cells in the "-T3SS" condition, activity from the *sicA* promoter increases between three and four hours post-induction, while cells in the "+T3SS" condition have increased *sicA* promoter activity between two and three hours post-induction (Figure 3.5). For all promoters tested, the leaky expression from the overnight culture was diluted by cell division early in the experiment, causing a decrease in cellular fluorescence. When promoter activity was increased in the log-phase cultures, the level of fluorescence increased as GFP production was greater than dilution of GFP.
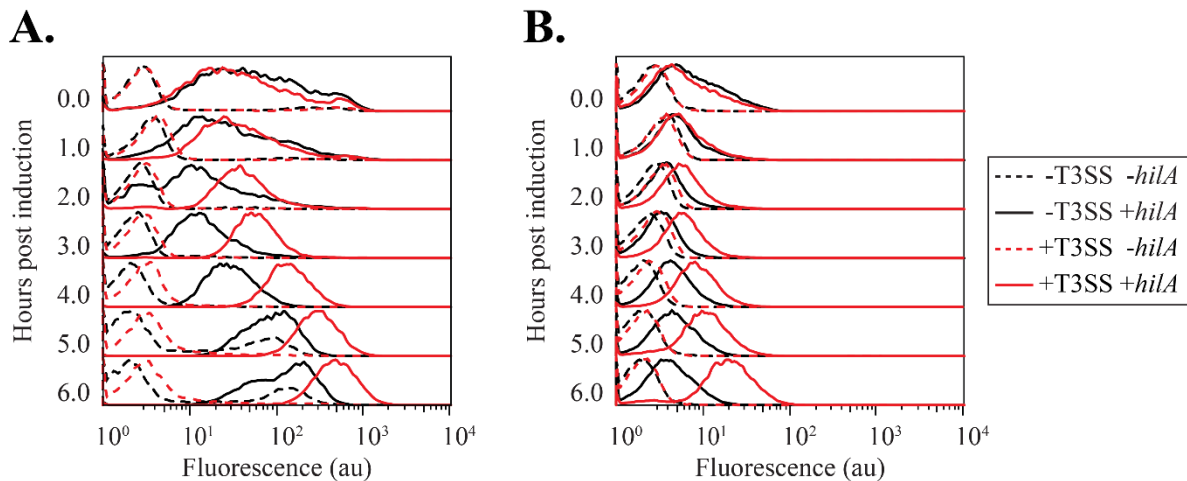
Figure 3.3 Flow cytometry analysis of **A.** P_{invF} and **B.** P_{prgH} activity. Representative histogram of samples grown in different growth conditions with and without *hilA* overexpression. Cultures carrying the upregulation vector were induced with 100 µM IPTG at the time of subculture and denoted "+*hilA*". Cultures that did not carry the upregulation vector are denoted "-*hilA*". Cultures grown in the "-T3SS" condition without and with *hilA* overexpression are denoted with a black dash line and a black solid line, respectively. Cultures grown in the "+T3SS" condition without and with *hilA* overexpression are denoted with a red dash line and a red solid line, respectively.
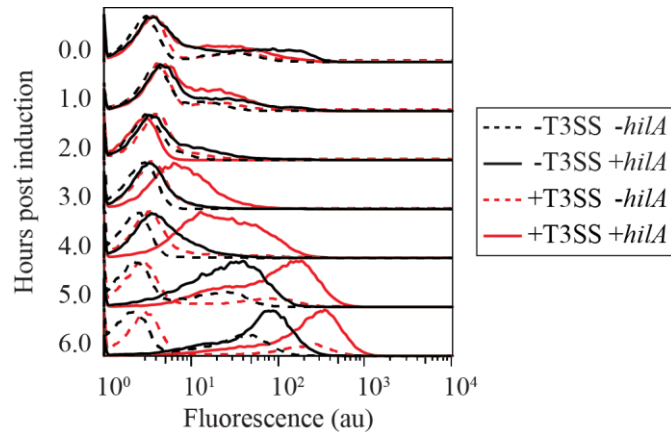
Figure 3.4 Flow cytometry analysis of P_{sicA} activity. Representative histogram of samples grown in different growth conditions with and without *hilA* overexpression. Cultures carrying the upregulation vector were induced with 100 µM IPTG at the time of subculture and denoted "*+hilA*". Cultures that did not carry the upregulation vector are denoted "*-hilA*". Cultures grown in the "-T3SS" condition without and with *hilA* overexpression are denoted with a black dash line and a black solid line, respectively. Cultures grown in the "+T3SS" condition without and with *hilA* overexpression are denoted with a red dash line and a red solid line, respectively.
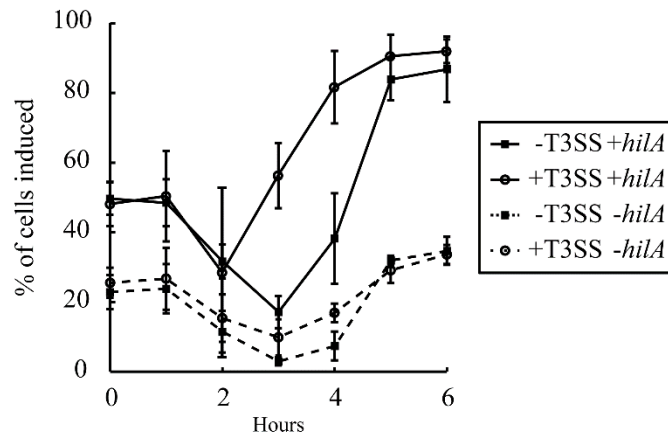
Figure 3.5 Plot of fraction of cells exhibiting P_{sicA} activity from culture with and without *hilA* overexpression in different growth conditions. Cultures carrying the upregulation vector were induced with 100 µM IPTG at the time of subculture and denoted "*+hilA*". Cultures that did not carry the upregulation vector are denoted "*-hilA*". Cultures grown in the "-T3SS" condition without and with *hilA* overexpression are denoted with solid black squares, and a black dash line and a black solid line, respectively. Cultures grown in the "+T3SS" condition without and with *hilA* overexpression are denoted with open black circles, and a red dash line and a red solid line, respectively. The experiment was performed on different days in biological triplicate. Error bars represent one standard deviation.

3.3.3 Secreted protein titer is a function of expression of both the POI and the secretion system

In the previous experiments, the production and secretion of the target protein is coupled through the use of the *sicA* promoter. To probe the dependence of expression timing and level on the protein secretion phenotype, the *tet* promoter, which is controlled by anhydrotetracycline (aTc), was employed to drive the expression of the POI, SptP-DH-2xF-6xH. Thus, expression of the gene encoding the POI is induced orthogonally to SPI-1. First, the secreted protein titer was quantified for the SptP-DH-2xF-6xH fusion protein under P_{tet} control (Figure 3.6A). The highest titer for this genetic construct was 16 ± 6 mg/L in the "-T3SS" condition with *hilA* overexpression. This titer is on the same order as that for the P_{sicA} construct (Figure 3.2B).

A.

T3SS	+	-	+
hilA	-	+	+

$P_{tet} DH$

B.

C.

D.
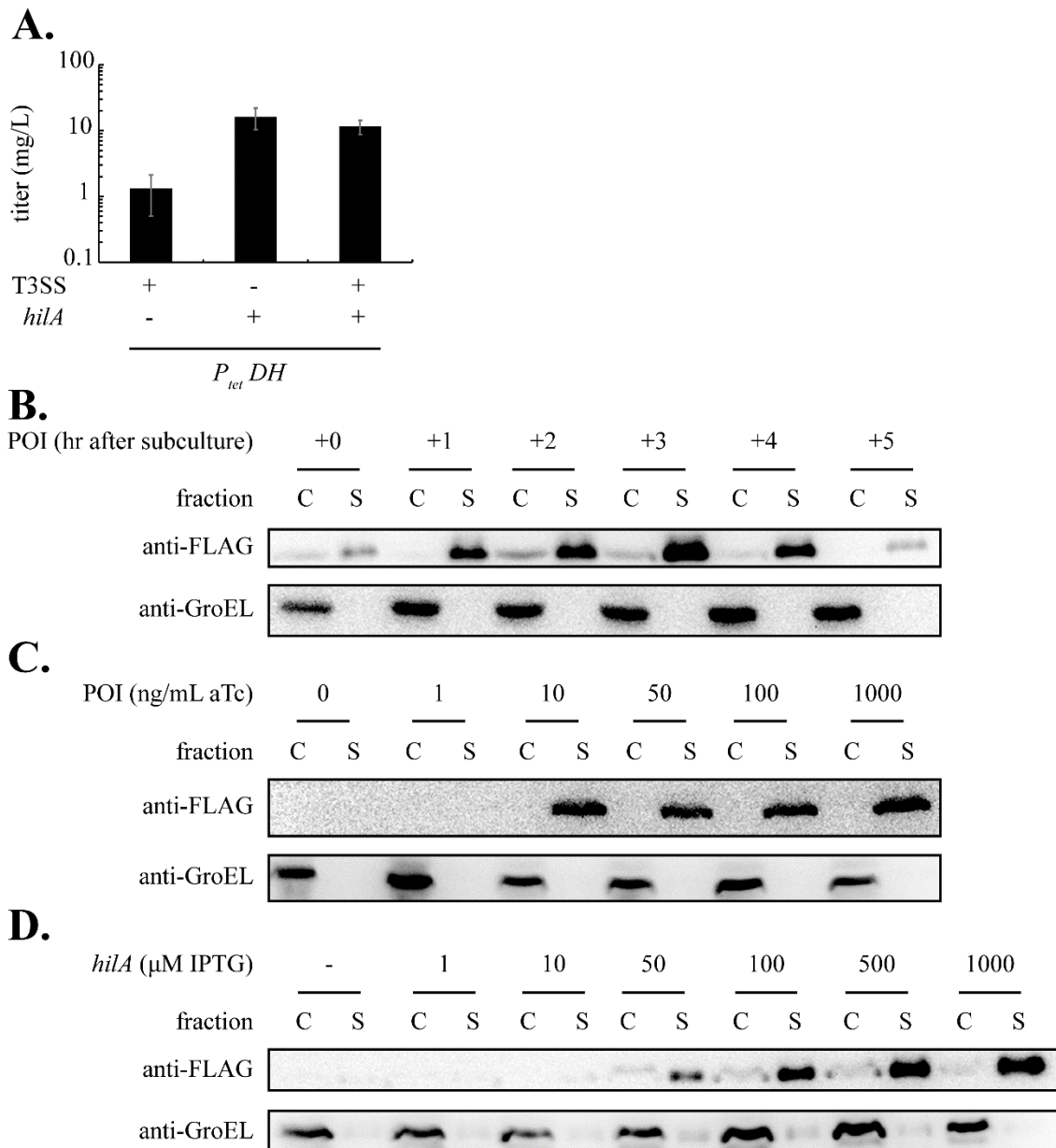
Figure 3.6 Effect of *hilA* overexpression on secreted protein titer when controlling SptP-DH-2xF-6xH and SPI-1 production orthogonally (*from previous page*). **A.** Quantification of secreted protein titer for the P_{tet} *DH* construct using a quantitative western blot. Cultures carrying the upregulation vector were induced with 100 μM IPTG at the time of subculture and denoted "+*hilA*". Cultures that did not carry the upregulation vector are denoted "-*hilA*". The experiment was performed on different days in biological triplicate. Error bars represent one standard deviation. **B-D.** All samples are "-T3SS" growth condition. Samples denoted "C" are soluble cell pellet samples, and "S" are supernatant samples. All samples are loaded equal OD_{600}. **B.** Western blot of culture fractions with varying time of addition of inducer (100 ng/mL aTc) for SptP-DH-2xF-6xH, the protein of interest (POI), relative to subculture and induction of the upregulation vector (100 μM IPTG) at the time of the subculture. **C.** Western blot of culture fractions with varying amount of POI inducer (aTc) added three hours after subculture and induction of the upregulation vector (100 μM IPTG) at the time of the subculture. **D.** Western blot of culture fractions with varying amount of *hilA* inducer (IPTG) added at the time of subculture. POI in this experiment is under P_{sicA} control, which is *hilA*-dependent. The sample denoted "-*hilA*" did not carry the upregulation vector.

To determine where the POI localizes, cultures were fractionated into culture supernatant ("S") and soluble cell pellet ("C") samples and analyzed by western blotting. The inducer aTc was added at various times and concentrations such that the expression timing and level of the POI could be manipulated. Delaying induction of the secreted protein for three hours (OD_{600} ~ 0.6) resulted in the greatest secreted titer, with minimal detectable SptP-DH-2xF-6xH protein remaining in soluble form in the cell, when grown in the "-T3SS" condition (Figure 3.6B). As expected, the overall production of the POI decreased with increasing induction delay (Figure 3.7A). The maximum secreted protein titer for cells grown in the "+T3SS" condition occurred when the POI was induced at or before two hours post-subculture (Figure 3.8A), earlier than in the "-T3SS" condition (Figure 3.6A). This result agrees with the induction dynamics of the *sicA* promoter in the two growth conditions, in which P_{sicA} activity on a per cell basis increases after two hours in the "+T3SS" condition and after three hours in the "-T3SS" condition (Figure 3.5). Thus, maximal secreted protein titer is context-dependent and does not monotonically increase with increasing expression (Figure 3.6 and Figure 3.7).

A.

POI (hr after subculture) +0 +1 +2 +3 +4 +5

anti-FLAG

anti-GroEL

B.

POI (ng/mL aTc) 0 1 10 50 100 1000

anti-FLAG

anti-GroEL

C.

hilA (μM IPTG) - 1 10 50 100 500 1000

anti-FLAG

anti-GroEL
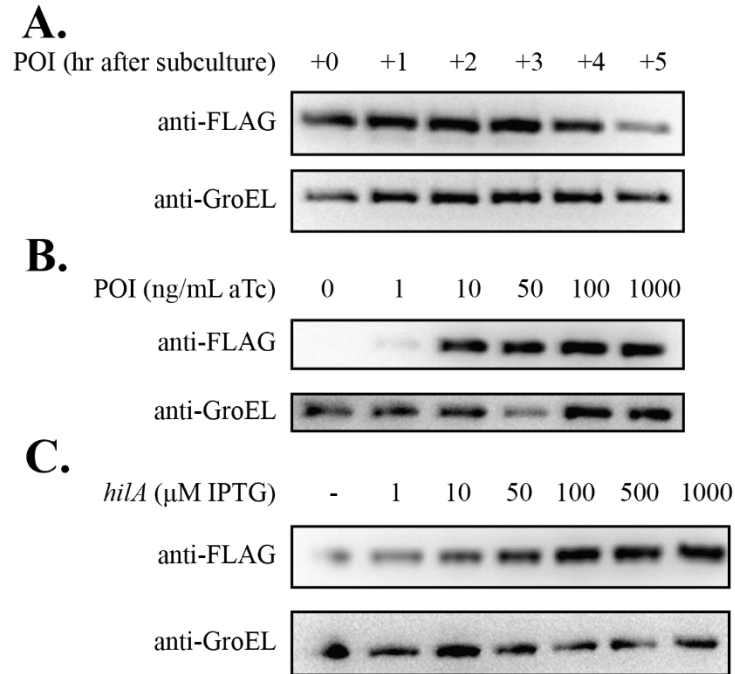
Figure 3.7 Western blot of whole culture lysate samples grown in the "-T3SS" condition. **A.** Effect of timing of POI induction (100 ng/mL aTc), with induction of the upregulation vector (100 μM IPTG) at the time of the subculture. **B.** Effect of POI induction level, with induction of the upregulation vector (100 μM IPTG) at the time of the subculture. **C.** Effect of *hilA* expression from the upregulation vector. POI in this experiment is under P_{sicA} control, which is *hilA*-dependent. The sample denoted "-*hilA*" did not carry the upregulation vector. For all blots, samples were loaded equal OD_{600}.

A. i.

POI (hr after subculture)	+0		+1		+2		+3		+4		+5	
fraction	C	S	C	S	C	S	C	S	C	S	C	S
anti-FLAG												
anti-GroEL												

ii.

whole culture

POI (hr after subculture)	+0	+1	+2	+3	+4	+5
anti-FLAG						
anti-GroEL						

B. i.

POI (ng/mL aTc)	0		1		10		50		100		1000	
fraction	C	S	C	S	C	S	C	S	C	S	C	S
anti-FLAG												
anti-GroEL												

ii.

whole culture

POI (ng/mL aTc)	0	1	10	50	100	1000
anti-FLAG						
anti-GroEL						

C. i.

hilA (μM IPTG)	-		1		10		50		100		500		1000	
fraction	C	S	C	S	C	S	C	S	C	S	C	S	C	S
anti-FLAG														
anti-GroEL														

ii.

whole culture

hilA (μM IPTG)	-	1	10	50	100	500	1000
anti-FLAG							
anti-GroEL							

56

Figure 3.8 Control of *hilA* and POI expression in "+T3SS" growth condition (*from previous page*). All samples are "+T3SS" growth condition. Samples denoted "C" are soluble cell pellet samples, and "S" are supernatant samples. All samples are loaded equal OD_{600}. **A.** Western blots from cultures with varying time of addition of POI inducer (100 ng/mL aTc), relative to subculture and induction of the upregulation vector (100 μM IPTG) at the time of the subculture. **i.** Culture fractions **ii.** Whole culture lysate. **B.** Western blot of cultures with varying amount of POI inducer (aTc) added three hours after subculture and induction of the upregulation vector (100 μM IPTG) at the time of the subculture. **i.** Culture fractions. **ii.** Whole culture lysate. **C.** Western blot of cultures with varying amount of *hilA* inducer (IPTG) added at the time of subculture. POI in this experiment is under P_{sicA} control, which is *hilA*-dependent. The sample denoted "-*hilA*" did not carry the upregulation vector. **i.** Culture fractions. **ii.** Whole culture lysate.

Nonetheless, secretion of the target protein has a dependency on the expression level of the POI (Figure 3.6C). Similar secreted titers were observed for inducer concentrations between 10 and 1000 ng/mL aTc, but overall expression also does not change for aTc concentrations over the same range (Figure 3.7B), indicating that promoter activity may be limiting rather than secretion capacity. The observed dose-dependency of secreted protein titer on aTc concentration is consistently stronger for cultures grown in the "+T3SS" condition (Figure 3.8B) than in the "-T3SS" condition.

The upregulation vector allows for control of SPI-1 expression by controlling *hilA* expression with IPTG. The export plasmid encoding SptP-DH-2xF-6xH under P_{sicA} control was used to measure the effect of *hilA* expression from the upregulation vector on secreted protein titer. The titer increases with increasing IPTG concentration up to 1 mM IPTG (Figure 3.6D). Increasing secreted protein titer with increasing IPTG concentration for the "+T3SS" growth condition was also observed, though the maximum secreted protein titer is greatest in the presence of 100 μM IPTG (Figure 3.8C). The timing of *hilA* overexpression was also modified and examined (Figure 3.9). Addition of IPTG between zero and two hours after subculture resulted in similar secreted titer. Addition of IPTG more than two hours after subculture yielded much lower secretion titers.
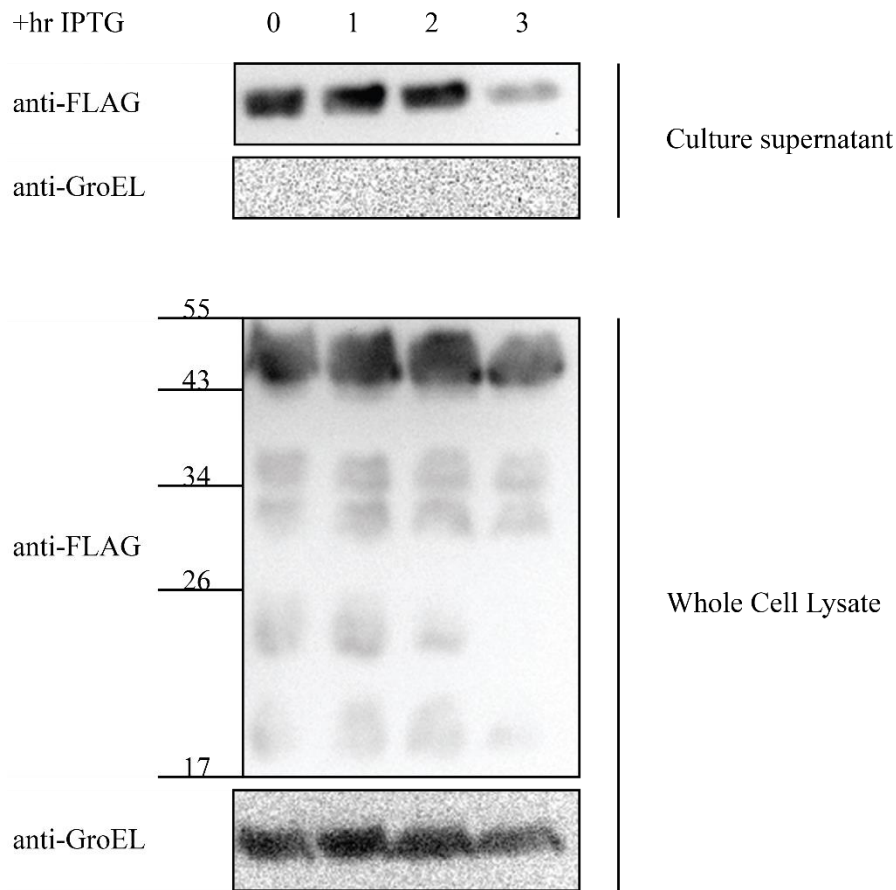
Figure 3.9 Effect of *hilA* induction on secreted protein titer. Strains carrying the upregulation vector and the P$_{sicA}$ *sicP sptP-DH-2xF-6xH* export vector were grown. All samples are "-T3SS" growth condition with *hilA* overexpression (100 µM IPTG) added at different times, with respect to the whole culture lysate. Cultures were fractionated into culture supernatant and whole cell lysates. All samples are loaded equal culture volume, and no significant differences in final cell density was observed for these cultures. These cultures were grown in 30 mL of media in 125 mL shake flasks for 6.25 hours. Ladder shows apparent molecular weight in kDa.

3.3.4 *hilA* overexpression increases the secreted protein titer for diverse classes of proteins and yields an active secreted enzyme

We next set out to test the generality of the *hilA* overexpression effect with respect to the heterologous protein targeted for export. In addition to the model protein, DH, which is a domain from an enzyme of human origin, the impact of HilA production was tested on the secretion of two spider silk proteins (ADF3 and ADF4) and a bacterial enzyme (beta-lactamase, Bla). The export vector was altered to include fusions to the appropriate genes for these proteins, and overexpression of *hilA* indeed results in improvements in secreted protein titer for each protein tested. Secreted protein was not detected by western blotting in the "+T3SS" condition, either with or without *hilA* overexpression (data not shown). Additionally, increased secreted protein titer from *hilA* overexpression is T3SS-dependent, as protein was not detected in the culture supernatant fraction in the *prgI* mutant cells (Figure 3.10). The titer appears to be protein-dependent (Figure 3.11).
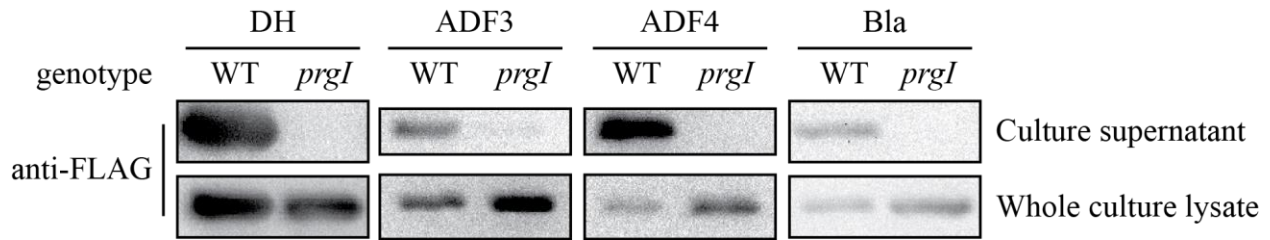
Figure 3.10 Secretion of all proteins tested in WT and *prgI* strains. All samples are "-T3SS" growth condition with *hilA* overexpression (100 μM IPTG). Cultures were fractionated into culture supernatant and whole culture lysates. All samples are loaded equal OD_{600}.
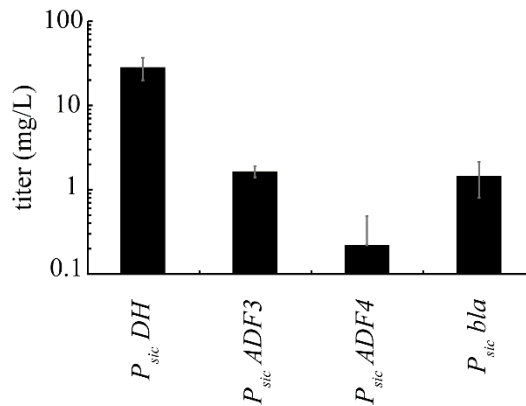
Figure 3.11 Quantification of secreted protein titer for different POIs by quantitative western blotting. Samples were grown in the "-T3SS" condition and with induction of the upregulation vector (100 μM IPTG) at the time of the subculture. The experiment was performed on different days in biological triplicate. Error bars represent one standard deviation.

The culture supernatant of samples expressing the SptP-Bla-2xF-6xH fusion was next probed for enzyme activity as a proxy for protein folding after secretion. Fusions of Bla to the other native effectors SipA and SopD are secreted and active *in vivo* (Raffatellu et al., 2005). Secretion of SptP-Bla-2xF-6xH also results in detectable enzymatic activity in the culture supernatant *in vitro* (Figure 3.12). When SptP-Bla-2xF-6xH is expressed in a *prgI* mutant, which is incapable of SPI-1 secretion (Kimbrough and Miller, 2000), the protein is still expressed (Figure 3.13B), but the protein is not detected in the culture supernatant by western blotting (Figure 3.13A) or by enzyme activity (Figure 3.12). Because secretion of protein from the SPI-1 T3SS requires unfolding of the protein during translocation (Radics et al., 2013), these results suggest that the SptP-Bla-2xF-6xH enzyme refolds after secretion to adopt an active conformation in the culture supernatant post-secretion.
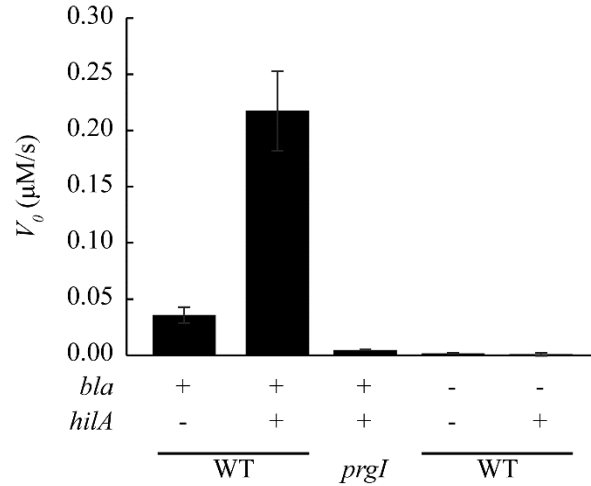
Figure 3.12 Plot of the initial reaction velocity (V_0) for culture supernatant samples. Samples denoted "-*bla*" are cultures carrying the P_{sicA} *gfp* plasmid. Samples were grown in the "-T3SS" condition. Cultures carrying the upregulation vector were induced with 100 µM IPTG at the time of subculture and denoted "+*hilA*". Cultures that did not carry the upregulation vector are denoted "-*hilA*". The experiment was performed on different days in biological triplicate. Error bars represent one standard deviation.
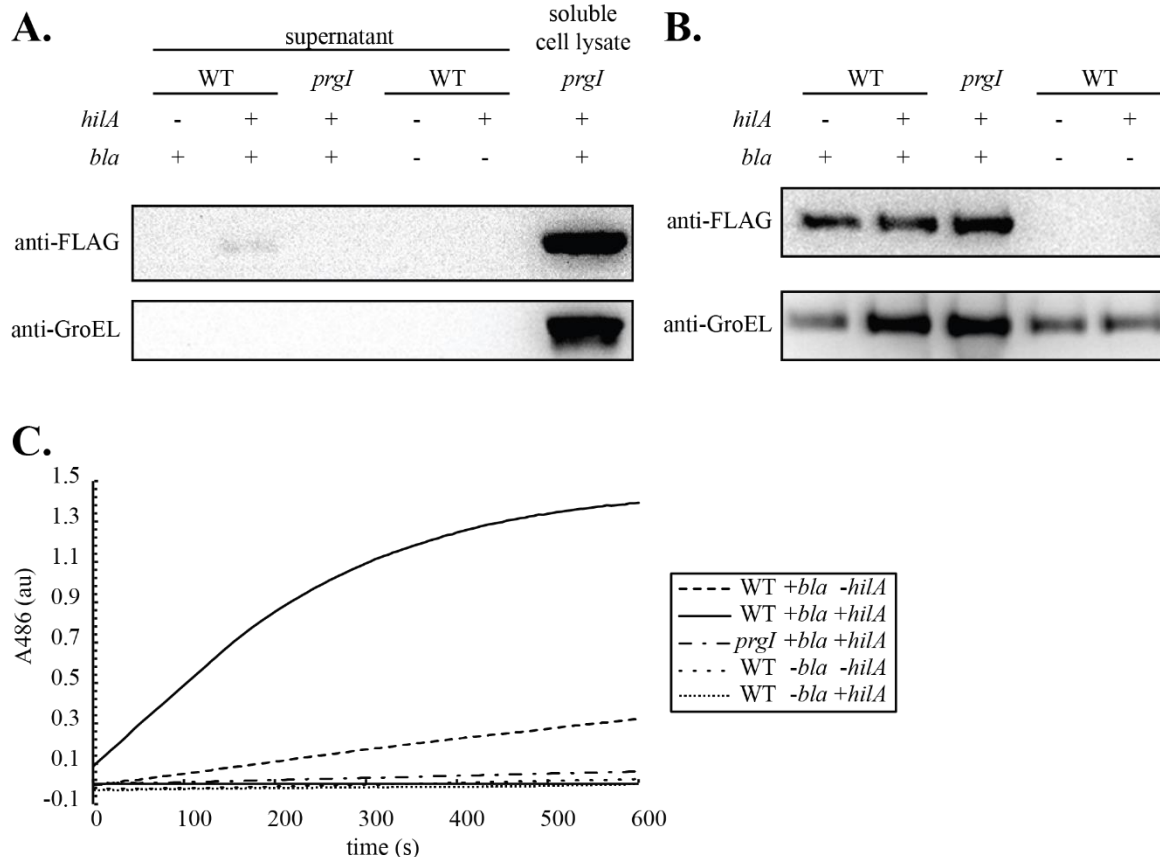
Figure 3.13 Supporting data from activity assay for secreted beta-lactamase as presented in Figure 3.12. Samples denoted "-*bla*" are cultures carrying the P_{sicA} *gfp* plasmid. Samples were grown in the "-T3SS" condition. Cultures carrying the upregulation vector were induced with 100 µM IPTG at the time of subculture and denoted "+*hilA*". Cultures that did not carry the upregulation vector are denoted "-*hilA*". **A.** Western blot of samples analyzed for beta-lactamase activity. Soluble cell lysate sample added as a control for the western. Samples are loaded equal culture volume. **B.** Western blot of whole culture lysate samples. Samples are loaded equal culture volume. **C.** Representative raw activity assay data.

Given the presence of detectable enzyme activity only in cultures containing secretion-capable cells, we reasoned that activity levels may correlate with the concentration of secreted enzyme. Indeed, *hilA* overexpression resulted in an increased initial rate of reaction in the culture supernatant relative to cultures grown in "-T3SS" conditions without *hilA* overexpression. Moreover, in the absence of *hilA* overexpression, enzyme activity is greater than that from cultures of the secretion-deficient *prgI* mutant strain. It should be noted that the level of secretion is too low to detect by western blotting when *hilA* is not overexpressed in "-T3SS" conditions (Figure 3.13A), indicating that the enzymatic activity assay is more sensitive.

3.4 Discussion

Protein secretion to the extracellular space offers numerous advantages over recovery from the cytosol. In particular this strategy minimizes proteolytic degradation and protein aggregation, which are inherently less common in the dilute extracellular space. Moreover, secretion provides

an initial purification event, and thus may simplify downstream sample purification (Choi and Lee, 2004; Georgiou and Segatori, 2005; Wurm, 2004). Previous attempts to secrete proteins with bacteria are not robust for many different types of proteins, in part due to limitations of the secretion machinery (Stader and Silhavy, 1990). Our results demonstrate that the use of the native SPI-1 T3SS of *S. enterica*, coupled with the overproduction of the native transcription factor HilA, results in a level of protein secretion up to 28 mg/L, over 10-fold higher than titers in the absence of *hilA* overexpression in the same strain. Overexpression of *hilA* increases the number of cells that express secretion system genes, the time during which cells expressed these secretion system genes, and the number of secretion systems per cell (Carleton et al., 2013). Further optimization of the secretion system in production culture conditions will likely result in even higher levels of recovered protein in the culture supernatant.

Several differences between cellular phenotypes were observed in the "-T3SS" and "+T3SS" growth conditions. The growth was much slower in the "+T3SS" condition, a phenomenon observed previously (Sturm et al., 2011). This further highlights differences between the two growth conditions as physiologically distinct and supports the use of *hilA* overexpression to induce protein secretion in conditions that are conducive to cell growth, but not to endogenous expression of the SPI-1 T3SS. For these experiments, *hilA* alleles are present both on the genome, which permits induction by environmental conditions (Tartera and Metcalf, 1993), and on the upregulation vector, which permits induction by addition of IPTG. Thus, the "+T3SS" conditions induce greater expression of genomic *hilA*, relative to the "-T3SS" conditions, while *hilA* from the upregulation vector is induced with IPTG in either condition. It is as yet unclear whether the slower growth rate observed in the "+T3SS" condition is important for SPI-1 expression and protein secretion in the native system. By expressing *hilA* from a plasmid, the retarded growth and protein secretion phenotypes are decoupled to achieve high titer secretion of heterologous proteins.

As we and others observe, the native SPI-1 system is very sensitive to many different growth parameters, such as dissolved oxygen, osmolarity, small molecules, and growth phase (Tartera and Metcalf, 1993). By controlling the expression level of *hilA*, the secretion phenotype can be manipulated as desired. It will likely be important to tune both HilA and POI production to minimize protein aggregation and maximize secreted protein titer, particularly considering the propensity of overproduced foreign proteins to aggregate (Schein, 1989). Previous reports on the link between solubility and secretability support this hypothesis (Schein, 1993). In these experiments, we hypothesized that production of a protein before the secretion machinery is secretion-active results in a greater amount of aggregation in the cytosol, which decreases the amount of soluble cellular protein to be secreted. It is important to note that we assume that aggregated protein cannot be secreted and is not solubilized and secreted on the time scales in which our experiments were conducted. Indeed, maximal secretion is observed for the POI under the control of the synthetic *tet* promoter when the inducer molecule, aTc, was added three hours after induction of *hilA* in the "-T3SS" condition (Figure 3.6B), mimicking the dynamics of the endogenous regulation of the *sicA* locus. A mathematical model of *sicA* promoter activity supports the hypothesis that this promoter is only active when cells are secretion-active, such that expression of *sptP* and other effectors occurs only after the needle apparatus is actively secreting protein (Temme et al., 2008). Also, given that the *sicA* promoter activity increases between three and four hours post-induction (Figure 3.5), it logically follows that production of a secreted protein should mimic the timing of the natural system, such that proteins secreted by the SPI-1 T3SS in the native system, termed effectors, are only produced after the secretion system is assembled and active

(Darwin and Miller, 2001; Darwin and Miller, 1999; Widmaier et al., 2009). This strategy would limit production of effectors in non-secretion-active cells and decrease accumulation of effectors in the cytosol of *S. enterica* (Darwin and Miller, 2001; Darwin and Miller, 1999; Widmaier and Voigt, 2010). As observed in Figure 3.11, the effect of *hilA* expression on secreted protein titer is protein-dependent. This may be explained by differences in the gene products. If a protein is more aggregation-prone, then the increase in secreted protein titer from controlled *hilA* expression may not be as large.

We also show that an enzyme, Bla, is secreted and active in the culture media. Secretion by the T3SS requires unfolding and full linearization of the polypeptide (Radics et al., 2013). Thus secreted Bla must refold and adopt an active conformation post-secretion in the culture media. This is somewhat surprising given the differences in macromolecular crowding (macromolecule density in the cytoplasm is ~100-400 mg/mL and the culture media density is ~1 mg/mL) and absence of folding chaperones in the extracellular space (Ellis, 2001; Hingorani and Gierasch, 2014; Moran et al., 2010). It is worth noting that Bla is natively secreted to the periplasm by the general secretory (Sec) pathway (Kadonaga et al., 1984), and the mechanism of the Sec pathway also requires the protein to unfold during translocation and subsequently refold in the periplasm (Driessen et al., 1998). However, the periplasm, like the cytoplasm, is also a very crowded, gel-like environment (Wülfing and Plückthun, 1994b), so refolding of the secreted protein in the periplasm occurs in a very different environment from the extracellular space. Enzyme activity-based detection is specific, sensitive, and robust. The activity assay detected activity above background for the sample without *hilA* overexpression, indicating that the enzymatic activity assay is more sensitive than a western blot. The use of a simple enzyme-based spectrophotometric assay can greatly increase the throughput of *in vitro* secretion experiments.

3.5 Acknowledgements

3.6 References

Ahmad, K. Farid, and Wendell A. Lim. 2010. "The Minimal Autoinhibited Unit of the Guanine Nucleotide Exchange Factor Intersectin." *PLoS ONE* 5 (6): e11291. doi:10.1371/journal.pone.0011291.

Anderson, J. Christopher, John E. Dueber, Mariana Leguia, Gabriel C. Wu, Jonathan A. Goler, Adam P. Arkin, and Jay D. Keasling. 2010. "BglBricks: A Flexible Standard for Biological Part Assembly." *Journal of Biological Engineering* 4 (1): 1–12. doi:10.1186/1754-1611-4-1.

Azam, Anum, Cheng Li, Kevin J. Metcalf, and Danielle Tullman-Ercek. 2015. "Type III Secretion as a Generalizable Strategy for the Production of Full-Length Biopolymer-Forming Proteins." *Biotechnology and Bioengineering*, June. doi:10.1002/bit.25656.

Bajaj, Vivek, Clara Hwang, and Catherine A. Lee. 1995. "*hilA* Is a Novel *ompR/toxR* Family Member That Activates the Expression of *Salmonella* Typhimurium Invasion Genes." *Molecular Microbiology* 18 (4): 715–27. doi:10.1111/j.1365-2958.1995.mmi_18040715.x.

Bajaj, Vivek, Robin L Lucas, Clara Hwang, and Catherine A Lee. 1996. "Co-ordinate Regulation of *Salmonella* Typhimurium Invasion Genes by Environmental and Regulatory Factors Is Mediated by Control of *hilA* Expression." *Molecular Microbiology* 22 (4): 703–14. doi:10.1046/j.1365-2958.1996.d01-1718.x.

Baneyx, François. 1999. "Recombinant Protein Expression in *Escherichia coli*." *Current Opinion in Biotechnology* 10 (5): 411–21. doi:10.1016/S0958-1669(99)00003-8.

Carleton, Heather A., María Lara-Tejero, Xiaoyun Liu, and Jorge E. Galán. 2013. "Engineering the Type III Secretion System in Non-Replicating Bacterial Minicells for Antigen Delivery." *Nature Communications* 4 (March): 1590. doi:10.1038/ncomms2594.

Choi, J H, and S Y Lee. 2004. "Secretory and Extracellular Production of Recombinant Proteins Using *Escherichia coli*." *Applied Microbiology and Biotechnology* 64 (5): 625–35. doi:10.1007/s00253-004-1559-9.

Cormack, Brendan P., Raphael H. Valdivia, and Stanley Falkow. 1996. "FACS-Optimized Mutants of the Green Fluorescent Protein (GFP)." *Gene* 173 (1 Spec No): 33–38.

Cornelis, Guy R. 2006. "The Type III Secretion Injectisome." *Nature Reviews Microbiology* 4 (11): 811–25. doi:10.1038/nrmicro1526.

Darwin, K. Heran, and Virginia L. Miller. 1999. "InvF Is Required for Expression of Genes Encoding Proteins Secreted by the SPI1 Type III Secretion Apparatus in *Salmonella* Typhimurium." *Journal of Bacteriology* 181 (16): 4949–54.

Darwin, K. Heran, and Virginia L. Miller. 2001. "Type III Secretion Chaperone-Dependent Regulation: Activation of Virulence Genes by SicA and InvF in *Salmonella* Typhimurium." *The EMBO Journal* 20 (8): 1850–62. doi:10.1093/emboj/20.8.1850.

Datsenko, Kirill A., and Barry L. Wanner. 2000. "One-Step Inactivation of Chromosomal Genes in *Escherichia coli* K-12 Using PCR Products." *Proceedings of the National Academy of Sciences* 97 (12): 6640–45. doi:10.1073/pnas.120163297.

Desvaux, Mickaël, Michel Hébraud, Ian R. Henderson, and Mark J. Pallen. 2006. "Type III Secretion: What's in a Name?" *Trends in Microbiology* 14 (4): 157–60. doi:10.1016/j.tim.2006.02.009.

Driessen, Arnold J. M., Peter Fekkes, and Jeroen P. W. van der Wolk. 1998. "The Sec System." *Current Opinion in Microbiology* 1 (2): 216–22. doi:10.1016/S1369-5274(98)80014-3.

Eichelberg, Katrin, and Jorge E. Galán. 1999. "Differential Regulation of *Salmonella* Typhimurium Type III Secreted Proteins by Pathogenicity Island 1 (SPI-1)-Encoded Transcriptional Activators InvF and HilA." *Infection and Immunity* 67 (8): 4099–4105.

Ellis, R. John. 2001. "Macromolecular Crowding: Obvious but Underappreciated." *Trends in Biochemical Sciences* 26 (10): 597–604. doi:10.1016/S0968-0004(01)01938-7.

Galán, Jorge E., and Alan Collmer. 1999. "Type III Secretion Machines: Bacterial Devices for Protein Delivery into Host Cells." *Science* 284 (5418): 1322–28. doi:10.1126/science.284.5418.1322.

Gassmann, Max, Beat Grenacher, Bianca Rohde, and Johannes Vogel. 2009. "Quantifying Western Blots: Pitfalls of Densitometry." *ELECTROPHORESIS* 30 (11): 1845–55. doi:10.1002/elps.200800720.

Georgiou, George, and Laura Segatori. 2005. "Preparative Expression of Secreted Proteins in Bacteria: Status Report and Future Prospects." *Current Opinion in Biotechnology* 16 (5): 538–45. doi:10.1016/j.copbio.2005.07.008.

Hingorani, Karan S., and Lila M. Gierasch. 2014. "Comparing Protein Folding in Vitro and in Vivo: Foldability Meets the Fitness Challenge." *Current Opinion in Structural Biology* 24 (February): 81–90. doi:10.1016/j.sbi.2013.11.007.

Hochkoeppler, Alejandro. 2013. "Expanding the Landscape of Recombinant Protein Production in *Escherichia coli*." *Biotechnology Letters* 35 (12): 1971–81. doi:10.1007/s10529-013-1396-y.

Hoiseth, Susan K., and B. A. D. Stocker. 1981. "Aromatic-Dependent *Salmonella* Typhimurium Are Non-Virulent and Effective as Live Vaccines." *Nature* 291 (5812): 238–39. doi:10.1038/291238a0.

Kadonaga, James T., Antoine E. Gautier, Donald R. Straus, Andrew D. Charles, Michael D. Edge, and Jeremy R. Knowles. 1984. "The Role of the Beta-Lactamase Signal Sequence in the Secretion of Proteins by *Escherichia coli*." *Journal of Biological Chemistry* 259 (4): 2149–54.

Kimbrough, Tyler G., and Samuel I. Miller. 2000. "Contribution of *Salmonella* Typhimurium Type III Secretion Components to Needle Complex Formation." *Proceedings of the National Academy of Sciences of the United States of America* 97 (20): 11008–13. doi:10.1073/pnas.200209497.

Laemmli, U. K. 1970. "Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4." *Nature* 227 (5259): 680–85. doi:10.1038/227680a0.

Lee, Catherine A., Bradley D. Jones, and Stanley Falkow. 1992. "Identification of a *Salmonella* Typhimurium Invasion Locus by Selection for Hyperinvasive Mutants." *Proceedings of the National Academy of Sciences* 89 (5): 1847–51.

Lim, Sangyong, Boyoung Lee, Minjeong Kim, Dongho Kim, Hyunjin Yoon, Kyeonghwa Yong, Dong-Hyun Kang, and Sangryeol Ryu. 2012. "Analysis of HilC/D-Dependent *invF* Promoter Expression under Different Culture Conditions." *Microbial Pathogenesis* 52 (6): 359–66. doi:10.1016/j.micpath.2012.03.006.

Lostroh, C. Phoebe, Vivek Bajaj, and Catherine A. Lee. 2000. "The Cis Requirements for Transcriptional Activation by HilA, a Virulence Determinant Encoded on SPI-1." *Molecular Microbiology* 37 (2): 300–315. doi: 10.1046/j.1365-2958.2000.01991.x.

Lostroh, C. Phoebe, and Catherine A. Lee. 2001a. "The HilA Box and Sequences Outside It Determine the Magnitude of HilA-Dependent Activation of P$_{prgH}$ from *Salmonella* Pathogenicity Island 1." *Journal of Bacteriology* 183 (16): 4876–85. doi:10.1128/JB.183.16.4876-4885.2001.

Lostroh, C. Phoebe, and Catherine A. Lee. 2001b. "The *Salmonella* Pathogenicity Island-1 Type III Secretion System." *Microbes and Infection* 3 (14–15): 1281–91. doi:10.1016/S1286-4579(01)01488-5.

Majander, Katariina, Lena Anton, Jenni Antikainen, Hannu Lång, Mirko Brummer, Timo K. Korhonen, and Benita Westerlund-Wikström. 2005. "Extracellular Secretion of Polypeptides Using a Modified *Escherichia coli* Flagellar Secretion Apparatus." *Nature Biotechnology* 23 (4): 475–81. doi:10.1038/nbt1077.

Miroux, Bruno, and John E. Walker. 1996. "Over-Production of Proteins in *Escherichia coli*: Mutant Hosts That Allow Synthesis of Some Membrane Proteins and Globular Proteins at High Levels." *Journal of Molecular Biology* 260 (3): 289–98. doi:10.1006/jmbi.1996.0399.

Moran, Uri, Rob Phillips, and Ron Milo. 2010. "SnapShot: Key Numbers in Biology." *Cell* 141 (7): 1262–1262.e1. doi:10.1016/j.cell.2010.06.019.

Radics, Julia, Lisa Königsmaier, and Thomas C. Marlovits. 2013. "Structure of a Pathogenic Type 3 Secretion System in Action." *Nature Structural & Molecular Biology* 21 (December): 82–87. doi:10.1038/nsmb.2722.

Raffatellu, Manuela, Yao-Hui Sun, R. Paul Wilson, Quynh T. Tran, Daniela Chessa, Helene L. Andrews-Polymenis, Sara D. Lawhon, et al. 2005. "Host Restriction of *Salmonella enterica* Serotype Typhi Is Not Caused by Functional Alteration of SipA, SopB, or SopD." *Infection and Immunity* 73 (12): 7817–26. doi:10.1128/IAI.73.12.7817-7826.2005.

Schein, Catherine H. 1989. "Production of Soluble Recombinant Proteins in Bacteria." *Nature Biotechnology* 7 (11): 1141–49. doi:10.1038/nbt1189-1141.

Schein, Catherine H. 1993. "Solubility and Secretability." *Current Opinion in Biotechnology* 4 (4): 456–61. doi:10.1016/0958-1669(93)90012-L.

Schlumberger, Markus C., Andreas J. Müller, Kristin Ehrbar, Brit Winnen, Iwan Duss, Bärbel Stecher, and Wolf-Dietrich Hardt. 2005. "Real-Time Imaging of Type III Secretion: *Salmonella* SipA Injection into Host Cells." *Proceedings of the National Academy of Sciences of the United States of America* 102 (35): 12548–53. doi:10.1073/pnas.0503407102.

Schneider, Caroline A., Wayne S. Rasband, and Kevin W. Eliceiri. 2012. "NIH Image to ImageJ: 25 Years of Image Analysis." *Nature Methods* 9 (7): 671–75. doi:10.1038/nmeth.2089.

Singer, Hanna M, Marc Erhardt, Andrew M Steiner, Min-Min Zhang, Doju Yoshikami, Grzegorz Bulaj, Baldomero M Olivera, and Kelly T Hughes. 2012. "Selective Purification of Recombinant Neuroactive Peptides Using the Flagellar Type III Secretion System." *mBio* 3 (3). doi:10.1128/mBio.00115-12.

Stader, Joan A., and Thomas J. Silhavy. 1990. "Engineering *Escherichia coli* to Secrete Heterologous Gene Products." *Methods in Enzymology* 185:166–87. doi:10.1016/0076-6879(90)85017-I.

Stebbins, C. Erec, and Jorge E. Galán. 2001. "Maintenance of an Unfolded Polypeptide by a Cognate Chaperone in Bacterial Type III Secretion." *Nature* 414 (6859): 77–81. doi:10.1038/35102073.

Sturm, Alexander, Matthias Heinemann, Markus Arnoldini, Arndt Benecke, Martin Ackermann, Matthias Benz, Jasmine Dormann, and Wolf-Dietrich Hardt. 2011. "The Cost of Virulence: Retarded Growth of *Salmonella* Typhimurium Cells Expressing Type III Secretion System 1." *PLoS Pathog* 7 (7): e1002143. doi:10.1371/journal.ppat.1002143.

Swartz, James R. 2001. "Advances in *Escherichia coli* Production of Therapeutic Proteins." *Current Opinion in Biotechnology* 12 (2): 195–201. doi:10.1016/S0958-1669(00)00199-3.

Tartera, Carmen, and Eleanor S. Metcalf. 1993. "Osmolarity and Growth Phase Overlap in Regulation of *Salmonella* Typhi Adherence to and Invasion of Human Intestinal Cells." *Infection and Immunity* 61 (7): 3084–89.

Temme, Karsten, Howard Salis, Danielle Tullman-Ercek, Anselm Levskaya, Soon-Ho Hong, and Christopher A. Voigt. 2008. "Induction and Relaxation Dynamics of the Regulatory Network Controlling the Type III Secretion System Encoded within *Salmonella* Pathogenicity Island 1." *Journal of Molecular Biology* 377 (1): 47–61. doi:10.1016/j.jmb.2007.12.044.

Terpe, Kay. 2006. "Overview of Bacterial Expression Systems for Heterologous Protein Production: From Molecular and Biochemical Fundamentals to Commercial Systems." *Applied Microbiology and Biotechnology* 72 (2): 211–22. doi:10.1007/s00253-006-0465-8.

Widmaier, Daniel M., Danielle Tullman-Ercek, Ethan A. Mirsky, Rena Hill, Sridhar Govindarajan, Jeremy Minshull, and Christopher A. Voigt. 2009. "Engineering the *Salmonella* Type III Secretion System to Export Spider Silk Monomers." *Molecular Systems Biology* 5 (1): 309. doi:10.1038/msb.2009.62.

Widmaier, Daniel M., and Christopher A. Voigt. 2010. "Quantification of the Physiochemical Constraints on the Export of Spider Silk Proteins by *Salmonella* Type III Secretion." *Microbial Cell Factories* 9 (1): 78. doi:10.1186/1475-2859-9-78.

Wülfing, Christoph, and Andreas Plückthun. 1994. "Protein Folding in the Periplasm of *Escherichia coli*." *Molecular Microbiology* 12 (5): 685–92. doi:10.1111/j.1365-2958.1994.tb01056.x.

Wurm, Florian M. 2004. "Production of Recombinant Protein Therapeutics in Cultivated Mammalian Cells." *Nature Biotechnology* 22 (11): 1393–98. doi:10.1038/nbt1026.

Yeh, Brian J., Robert J. Rutigliano, Anrica Deb, Dafna Bar-Sagi, and Wendell A. Lim. 2007. "Rewiring Cellular Morphology Pathways with Synthetic Guanine Nucleotide Exchange Factors." *Nature* 447 (7144): 596–600. doi:10.1038/nature05851.

CHAPTER 4

APPARATUS ENGINEERING FOR INCREASED SECRETED PROTEIN TITER

4.1 Introduction

Proteins that are secreted by the type III secretion system are secreted by a complex, multi-component protein structure that is both inner- and outer-membrane embedded (Cornelis 2006). This structure is highly regulated and the mechanism of this control is not well understood (Galán et al. 2014). However, the secretion apparatus achieves very high secretion rates when in contact with host cells that is not observed when grown in the absence of host cells (Schlumberger et al. 2005; Enninga et al. 2005). These data suggest that the secretion apparatus is activated for a higher rate of secretion by an unknown mechanism (Galán et al. 2014).

The secretion apparatus itself is thought to play a role in controlling the identity and rate of proteins secreted. The portion of the secretion machine structure that extends outward from the outer membrane into the extracellular space is primarily composed of one protein, PrgI, that self-assembles into a tubular, needle-like structure (Loquet et al. 2012). A cartoon model of PrgI monomer, self-assembled needle, and the whole secretion apparatus is given in Figure 4.1. This needle structure interacts with host cell membrane in the native context of pathogenicity of the type III secretion system. It is thought that the needle regulates secretion activity of the secretion apparatus. In this model, the secretion apparatus is activated via a conformational change in the needle structure upon contact with a host cell membrane (Galán et al. 2014). The order of secretion of different proteins is ordered and hierarchical (Lara-Tejero et al. 2011), and the ability to sense host cell membranes may enable the controlled secretion of effector proteins only at relevant times.
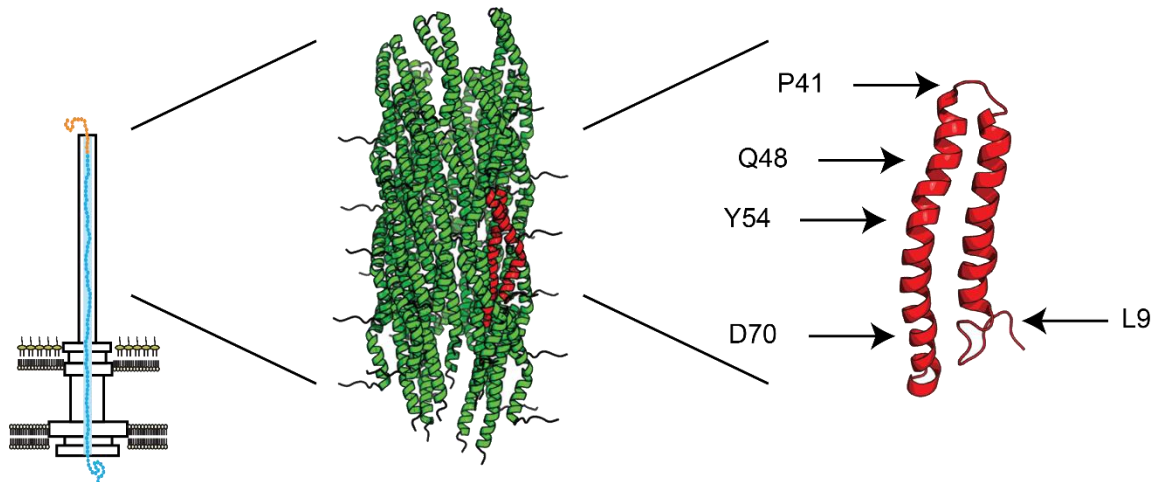
Figure 4.1 Cartoon of secretion machine structure. PDB: 2LPZ and 2KV7. Cartoon on the left modified from Radics, Königsmaier, and Marlovits 2013.

Activation of the secretion apparatus represents a possible mechanism by which bacteria can regulate protein secretion *in vivo*. However, previous work has shown that the type III secretion system is still able to secrete protein, including heterologous proteins, in the absence of host cells (Metcalf et al. 2014; Azam et al. 2015; Widmaier and Voigt 2010; Widmaier et al. 2009; Fu and Galán 1998). The secreted protein titers in these pure cultures do not achieve the secretion rate predicted by direct observation of secretion into host cells (Enninga et al. 2005; Schlumberger et al. 2005). In fact, the secreted protein titer that is experimentally achieved is on the very lower end of the range predicted from experimental values (Section 1.7). These data suggest that the secretion machine is functioning suboptimally in the absence of host cells. However, production of heterologous proteins with bacteria is not cost effective with the addition of host cells. Artificial methods to activate the secretion apparatus in the absence of host cells may increase secreted protein titer.

Activation of the secretion apparatus has been suggested to occur via conformational changes in the needle structure that are induced by interaction of the needle tip with host cell membranes (Barison, Gupta, and Kolbe 2013; Blocker et al. 2008; Galán et al. 2014). Indeed, in the type III secretion system of *Shigella flexneri*, mutations to the gene that codes for the needle protein, *mxiH*, resulted in different secretion phenotypes, such as: constitutive secretion; higher secreted protein titer; and differential secreted protein titer (Kenjale et al. 2005). The MxiH needle has structural and function homology to the PrgI needle of *S. enterica* (Cornelis 2006). I hypothesized that mutations to the gene that codes for the needle protein exist that will increase the titer of secreted heterologous proteins. To test this hypothesis, *prgI* mutations were studied that conferred new secretion phenotypes in the *Shigella* secretion system. In this chapter, I describe several *prgI* mutations that give increased secreted protein titer.

4.2 Materials and Methods

4.2.1 Strains and growth conditions

All *S. enterica* experiments used derivatives of the SL1344 strain (Hoiseth and Stocker 1981). The *prgI* deletion strain was constructed by the methods of Datsenko and Wanner and is described in greater detail in Section 3.2.1. Strains used in this study are listed in Table 4.1.

Table 4.1 List of strains used in this chapter.

Strain name	Comment	Strain ID	Reference
Wild-type	SL1344-derived lab strain	sKJM002	Widmaier et al., 2009
SL1344 *prgI⁻*	Deletion of *prgI*$_{1\text{-}231}$ with pKD13 and FLP-out	sKJM085	Metcalf et al., 2014
SL1344 *prgI*::*cat-sacB*	Recombineered by method of Thomason et al. 2014	sKJM134	This study
SL1344 *prgI*^{L9A}	Recombineered by method of Thomason et al. 2014	EV26	This study
SL1344 *prgI*^{P41A}	Recombineered by method of Thomason et al. 2014	EV13	This study
SL1344 *prgI*^{Q48A}	Recombineered by method of Thomason et al. 2014	EV14	This study
SL1344 *prgI*^{Y54A}	Recombineered by method of Thomason et al. 2014	EV27	This study
SL1344 *prgI*^{D70A}	Recombineered by method of Thomason et al. 2014	EV15	This study
E. coli TUC01	genomic *cat-sacB* cassette	eKJM118	Thomason et al., 2014

All strains carried an upregulation vector (P$_{lacUV5}$ *hilA*) and 100 µM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the growth media at the time of subculture. Electroporation was used for all transformations.

Cell growth was performed in 24-well blocks (Axygen). Cells were grown in an orbital shaker at 37 °C at 225 rpm for 12–16 hr in LB-Lennox with appropriate antibiotics from colonies on LB-Lennox Agar plate of freshly streaked frozen stocks of each strain. Cells were subcultured 1:100 into fresh LB-Lennox with appropriate antibiotics and IPTG and grown in an orbital shaker for 8 hr at 37 °C and 225 rpm. Culture supernatant samples were harvested from the cell culture by two sequential centrifugation steps of 2,272g for 10 minutes. Where indicated, samples were precipitated in 20% trichloroacetic acid (TCA) overnight at 4 °C, washed twice with cold acetone and dried by heating. Laemmli buffer was added to all samples to prepare for separation by SDS-PAGE.

4.2.2 DNA manipulations

The P$_{lacUV5}$ *hilA* upregulation plasmids used in this chapter were both derived from the BglBrick plasmid collection (Anderson et al. 2010). The p15a origin vector is described in Section 3.2.2. The colE1 origin vector was constructed as in Section 3.2.2, but the *hilA* insert was cloned into the *bglII* and *xhoI* restriction sites of the P$_{lacUV5}$ *rfp* vector (pKJM007).

All *prgI* point mutations were performed on a plasmid copy of *prgI* using quickchange mutagenesis. The WT *prgI* gene was amplified from the *S. enterica* str. SL1344 genome using the primers listed in Table 4.2. The amplified *prgI* fragment was cloned into the *bglII* and *bamHI* restriction sites of the P$_{lacUV5}$ *rfp* kan^R colE1 plasmid from the BglBrick collection (Anderson et al. 2010). Then, quickchange mutagenesis was used to mutate specific nucleotides in the *prgI* gene. A list of primers used to perform *prgI* mutations is given in Table 4.3. The *prgI* plasmids created are listed in Table 4.4

Table 4.2 Primers used to amplify WT *prgI* from *S. enterica* genome.

Sequence	Used for the construction of:
FWD: ATTAAGATCTTTAAAGAGGAGAAAGGTCATGGCAACACCTTGGTCAG REV: taatggatccTTAACGGAAGTTCTGAATAATGGCAG	P$_{lacUV5}$ *prgI*

Table 4.3 Primers used to construct *prgI* site-directed mutations using quickchange mutagenesis.

Sequence	Used for the construction of:
FWD: CTTGGTCAGGCTATGCGGATGACGTCTCAG REV: CTGAGACGTCATCCGCATAGCCTGACCAAG	*prgI*^{L9A} mutant
FWD: CAAAACCCTCCGATGCGGCGCTACTGGC REV: GCCAGTAGCGCCGCATCGGAGGGTTTTG	*prgI*^{P41A} mutant
FWD: CTGGCGGCGTATGCGAGTAAGCTCTCG REV: CGAGAGCTTACTCGCATACGCCGCCAG	*prgI*^{Q48A} mutant
FWD: GAGTAAGCTCTCGGAAGCGAACTTGTACCGTAACGC REV: GCGTTACGGTACAAGTTCGCTTCCGAGAGCTTACTC	*prg*^{Y54A} mutant
FWD: CGGTAAAAGTCTTTAAGGCGATTGATGCTGCCATTATTC REV: GAATAATGGCAGCATCAATCGCCTTAAAGACTTTTACCG	*prgI*^{D70A} mutant

Table 4.4 Plasmids used in this chapter.

Plasmid name	ORFs under inducible control	ORI	abR	Plasmid ID	Reference
P_{lacUV5} *hilA*	*hilA*	p15a	kan	pKJM035	1
P_{lacUV5} *hilA*	*hilA*	colE1	kan	pKJM010	This study
P_{sicA} *bla*	*sicP; sptP-bla-2xF-6xH*	colE1	cam	pKJM112	1
P_{sicA} *DH*	*sicP; sptP-DH-2xF-6xH*	colE1	cam	pKJM026	1
P_{lacUV5} *rfp*	*rfp*	colE1	kan	pKJM007	2
P_{lacUV5} *prgI*	*prgI*	colE1	kan	pKJM020	This study
P_{lacUV5} *prgI*^{L9A}	*prgI*^{L9A}	colE1	kan	-	This study
P_{lacUV5} *prgI*^{P41A}	*prgI*^{P41A}	colE1	kan	pKJM056	This study
P_{lacUV5} *prgI*^{Q48A}	*prgI*^{Q48A}	colE1	kan	pKJM051	This study
P_{lacUV5} *prgI*^{Y54A}	*prgI*^{Y54A}	colE1	kan	-	This study
P_{lacUV5} *prgI*^{D70A}	*prgI*^{D70A}	colE1	kan	pKJM052	This study
P_{prgH} *gfpmut2*	*gfpmut2*	colE1	cam	pKJM002	3
P_{lacUV5} *rfp*	*rfp*	p15a	kan	pKJM024	2
P_{prgH} no CDS	-	p15a	kan	pKJM054	This study
P_{prgH} *prgI*	*prgI*	p15a	kan	pKJM071	This study
P_{lacUV5} *prgI*^{P41A}	*prgI*^{P41A}	p15a	kan	pKJM065	This study
P_{lacUV5} *prgI*^{Q48A}	*prgI*^{Q48A}	p15a	kan	pKJM066	This study
P_{lacUV5} *prgI*^{D70A}	*prgI*^{D70A}	p15a	kan	pKJM074	This study
pSIM6	*gam; beta; exo*	pSC101^{ts}	carb	pKJM105	4

Table references:

1. Metcalf, Kevin J., Casey Finnerty, Anum Azam, Elias Valdivia, and Danielle Tullman-Ercek. 2014. "Using Transcriptional Control to Increase Titers of Secreted Heterologous Proteins by the Type III Secretion System." *Applied and Environmental Microbiology* 80 (19): 5927–34. doi:10.1128/AEM.01330-14.

2. Anderson, J. Christopher, John E. Dueber, Mariana Leguia, Gabriel C. Wu, Jonathan A. Goler, Adam P. Arkin, and Jay D. Keasling. 2010. "BglBricks: A Flexible Standard for Biological Part Assembly." *Journal of Biological Engineering* 4 (1): 1–12. doi:10.1186/1754-1611-4-1.

3. Temme, Karsten, Howard Salis, Danielle Tullman-Ercek, Anselm Levskaya, Soon-Ho Hong, and Christopher A Voigt. 2008. "Induction and Relaxation Dynamics of the Regulatory Network Controlling the Type III Secretion System Encoded within Salmonella Pathogenicity Island 1." *Journal of Molecular Biology* 377 (1): 47–61. doi:10.1016/j.jmb.2007.12.044.

4. Datta, Simanti, Nina Costantino, and Donald L. Court. 2006. "A Set of Recombineering Plasmids for Gram-Negative Bacteria." *Gene* 379 (September): 109–15. doi:10.1016/j.gene.2006.04.018.

The P$_{prgH}$ no CDS plasmid (pKJM054) was constructed using the *ecoRI* and *bamHI* restriction sites into a modified BglBrick vector that had the promoter removed via linearization of the backbone and recircularization via the *ecoRI* site (gift from Sergey Boyarskiy, UC, Berkeley). The P$_{prgH}$ *prgI* plasmids were constructed by amplification of each of the *prgI* alleles carried in the P$_{lacUV5}$ vectors listed in Table 4.4 with the primers listed in Table 4.2. The PCR inserts were cloned into the *bglII* and *bamHI* restriction sites of the P$_{prgH}$ no CDS vector.

```
GAATTCaaaagatcttttaagaaggagatatacatatggcgagtagcgaagacgttatcaaagagttcatgcgtttc
aaagttcgtatggaaggttccgttaacggtcacgagttcgaaatcgaaggtgaaggtgaaggtcgtccgtacgaagg
tacccagaccgctaaactgaaagttaccaaaggtggtccgctgccgttcgcttgggacatcctgtccccgcagttcc
agtacggttccaaagcttacgttaaacacccggctgacatcccggactacctgaaactgtccttcccggaaggtttc
aaatgggaacgtgttatgaacttcgaagacggtggtgttgttaccgttacccaggactcctccctgcaagacggtga
gttcatctacaaagttaaactgcgtggtaccaacttcccgtccgacggtccggttatgcagaaaaaaaccatgggtt
gggaagcttccaccgaacgtatgtacccggaagacggtgctctgaaaggtgaaatcaaaatgcgtctgaaactgaaa
gacggtggtcactacgacgctgaagttaaaaccacctacatggctaaaaaaccggttcagctgccgggtgcttacaa
aaccgacatcaaactggacatcacctcccacaacgaagactacaccatcgttgaacagtacgaacgtgctgaaggtc
gtcactccaccggtgcttaaggatccaaactcgagtaaggatctccaggcatcaaataaaacgaaaggctcagtcga
aagactgggcctttcgttttatctgttgtttgtcggtgaacgctctctactagagtcacactggctcaccttcgggt
gggcctttctgcgtttatacctagggatatattccgcttcctcgctcactgactcgctacgctcggtcgttcgactg
cggcgagcggaaatggcttacgaacggggcggagatttcctggaagatgccaggaagatacttaacagggaagtgag
agggccgcggcaaagccgtttttccataggctccgcccccctgacaagcatcacgaaatctgacgctcaaatcagtg
gtggcgaaacccgacaggactataaagataccaggcgtttccccctggcggctccctcgtgcgctctcctgttcctg
cctttcggtttaccggtgtcattccgctgttatggccgcgtttgtctcattccacgcctgacactcagttccgggta
ggcagttcgctccaagctggactgtatgcacgaaccccccgttcagtccgaccgctgcgccttatccggtaactatc
gtcttgagtccaacccggaaagacatgcaaaagcaccactggcagcagccactggtaattgatttagaggagttagt
cttgaagtcatgcgccggttaaggctaaactgaaaggacaagttttggtgactgcgctcctccaagccagttacctc
ggttcaaagagttggtagctcagagaaccttcgaaaaaccgccctgcaaggcggttttttcgttttcagagcaagag
attacgcgcagaccaaaacgatctcaagaagatcatcttattaatcagataaaatatttctagatttcagtgcaatt
tatctcttcaaatgtagcacctgaagtcagccccatacgatataagttgttactagtgcttggattctcaccaataa
aaaacgcccggcggcaaccgagcgttctgaacaaatccagatggagttctgaggtcattactggatctatcaacagg
agtccaagcgagctcgatatcaaattacgccccgccctgccactcatcgcagtactgttgtaattcattaagcattc
tgccgacatggaagccatcacaaacggcatgatgaacctgaatcgccagcggcatcagcaccttgtcgccttgcgta
taatatttgcccatggtgaaaacggggcgaagaagttgtccatattggccacgtttaaatcaaaactggtgaaact
cacccagggattggctgagacgaaaaacatattctcaataaacccttagggaaataggccaggttttcaccgtaac
acgccacatcttgcgaatatatgtgtagaaactgccggaaatcgtcgtggtattcactccagagcgatgaaaacgtt
tcagtttgctcatggaaaacggtgtaacaagggtgaacactatcccatatcaccagctcaccgtctttcattgccat
acgaaattccggatgagcattcatcaggcgggcaagaatgtgaataaaggccggataaaacttgtgcttatttttct
ttacggtctcttaaaaaggccgtaatatccagctgaacggtctggttataggtacattgagcaactgactgaaatgcc
tcaaaatgttctttacgatgccattgggatatatcaacggtggtatatccagtgatttttttctccattttagcttc
cttagctcctgaaaatctcgataactcaaaaaatacgcccggtagtgatcttatttcattatggtgaaagttggaac
ctcttacGTGCCGATCAACGTCTCATTT
```
Figure 4.2 Plasmid sequence for modified BglBrick vector used to construct P$_{prgH}$ *prgI* plasmids.

4.2.3 Recombineering

Recombineering was performed by the methods of Thomason et al. 2014. See the Appendix for a more detailed protocol. Briefly, a strain of *S. enterica* was transformed with the pSIM6 plasmid, which contains the lamba Red recombinase genes and has a temperature-sensitive replicon (Datta, Costantino, and Court 2006). Next, a linear dsDNA insert was prepared by PCR amplification of the genome from the strain *E. coli* TUC01 to create a ~3.5 kb amplicon that was subsequently desalted with a PCR clean-up kit (Promega). This insert was designed to have ~50 bp regions of homology to the desired *S. enterica* locus on the ends of the insert. The interior of the insert contained a sequence for the *cat-sacB* cassette, which allows for positive and negative selection. The primers used to amplify the *cat-sacB* cassette are listed in Table 4.5.

73

Table 4.5 Primers used to amplify the *cat-sacB* cassette from the TUC01 genome.

Sequence	Used for the construction of:
FWD: AGGCCATTGGTATTTCCCAAGCCCACTTTAATTTAACGTAAATAAGGAAG TCATTATCAAAGGGAAAACTGTCCATAT REV: TAACGGCATTCTCAGGGACAATAGTTGCAATCGACATAATCCACCTTATA ACTGATGTGACGGAAGATCACTTCG	*cat-sacB* cassette

   The strain carrying the pSIM6 plasmid was grown to an OD_{600} of 0.4, shifted to 42°C for 15 minutes, and then rapidly cooled in an ice-water bath. The cells were then prepared for electroporation and the strain was transformed with the cleaned-up PCR product. Cells were recovered in SOC media for 1 hr and plated on LB agar plates containing 10 µg/mL chloramphenicol. Colonies were checked by PCR and DNA sequencings for correct genomic modification.

   To replace the *cat-sacB* cassette, the new *prgI* allele was amplified from a plasmid carrying the correct gene. Primers were designed to have ~50 bp regions of homology to the desired *S. enterica* locus. Primers used to amplify the different *prgI* alleles from the plasmids listed in Table 4.4 are listed in Table 4.6. The procedure was performed as above, but electroporated cells were recovered with SOC media and transferred to culture tubes with 10 mL LB-Lennox. Cells were grown for 4 hr and plated on LB agar plates containing 6 % w/w sucrose without NaCl. Colonies were checked by PCR and DNA sequencing for correct genomic modification. Strains created are listed in Table 4.1.

Table 4.6 Primers used to amplify mutant *prgI* alleles from the plasmids listed in Table 4.4.

Sequence	Used for the construction of:
FWD: CCCAAGCCCACTTTAATTTAACGTAAATAAGGAAGTCATTATGGCAACAC CTTGGTCAGG REV: GGACAATAGTTGCAATCGACATAATCCACCTTATAACTGATTAACGGAAG TTCTGAATAATGGC	*prgI* inserts

4.2.4 Protein separation, staining, and western blotting

   Culture samples were separated by SDS-PAGE with homemade 12.5% acrylamide gels. Samples were run with equal culture volumes in the same gel, note that the OD_{600} were similar between all samples. Gels were stained with Sypro Ruby per manufacturer's instructions (Thermo Fisher).

   For western blots, proteins were transferred to a polyvinylidene fluoride (Millipore) using the TransBlot SD unit or the Mini Trans-Blot Cell (Bio-Rad). No difference in transfer was detected between the two methods. Membranes were interrogated with Mouse anti-FLAG antibodies per manufacturer's instructions (Sigma). A secondary labeling step was carried out with Goat anti-Mouse IgG (H+L), HRP conjugate antibodies, per manufacturer's instructions (Thermo). Bands were visualized with west-pico or west-femto chemiluminescent substrate (Thermo) and imaged with a ChemiDoc XRS+ unit (Bio-Rad).

4.3 Results

4.3.1 PrgI is homolog to MxiH

Kenjale, et al. reported new secretion phenotypes for MxiH mutants in *S. flexneri*. MxiH is a PrgI homolog and the alignment of the protein sequences is given in Figure 4.3. PrgI has 65% identity to MxiH, and the C-terminal domain has greater identity than the N-terminal domain. This is not surprising, as the N-terminal domain is more structurally different between these two proteins (Loquet et al. 2012).

```
PrgI     1   MATPWSG---YLDDVSAKFDTGVDNLQTQVTEALDKLAAKPSDPALLAAYQSKLSEYNLY 57
MxiH     1   MSVTVPNDDWTLSSLSETFDDGTQTLQGELTLALDKLAKNPSNPQLLAEYQSKLSEYTLY 60
             *:.. ..    *..:* .** *.:.** ::* ****** :**:* *** ********.**

PrgI    58   RNAQSNTVKVFKDIDAAIIQNFR 80
MxiH    61   RNAQSNTVKVIKDVDAAIIQNFR 83
             **********:**:*********
```

Figure 4.3 Sequence alignment of MxiH of *S. flexneri* str. M90T and PrgI of *S. enterica* str. SL1344. Alignment was performed with the ClustalW2 multiple sequence alignment program (Larkin et al. 2007).

From the study by Kenjale et al., I selected three mutations (Table 4.7) identified to give higher secretion of Ipa and Ipg effectors in uninduced overnight cultures and in congo-red-induced cultures. These point mutations are given in Table 4.7, along with the corresponding mutation in PrgI, as identified from the sequence alignment in Figure 4.3. These mutations would be tested as an initial screen of the effect of PrgI sequence on secreted protein titer.

Table 4.7 Mutations that gave an increased secretion phenotype in *Shigella*, and the corresponding mutation that was achieved in PrgI.

MxiH	PrgI
P44A	P41A
Q51A	Q48A
D73A	D70A

Two further mutations were tested to serve as negative controls. The mutation MxiH^{L12A}, which corresponds to PrgI^{L9A}, resulted in a secretion-deficient strain (Kenjale et al. 2005). The mutation MxiH^{Y57A}, which corresponds to PrgI^{Y54A}, resulted in a secretion-defective mutant due to a defect in needle self-assembly. These two mutations are used to demonstrate the ability to modulate secreted protein titer both above and below wild type levels.

4.3.2 *prgI* does not complement on a plasmid

First, a plasmid-based strategy was attempted. This strategy had precedence; the Kenjale et al. study used a *mxiH*⁻ strain with a plasmid-based *mxiH* mutant allele (Kenjale et al. 2005), and secretion of native effectors has been complemented in a *prgI*⁻ strain with a plasmid-borne *prgI* (Kimbrough and Miller 2000). To replicate the study by Kimbrough and Miller, *prgI* alleles were expressed on a plasmid under the control of P$_{prgH}$, which is the native promoter that controls *prgI* expression (Lostroh and Lee 2001). All strains carried the pKJM010 upregulation vector to enable transcriptional control of SPI-1 genes (Metcalf et al. 2014). Samples were taken 8 hr after

subculture. Culture supernatant samples were separated by SDS-PAGE and the gel was stained with Sypro Ruby to visualize proteins in the gel and is presented in Figure 4.4.
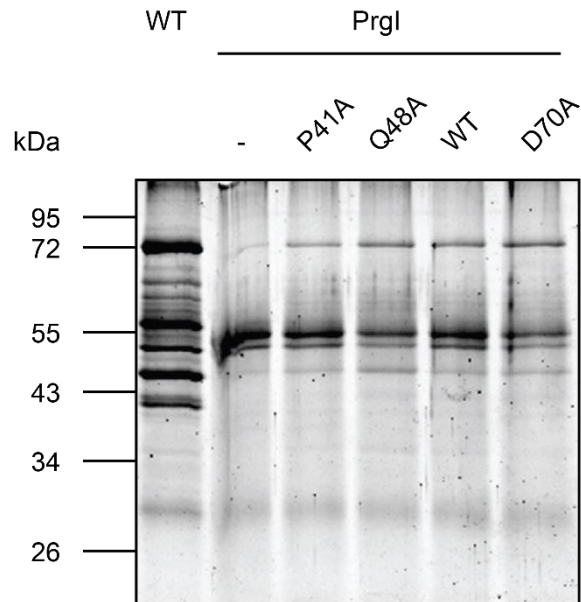
Figure 4.4 Sypro Ruby stained SDS-PAGE gel of culture supernatants. All strains carried the pKJM010 upregulation vector and a P_{prgH} *prgI* plasmid with the corresponding allele. Samples were loaded equal culture volume. Samples were precipitated with trichloroacetic acid and loaded equal culture volume.

The WT sample serves as a positive control. The bands present in this lane are characteristic of native effectors found in the culture supernatant. The band at 72 kDa can be assigned to SipA, a native effector of the SPI-1 T3SS (Kimbrough and Miller 2000; Widmaier et al. 2009). The second lane comes from a culture that did not carry a *prgI* gene and serves as a negative control, as this genotype is secretion-deficient (Kimbrough and Miller 2000). All complemented strains had similar banding patterns to the negative control. This indicates that the plasmid-based system was not able to complement protein secretion of native effectors in my system.

4.3.3 Mutation of genomic *prgI* gives increased secreted protein titer
Given the inability to complement the *prgI* deletion strain using a plasmid carrying *prgI*, a recombineering-based approach was taken (Datta, Costantino, and Court 2006). Here, the genomic copy of *prgI* was exchanged for a new allele in a scarless and markerless fashion. This procedure proceeded in two steps, where the *prgI* gene was first replaced with a two-component selectable marker allowing for both positive and negative selections, the *cat-sacB* cassette. Next, the new *prgI* allele was recombined into the *prgI* locus, removing the *cat-sacB* cassette in the process. Verification of allelic replacement was performed by amplification of the locus and Sanger sequencing of the amplicon.

The effect of *prgI* allele on secreted protein titer was tested by western blot. Note that all cultures carried the export plasmid and the upregulation plasmid (pKJM035). First, the test protein DH, a domain of the human protein intersectin (Section 3.3.1), was tested. The production of this protein in the whole culture lysate was similar in the strains carrying different *prgI* alleles (Figure

4.5). This indicates that expression of the DH fusion protein does not vary with *prgI* allele. However, the culture supernatant shows a higher signal in the PrgI^{P41A} and PrgI^{Q48A} mutants. This indicates that the secreted protein titer is greater in these mutant strains, relative to wild-type *prgI*. The PrgI^{D70A} and PrgI^{Y54A} mutants did not give secreted protein titer different from the WT strain. No secretion of the DH fusion was detected in the PrgI^{L9A} mutant.
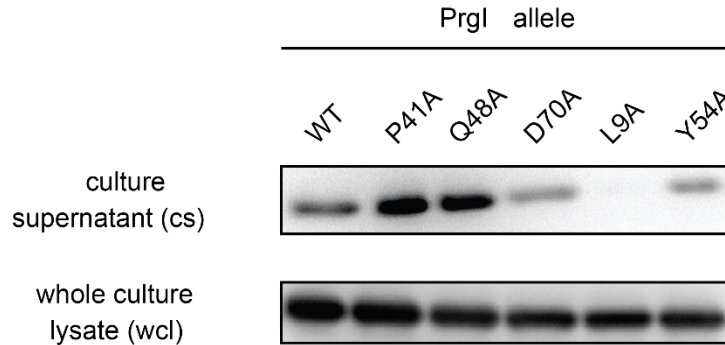
Figure 4.5 Western blot of culture supernatant and whole culture lysate samples from cultures producing DH fusion.

The effect of *prgI* allele on beta-lactamase (Bla) secretion was determined to test for the generality of the effect on secretion. This protein is of bacterial origin and is structurally and phylogenetically distinct from the DH protein. Indeed, increased secreted protein titer is seen for the Bla fusion with the PrgI^{P41A} and PrgI^{Q48A} mutants (Figure 4.6). Again, this effect is independent of protein production, as the amount of fusion protein in the whole culture lysate is similar for all *prgI* mutants tested. The trend of the effect of PrgI mutants on secreted titer held for Bla fusion, as the PrgI^{D70A} and PrgI^{Y54A} mutants showed secreted protein titers similar to WT, and no secreted protein was observed for the PrgI^{L9A} mutant.
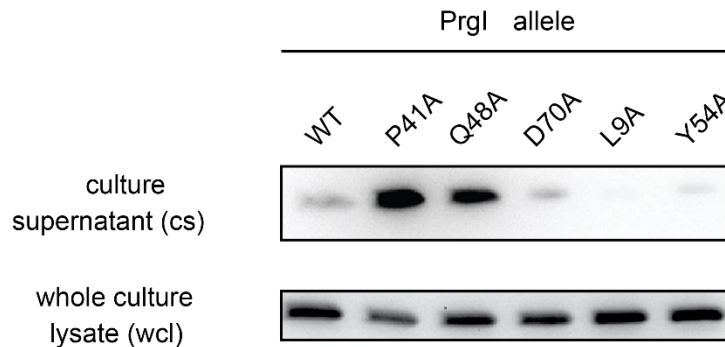
Figure 4.6 Western blot of culture supernatant and whole culture lysate samples from cultures producing Bla fusion.

4.4 Discussion

Increased protein secretion was observed in PrgI^{P41A} and PrgI^{Q48A} mutants (Figure 4.5 and Figure 4.6). Similar production of the protein of interest indicates that *prgI* mutations did not change protein production, but instead changed protein secretion. This effect was found with the

secretion of both DH and Bla fusions, indicating that the effect may be general. Further, the PrgI^{L9A} mutation resulted in undetectable secretion, indicating that mutation to the *prgI* gene can result in both increased and decreased secreted protein titer, relative to the wild type.

The effect of increased secreted protein titer from mutations to *prgI* may result from two mechanisms: 1) increased duration in a secretion-active state; and 2) increased effective secretion rate. These two mechanisms are not necessarily mutually exclusive, as the secretion-active state may simply be a state where the secretion apparatus increases the effective secretion rate. However, below I consider these two effects as distinct, but potentially related mechanisms. Importantly, I rule out changes in protein production as explaining the increased secreted protein effect, as there was no differences observed in overall production of the protein of interest in the culture (Figure 4.5 and Figure 4.6).

Increased duration in a secretion-active state would increase the secreted protein titer due to a longer amount of time spent actively secreting protein. To simplify the discussion here, I consider a three-state system: 1) no secretion apparatus present; 2) low-secretion state, apparatus present; and 3) high-secretion state, apparatus present (Section 1.6.1). In state 1, no secretion occurs because the machinery that is necessary for secretion is not present. This may be the situation at early times in the culture, before SPI-1 expression. State 2 may represent a basal secretion rate, and this is the expected condition in the absence of host cells. When the secretion apparatus is activated, it enters state 3, which is a highly active secretion state. If a *prgI* mutant were to increase the duration in a secretion-active state, it would increase the proportion of time spent in state 3. To test this hypothesis, one will likely need to be able to differentiate between states 2 and 3. To date, no method exists to differentiate different secretion states. However, the secretion apparatus is activated for secretion by contact with host cells (Zierler and Galán 1995) and controls the sequential secretion order of several different natively secreted proteins (Lara-Tejero et al. 2011). These data suggest maturation of the secretion apparatus and distinct functional states. A potential indicator of distinct functional states is the maturation of the needle tip through the addition of translocases that mediate attachment to host cells. The presence of the early needle tip protein, SipD, and the later needle tip proteins, SipB and SipC, has been detected on live cells using immunofluorescence microscopy (Lara-Tejero and Galán 2009). The presence of SipB and SipC on the tip of the needle domain of the secretion apparatus may be an indicator of a high-secretion state. However, this phenotype does not necessarily indicate a difference in functional secretion states and would need further evidence to support this hypothesis.

The effective rate of secretion depends on the individual steps of secretion. Secretion is known to initiate on the cytosolic side of the inner membrane via an unfolding event that is catalyzed by the InvC unfoldase (Eichelberg, Ginocchio, and Galán 1994). The N-terminus of the unfolded protein enters the lumen of the secretion apparatus first and the protein is translocated to the extracellular space, where the unfolded protein is ejected. The energy that powers this chain of events is not clear; ATP hydrolysis drives protein unfolding, but it is not known the energy that drives unfolded protein translocation through the secretion apparatus (Galán et al. 2014; Barison, Gupta, and Kolbe 2013; Lee and Rietsch 2015). Further, it is not clear how changes in the PrgI sequence could result in differences in secretion rate, beyond activation of the needle to a high-secretion state. Thus, I propose that mutations to PrgI modulate secretion by changing the activation of the apparatus to a high-secretion state, and is not due to changing the intrinsic secretion rate in this state.

A recombineering-based approach was taken to introduce *prgI* mutations *in situ*. This was done due to the inability to complement the *prgI* deletion strain with a plasmid-borne *prgI* (Figure 4.4). The ability to complement a *prgI* deletion strain has been reported previously (Kimbrough and Miller 2000), so I discuss here differences between that study and mine that could explain this discrepancy. I created a *prgI* deletion strain using the methods of Datsenko and Wanner (Datsenko and Wanner 2000). This strain was created with the pKD13-derived insert, which may introduce a polar effect (Datsenko and Wanner 2000). Note that the *prgI* gene is the second gene from the 5' end in the seven-gene *prg* operon (Lostroh and Lee 2001). In the study by Kimborough and Miller, an in-frame deletion of *prgI* was achieved by allelic exchange. Further, they showed complementation of *prgI* in *prgI* deletion strains under the control of both P_{prgH} and P_{BAD} (Kimbrough and Miller 2000). However, the phenotype that was complemented in that study was secretion of native effectors without *hilA* overexpression. This is an important distinction, as I did not seek to replicate the Kimborough and Miller study exactly. For the purpose of production and secretion of heterologous proteins, culturing conditions and growth strategies are used to maximize product yield.

The results in this chapter present a strategy for genome engineering of a bacterial strain for increased secreted protein titer. The method described here is complementary to and distinct from other approaches to engineer genomic DNA for improved phenotypes (Wang et al. 2009). This recombineering strategy does not require inactivation of methyl-directed mismatch repair or introduction of silent mutations. Further, longer sequences of DNA can be recombined when using dsDNA. ssDNA-based strategies experience an upper limit of ~90 nucleotides per strand for efficient recombination (Sawitzke et al. 2011; Wang et al. 2009). This strategy may be an efficient method for the introduction of libraries of a given allele site-specifically, which has been shown to be an efficient strategy in *Saccharomyces cerevisiae* (Ryan et al. 2014).

4.5 Acknowledgements

4.6 References

Anderson, J. Christopher, John E. Dueber, Mariana Leguia, Gabriel C. Wu, Jonathan A. Goler, Adam P. Arkin, and Jay D. Keasling. 2010. "BglBricks: A Flexible Standard for Biological Part Assembly." *Journal of Biological Engineering* 4 (1): 1–12. doi:10.1186/1754-1611-4-1.

Azam, Anum, Cheng Li, Kevin J. Metcalf, and Danielle Tullman-Ercek. 2015. "Type III Secretion as a Generalizable Strategy for the Production of Full-Length Biopolymer-Forming Proteins." *Biotechnology and Bioengineering*, June. doi:10.1002/bit.25656.

Barison, Nicola, Rashmi Gupta, and Michael Kolbe. 2013. "A Sophisticated Multi-Step Secretion Mechanism: How the Type 3 Secretion System Is Regulated." *Cellular Microbiology*. doi:10.1111/cmi.12178.

Blocker, Ariel J., Janet E. Deane, Andreas K. J. Veenendaal, Pietro Roversi, Julie L. Hodgkinson, Steven Johnson, and Susan M. Lea. 2008. "What's the Point of the Type III Secretion System Needle?" *Proceedings of the National Academy of Sciences* 105 (18): 6507–13. doi:10.1073/pnas.0708344105.

Cornelis, Guy R. 2006. "The Type III Secretion Injectisome." *Nature Reviews Microbiology* 4 (11): 811–25. doi:10.1038/nrmicro1526.

Datsenko, Kirill A., and Barry L. Wanner. 2000. "One-Step Inactivation of Chromosomal Genes in *Escherichia coli* K-12 Using PCR Products." *Proceedings of the National Academy of Sciences* 97 (12): 6640–45. doi:10.1073/pnas.120163297.

Datta, Simanti, Nina Costantino, and Donald L. Court. 2006. "A Set of Recombineering Plasmids for Gram-Negative Bacteria." *Gene* 379 (September): 109–15. doi:10.1016/j.gene.2006.04.018.

Eichelberg, Katrin, Christine C. Ginocchio, and Jorge E. Galán. 1994. "Molecular and Functional Characterization of the *Salmonella* Typhimurium Invasion Genes *invB* and *invC*: Homology of InvC to the F_0F_1 ATPase Family of Proteins." *Journal of Bacteriology* 176 (15): 4501–10.

Enninga, Jost, Joëlle Mounier, Philippe Sansonetti, and Guy Tran Van Nhieu. 2005. "Secretion of Type III Effectors into Host Cells in Real Time." *Nature Methods* 2 (12): 959–65. doi:10.1038/nmeth804.

Fu, Yixin, and Jorge E. Galán. 1998. "Identification of a Specific Chaperone for SptP, a Substrate of the Centisome 63 Type III Secretion System of *Salmonella* Typhimurium." *Journal of Bacteriology* 180 (13): 3393–99.

Galán, Jorge E., Maria Lara-Tejero, Thomas C. Marlovits, and Samuel Wagner. 2014. "Bacterial Type III Secretion Systems: Specialized Nanomachines for Protein Delivery into Target Cells." *Annual Review of Microbiology* 68 (1): 415–38. doi:10.1146/annurev-micro-092412-155725.

Hoiseth, Susan K., and B. A. D. Stocker. 1981. "Aromatic-Dependent *Salmonella* Typhimurium Are Non-Virulent and Effective as Live Vaccines." *Nature* 291 (5812): 238–39. doi:10.1038/291238a0.

Kenjale, Roma, Justin Wilson, Sebastian F. Zenk, Saroj Saurya, Wendy L. Picking, William D. Picking, and Ariel Blocker. 2005. "The Needle Component of the Type III Secreton of *Shigella* Regulates the Activity of the Secretion Apparatus." *The Journal of Biological Chemistry* 280 (52): 42929–37. doi:10.1074/jbc.M508377200.

Kimbrough, Tyler G., and Samuel I. Miller. 2000. "Contribution of *Salmonella* Typhimurium Type III Secretion Components to Needle Complex Formation." *Proceedings of the National Academy of Sciences of the United States of America* 97 (20): 11008–13. doi: 10.1073/pnas.200209497.

Lara-Tejero, María, and Jorge E. Galán. 2009. "*Salmonella enterica* Serovar Typhimurium Pathogenicity Island 1-Encoded Type III Secretion System Translocases Mediate Intimate Attachment to Nonphagocytic Cells." *Infection and Immunity* 77 (7): 2635–42. doi:10.1128/IAI.00077-09.

Lara-Tejero, María, Junya Kato, Samuel Wagner, Xiaoyun Liu, and Jorge E. Galán. 2011. "A Sorting Platform Determines the Order of Protein Secretion in Bacterial Type III Systems." *Science* 331 (6021): 1188–91. doi:10.1126/science.1201476.

Larkin, M. A., G. Blackshields, N. P. Brown, R. Chenna, P. A. McGettigan, H. McWilliam, F. Valentin, I. M. Wallace, A. Wilm, R. Lopez, J. D. Thompson, T. J. Gibson, and D. G.

Higgins. 2007. "Clustal W and Clustal X Version 2.0." *Bioinformatics* 23 (21): 2947–48. doi:10.1093/bioinformatics/btm404.

Lee, Pei-Chung, and Arne Rietsch. 2015. "Fueling Type III Secretion." *Trends in Microbiology*. Accessed March 28. doi:10.1016/j.tim.2015.01.012.

Loquet, Antoine, Nikolaos G. Sgourakis, Rashmi Gupta, Karin Giller, Dietmar Riedel, Christian Goosmann, Christian Griesinger, Michael Kolbe, David Baker, Stefan Becker, and Adam Lange. 2012. "Atomic Model of the Type III Secretion System Needle." *Nature*. doi:10.1038/nature11079.

Lostroh, C. Phoebe, and Catherine A. Lee. 2001. "The *Salmonella* Pathogenicity Island-1 Type III Secretion System." *Microbes and Infection* 3 (14–15): 1281–91. doi:10.1016/S1286-4579(01)01488-5.

Metcalf, Kevin J., Casey Finnerty, Anum Azam, Elias Valdivia, and Danielle Tullman-Ercek. 2014. "Using Transcriptional Control To Increase Titers of Secreted Heterologous Proteins by the Type III Secretion System." *Applied and Environmental Microbiology* 80 (19): 5927–34. doi:10.1128/AEM.01330-14.

Radics, Julia, Lisa Königsmaier, and Thomas C. Marlovits. 2013. "Structure of a Pathogenic Type 3 Secretion System in Action." *Nature Structural & Molecular Biology* 21 (December): 82–87. doi:10.1038/nsmb.2722.

Ryan, Owen W., Jeffrey M. Skerker, Matthew J. Maurer, Xin Li, Jordan C. Tsai, Snigdha Poddar, Michael E. Lee, et al. 2014. "Selection of Chromosomal DNA Libraries Using a Multiplex CRISPR System." *eLife* 3 (September): e03703. doi:10.7554/eLife.03703.

Sawitzke, James A., Nina Costantino, Xin-tian Li, Lynn C. Thomason, Mikhail Bubunenko, Carolyn Court, and Donald L. Court. 2011. "Probing Cellular Processes with Oligo-Mediated Recombination and Using the Knowledge Gained to Optimize Recombineering." *Journal of Molecular Biology* 407 (1): 45–59. doi:10.1016/j.jmb.2011.01.030.

Schlumberger, Markus C., Andreas J. Müller, Kristin Ehrbar, Brit Winnen, Iwan Duss, Bärbel Stecher, and Wolf-Dietrich Hardt. 2005. "Real-Time Imaging of Type III Secretion: *Salmonella* SipA Injection into Host Cells." *Proceedings of the National Academy of Sciences of the United States of America* 102 (35): 12548–53. doi:10.1073/pnas.0503407102.

Thomason, Lynn C., James A. Sawitzke, Xintian Li, Nina Costantino, and Donald L. Court. 2014. "Recombineering: Genetic Engineering in Bacteria Using Homologous Recombination." In *Current Protocols in Molecular Biology*. John Wiley & Sons, Inc. doi: 10.1002/0471142727.mb0116s106.

Wang, Harris H., Farren J. Isaacs, Peter A. Carr, Zachary Z. Sun, George Xu, Craig R. Forest, and George M. Church. 2009. "Programming Cells by Multiplex Genome Engineering and Accelerated Evolution." *Nature* 460 (7257): 894–98. doi:10.1038/nature08187.

Widmaier, Daniel M., Danielle Tullman-Ercek, Ethan A. Mirsky, Rena Hill, Sridhar Govindarajan, Jeremy Minshull, and Christopher A. Voigt. 2009. "Engineering the *Salmonella* Type III Secretion System to Export Spider Silk Monomers." *Molecular Systems Biology* 5 (1): 309. doi:10.1038/msb.2009.62.

Widmaier, Daniel M., and Christopher A. Voigt. 2010. "Quantification of the Physiochemical Constraints on the Export of Spider Silk Proteins by *Salmonella* Type III Secretion." *Microbial Cell Factories* 9 (1): 78. doi:10.1186/1475-2859-9-78.

Zierler, Michael K., and Jorge E. Galán. 1995. "Contact with Cultured Epithelial Cells Stimulates Secretion of *Salmonella* Typhimurium Invasion Protein InvJ." *Infection and Immunity* 63 (10): 4024–28.

CHAPTER 5

PROTEIN FOLDING AFTER TYPE III SECRETION

5.1 Introduction

Heterologous protein production is used to make protein products, such as therapeutics and industrial enzymes, and enables researchers to study proteins that would otherwise be difficult to isolate from their native source. In order for a protein to perform its function, the protein must adopt a three dimensional structure that allows for proper function. When producing a heterologous protein, it is desired to maximize both product titer and proper folding of the protein of interest. Secretion of heterologous proteins to the extracellular space holds several advantages over intracellular production: proteins accumulate outside the cell, limiting cytotoxicity associated with intracellular accumulation; secretion serves as a first step of purification, as the cell selectively secretes proteins to the extracellular space; and lysis of the production organism is not required, enabling continuous protein production (Georgiou and Segatori 2005; Stader and Silhavy 1990). These advantages are apparent in the use of secretion in protein production processes that use eukaryotic host organisms with a native propensity to secrete heterologous proteins, such as *Saccharomyces cerevisiae* and Chinese hamster ovary cells.

The type III secretion system (T3SS) is a protein secretion machine found in Gram-negative pathogenic bacteria. This multimeric heteroprotein structure is characterized by a long passageway that is 2-3 nm in internal diameter, termed the needle (Cornelis 2006). Given the diameter of a typical folded protein, considerable unfolding of the protein is required in order to fit through the needle. It is hypothesized that only secondary structures could exist in the secreted protein during translocation. Indeed, cryo-electron microscopy of secretion suggests that proteins are fully linearized before being ejected into the extracellular space (Radics, Königsmaier, and Marlovits 2013). Proteins secreted by a T3SS have been previously shown to adopt a native conformation after secretion, both in the extracellular space and when delivered to the cytoplasm of a neighboring cell (Metcalf et al. 2014; Derouazi et al. 2008; Majander et al. 2005).

The constraints of this system present a unique condition for protein folding. Proteins are secreted by the T3SS at a rate of 10^3-10^4 amino acids per second (Singer et al. 2012; Schlumberger et al. 2005) (about 1-10 proteins per second) and must be unfolded in order to pass through the T3SS (Radics, Königsmaier, and Marlovits 2013). Thus, proteins are released rapidly into the extracellular space in an unfolded and extended confirmation, in contrast the mechanism of co-translational folding. Additionally, the extracellular space has a much lower macromolecule concentration compared to inside the cell (Hingorani and Gierasch 2014). As a result, protein folding post-secretion may resemble in vitro refolding in dilute solution. By capitalizing on this feature of protein folding and coupling production with secretion, this T3SS-based approach may hold advantages over industrial approaches that are based on inclusion body formation that requires a separate refolding step. (Clark 2001).

In this study, we tested the biochemical requirements for protein function to understand protein folding following secretion by the T3SS. We used protein function (e.g., enzymatic activity or antigen binding) as a proxy for folding. We investigated the ability two enzymes (beta-lactamase and alkaline phosphatase) and one single-chain variable fragment (scFv) of an antibody to adopt an active conformation after secretion. We found in all cases that protein secretion to the extracellular space allows the production of functional, correctly folded protein product. Moreover, we found that the concentration of sodium chloride in the culture medium could affect both secreted protein titer and the fraction of secreted proteins that are correctly folded, allowing for simultaneous optimization of both protein titer and folding.

5.2 Methods

5.2.1 Strains and growth conditions

All *S. enterica* experiments used derivatives of the SL1344 strain (Hoiseth and Stocker 1981). The *prgI* deletion strain was described by Metcalf et al. 2014. All strains were grown from colonies from fresh transformations or fresh streaks from frozen stock in lysogeny broth (LB-Lennox, LB-L) (10 g/L tryptone, 5 g/L yeast extract, and 5 g/L NaCl) (VWR #EM1.00547.5007) with appropriate antibiotics (34 µg/mL chloramphenicol and/or 50 µg/mL kanamycin) for 12-16 hr at 37 °C and 225 rpm in an orbital shaker overnight. Overnight cultures were subcultured into fresh LB-L media supplemented with 100 µM isopropyl β-D-1-thiogalactopyranoside (IPTG) and the appropriate antibiotics. All culturing steps were performed in 24-well blocks (Axygen).

S. enterica strains were transformed with the plasmids listed in Table 5.1 using electroporation. All experiments were performed using a two-plasmid system, with the upregulation vector (P_{lacUV5} *hilA*), as reported by Metcalf et al. 2014, in addition to the export vector that carried the gene coding for the protein of interest (Widmaier et al. 2009). Controlled overexpression of *hilA* allows for controlled expression of genes coding for both the secretion apparatus and the protein of interest (Metcalf et al. 2014).

Table 5.1 Plasmids used in this study.

Plamid name	ORFs under inducible control	ORI	ab^R	Plasmid ID	Reference
P$_{lacUV5}$ *hilA*	*hilA*	p15a	kan	pKJM035	Metcalf et al, 2014
P$_{sicA}$ *bla*	*sicP; sptP-bla-2xF-6xH*	colE1	cam	pKJM112	Metcalf et al, 2014
P$_{sicA}$ *bla^{ST71TS}*	*sicP; sptP-bla^{ST71TS}-2xF-6xH*	colE1	cam	pKJM129	This study
P$_{sicA}$ *bla^{C75S}*	*sicP; sptP-bla^{C75S}-2xF-6xH*	colE1	cam	pKJM142	This study
P$_{sicA}$ *bla^{C121S}*	*sicP; sptP-bla^{C121S}-2xF-6xH*	colE1	cam	pKJM130	This study
P$_{sicA}$ *phoA*	*sicP; sptP-phoA-2xF-6xH*	colE1	cam	pKJM153	This study
P$_{sicA}$ *phoA^{S102A}*	*sicP; sptP-phoA^{S102A}-2xF-6xH*	colE1	cam	pKJM225	This study
P$_{sicA}$ *phoA^{C168S}*	*sicP; sptP-phoA^{C168S}-2xF-6xH*	colE1	cam	pKJM221	This study
P$_{sicA}$ *phoA^{C178S}*	*sicP; sptP-phoA^{C178S}-2xF-6xH*	colE1	cam	pKJM222	This study
P$_{sicA}$ *phoA^{C286S}*	*sicP; sptP-phoA^{C286S}-2xF-6xH*	colE1	cam	pKJM223	This study
P$_{sicA}$ *phoA^{C336S}*	*sicP; sptP-phoA^{C336S}-2xF-6xH*	colE1	cam	pKJM224	This study
P$_{sicA}$ *phoA^{T60R}*	*sicP; sptP-phoA^{T60R}-2xF-6xH*	colE1	cam	pKJM323	This study
P$_{sicA}$ *14B7**	*sicP; sptP-14B7*-2xF-6xH*	colE1	cam	pKJM175	This study
P$_{sicA}$ *14B7*^{C40S}*	*sicP; sptP-14B7*^{C40S}-2xF-6xH*	colE1	cam	pKJM261	This study
P$_{sicA}$ *14B7*^{C105S}*	*sicP; sptP-14B7*^{C105S}-2xF-6xH*	colE1	cam	pKJM262	This study
P$_{sicA}$ *14B7*^{C167S}*	*sicP; sptP-14B7*^{C167S}-2xF-6xH*	colE1	cam	pKJM263	This study
P$_{sicA}$ *14B7*^{C241S}*	*sicP; sptP-14B7*^{C241S}-2xF-6xH*	colE1	cam	pKJM264	This study

5.2.2 DNA manipulations

PCR was performed with Pfu DNA polymerase and the primers listed in Table 5.2. Restriction enzymes and ligase (NEB) were used according to the manufacturer's instructions. For all cloning, *E. coli* DH10B cells were used. Mutations to Bla, PhoA, and 14B7* are specified with respect to the full-length mature wild type protein. The *bla* gene was amplified from the plasmid pTrc99A (Amann, Ochs, and Abel 1988). The *phoA* gene was amplified from *E. coli* MG1655. The *14b7** gene was amplified from the plasmid pFLAG-APEx 14B7* (gift from the Georgiou lab).

Table 5.2 Primers used in this study. For each pair, the top row is the forward primer and the bottom row is the reverse primer.

Sequence	Used for the construction of:
FWD: GAACGTTTTCCAATGATGACCTCTTTTAAAGTTCTGCTATG REV: CATAGCAGAACTTTAAAAGAGGTCATCATTGGAAAACGTTC	bla^{ST71TS} mutant
FWD: CTTTTAAAGTTCTGCTAAGCGGCGCGGTATTATCCCG REV: CGGGATAATACCGCGCCGCTTAGCAGAACTTTAAAAG	bla^{C75S} mutant
FWD: GACAGTAAGAGAATTAAGCAGTGCTGCCATAAC REV: GTTATGGCAGCACTGCTTAATTCTCTTACTGTC	bla^{C121S} mutant
FWD: attaggtctcaGCTTCGGACACCAGAAATGCCTG REV: attaggtctcaCGCTTTTCAGCCCCAGAGCGG	P_{sicA} $sicP$ $sptP$-$phoA$-$2xF$-$6xH$ plasmid
FWD: CTACGTCACCGACGCGGCTGCATCAG REV: CTGATGCAGCCGCGTCGGTGACGTAG	$phoA^{S102A}$ mutant
FWD: GACCTCGCGCAAAAGCTACGGTCCGAG REV: CTCGGACCGTAGCTTTTGCGCGAGGTC	$phoA^{C168S}$ mutant
FWD: GCGACCAGTGAAAAAAGCCCGGGTAACGCTCTG REV: CAGAGCGTTACCCGGGCTTTTTTCACTGGTCGC	$phoA^{C178S}$ mutant
FWD: GCCCGCAGTCACCAGCACGCCAAATCCGC REV: GCGGATTTGGCGTGCTGGTGACTGCGGGC	$phoA^{C286S}$ mutant
FWD: CATGCTGCGAATCCTAGCGGGCAAATTGGCGAG REV: CTCGCCAATTTGCCCGCTAGGATTCGCAGCATG	$phoA^{C336S}$ mutant
FWD: GGGGACTCGGAAATTCGCGCCGCACGTAATTATG REV: CATAATTACGTGCGGCGCGAATTTCCGAGTCCCC	$phoA^{T60R}$ mutant
FWD: aggtctcaGCTTGAGGCCCAGCCGGCCATG REV: aggtctcaCGCTTGCGGCCGCGAATTCGG	P_{sicA} $sicP$ $sptP$-$14B7*$-$2xF$-$6xH$ plasmid
FWD: GAGTCACCATCAGTAGCAGGGCAAGTCA REV: CTGACTTGCCCTGCTACTGATGGTGACTC	$14B7*^{C40S}$ mutant
FWD: GATATTGGCACTTACTTTAGCCAACAGGGTAATACG REV: CGTATTACCCTGTTGGCTAAAGTAAGTGCCAATATC	$14B7*^{C105S}$ mutant
FWD: CTCAGTGAAGATTTCCAGCAAAGATTCTGGCTAC REV: GTAGCCAGAATCTTTGCTGGAAATCTTCACTGAG	$14B7*^{C167S}$ mutant
FWD: GCGGTCTATTTCAGTGCAAGGTCGGG REV: CCCGACCTTGCACTGAAATAGACCGC	$14B7*^{C241S}$ mutant

5.2.3 Protein separation and western blotting

Samples were separated by SDS-PAGE. Proteins were transferred to a polyvinylidene fluoride (Millipore) membrane for chemiluminescence detection, using the TransBlot SD unit (Bio-Rad). Membranes were interrogated with Mouse anti-FLAG antibodies per manufacturer's instructions (Sigma). A secondary labeling step was carried out with Goat anti-Mouse IgG (H+L), HRP conjugate antibodies, per manufacturer's instructions (Thermo). Bands were visualized with west-pico or west-femto chemiluminescent substrate (Thermo) and imaged with a ChemiDoc XRS+ unit (Bio-Rad).

5.2.4 Protein purification

Culture homogenate was purified using a His GraviTrap column (GE Healthcare # 11-0033-99). Eluted protein sample was separated by SDS-PAGE, stained with Coomassie G-250, and quantified using densitometry relative to a bovine serum albumin standard (Thermo). Purified protein samples were diluted in LB-L and stored at 4 °C for enzyme activity assays.

5.2.5 Protein quantification

Supernatant samples were harvested from the cell culture as described earlier. Purified protein samples were used to create four standards that were included with each blot to construct a standard curve. Dried supernatant samples were resuspended in an appropriate volume of Laemmli buffer, separated by SDS-PAGE, and transferred to a polyvinylidene fluoride (Millipore). A linear least-squares regression of the standard samples were used to calculate the concentration of each supernatant sample.

5.2.6 Beta-lactamase activity assay

Samples were grown overnight in LB-L media, then subcultured 1:100 in LB-L media and grown for eight hours at 37 °C and 225 rpm. The cultures were pelleted by one centrifugation step of 2,272 x g for 10 minutes and the supernatant was passed through a 0.45 μm filter. Samples were then subjected to a nitrocefin hydrolysis assay, per the substrate vendor (Sigma). 100 μL of reaction buffer (0.1 M phosphate, 1 mM ethylenediaminetetraacetic acid, 50 g/mL nitrocefin (EMD Millipore), 0.5% dimethyl sulfoxide, pH 7) was mixed with 10 μL culture supernatant and the absorbance at 486 nm was observed over time. The reaction was performed in a disposible UV-Transparent cuvette (BrandTech, part# 759215) without stirring at 37 °C. The absorbance at 486 nm was measured every 5 seconds for 60 seconds with a UV-Vis spectrophotometer (Nanodrop, part# 2000c). The reaction was linear for the first 40 seconds of all reactions tested and the change in the absorbance as a function of time was determined using a linear least-squares fit. The initial reaction velocity was calculated using an extinction coefficient of 20,500 M^{-1} cm^{-1}, as specified by the vendor. The experiment was performed on different days in biological triplicate. Error bars represent one standard deviation.

5.2.7 Alkaline phosphatase activity assay

Samples were grown overnight in LB-L media, then subcultured 1:100 in LB-L media and grown for eight hours at 37 °C and 225 rpm. The cultures were pelleted by one centrifugation step of 2,272 x g for 10 minutes and the supernatant was passed through a 0.45 μm filter. Samples were then subjected to a para-nitrophenolphosphate assay, modified from Glasgow, et al. (Glasgow et al. 2012). Briefly, supernatant samples were mixed with the appropriate volume of 1 M Tris (base), pH 8.0 and 0.4 w/w% para-nitrophenolphosphate. For endpoint assays, multiple equal-volume reactions were performed simultaneously in a 96-well microtiter plate and incubated at 37 °C without shaking for at least 1 hour. The absorbance at 405 nm of each well was measured using a Synergy HTX Multi-Mode Reader spectrophotometer (Bio-Tek). For kinetic assays, the reaction was performed in a disposible UV-Transparent cuvette (BrandTech, part# 759215) without stirring at 37 °C. The absorbance at 405 nm was measured every 5 seconds for 60 seconds with a UV-Vis spectrophotometer (Nanodrop, part# 2000c). The reaction was linear for the first 40 seconds of all reactions tested and the change in the absorbance as a function of time was determined using a linear least-squares fit. The initial reaction velocity was calculated using an extinction coefficient of 18,000 M^{-1} cm^{-1}, as specified by the vendor. The experiment was performed on different days in biological triplicate. Error bars represent one standard deviation.

5.2.8 Enzyme-linked immunosorbent assay (ELISA)

Samples were grown overnight in LB-L media, then subcultured 1:100 in LB-L media and grown for eight hours at 37 °C and 225 rpm. The cultures were pelleted by one centrifugation step of 2,272 x g for 10 minutes and the supernatant was passed through a 0.45 μm filter. The wells of a 96-well microtiter plate (Santa Cruz Biotechnology, Inc., part# sc-204463) was coated with 100

µL of a solution of 4 µg/mL protective antigen (PA) of the anthrax toxin (List Biological Laboratories, part# 171E) in 5 mM HEPES, 50 mM NaCl, pH 7.5 covered at 4 °C overnight. Liquid was removed by inversion and wells were incubated with a 200 µL blocking solution (200 µL 2% milk, 0.05% TBST) at room temperature for one hour. Liquid was removed by inversion and 100 µL filtered culture supernatant was added to the wells. Samples were incubated at room temperature for 2 hours. Liquid was removed by inversion and wells were rinsed with 0.05% TBST three times. Next, 100 µL of primary labeling solution (1:10,000 dilution of Mouse anti-FLAG antibody (Sigma) diluted in 0.05% TBST) was added to the wells and incubated 1 hour at room temperature. Liquid was removed by inversion and wells were rinsed with 0.05% TBST five times. Then, 100 µL of secondary labeling solution (1:5,000 dilution of Goat anti-Mouse IgG (H+L), HRP conjugate antibodies (Thermo) diluted in 0.05% TBST) was added to the wells and incubated 1 hour at room temperature. Liquid was removed by inversion and wells were rinsed with 0.05% TBST five times. 100 µL of 3,3′,5,5′-Tetramethylbenzidine (TMB) Liquid Substrate (Thermo) was added to the wells and incubated for approximately 20 minutes at room temperature. The reaction was quenched with 100 µL 2 M H_2SO_4. Absorbance at 450 nm was measured using a Synergy HTX Multi-Mode Reader spectrophotometer (Bio-Tek).

5.2.9 Cysteine alkylation

Filtered supernatant samples were precipitated with trichloroacetic acid (20% w/w final concentration) overnight at 4 °C, washed twice with cold acetone, and dried by heating. Dried samples were resuspended in 50 µL resuspension buffer (1 M Tris (base), pH 7.5, 3% w/w sodium dodecyl sulfate). Resuspension buffer was supplemented with 10 mM tris(2-carboxyethyl)phosphine (TCEP) for reduced samples, as necessary. Samples were incubated at room temperature for 10 minutes. Next, 8.8 µL of 100 mM 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid (AMS) was added to samples (final concentration is 15 mM), as necessary. Samples to which AMS was not added were diluted with equal volume distilled water. Samples were incubated at room temperature for 2 hours, protected from light. Samples were then mixed with Laemmli buffer, boiled, and separated by SDS-PAGE, and bands were detected by a western blot, as specified above.

5.2.10 Error estimation of Michaelis-Menten model

Error estimation was conducted using Matlab (Mathworks, R2014a). Biological replicates were treated as independent samples. Three independent replicates were then analyzed for both purified and secreted samples. Least-squares minimization was used to fit a modified Michaelis-Menton model (Eq. 5.1) to the measured values and determine the parameters k_{cat}^{app} and K_M.

$$\frac{V_0}{[E]_T} = \frac{k_{cat}^{app} \cdot [S]}{K_M + [S]} \tag{5.1}$$

where V_0 is the initial reaction rate, $[E]_T$ is the total enzyme concentration, $[S]$ is the substrate concentration, and k_{cat}^{app} and K_M are fitting parameters. In this formulation, k_{cat}^{app} refers to the apparent rate constant, such that increasing the percentage of misfolded protein will decrease the value of k_{cat}^{app}. To compare the parameters from different treatments, a t-test (Eq. 5.2) was used where the degrees of freedom was determined by the pooled sample size.

$$t = \frac{\beta_1 - \beta_2}{\sqrt{Se_1^2 - Se_2^2}} \qquad (5.2)$$

where t is the t-statistic, β is the fitting parameter of interest (i.e., k_{cat}^{app} and K_M), and Se is the standard error of the estimate calculated from the nonlinear regression. A p-value of 0.05 was used to define significance.

5.2.11 Calculation of f_{fold}

First, we assume that secreted proteins exist in two states: state 1) catalytically active; and state 2) catalytically inactive. Yet, the quantitative western blot assay experimentally determines the total secreted protein concentration, $[E]_T$, which includes both active (state 1) and inactive (state 2) forms of the secreted enzyme (Eq. 5.3):

$$[E]_T = [E]_{active} + [E]_{inactive} \qquad (5.3)$$

where $[E]_{active}$ is the concentration of secreted enzyme that is catalytically active (state 1) and $[E]_{inactive}$ is the concentration of secreted enzyme that is not catalytically active (state 2). We then define a parameter, f_{fold}, that is the fraction of secreted enzyme that is active:

$$f_{fold} = \frac{[E]_{active}}{[E]_T} \qquad (5.4)$$

In this two state model, secreted enzymes in state 1 catalyze reactions with the rate constant k_{cat}, while secreted enzymes in state 2 do not catalyze reactions, and thus have a rate constant, $k_{cat}^{inactive}$, equal to zero. We then apply this assumption to a modified Michaelis-Menten equation, yielding Eq. 5.5:

$$\frac{dP}{dt} = \frac{k_{cat}[E]_{active}[S]}{K_M + [S]} + \frac{k_{cat}^{inactive}[E]_{inactive}[S]}{K_M + [S]} = \frac{k_{cat}[E]_T[S]}{K_M + [S]} f_{fold} \qquad (5.5)$$

We then define an apparent rate constant, k_{cat}^{app}, that is the observed reaction rate constant:

$$k_{cat}^{app} = f_{fold} k_{cat} \qquad (5.6)$$

We combine Eqs. 5.5 and 5.6 to yield Eq. 5.7:

$$\frac{dP}{dt} = \frac{k_{cat}^{app}[E]_T[S]}{K_M + [S]} \qquad (5.7)$$

Thus, we calculate the value of f_{fold} by taking the ratio of the values of the fitted parameters k_{cat}^{app} and k_{cat}. This allows for calculation of the fraction of total secreted protein that is folded, using protein purified from the cytosol as a reference state that we assume to be exclusively in state 1.

5.3 Results

5.3.1 Secreted proteins are functional after secretion

Beta-lactamase (EC:3.5.2.6, class A) is a monomeric enzyme that forms one intrachain disulfide bond, but is not required for activity (Schultz et al. 1987). No cofactors are required for activity (Expasy). We previously reported that the enzyme beta-lactamase adopts a catalytically active conformation after secretion by the T3SS (Metcalf et al. 2014). We confirmed that beta-lactamase was indeed active in the extracellular space after secretion by the T3SS, and found that enzymatic activity in the extracellular space was both enzyme- and secretion-dependent (Figure 5.1A). No secretion or activity was detected when secretion was prevented by deletion of the *prgI* gene, which codes for an essential component of the SPI-1 T3SS (Kimbrough and Miller 2000). No activity was detected when the catalytic site of the enzyme was knocked out (ST71TS) (Dalbadie-McFarland, Neitzel, and Richards 1986), though the protein was still secreted. These results indicate that detected activity in the extracellular space was due to a catalytically active beta-lactamase. We mutated the two cysteine residues in beta-lactamase to serine to prevent disulfide bond formation. Both mutant enzymes were secreted, and the C121S mutation resulted in a catalytic activity similar to the wild type. Interestingly, the C75S mutation was not catalytically active, in contrast to previous reports in the literature (Schultz et al. 1987). Differences in N- and C-terminal modification may explain this difference—our secreted beta-lactamase bears a substantial N-terminal secretion signal and C-terminal epitopes that may affect the essentiality of Cys75.

The enzyme alkaline phosphatase (EC 3.1.3.1, isozyme 1) requires the acquisition of two Zn^{2+} and one Mg^{2+} cofactors, dimerization, and the formation of two intrachain disulfide bonds for catalytic activity (Stec, Holtz, and Kantrowitz 2000). Catalytic activity in the extracellular space was detected, indicating that alkaline phosphatase folded and satisfied all structural requirements for activity in the extracellular space (Figure 5.1B). No secretion or activity was detected when secretion was prevented by deletion of the *prgI* gene, and no activity was detected when the catalytic site of is knocked out (S102A) (Butler-Ransohoff et al. 1992), though the protein was still secreted. Systematic mutation of each of the four cysteines to serine to prevent disulfide bond formation resulted in secreted but catalytically inactive enzyme. In addition, no activity was detected after chemical reduction of wild type alkaline phosphatase with 10% v/v 2-mercaptoethanol. Further, no activity was detected in a monomeric alkaline phosphatase mutant (T60R) (Boulanger and Kantrowitz 2003), though this mutant protein was still secreted. Together, these data indicate that alkaline phosphatase folds into a catalytically active conformation, including the correct formation of disulfide bonds, in the extracellular space after secretion by the SPI-1 T3SS.

A single-chain variable fragment (scFv) of an antibody is a monomeric protein that forms, but does not necessarily require, two intrachain disulfide bonds. 14B7* is an scFv of a mouse IgG antibody that binds to the protective antigen (PA) of the anthrax toxin (Leysath et al. 2009; Little, Leppla, and Cora 1988). Binding of secreted 14B7* to PA was detected by ELISA (Figure 5.1C). No secretion or activity was detected when secretion is prevented by deletion of the *prgI* gene. Systematic mutation of each of the four cysteines to serine to prevent disulfide bond formation resulted in secretion and antigen binding, though each of the four mutants exhibited lower binding than wild type. Binding activity equivalent to wild type was detected after chemical reduction of wild type 14B7* with 10% 2-mercaptoethanol.

A.

Strain	WT	*prgI*	WT	
Bla allele	WT	WT	ST71TS C75S	C121S

B.

10% BME	−	−	−	−	−	−	−	+
Strain	WT	*prgI*			WT			
PhoA allele	WT	WT	S102A	C168S	C178S	C268S	C336S	T60R	WT

C.

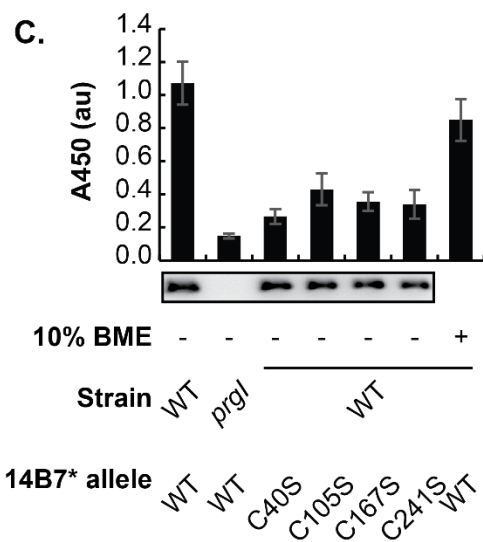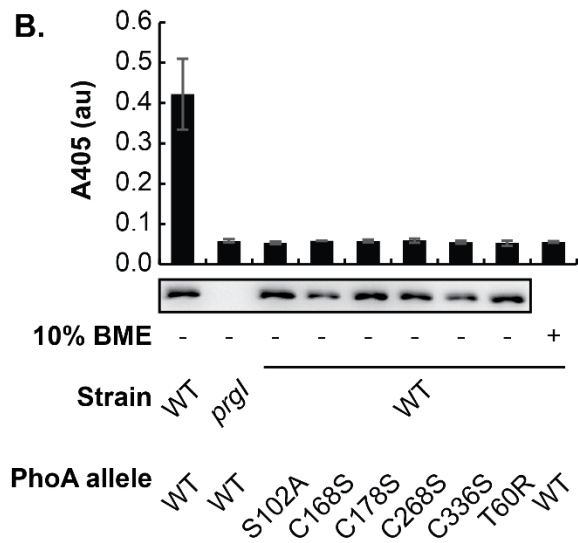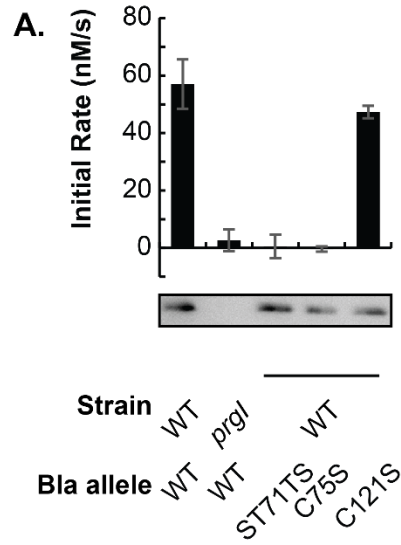|						
10% BME	−	−	−	−	−	+
Strain	WT	*prgI*		WT		
14B7* allele	WT	WT	C40S	C105S	C167S	C241S	WT

Figure 5.1 Secreted proteins adopt functional conformations (*from previous page*). Activity or ELISA signal is given for samples analyzed from the culture supernatant. Genetic modifications described are with respect to the mature native protein sequence of the POI in the fusion. All proteins are of the format SptP-POI-2xFLAG-6xHIS. Results are plotted for the POIs **A.** Bla **B.** PhoA **C.** 14B7*. The mean is plotted from three biological replicate experiments and the error bars represent one standard deviation. Western blots are representative of the samples analyzed in the functional assays.

5.3.2 Secreted proteins form disulfide bonds

The presence of disulfide bonds in secreted proteins was confirmed by selective cysteine alkylation with the reagent 4'-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid (AMS). AMS selectively adds to free thiols, adding ~500 Da of mass with each addition. It will not covalently modify cysteines that participate in a disulfide bond. Reduction of the protein sample with tris(2-carboxyethyl)phosphine (TCEP) will reduce disulfide bonds and convert all cysteines to the free thiol form. Thus, we can observe the disulfide bond state of a protein by detecting changes in molecular weight resulting from redox-dependent protein modification by AMS (Sechi and Chait 1998). Greater cysteine modification will result in a protein that migrates more slowly in a denaturing polyacrylamide gel. For all proteins tested, the N-terminal SptP secretion signal sequence contains a cysteine residue at position 112 that is not expected to participate in a disulfide bond and is thus a free thiol. Indeed, a shift in migration was detected when all proteins are modified with AMS without TCEP pretreatment, indicating that the cysteine in the SptP secretion signal sequence is modified (Figure 5.2, lane 3).

Disulfide bonds were detected in beta-lactamase (Figure 5.2A). This protein contains one intrachain disulfide bond in the native protein, giving a total of three cysteine residues in the fusion protein. An increase in apparent molecular weight was observed when the protein was modified with AMS after TCEP pretreatment, indicating that the protein contained a disulfide bond in the extracellular space. Disulfide bonds were also detected in both alkaline phosphatase and the 14B7* scFv (Figure 5.2B and C). Both of these proteins contain two intrachain disulfide bonds in the native protein, giving a total of five cysteine residues in the fusion protein. When the sample was pretreated with TCEP before modification with AMS, a further increase in apparent molecular weight was observed, indicating that disulfides were present in the secreted protein.

A.

Bla

TCEP	-	+	-	+
AMS	-	-	+	+

55 ―

43 ―

B.

PhoA

TCEP	-	+	-	+
AMS	-	-	+	+

72 ―

55 ―

C.

14B7*

TCEP	-	+	-	+
AMS	-	-	+	+

72 ―

55 ―
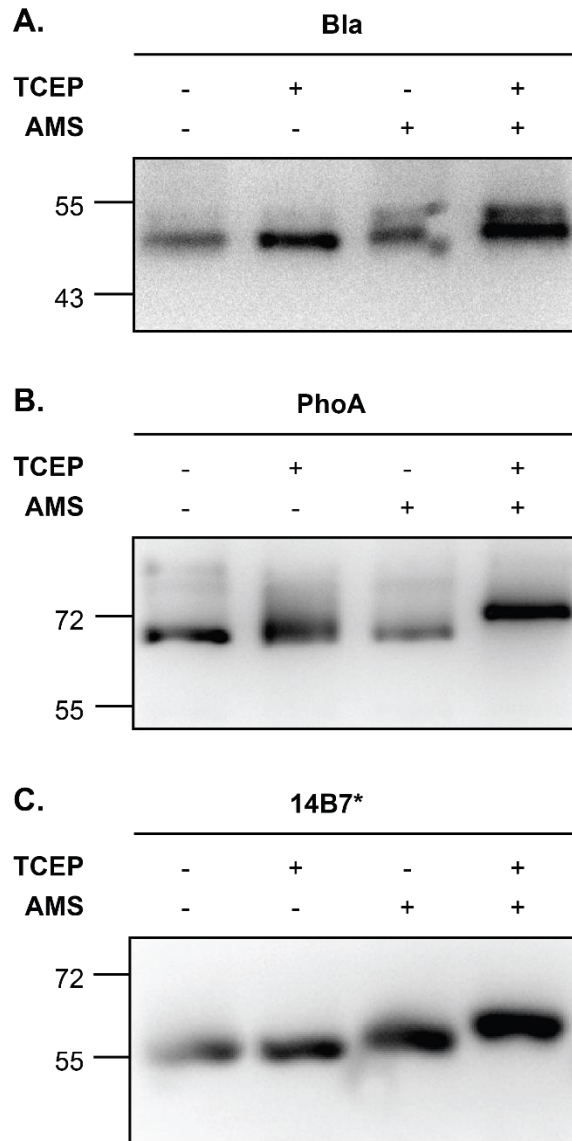
Figure 5.2 Western blots of secreted fusion protein samples subjected to the selective alkylation procedure separated by SDS-PAGE. All proteins are of the format SptP-POI-2xFLAG-6xHIS. Representative images are presented from a western blot for the POIs **A.** Bla **B.** PhoA **C.** 14B7*.

5.3.3 Specific activity of secreted enzymes is affected by salt concentration in growth medium

Activity of the secreted enzymes was compared with enzyme purified from the cytosol. While activity of the secreted enzymes was detected as shown in Figure 5.1, it was not clear what fraction of the secreted enzymes were active. We define the parameter f_{fold} as the fraction of functional secreted protein, relative to the same protein fusion purified from the cytoplasm. Briefly, we assume that secreted enzymes that are folded are also active and catalyze reactions with rate k_{cat}, while misfolded secreted enzymes do not contribute to catalysis. The sample thus catalyzes reaction with an apparent rate constant, k_{cat}^{app}, that is less than or equal to k_{cat} (see Section 5.2.11 for a thorough description of the f_{fold} parameter and the apparent rate constant k_{cat}^{app}).

Beta-lactamase and alkaline phosphatase were purified from the cytosol and the enzyme concentration, $[E]_T$, and the kinetic parameters K_M, k_{cat}^{app}, and V_{max} were calculated for each sample. In addition, the same kinetic parameters were calculated for secreted enzyme (Table 5.3). First, this analysis was performed in standard production media (Lysogeny Broth, Lennox; 5 g/L NaCl) (Metcalf et al. 2014).

Table 5.3 Analysis of refolding efficiency of secreted enzyme in the culture supernatant, relative to purified, soluble cellular enzyme. Uncertainty is given as standard error of the estimate and is propagated for the f_{fold} calculation. All experiments were performed three times in biological replicate. Parameters for secreted samples were calculated for samples generated in LB media with 5 g/L NaCl.

Protein	secreted		purified		
	k_{cat}^{app} (s^{-1})	K_M (μM)	k_{cat}^{app} (s^{-1})	K_M (μM)	f_{fold}
Bla	38 ± 4	28 ± 11	248 ± 11	42 ± 6	0.15 ± 0.02
PhoA	22 ± 2	100 ± 40	26 ± 2	230 ± 80	0.85 ± 0.10

The fraction of secreted beta-lactamase enzymes that are active was 15 ± 2 %, relative to the purified form. The kinetic parameters K_M and k_{cat}^{app} of the purified beta-lactamase fusion compared well with published values for the wild type enzyme for the nitrocefin substrate (110 μM and 900 sec^{-1}, respectively) (Sigal et al. 1984). No statistically significant difference in the value of K_M was found between purified and secreted beta-lactamase fusion. However, the secreted and purified forms of beta-lactamase significantly differed in the calculated apparent rate constant, k_{cat}^{app} ($p < 0.05$), suggesting a folding defect in the secreted enzyme, relative to the enzyme purified from the cell.

The fraction of secreted alkaline phosphatase enzymes that are active was 85 ± 10 %, relative to the purified form. Both kinetic parameters, K_M and k_{cat}^{app}, of the purified alkaline phosphatase fusion were significantly different from the published values for the wild type enzyme for the para-nitrophenylphosphate substrate (35 ± 5 μM and 176 ± 6 sec^{-1}, respectively) (Wojciechowski and Kantrowitz 2002). It should be noted that these reported kinetic parameters reported by Wojciechowski and Kantrowitz were calculated for reactions performed at 25 °C in a Tris-buffered solution, while all reactions with alkaline phosphatase in this study were conducted at 37 °C in LB media, prohibiting a direct comparison of the values. No statistically significant differences in the values of K_M and k_{cat}^{app} were found between purified and secreted alkaline phosphatase fusion. Thus, the activity of the alkaline phosphatase fusion studied in this work did not experience a significant folding defect after secretion, compared to protein purified from soluble cytosolic fraction. It should be noted that while the value of K_M for the secreted and purified samples was not statistically significantly different, the large difference in K_M between the samples may indicate that the folding of alkaline phosphatase is not well described by our simple two-state model (see Section 5.2.11 for details on two-state model).

We attempted to increase the parameter f_{fold} by changing culturing conditions. By changing the components in the growth medium, we hypothesized that the folding of secreted protein could be modulated. The ionic strength of a solution is known to affect protein folding, likely through

charge-charge interactions (Song, Cho, and Raleigh 2007). The concentration of NaCl in the growth medium was varied between 5 and 17 g/L (0.09 and 0.3 M, respectively). The activity was then calculated using activity assays and quantitative western blotting, as above. Interestingly, activity of secreted beta-lactamase monotonically increased with increasing [NaCl]. However, secreted protein titer also increased monotonically with increasing [NaCl] (Figure 5.3A.i). As a result, the activity of beta-lactamase at saturating concentrations of substrate increased with NaCl concentration in the growth medium, but the increased activity in Figure 5.3A.i was due to both increased enzyme concentration and increased f_{fold} (Figure 5.3A.ii). Further, the calculated value of K_M for secreted beta-lactamase was not significantly different between the three media conditions tested (Figure 5.3A.iii).

The effect of NaCl in the growth media had an opposite effect on alkaline phosphatase. No change was observed in secreted protein titer or activity at saturating concentrations of substrate (Figure 5.3B.i). The value of f_{fold} did not change at low concentrations of NaCl but decreased at the highest salt concentration (Figure 5.3B.ii). The calculated value of K_M for secreted alkaline phosphatase was not significantly different between the three conditions tested (Figure 5.3B.iii).
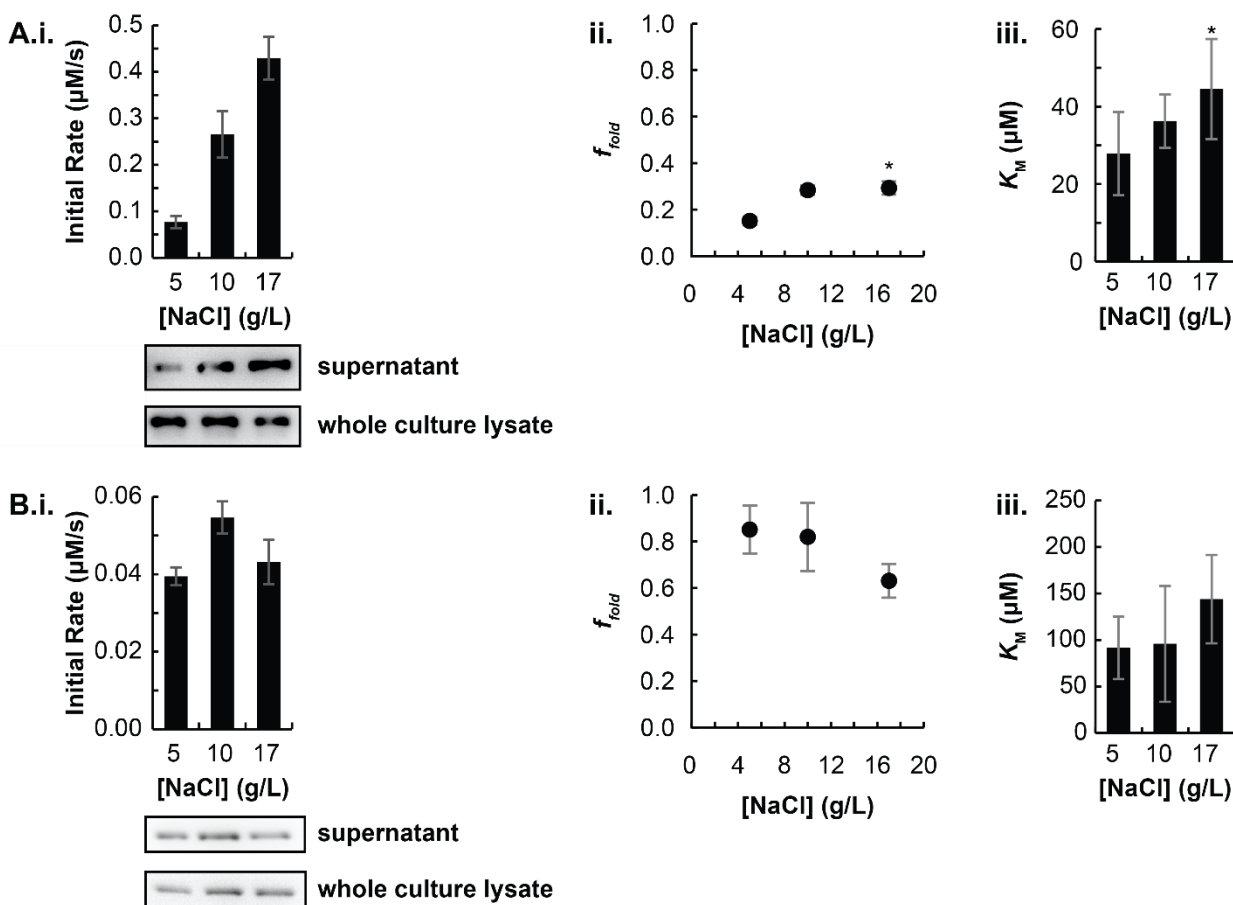
Figure 5.3 Salt and media can change secreted protein titer and folding efficiency (*from previous page*). Both proteins studied are in the fusion format SptP-POI-2xFLAG-6xHIS. For all plots, unless specified, the mean of three biological replicates is plotted, except where noted by the symbol * to indicate two biological replicates. Error bars represent one standard deviation, unless noted. **A. i.** Plot of raw activity of secreted Bla as a function of growth media with representative western blot of analyzed samples. **ii.** Plot of f_{fold} for secreted beta-lactamase as a function of growth media. **iii.** Plot of K_M for secreted beta-lactamase as a function of growth media. Error bars represent the standard error of the estimate. **B. i.** Plot of raw activity of secreted PhoA as a function of growth media with representative western blot of analyzed samples. **ii.** Plot of f_{fold} for secreted alkaline phosphatase as a function of growth media. **iii.** Plot of K_M for secreted alkaline phosphatase as a function of growth media. Error bars represent the standard error of the estimate.

5.4 Discussion

Pure and correctly folded protein is desired when producing a heterologous protein. Production of heterologous proteins via secretion to the extracellular space holds many advantages over intracellular accumulation: purification is simplified; cytotoxicity is alleviated; and cell lysis is not required (Georgiou and Segatori 2005; Stader and Silhavy 1990). The production and secretion of heterologous proteins can be achieved using the T3SS of various Gram-negative bacteria (Majander et al. 2005; Widmaier et al. 2009; Singer et al. 2012; Derouazi et al. 2008; Azam et al. 2015). However, the mechanism of protein secretion requires an unfolding event during translocation (Radics, Königsmaier, and Marlovits 2013). For this production strategy to be effective, the protein should fold into a functional conformation after secretion. This event must occur in the extracellular space, a region of the cell culture thought to be devoid of molecular chaperones that assist in protein folding. By testing for protein function, the folded state of the protein is probed, as only folded proteins are expected to be functionally active. The protein function assays are sensitive enough to give information on the folded state of the protein of interest in a heterogeneous, dilute protein mixture.

Previous studies have demonstrated the ability of heterologous proteins secreted by a T3SS to adopt active conformations (Majander et al. 2005; Metcalf et al. 2014; Derouazi et al. 2008). In this study, we studied the ability of secreted proteins with different chemical and structural requirements to adopt active conformations. The enzyme beta-lactamase folds into a catalytically active tertiary structure (Figure 5.1A). The enzyme alkaline phosphatase adopts a catalytically active tertiary structure, forms a dimer and two intrachain disulfide bonds, and acquires one Mg^{2+} and two Zn^{2+} ions per chain (Figure 5.1B). The scFv 14B7* folds into a tertiary structure that permits antigen binding (Figure 5.1C). These data demonstrate that proteins can adopt active confirmations after secretion. Furthermore, in the extracellular space—an area thought to be devoid of molecular chaperones that aid in folding inside the cell—formation of disulfide bonds, multimerization, and acquisition of metal ion cofactors can still occur. We hypothesize that these interactions occur spontaneously in the extracellular space. The cultures are grown aerobically, and it is likely that the oxidizing environment of the culture medium allows for disulfide bond formation. Further, the growth media is not chemically defined and likely contains trace metals. Nonetheless, it is surprising that these proteins are able to adopt active conformations in the extracellular space after secretion.

A comparison of the specific activity of secreted and purified cellular enzyme was performed to compare the fraction of secreted proteins that adopt active conformations in the extracellular space. Secreted enzyme that is catalytically inactive will decrease the apparent rate constant, k_{cat}^{app}. Indeed, beta-lactamase had a lower value of k_{cat}^{app} in the secreted sample, compared to the purified (Table 5.3). This suggests that only a fraction of the secreted enzyme adopts an active conformation after secretion. We speculate that this is due to misfolding or aggregation. Increasing the ionic strength of a solution has been shown to increase the thermodynamic stability of a folded monomeric protein (Song, Cho, and Raleigh 2007), increasing the concentration of NaCl in the growth medium from 5 to 17 g/L increased f_{fold} of secreted beta-lactamase by almost three-fold (Figure 5.3A.ii). This indicates that the ability of the secreted protein to adopt an active conformation after secretion may be dependent on the chemical environment in which it folds. Secreted alkaline phosphatase had a k_{cat}^{app} value similar to the purified sample, such that f_{fold} is 85 $\pm$ 10% for the 5 g/L NaCl sample (Table 5.3). This indicates that the fraction of folding for secreted alkaline phosphatase is similar to the folding when the enzyme is purified from the soluble cellular fraction. Increasing the concentration of NaCl from 5 to 10 g/L in the culture medium did not increase the value of f_{fold} (Figure 5.3B.ii). However, the value of f_{fold} decreased at the highest media NaCl concentration. This effect could be due to increased charge screening at high solution ionic strength, as charge-charge interactions are known to be important in dimerization of alkaline phosphatase (Boulanger and Kantrowitz 2003; Torriani 1968).

Understanding protein folding after secretion is important for improving protein production. Large-scale protein production often involves production of the protein of interest in inclusion bodies, which are then solubilized and refolded. Parallels exist between protein folding in the extracellular space and inclusion body solubilization followed by protein refolding in vitro. In an in vitro refolding procedure, the inclusion body is solubilized in a high concentration solution of a chaotrope, such as guanidinium chloride. This also unfolds the proteins in the inclusion body. The solution is then diluted to allow for proteins to fold into a native conformation. Dilution decreases the concentration of protein in solution in addition to decreasing the concentration of chaotrope. The lower concentration of protein in solution improves protein refolding, as each chain is less likely to form interchain aggregates. Secretion of protein to the extracellular space mimics this process, as the extracellular space also has a lower protein concentration, relative to inside the bacterial cell. The needle structure of the T3SS extends ~50 nm from the outer membrane (Kubori et al. 1998), potentially beyond any extracellular cellular structures, such as the lipopolysaccharide layer. Thus, we speculate that proteins secreted by the T3SS to the extracellular space will experience a folding environment that, as in in vitro refolding, can be tailored to increase the folding of the secreted protein. In addition to allowing the facile production of correctly folded heterologous protein in the supernatant of bacterial culture, protein secretion by the T3SS offers a unique condition to study protein folding. Refolding of proteins after secretion may access different folding trajectories than found in co-translational or in vitro folding conditions. Given the rate and directionality of secretion, proteins may fold in a unique vectorial folding pathway. Further, the unfolded conformations that are accessible to the protein during secretion are likely constrained, which in turn may change the conformations that are accessible after secretion. We anticipate that this system will provide a unique folding environment for future study.

5.5 Acknowledgements

I would like to acknowledge the great work that James Bevington and Sandy Rosales performed in this section. James helped with statistical analysis and Sandy performed many enzyme activity assays with beta-lactamase and alkaline phosphatase.

5.6 References

Amann, Egon, Birgit Ochs, and Karl-Josef Abel. 1988. "Tightly Regulated Tac Promoter Vectors Useful for the Expression of Unfused and Fused Proteins in *Escherichia coli*." *Gene* 69 (2): 301–15. doi:10.1016/0378-1119(88)90440-4.

Azam, Anum, Cheng Li, Kevin J. Metcalf, and Danielle Tullman-Ercek. 2015. "Type III Secretion as a Generalizable Strategy for the Production of Full-Length Biopolymer-Forming Proteins." *Biotechnology and Bioengineering*, June. doi:10.1002/bit.25656.

Boulanger, Robert R., and Evan R. Kantrowitz. 2003. "Characterization of a Monomeric *Escherichia coli* Alkaline Phosphatase Formed upon a Single Amino Acid Substitution." *Journal of Biological Chemistry* 278 (26): 23497–501. doi:10.1074/jbc.M301105200.

Butler-Ransohoff, John E., Steven E. Rokita, Debra A. Kendall, Jennifer A. Banzon, Kristin S. Carano, Emil Thomas Kaiser, and Albert R. Matlin. 1992. "Active-Site Mutagenesis of *E. coli* Alkaline Phosphatase: Replacement of Serine-102 with Nonnucleophilic Amino Acids." *The Journal of Organic Chemistry* 57 (1): 142–45. doi:10.1021/jo00027a027.

Clark, Eliana De Bernardez. 2001. "Protein Refolding for Industrial Processes." *Current Opinion in Biotechnology* 12 (2): 202–7. doi:10.1016/S0958-1669(00)00200-7.

Cornelis, Guy R. 2006. "The Type III Secretion Injectisome." *Nature Reviews Microbiology* 4 (11): 811–25. doi:10.1038/nrmicro1526.

Dalbadie-McFarland, Gloria, James J. Neitzel, and John H. Richards. 1986. "Active-Site Mutants of Beta-Lactamase: Use of an Inactive Double Mutant to Study Requirements for Catalysis." *Biochemistry* 25 (2): 332–38. doi: 10.1021/bi00350a008.

Derouazi, M., B. Toussaint, L. Quénée, O. Epaulard, M. Guillaume, R. Marlu, and B. Polack. 2008. "High-Yield Production of Secreted Active Proteins by the *Pseudomonas aeruginosa* Type III Secretion System." *Applied and Environmental Microbiology* 74 (11): 3601–4. doi:10.1128/AEM.02576-07.

Expasy. http://enzyme.expasy.org/EC/3.5.2.6. Accessed on Feb 3, 2016.

Georgiou, George, and Laura Segatori. 2005. "Preparative Expression of Secreted Proteins in Bacteria: Status Report and Future Prospects." *Current Opinion in Biotechnology* 16 (5): 538–45. doi:10.1016/j.copbio.2005.07.008.

Glasgow, Jeff E., Stacy L. Capehart, Matthew B. Francis, and Danielle Tullman-Ercek. 2012. "Osmolyte-Mediated Encapsulation of Proteins inside MS2 Viral Capsids." *ACS Nano* 6 (10): 8658–64. doi:10.1021/nn302183h.

Hingorani, Karan S., and Lila M. Gierasch. 2014. "Comparing Protein Folding *In Vitro* and *In Vivo*: Foldability Meets the Fitness Challenge." *Current Opinion in Structural Biology* 24 (February): 81–90. doi:10.1016/j.sbi.2013.11.007.

Hoiseth, Susan K., and B. A. D. Stocker. 1981. "Aromatic-Dependent *Salmonella* Typhimurium Are Non-Virulent and Effective as Live Vaccines." *Nature* 291 (5812): 238–39. doi:10.1038/291238a0.

Kimbrough, Tyler G., and Samuel I. Miller. 2000. "Contribution of *Salmonella* Typhimurium Type III Secretion Components to Needle Complex Formation." *Proceedings of the*

National Academy of Sciences of the United States of America 97 (20): 11008–13. doi: 10.1073/pnas.200209497.

Kubori, Tomoko, Yukiyasu Matsushima, Dai Nakamura, Jaimol Uralil, Maria Lara-Tejero, Anand Sukhan, Jorge E. Galán, and Shin-Ichi Aizawa. 1998. "Supramolecular Structure of the *Salmonella* Typhimurium Type III Protein Secretion System." *Science* 280 (5363): 602–5. doi:10.1126/science.280.5363.602.

Leysath, Clinton E., Arthur F. Monzingo, Jennifer A. Maynard, Jason Barnett, George Georgiou, Brent L. Iverson, and Jon D. Robertus. 2009. "Crystal Structure of the Engineered Neutralizing Antibody M18 Complexed to Domain 4 of the Anthrax Protective Antigen." *Journal of Molecular Biology* 387 (3): 680–93. doi:10.1016/j.jmb.2009.02.003.

Little, Stephen F., Stephen H. Leppla, and Elsa Cora. 1988. "Production and Characterization of Monoclonal Antibodies to the Protective Antigen Component of Bacillus Anthracis Toxin." *Infection and Immunity* 56 (7): 1807–13.

Majander, Katariina, Lena Anton, Jenni Antikainen, Hannu Lång, Mirko Brummer, Timo K. Korhonen, and Benita Westerlund-Wikström. 2005. "Extracellular Secretion of Polypeptides Using a Modified *Escherichia coli* Flagellar Secretion Apparatus." *Nature Biotechnology* 23 (4): 475–81. doi:10.1038/nbt1077.

Metcalf, Kevin J., Casey Finnerty, Anum Azam, Elias Valdivia, and Danielle Tullman-Ercek. 2014. "Using Transcriptional Control To Increase Titers of Secreted Heterologous Proteins by the Type III Secretion System." *Applied and Environmental Microbiology* 80 (19): 5927–34. doi:10.1128/AEM.01330-14.

Radics, Julia, Lisa Königsmaier, and Thomas C. Marlovits. 2013. "Structure of a Pathogenic Type 3 Secretion System in Action." *Nature Structural & Molecular Biology* 21 (December): 82–87. doi:10.1038/nsmb.2722.

Schlumberger, Markus C., Andreas J. Müller, Kristin Ehrbar, Brit Winnen, Iwan Duss, Bärbel Stecher, and Wolf-Dietrich Hardt. 2005. "Real-Time Imaging of Type III Secretion: *Salmonella* SipA Injection into Host Cells." *Proceedings of the National Academy of Sciences of the United States of America* 102 (35): 12548–53. doi:10.1073/pnas.0503407102.

Schultz, Steve C., Gloria Dalbadie-McFarland, James J. Neitzel, and John H. Richards. 1987. "Stability of Wild-Type and Mutant RTEM-1 Beta-Lactamases: Effect of the Disulfide Bond." *Proteins* 2 (4): 290–97. doi:10.1002/prot.340020405.

Sechi, Salvatore, and Brian T. Chait. 1998. "Modification of Cysteine Residues by Alkylation. A Tool in Peptide Mapping and Protein Identification." *Analytical Chemistry* 70 (24): 5150–58. doi:10.1021/ac9806005.

Sigal, Irving S., William F. DeGrado, Barbara J. Thomas, and Stephen R. Petteway. 1984. "Purification and Properties of Thiol Beta-Lactamase. A Mutant of pBR322 Beta-Lactamase in Which the Active Site Serine Has Been Replaced with Cysteine." *Journal of Biological Chemistry* 259 (8): 5327–32.

Singer, Hanna M., Marc Erhardt, Andrew M. Steiner, Min-Min Zhang, Doju Yoshikami, Grzegorz Bulaj, Baldomero M. Olivera, and Kelly T. Hughes. 2012. "Selective Purification of Recombinant Neuroactive Peptides Using the Flagellar Type III Secretion System." *mBio* 3 (3). doi:10.1128/mBio.00115-12.

Song, Benben, Jae-Hyun Cho, and Daniel P. Raleigh. 2007. "Ionic-Strength-Dependent Effects in Protein Folding: Analysis of Rate Equilibrium Free-Energy Relationships and Their Interpretation." *Biochemistry* 46 (49): 14206–14. doi:10.1021/bi701645g.

Stader, Joan A., and Thomas J. Silhavy. 1990. "Engineering *Escherichia coli* to Secrete Heterologous Gene Products." *Methods in Enzymology* 185:166–87. doi:10.1016/0076-6879(90)85017-I.

Stec, Boguslaw, Kathleen M. Holtz, and Evan R. Kantrowitz. 2000. "A Revised Mechanism for the Alkaline Phosphatase Reaction Involving Three Metal Ions." *Journal of Molecular Biology* 299 (5): 1303–11. doi:10.1006/jmbi.2000.3799.

Torriani, Annamarie. 1968. "Alkaline Phosphatase Subunits and Their Dimerization *In Vivo*." *Journal of Bacteriology* 96 (4): 1200–1207.

Widmaier, Daniel M., Danielle Tullman-Ercek, Ethan A. Mirsky, Rena Hill, Sridhar Govindarajan, Jeremy Minshull, and Christopher A. Voigt. 2009. "Engineering the Salmonella Type III Secretion System to Export Spider Silk Monomers." *Molecular Systems Biology* 5 (1): 309. doi:10.1038/msb.2009.62.

Wojciechowski, Cheryl L., and Evan R. Kantrowitz. 2002. "Altering of the Metal Specificity of *Escherichia coli* Alkaline Phosphatase." *Journal of Biological Chemistry* 277 (52): 50476–81. doi:10.1074/jbc.M209326200.

APPENDIX

RECOMBINEERING PROTOCOL

Protocol for recombineering adapted from Thomason et al. 2001 for genetic manipulations in *S. enterica*.

A.1 Reminder

Put sterile water in the refrigerator and turn on the water bath at the beginning of the day of recombineering. Double check that the water bath has enough water to cover the Erlenmeyer flask up to the level of the subcultures.

Always construct your desired ApE files for the first and second round before ordering primers to ensure that your genetic manipulations are as intended.

Court suggests the exclusive use of LB-Lennox for liquid media as there is better recombination efficiency, compared with LB-Miller. This protocol reflects that recommendation, though I bet that LB-Miller might work in a pinch.

A.2 Materials

LB-Lennox
>5 g/L NaCl
>5 g/L yeast extract
>10 g/L tryptone

LB-sucrose-agar
>5 g/L yeast extract
>10 g/L tryptone
>60 g/L sucrose
>15 g/L agar

A.3 DNA sequence of *cat-sacB* cassette

```
TGTGACGGAAGATCACTTCGCAGAATAAATAAATCCTGGTGTCCCTGTTGATACCGGGAAGCCCTGGGCCAACTTTT
GGCGAAAATGAGACGTTGATCGGCACGTAAGAGGTTCCAACTTTCACCATAATGAAATAAGATCACTACCGGGCGTA
TTTTTTGAGTTATCGAGATTTTCAGGAGCTAAGGAAGCTAAAATGGAGAAAAAAATCACTGGATATACCACCGTTGA
TATATCCCAATGGCATCGTAAAGAACATTTTGAGGCATTTCAGTCAGTTGCTCAATGTACCTATAACCAGACCGTTC
AGCTGGATATTACGGCCTTTTTAAAGACCGTAAAGAAAAATAAGCACAAGTTTTATCCGGCCTTTATTCACATTCTT
GCCCGCCTGATGAATGCTCATCCGGAATTCCGTATGGCAATGAAAGACGGTGAGCTGGTGATATGGGATAGTGTTCA
CCCTTGTTACACCGTTTTCCATGAGCAAACTGAAACGTTTTCATCGCTCTGGAGTGAATACCACGACGATTTCCGGC
AGTTTCTACACATATATTCGCAAGATGTGGCGTGTTACGGTGAAAACCTGGCCTATTTCCCTAAAGGGTTTATTGAG
AATATGTTTTTCGTCTCAGCCAATCCCTGGGTGAGTTTCACCAGTTTTGATTTAAACGTGGCCAATATGGACAACTT
CTTCGCCCCCGTTTTCACCATGGGCAAATATTATACGCAAGGCGACAAGGTGCTGATGCCGCTGGCGATTCAGGTTC
ATCATGCCGTTTGTGATGGCTTCCATGTCGGCAGAATGCTTAATGAATTACAACAGTACTGCGATGAGTGGCAGGGC
GGGGCGTAATTTTTTTAAGGCAGTTATTGGTGCCCTTAAACGCCTGGTTGCTACGCCTGAATAAGTGATAATAAGCG
GATGAATGGCAGAAATTCGAAAGCAAATTCGACCCGGTCGTCGGTTCAGGGCAGGGTCGTTAAATAGCCGCTTATGT
CTATTGCTGGTCTCGGTACCCGGGGATCGCGGCCGCGGACCGGATCCCATCACATATACCTGCCGTTCACTATTATT
TAGTGAAATGAGATATTATGATATTTTCTGAATTGTGATTAAAAAGGCAACTTTATGCCCATGCAACAGAAACTATA
AAAAATACAGAGAATGAAAAGAAACAGATAGATTTTTTAGTTCTTTAGGCCCGTAGTCTGCAAATCCTTTTATGATT
TTCTATCAAACAAAAGAGGAAAATAGACCAGTTGCAATCCAAACGAGAGTCTAATAGAATGAGGTCGAAAAGTAAAT
CGCGCGGGTTTGTTACTGATAAAGCAGGCAAGACCTAAAATGTGTAAAGGGCAAAGTGTATACTTTGGCGTCACCCC
TTACATATTTTAGGTCTTTTTTTATTGTGCGTAACTAACTTGCCATCTTCAAACAGGAGGGCTGGAAGAAGCAGACC
GCTAACACAGTACATAAAAAAGGAGACATGAACGATGAACATCAAAAAGTTTGCAAAACAAGCAACAGTATTAACCT
TTACTACCGCACTGCTGGCAGGAGGCGCAACTCAAGCGTTTGCGAAAGAAACGAACCAAAAGCCATATAAGGAAACA
TACGGCATTTCCCATATTACACGCCATGATATGCTGCAAATCCCTGAACAGCAAAAAAATGAAAAATATCAAGTTCC
TGAGTTCGATTCGTCCACAATTAAAAATATCTCTTCTGCAAAAGGCCTGGACGTTTGGGACAGCTGGCCATTACAAA
ACGCTGACGGCACTGTCGCAAACTATCACGGCTACCACATCGTCTTTGCATTAGCCGGAGATCCTAAAAATGCGGAT
GACACATCGATTTACATGTTCTATCAAAAAGTCGGCGAAACTTCTATTGACAGCTGGAAAAACGCTGGCCGCGTCTT
TAAAGACAGCGACAAATTCGATGCAAATGATTCTATCCTAAAAGACCAAACACAAGAATGGTCAGGTTCAGCCACAT
TTACATCTGACGGAAAAATCCGTTTATTCTACACTGATTTCTCCGGTAAACATTACGGCAAACAAACACTGACAACT
GCACAAGTTAACGTATCAGCATCAGACAGCTCTTTGAACATCAACGGTGTAGAGGATTATAAATCAATCTTTGACGG
TGACGGAAAAACGTATCAAAATGTACAGCAGTTCATCGATGAAGGCAACTACAGCTCAGGCGACAACCATACGCTGA
GAGATCCTCACTACGTAGAAGATAAAGGCCACAAATACTTAGTATTTGAAGCAAACACTGGAACTGAAGATGGCTAC
CAAGGCGAAGAATCTTTATTTAACAAAGCATACTATGGCAAAAGCACATCATTCTTCCGTCAAGAAAGTCAAAAACT
TCTGCAAAGCGATAAAAAACGCACGGCTGAGTTAGCAAACGGCGCTCTCGGTATGATTGAGCTAAACGATGATTACA
CACTGAAAAAAGTGATGAAACCGCTGATTGCATCTAACACAGTAACAGATGAAATTGAACGCGCGAACGTCTTTAAA
ATGAACGGCAAATGGTACCTGTTCACTGACTCCCGCGGATCAAAAATGACGATTGACGGCATTACGTCTAACGATAT
TTACATGCTTGGTTATGTTTCTAATTCTTTAACTGGCCCATACAAGCCGCTGAACAAAACTGGCCTTGTGTTAAAAA
TGGATCTTGATCCTAACGATGTAACCTTTACTTACTCACACTTCGCTGTACCTCAAGCGAAAGGAAACAATGTCGTG
ATTACAAGCTATATGACAAACAGAGGATTCTACGCAGACAAACAATCAACGTTTGCGCCAAGCTTCCTGCTGAACAT
CAAAGGCAAGAAACATCTGTTGTCAAAGACAGCATCCTTGAACAAGGACAATTAACAGTTAACAAATAAAAACGCA
AAAGAAAATGCCGATATTGACTACCGGAAGCAGTGTGACCGTGTGCTTCTCAAATGCCTGATTCAGGCTGTCTATGT
GTGACTGTTGAGCTGTAACAAGTTGTCTCAGGTGTTCAATTTCATGTTCTAGTTGCTTTGTTTTACTGGTTTCACCT
GTTCTATTAGGTGTTACATGCTGTTCATCTGTTACATTGTCGATCTGTTCATGGTGAACAGCTTTAAATGCACCAAA
AACTCGTAAAAGCTCTGATGTATCTATCTTTTTTACACCGTTTTCATCTGTGCATATGGACAGTTTTCCCTTTGAT
```

cat-*sacB*

A.4 *cat-sacB* cassette preparation

Day 1

1. Design primers with regions of homology to the *cat-sacB* (eKJM118 TUC01) cassette and to the target in the genome. ~25 base pair homology to *cat-sacB* (see below) and 40 base pair homology to genomic target. The cassette size should be ~3 kb. Note that either orientation seems to work for us, don't worry about getting the orientation of the *cat-sacB* cassette correct.

a. The homology sequence we use to the TUC01 template for ALL amplifications

cat FWD homology (Tm = 56 °C)
TGTGACGGAAGATCACTTCG

sacB REV homology (Tm = 57 °C)
 ATCAAAGGGAAAACTGTCCATAT

b. The more homology to the genome, the better. For a tricky locus (*prgI* in SL1344), we used 50 bp homology, as 40 bp homology did not seem to work.

2. Run PCR with primers and TUC01 template. For best results, miniprep the genome from an overnight culture, resuspend the pellet in 600 µL 10% SDS, add lysis buffer, neutralization buffer, 600 µL ddH$_2$O, and then proceed as normal with miniprep.

3. Run a small sample of *cat-sacB* PCR product on electrophoresis gel. If the correct PCR product amplified, then do a cleanup with the kit and obtain DNA concentration with by A260 (Nanodrop)

1st round of recombineering: Knocking out the targeted gene from genome

pSIM6 is temperature sensitive, so always grow cells with pSIM6 at 30 °C!!!

4. Transform cell with pSIM6 (λ red). Cells can still grow on plates with 50 µg/mL carbenicillin. Then make freezer stock for later use.

a. We typically use electroporation, but chemical transformation will work here, as well.

Day 2
5. From a plate with a streaked out freezer stock or fresh transform, pick a colony and inoculate into 5mL of LB-L + 30 µg/mL carb, and grow in 30 °C shaking incubator overnight.

a. We use a lower concentration of carb because the antibiotic resistance cassette is on a low copy plasmid, per Court.

Day 3
6. Subculture from overnight 0.5 mL into 35 mL of LB-L (250 mL Erlenmeyer flask) and grow in 30 °C shaking incubator until cells reach an OD of 0.4-0.6. Recombineering fails when OD is higher than 0.6. Also, **do not add carbenicillin to the 35 mL of LB-L** because antibiotics may inhibit recombination efficiency, per Court lab.

a. This is enough for ~3 transformations plus one control. Scale up culture as necessary.

b. This subculture takes 2.5 hrs to reach OD.

c. This is enough for 4-10 transformations (depending on how much you use)

7. Transfer have of the culture into a water bath at 42 °C with shaking on maximum (if using the old water bath shaker) for induction of the λ red system. It is very important, to **induce for only 15 minutes!**

8. After exactly 15 minutes have passed, cool in iced water bath for 10 minutes with constant gentle swirling. More than 15 minutes of induction will cause calls to die.

A.5 Uptake of the *cat-sacB* cassette

From this point on cells must remain **COLD**. Cool down the floor centrifuge before spinning. Place falcon tubes, tube holders, and electroporation cuvettes in the freezer. Place sterile water in the refrigerator.

9. Prepare cells for electroporation. Transfer the cultures into falcon tubes. Spin for 3 minutes at 4 °C and 4,600 X *g*. After spin, decant supernatant carefully and slowly so the cell pellet does not flow away. Rinse with 30 mL of iced cold water. Pippette up and down at least 3 times and until the whole cell pellet is resuspended. Spin again and rinse for a total of 2 washes with iced cold water. At the end, resuspend with only 200 μL of ice cold water.

a. To make the cells go further, can add a larger volume of ice cold water here, but I don't know how dilute is too dilute.

10. Add 50 μL of cell solution and 100 ng of PCR product. Electroporate at 1900 V for 5 msec. Recover quickly with 1 mL of SOC, and then transfer into 1.5 mL eppendorf tubes. Always include a control of induced culture without DNA. Put the tubes in the 30 °C shaking incubator for 1-2 hours.

11. Spread 200 μL of recovered cells on an agar plate. The plates need to have a low concentration of chloramphenicol (10 μg/mL) because of low resistance gene copy number. Place the plates in the 30 °C incubator if planning to do 2nd round of recombineering. This way the pSIM6 does not need to be retransformed.

Day 4-5

12. In our hands, we have on the order of ~10 colonies on the +DNA plate and ~0 colonies on the –DNA control plate.

13. **OPTIONAL** (and not necessary). The next day, to check for correct insertion, check for sucrose sensitivity or for genotype (by PCR).

a. To test for sucrose sensitivity: Grow two liquid cultures, the knockout and WT overnight. Do a 10-fold serial dilution series and spot plate 5 microliters of each parallel to each other out to 10^{-8}. You should notice a 3-orders of magnitude more

growth with WT than the *cat-sacB* knockout on sucrose plates. BE MINDFUL OF PERMISSIVE GROWTH TEMPERATURE FOR ALL CELL GROWTH STEPS!

    b. You can also screen for insertion by performing colony PCR. You will need primers outside of the region that you are targeting. You can use the following primers that anneal to the 5' and 3' ends of the *cat-sacB* cassette, or just amplify across the whole cassette. It is important that you do not use the same primers that you used to amplify the *cat-sacB* cassette, but instead primers that are outside of the cassette at your genome locus. Miniprep the genome for best template (see step 1).

        oKJM343 REV *cat* (Tm = 62 °C)
        GAATAAAGGCCGGATAAAACTTGTG

        oKJM346 FWD *sacB* (Tm = 65 °C)
        TCAAAGACAGCATCCTTGAACAAGG

    c. Don't patch to check for sucrose sensitivity. Because you can accumulate loss-of-function mutations, you are likely to see qualitatively the same phenotype. We don't see false positives at this point, so you can skip this step and proceed with the second round. To be really sure, you can check by PCR or colony counting form liquid culture dilutions.

A.6 2nd round of recombineering

14. Amplify the gene of interest with primers having homology to the new allele and targeted genomic sequence. Use the same genome homology regions as before. Check PCR product on gel for correct length and followed by a PCR cleanup.

15. Follow step 4-9

16. Add 50 µL of cell mixture and 100 ng of DNA. Electroporate at 1900 V for 5 msec. Recover in 1 mL SOC. Transfer all recovered cells to 10 mL of LB-L in the 37 °C shaker for 4 hours. Do not cut this step short, because full allele segregation is required. Use test tubes with the green tops. Always include induced culture without DNA.

    a. green tubes are 20 mm diameter

17. Do a 10-fold serial dilution series and plate 200 µL of the 10^{-2} and 10^{-3} dilutions on a LB-sucrose agar plate. Grow plates in the 37 °C incubator

Day 6

18. In our hands, we have on the order of ~100 colonies on the +DNA 10^{-2} dilution plate and ~10 colonies on the –DNA 10^{-2} dilution plate.

19. To check for correct insertion it would be best to sequence. Use primers from the genetic locus outside of the region you manipulated. Miniprep the genome as in step 12 for a good template. Amplify your locus, and submit the purified PCR product for sequencing.

20. To make a freezer stock, patch single colonies onto LB-cam[10], LB-carb[50], and LB agar plates. Your strain should be cam[s], carb[s]. The LB agar plate is to recover your strain.

A.7 References
Thomason, Lynn C., James A. Sawitzke, Xintian Li, Nina Costantino, and Donald L. Court. 2014. "Recombineering: Genetic Engineering in Bacteria Using Homologous Recombination." In *Current Protocols in Molecular Biology*. John Wiley & Sons, Inc. doi: 10.1002/0471142727.mb0116s106.