Characterization of TraT protein encoded in IncF resistance plasmids

Katelyn Ashley Lott

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CHARACTERIZATION OF TraT PROTEIN ENCODED IN IncF RESISTANCE PLASMIDS

by

KATELYN ASHLEY LOTT

A THESIS

Submitted in partial fulfillment of the requirements for the degree of Master of Science in The Department of Biological Sciences to The School of Graduate Studies of The University of Alabama in Huntsville

HUNTSVILLE, ALABAMA

2022
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We, the undersigned members of the Graduate Faculty of The University of Alabama in Huntsville, certify that we have advised and/or supervised the candidate on the work described in this thesis. We further certify that we have reviewed the thesis manuscript and approve it in partial fulfillment of the requirements for the degree of Master of Science in Biological Sciences.

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ABSTRACT
The School of Graduate Studies
The University of Alabama in Huntsville

Degree  Master of Science  College/Dept.  Biological Sciences
Name of Candidate  Katelyn Lott
Title  Characterization of TraT Protein Encoded in IncF Resistance Plasmids

Drug-resistant infections pose serious medical threats, and causative resistance genes spread through plasmid conjugation. The TraT protein is a virulence factor encoded in drug-resistance F-plasmids. TraT itself provides protection from conjugation, serum complement, and bacteriophages, but the protective mechanism is unknown. To facilitate mechanism studies, I developed improved purification protocols for isolation of the soluble domain of F-plasmid TraT from *Escherichia coli*. Wild-type TraT and two variants were obtained. TraT domain forms several types of oligomers *in vitro*, also observed in full-length TraT. This oligomerization is stable without membrane attachment and in broad range of salt concentrations. Purified recombinant TraT was not found to interact with other proteins in conducted pull-down assays but it is able to protect *E. coli* cells from serum killing when added *in trans*. New purification protocols, protein variants, and oligomerization knowledge will be used to address knowledge gaps regarding structure and mechanisms of function.

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CHAPTER 1: INTRODUCTION

1.1 Horizontal Gene Transfer

Horizontal gene transfer (HGT) is the term given to the varied processes by which bacteria can gain genetic information aside from through asexual reproduction. HGT in bacteria encompasses three different methods: transduction, transformation, and conjugation. In transduction, DNA is transferred between bacteria by a bacterial virus called a bacteriophage – as it replicates itself, it picks up bits of bacterial DNA to transfer. Transformation is considered to be the rarest of the methods and involves bacterial uptake of DNA from its environment. Finally, conjugation involves direct DNA (plasmid) transfer from one bacterial cell to another (Thomas & Nielsen, 2005). HGT is an outstanding system for the continued evolution of bacteria as it allows for the acquisition of hundreds of new genes faster than the process of random mutation alone and is thought to be heavily involved in the propagation of antibiotic resistance (Thomas & Nielsen, 2005).

1.2 Antimicrobial Resistance

Antimicrobial resistance is considered to have occurred when a microbe – such as a bacterium – is no longer affected by a substance designed to kill it – such as an antibiotic. This often occurs through the bacterial acquisition of a plasmid carrying a gene that encodes for resistance to a specific antibiotic. While exploiting this phenomenon can be useful to scientists in a research setting, outside of the lab, antibiotic resistance poses a
serious health threat. As of 2019, antibiotic resistance claims an estimated 1.27 million deaths annually worldwide (Murray et al., 2022). This issue is only exacerbated when multi-drug resistant strains come into play. By better understanding the methods of gene transfer that lead to antibiotic resistance, it is hoped that the overall spread of these potentially deadly microbes can be curbed.

1.3 Conjugation and Plasmids

Conjugation, the type of HGT in which a bacterium directly transfers genetic information to another bacterium via contact by a conjugative pilus, seems to be a major mechanism used for transfer of antibiotic resistant traits (Thomas & Nielsen, 2005). Both Gram-positive and Gram-negative bacterial cells often have cell surface appendages besides flagella which are known as pili. There are various functions for pili, but the primary ones deal with adhesion and interaction with other cells, whether eukaryotic host or bacterium.
Figure 1: Bacterial conjugation. The process is shown starting from initial pilus contact, to formation of a mating pair, to plasmid replication, to the production of a transconjugant cell.

Conjugation starts with both a donor cell with a transferable plasmid and a recipient cell. During conjugation, the donor cell first contacts the recipient cell through a conjugative pilus (Figure 1). The donor cell then attaches its conjugative pilus to the recipient cell and the pilus retracts; this brings the cells together and is known as formation of the ‘mating pair’. The plasmid is replicated and transferred into the recipient cell, resulting in what is now known as the transconjugant (Emamalipour et al., 2020).

Formation of the mating pair is a key step in conjugation and is mediated by the mating pair formation (Mpf) system, which is comprised of the conjugative pilus as well as a set of other conserved conjugative proteins (Schröder & Lanka, 2005). There are multiple conjugation systems, but the extracellular pilus is the ubiquitous factor among them all and is necessary for recognizing recipient cells (Will & Skurray, 1980).
Bacterial plasmids are double stranded extrachromosomal DNA molecules that can exist within a bacterial cell. Plasmids contain genes that provide various benefits to its cell, including genes coding for antibiotic resistance, increased virulence, increased stress tolerance or adaptation, and more (Lindsey et al., 2009). It is more metabolically challenging for a cell to keep a plasmid than not, but the benefit the plasmid provides can select for future survival of its host cell, or certain death if the host cell does not replicate the plasmid in the fascinating case of plasmid addiction modules through toxin-antitoxin systems (Smith & Magnuson, 2004), (Unterholzner et al., 2013). The diversity of plasmids is great, but one major group of plasmids known well for their commonality as well as conjugative ability are the IncF plasmids that are found in *Escherichia coli* and other *Enterobacteriaceae* species. Plasmids can be divided into incompatibility (Inc) groups, which are groups of plasmids that are not able to be stably propagated within the same bacterial host cell due to having a very similar or the same mechanism of replication (Humbert et al., 2019), (Lindsey et al., 2009). IncI and IncF plasmids, which produce type I pili and type F pili respectively, are discussed in this work.

Some of the plasmids carry conjugative genes and thus can be mobile. These mobile, transferable plasmids are classified into mobilizable and self-transmissible conjugative plasmids. The earliest discovered and most studied conjugative plasmid is the fertility factor of *E. coli* – F plasmid. Conjugative plasmids – including F-type plasmids – code for a variety of genes that have roles in the transfer of the plasmid from donor to recipient cell. These are referred to as the transfer (*tra*) genes, and are involved in stable mating pair formation, DNA transfer, as well as exclusion of conjugation.
Among different systems of classification of transferable plasmids aside from Inc grouping there is a method that involves grouping plasmids by their relaxase type. Relaxase is a tra-protein, DNA processing enzyme that is critical for recognition of a plasmid’s origin of transfer as well as initiation of the transfer itself. Relaxase-sequence classification is called mobility (MOB) grouping. There are currently 6 MOB groups identified: MOBF, MOBP, MOBH, MOBQ, MOBC, and MOBV. There is a fair amount of overlap with the replicon typing used for Inc grouping, so members of the same Inc group will generally be in the same MOB group. IncF plasmids are in MOBF, and IncI plasmids are in MOBP (Rozwandowicz et al., 2018).

1.4 Conjugation Exclusion

Exclusion is the diminished ability of a bacterial strain carrying a conjugative plasmid to act as the recipient cell in a conjugative transfer when the plasmids contained in both cells are the same or very similar (Sukupolvi & David O’connor, 1990). Both traS and traT genes are involved in exclusion, but through different mechanisms (Achtman et al., 1977). There are two different types of exclusion systems – entry exclusion and surface exclusion (Arutyunov & Frost, 2013) (Figure 2).
During entry exclusion, conjugative transfer is blocked after mating pair formation; the plasmid DNA is not able to enter the connected recipient cell. This process is mediated by the TraS protein in F-like plasmids. In surface exclusion, however, the initial stable mating pair formation is prevented; the donor and recipient cells are not able to properly join together, and so the plasmid DNA cannot transfer (Arutyunov & Frost, 2013). Surface exclusion is mediated by the TraT protein in F-like plasmids, which disrupts the mating pair formation (Mpf) system from forming stable aggregates (Schröder & Lanka, 2005). Although the end goal of these two tra genes is similar, the gene products are decidedly different, with the TraS protein lacking a cleavable signal sequence and being found in the cytoplasmic membrane, in contrast to the TraT protein’s signal sequence being cleaved upon localization to the outer membrane.
1.5 Unknowns in Conjugation

Some aspects of the process of conjugation are known – how conjugation is regulated, the process of DNA processing in donor cells, and how the actual transfer machinery works, for example. However, this initial step of mating pair formation via pilus contact is not heavily addressed in the literature. Due to this gap in knowledge, though much is known about the well-studied F plasmid, like most other systems (barring the MOBp/IncI plasmid R64, discussed later), it is not yet known how its conjugation is initiated. Nevertheless, learning how this initial binding step of donor pilus to recipient cell occurs would provide a critical piece of information. Preventing this step could prove useful in preventing conjugation itself, and therefore give the ability to hinder the spread of antibiotic resistant bacteria as an ultimate goal. When looking for potential substances for the F pili to bind, previous literature seems to indicate a few likely subjects present on the surface of the recipient cell, with Outer Membrane Protein A (OmpA) and lipopolysaccharides (LPS) prime among these (Manoil & Rosenbusch, 1982).

![Cell envelope of a Gram-negative bacterial cell. LPS, OmpA, and TraT are shown.](image)

Figure 3: Cell envelope of a Gram-negative bacterial cell. LPS, OmpA, and TraT are shown.
1.6 Lipopolysaccharides

These lipopolysaccharides – or LPS, also known as endotoxins – are a major component of the outer membrane (OM) of most Gram-negative bacteria, as well as creating a selectively permeable membrane and having an as of yet unknown role in conjugation. LPS are composed of three main segments: the O-antigen, a polysaccharide which comprises the most outward part of the structure; Lipid A, the hydrophobic lipid component responsible for anchoring the LPS into the outer membrane; and the outer and inner core, oligosaccharides which connect the two ends (Bertani & Ruiz, 2019).

![Lipopolysaccharide (LPS). Shown here are the O-antigen, core, and lipid A components.](image)

The O-antigen is a polysaccharide composed of a repeating oligosaccharide made of 2-8 sugars. There is great variety seen in the O-antigen both between strains as well as between species, approximately 186 different O-serotypes seen in *E. coli* alone (Fratamico et al., 2016). The O-antigen contributes antigenicity to the bacterial cell, as it
is often the first bacterial constituent recognized by the host’s immune system due to its location on the outside of the cell. The numerous possibilities of sugar combinations it is capable of, coupled with flagellar H-types, results in multiple antigenic types (Liu, Furevi, Perepelov, Guo, Cao, Wang, Reeves, Knirel, & Wang, 2020). On the other end of LPS exists Lipid A, a hexa-acylated, biphosphorylated glucosamine disaccharide. The attached fatty acid chains form the outer leaflet of the outer membrane. Lipid A contributes toxicity to the bacterial cell by stimulating the immune system and significantly contributes to disease symptoms. Both Lipid A and the O-antigen are involved in determining virulence. With Lipid A, the hexa-acylation of the two glucosamines provides the optimal level of toxicity for the bacterium; if more or less than six acyl chains are added or subtracted, toxicity of Lipid A decreases (Bertani & Ruiz, 2019). In regard to the O-antigen, it protects the cell from damage caused by antibody interactions, carries the toxic Lipid A, and helps the bacterial cell to adhere to host tissues.

The LPS core is a nonrepeating oligosaccharide. The core has less variation than O-antigen but is still variable to a degree especially in the outer core; this is seen in the five known core types of R1, R2, R3, R4, and K12. The inner core is more conserved and usually includes heptose and KDO. Positionally, the end of the inner core is linked to the glucosamines of Lipid A & the end of the outer core is attached to the O-antigen, if present (Clifton et al., 2016). LPS can be classified as “smooth” or “rough” based on their composition. Smooth LPS have an O-antigen and the entirety of their core, whereas rough LPS are missing the O-antigen and can lack part of the core. This can also often be seen phenotypically in colony formation (Fratamico et al., 2016).
There are different degrees of “rough” from “Ra” to “Re” based on how much of the structure is missing. The distinction between smooth and rough LPS can sometimes be seen phenotypically in the appearance of colony formation – cells with smooth LPS have a smoother appearance while strains with rough LPS can have a noticeably more irregular physical composition. Strains with deep rough LPS are more sensitive to detergents, antibiotics, and mutagens (Hitchcock et al., 1986).

1.7 Biosynthesis of Lipopolysaccharide

The first step in LPS biosynthesis is the formation of lipid A-KDO. This biosynthesis pathway involves 9 different enzymes (LpxA, LpxC, LpxD, LpxH, LpxB, LpxK, KdtA, LpxL, and LpxM) and occurs on the inner surface of the inner membrane (Wang & Quinn, 2010). The starting component of lipid A is UDP-GlcNAc. During the
first three reactions utilizing the first three enzymes listed, UDP-GlcNAc gains two 3-OH fatty acid chains placed on the 2- and 3-positions, which results in the formation of UDP-diacyl-GlcN. The first reaction involving LpxA can be reversed, but subsequent steps cannot. Next, LpxH hydrolyzes the UDP-diacyl-GlcN to form what is known as lipid X. Disaccharide-1-P is created through the condensation of lipid X and UDP-diacyl-GlcN by LpxB. LpxK phosphorylates this structure in the 4′-position and makes lipid IVA, and then two KDO residues are added at the 6′-position by KdtA. LpxL and LpxM aid in further editing of the resulting KDO2-lipid IVA – addition of a secondary lauroyl residue and addition of a myristoyl residue, respectively – to form the final KDO2-lipid A form (Bertani & Ruiz, 2018).

The structure of core oligosaccharides is more conserved than that of the O-antigen, but still has a degree of variation, with more variation being found in the outer core than the inner, which is typically largely composed of KDO and heptose. Three operons contain the genes that are needed for core biosynthesis in E. coli: gmhD, waaQ and kdtA. The gmhD operon holds the inner core biosynthesis genes gmhD-waaF-waaC-waaL (Wang & Quinn, 2010). The first three genes code for the production of certain proteins required for synthesizing and transferring heptose, while the latter gene codes for a ligase enzyme that is necessary in order to join the O-antigen to the LPS outer core. Seven to nine genes are contained in operon waaQ – these genes encode certain enzymes involved in the biosynthesis and modification of the outer core KdtA is encoded by the gene and in the operon of the same name and is necessary for the addition of the customary KDO residues to lipid A.
Genes involved in synthesis of the O-antigen are generally clustered in a specific location. In *E. coli*, this gene cluster can be found in the middle of galF and gnd, housekeeping genes (Liu, Furevi, Perepelov, Guo, Cao, Wang, Reeves, Knirel, Wang, et al., 2020). O-antigen gene clusters contain three main types of genes – glycosyltransferase genes that make specific linkages between donor and acceptor sugars, O-unit processing genes that polymerize and translocate the specific units that make up the O-antigen, and genes that synthesize sugar precursors for sugars found in a certain polysaccharide. Three pathways have been found that are involved in synthesizing and assembling an O-antigen structure: the Wzx/Wzy pathway, the ABC transporter, and pathways involved in synthesis of one or multiple genes for processing the individual O-units.

Each of these pathways begins by transferring a sugar phosphate from an NDP-sugar to undecaprenyl phosphate (Und-P). Typically, an initial transferase gene is included; in *Enterobacteriaceae*, this initial transferase is WecA, and it transfers a GlcNAcP residue from UDP-GlcNAc to Und-P. The Wzx/Wzy pathway is the most common synthesis pathway seen in *E. coli* – sugars will be transferred in sequence from their associated sugar nucleotides, initially UDP-N-acetylglucosamine (Und-PP-GlcNAc), by glycosyltransferases and added to each other to create the individual O-units (Wang & Quinn, 2010). The Und-PP-linked O units will be translocated by Wzx (a flippase protein) from the inner membrane to the periplasmic face of the inner membrane. Here the O-units will be polymerized by Wzy (a polymerase protein) and the polymer will be generated. Wzz, a chain length determinate, will determine the number of O-units in a particular type of O-antigen.
The presence of LPS is necessary for the survival of most Gram-negative bacteria. The finished LPS are positioned in the outer leaflet of the outer membrane, but are synthesized in the cytoplasm and periplasm, and so must be transported to their ultimate destination. This transport process must function properly if the cell is to survive, and numerous genes and the enzymes for which they encode are involved in this process. At the beginning of the transport process, the O-antigen and the core-lipid A are flipped from the cytoplasmic to periplasmic face of the inner membrane by Wzx and MsbA respectively. Here, Wzy and Wzz will polymerize the O-antigen unit to form the repeats. WaaL attaches these repeats to the core-lipid A, forming an entire LPS unit. The freshly made LPS is carried from the inner membrane’s periplasmic face to the inner surface of the outer membrane by the proteins LptA, LptB, LptC, LptF and LptG; the LPS is then assembled into the outer surface of the outer membrane by LptD and LptE (Wang & Quinn, 2010).

1.8 LPS and Conjugation

Inc I plasmid R64 produces a type IV pilus used for conjugation in a liquid environment. These thin, flexible pili are made up of PilS pilin, and PilV adhesins are believed to be found at the tip. PilV thin pili adhesins function via the recognition of a receptor found on the cell surface of the conjugative recipient. Through experimental mutation of the waa genes, it has been shown that the lipopolysaccharides of recipient bacterial cells comprise these specific receptors. It is proposed that the variable C-terminal domain of PilV adhesins is significantly involved with the ability to recognize and bind to the associated LPS, therefore determining specificity in Inc I plasmid R64.
liquid matings. There are five different LPS core structures: R1, R2, R3, R4, and K-12. Ishiwa and Komano (Ishiwa & Komano, 2004) used an assortment of waa mutants as the recipient cell in multiple liquid mating experiments in effort to determine the specificity of recognition. It has been demonstrated that *E. coli* R1 LPS is specifically recognized by PilVA adhesin, and that the PilVA receptor is the GlcNAc(β1–3)Glc structure. The GlcNAc(α1–2)Glc structure of *E. coli* R2 type LPS was demonstrated to be specifically recognized by the PilVBO adhesin. *E. coli* R3 LPS was able to be recognized by the PilVD0 adhesin. The *E. coli* K-12 LPS receptor structures GlcNAc(α1–2)Glc, GlcNAc(β1–7)Hep, and Glc(α 1–2)Glc or Glc(α1–2)Gal structures are specifically recognized by the PilVBO, PilVC, and PilVCO adhesins.

A study published in 2017 by Hong, et al. (Hong et al., 2017) shows that common polysaccharide antigen (CPA) lipopolysaccharide, a homopolymer of D-rhamnose, is necessary for initiating the transfer of large genomic island PAPI-1; this indicates that this CPA structure acts as a receptor for the conjugative type IV pilus in recipient strains. In this study, created mutations in locations involved in CPA synthesis showed significant loss of ability to acquire PAPI-1. However, mutations regarding the core LPS synthesis such as waaC, waaF and waaP did not show a significant decrease in PAPI-1 transfer. This suggests that CPA specifically, and thus the O antigen more broadly, is the main LPS structure driving the contact and interaction between donor and recipient in *P. aeruginosa*. Given the large number of O antigen varieties seen in *E. coli* - which at time of publication had been organized into 166 different O antigen serogroups - it is not impossible that the O antigen might be involved in conjugative binding for this bacterial species as well as *Pseudomonas*. 
Pérez-Mendoza et al. (Pérez-Mendoza & de la Cruz, 2009) screened for genes involved in bacterial conjugation through the use of two collections of mutants covering 99% of non-essential *E. coli* genes, and a lux-monitored conjugation assay. Some Keio collection mutants showed a decrease in conjugation, but generally the effect seen was minimal. However, fifteen of the Tn-insertion mutants showed a more definite negative effect regarding conjugation abilities. Eleven of these affected mutants had the transposon insertion located in genes that are involved in synthesis of LPS. One of the most noticeable effects was seen with a mutation to the *rfaC* gene, which is responsible for encoding a heptosyltransferase I that is needed for LPS inner core biosynthesis – adding a heptose to KDO. Mutations in *rfaC* result in a defective LPS core and lead to increased permeability to antibiotics, as well as a significant decrease in successful conjugation. This was especially noted in liquid mating conditions with F plasmids, suggesting that mutations in LPS result in reduced docking ability between the donor cell and the recipient cell.

Having stated that all conjugation-deficient mutants previously described were shown to have reduced conjugation abilities with F-type donor cells, Havekes et al. (Havekes & Hoekstra, 1976) set out to isolate mutants of *E. coli* K-12 that were conjugation-deficient with I-type donor cells. Lethal conjugation was chosen as an enrichment procedure for the isolation of ConI- mutants. Eight strains – AM4001, AM4011-12, AM4014, AM4017-18, AM4023, and AM4028 – were deemed to be ConI-. Upon further testing with these mutants, it was found that every mutant showed greater sensitivity to methylene blue and acridine orange dyes than did the parental strain, and every mutant aside from strains AM4014 and AM4018 could not grow on 1% SDS.
peptone-yeast agar. Also, unlike the parental AM4000 strain, most mutants showed sensitivity to phage C21 – this is believed to be due to an altered LPS that is deficient in galactose. Paper chromatography was used to compare the LPS of mutant and parental strains; the results confirmed that all of the mutants had some form of altered LPS, and all but strains AM4014 and AM4018 had LPS similar to that of a reference strain deficient in phosphate and galactose. Glucose and phosphate-deficient LPS results in poor recipient ability with both F and I types of donor strains, while heptose-deficient mutants seen so far are Con- with F type donors. Havekes et al. propose that LPS is very likely involved in conjugation with I-type donors and might even be the receptor for the I-pilus.

1.9 TraT Protein

TraT is a peripherally anchored outer membrane lipoprotein encoded by IncF plasmids and found in *E. coli*. As previously stated, F-type plasmids encode genes involved in the conjugative transfer (*tra* genes) of that plasmid from donor to recipient, stable mating pair formation itself, as well as the exclusion of conjugation; TraT is responsible for surface exclusion. TraT is prevalent in the cell, found in a copy number of around 21,000 (Moll et al., 1980). The mature form of the protein has 223 amino acids and a molecular weight of around 25 kDa, and the precursor molecule produced before translocation to the outer membrane is 244 amino acids long. Once transferred to its position in the outer membrane, the protein’s signal sequence is cleaved at the only cystine residue in the molecule – the 22\textsuperscript{nd} amino acid position (Figure 6).
Figure 6: Full length and mature forms of TraT protein. The cleavable signal sequence and lipidation site is marked in the full-length protein, as well as the NSAGA sequence specificity motif noted in both constructs.

Multiple modifications occur at the cysteine residue before and after the signal sequence is cleaved. These modifications are vital for the functioning of the protein, as they target it to the outer membrane. Before the signal sequence is cleaved, a thioether-linked diglyceride is added onto the cysteine residue, as well as palmitic fatty acids (Perumal & Minkley, 1984). The signal peptide is then cleaved by signal peptidase II and the cysteine is joined with an acyl group, forming the initial amino acid of the mature protein (Sukupolvi & David O’connor, 1990).

1.10 Exclusion and Sequence Specificity

To review, there are multiple types of exclusion including surface exclusion and entry exclusion, mediated by products of the tra genes which also mediate other factors involved in conjugation. Within the tra operon of the F plasmid, the traS gene is found directly upstream of the traT gene (Jalajakumari et al., 1987).
Surface exclusions are not without variation – four different systems have been distinguished in F-like plasmids: SfxI – SfxIV (Willetts & Maule, 1974). Unsurprisingly, it appears as though these systems share similar mechanisms, and exhibit exclusion when faced with hosts that contain plasmids within the same exclusion system. This implies that the *traS* and *traT* gene products bear exclusion group specificity (Willetts & Maule, 1974). The code of these surface exclusion systems was studied to determine their conservation and alterations to better understand their functioning, comparing plasmids F (Sfx I), ColB (Sfx II), and plasmid R100 (Sfx IV). It was found that there was a region of conservation from amino acid positions 116-120 (counting residues in the mature TraT protein) – NSAGA, amino acids asparagine, serine, alanine, glycine, and alanine, respectively. Single amino acid substitutions were found at position 116 in Sfx system II (asparagine to serine), and residue 120 in Sfx system I (alanine to glycine). These changes resulted in an altered surface exclusion system, disrupting the exclusion process. This finding of the conserved regions and changes, known as sequence specificity, is thought to be involved with how TraT enacts conjugative exclusion with each system with specificity (Sukupolvi & David O’connor, 1990).

As previously mentioned, one of the big unknowns in the process of conjugation is the identity of the receptor for the conjugative pilus. Since there are various different surface exclusion systems, it stands to reason that there are potentially multiple receptors. The exact mechanism by which exclusion is moderated by TraT is unknown as of yet, but literature suggests that the TraT protein may perform its exclusion function by interacting with the donor cell surface through binding to the end of the conjugative pilus, thereby blocking the pilus from interacting with its actual target on the recipient cell (Willetts &
Maule, 1974). Alternatively, the TraT protein can occlude the true receptor for conjugative pilus binding thus preventing interaction, as suggested for possible OmpA-TraT interaction.

1.11 Serum, Complement, and Bacterial Defense

Serum is a major component of blood; it consists of the liquid fraction of blood minus cells and clotting factors, which differentiates it from plasma (Sotelo-Orozco et al., 2021). Serum contains components that have bactericidal properties and is an important contributor to the host animal’s immune defense system against bacterial infection (Moll et al., 1980). Complement is the name for a set of serum proteins that perform organized attacks on extracellular pathogens such as bacteria and is considered to be an innate immune response. There are different pathways in the complement system, and depending on the pathway, complement can kill by promoting opsonization of the pathogen, or more directly though formation of pores in the invading bacterial cell by the Membrane Attack Complex (MAC). Activation of the complement system is due to a triggered-enzyme cascade, resulting in a significant amplification of complement proteins after each enzymatic reaction in the cascade. There are three complement pathways – classical, MB-lectin, and alternative. The classical pathway is activated by direct binding of C1q component to the bacterial cell surface, or to an antigen-antibody bound pair. This process interacts with the adaptive response of the immune system (Moll et al., 1980). The alternative complement pathway, on the other hand, is activated by direct contact with the pathogen’s cell surface constituents. Each pathway creates enzymatic proteolytic
activities that produce the complementary effector molecules that induce opsonization, direct killing, and inflammatory processes.

Although the complement cascade is an efficient process, some pathogens are resistant to killing by components of the host’s immune response and mount defenses against it. For example, consider uropathogenic *E. coli*, or UPEC strains. UPEC are the leading cause of urinary tract infections (UTI) worldwide, responsible for over 80% of all infections (Ulett et al., 2013). These bacteria have many virulence factors at play to promote their propagation and survival, including Type I fimbrial adhesins, toxins such as α-hemolysin and lipopolysaccharide, and iron-acquisition systems just to name a few. Additionally, UPEC strains can often be multidrug resistant, and infections with these bacteria can lead to recurrent UTI, urosepsis, and fatality. Along with their many offensive weapons, UPEC strains are also able to defend themselves by manipulating their host’s innate immune responses in a few different ways (Ulett et al., 2013). The aforementioned α-hemolysin is able to inhibit the cytokine production of epithelial cells and can also reduce inflammatory processes and adhesion of adversary cells. As well as promoting biofilm development, curli proteins found on the outside of UPEC strains are able to dampen early host inflammatory responses through interaction with cathelicidin LL-37, a peptide with an antimicrobial role. Certain UPEC strains are able to secrete a protein called TcpC, which is a “Toll/IL-1 receptor (TIR) domain-containing protein” that is structurally similar to host Toll-like Receptor 1. This protein can inhibit the TLR signaling and downstream pathways, thus reducing response. Interestingly, UPEC strains have also shown a propensity for regulating and inducing cyclooxygenase-2 (COX-2) and indoleamine 2,3-dioxygenase (IDO) respectively in uroepithelial cells, both of which are
factors involved in inflammatory response and T-cell-limiting immunosuppression. UPEC strains may also utilize autophagy pathways, which would affect the release of interleukins. (Ulett et al., 2013)

UPEC can also protect themselves against serum killing through overexcitation of IgG2 antibodies against the O-antigen of LPS found on the outside of UPEC cells. Instead of encouraging bacterial death, these “inhibitory” antibodies are overproduced and cover the surface of the invading bacterial cells, preventing serum killing by not allowing any place for the complement to bind and initiate MAC. This appears to be a titer-based effect, as reduction in O-antigen-specific-IgG2 concentration appears to restore normal complement functioning (Coggon et al., 2018). IgG2 is known to be the IgG subtype most heavily associated with response to endotoxin, as well as only binding one class of FcγR and having reduced availability of C1q binding sites (Wells et al., 2014). In infections where the inhibitory antibodies were seen, the causative bacteria tended to be more sensitive to serum than what is seen in urosepsis where inhibitory antibodies are not present. These findings suggest that this method of complement resistance adds a significant advantage to pathogenesis; the prevalence of this defense method is supported by the large number of patients in which it is found (Coggon et al., 2018).

Another method of UPEC defense against complement-mediated killing is the prevention of MAC formation. To kill invading bacterial cells, MAC forms a transmembrane pore which damages the cell envelope and leads to death. In order to accomplish this, C5 must be locally converted into C5b, which combines with other factors to form the pore. C7 must quickly interact with C5b to ensure adhesion to the
bacterial surface and complete pore formation. However, UPEC strains are able to interrupt the adhesion of the C5b-7 complex to the surface, preventing complement killing; the method by which this occurs is not yet confirmed (Doorduijn et al., 2020).

1.12 Serum Resistance Function of TraT

Although the main focus of this work describes TraT as being involved with surface exclusion determination, it is a multifunctional protein that serves other roles in the cell as well. TraT has been shown to protect the cell from a few different bacteriophages that typically bind to OmpA (Riede & Eschbach, 1986) as well as serving as a virulence factor by protecting its host cell from complement killing during bacterial exposure to animal sera (Moll et al., 1980). Literature states that E. coli cells containing a plasmid coding for the TraT protein exhibit an increased resistance to sera complement, showing better survivability than cells not containing a plasmid coding for TraT (Sukupolvi & David O’connor, 1990). Although – as in many other aspects of the functioning of TraT – the exact mechanism of serum resistance function is as of yet unknown, it is proposed that the interaction is very specific rather than simply quantity based, as it has been found that point mutations in the TraT protein result in reduced serum resistance ability, even with high quantities of that slightly altered TraT in the membrane. (Moll et al., 1980). It is thought that the TraT protein may block the interaction of some component of one or the other types of host immune system complement pathways. As TraT has been shown to prevent interaction of certain bacteriophages through blocking access to their target OmpA as previously mentioned, this theory is not unsubstantiated.
The two most likely types of complement pathways that could be involved here in response to bacterial contamination are the classical and alternative pathways. The changes in cell surface morphology provided by TraT presence have the potential to inhibit activation of the two aforementioned pathways by blocking their primary targets such as cell surface lipopolysaccharide for the alternative pathway, or other cell surface antigens from being reached by antibodies to be recognized by the classical pathway. TraT could perform its serum resistance function by altering deposition of C3 on the cell surface, or by changing the functioning or formation of the C5b6 complex. It could also possibly alter the cell membrane permeability, reducing efficacy of the MAC pore formation (Miajlovic & Smith, 2014). It has been observed that nonpathogenic bacteria typically show great sensitivity to serum, while the pathogenic bacteria are more often very resistant (Moll et al., 1980) – this shows the importance of serum resistance to the overall virulence of invasive pathogens and earmarks this trait as an important factor to consider in the study of TraT-containing bacteria.

1.13 TraT Predicted Structure

Currently, there is no definitive structure for the TraT protein solved through x-ray crystallography or other means. However, calculated predictive models have been created. TraT is a member of the PF05818 protein family; the PFAM (Protein Families) database, a collection of multiple sequence alignments (MSAs) representing related proteins, lists 187 sequences contained in this family. University of Washington’s Baker group was able to model each conserved PFAM through de novo in silico folding, using trRosetta – a deep learning software which functions through taking a MSA as the input,
and outputting information on the relative distances and inter-residue orientations of all amino acid pairs that compose the protein structure (Yang et al., 2020).

Figure 7: PF05818 protein family predicted structure. Approximate NSAGA region boxed in orange.

Seen in Figure 7 above is this predicted model for a PF05818 protein member. This structure is not the exact TraT protein, but an AlphaFold-derived model. This structure will prove very helpful for the advancement of structural knowledge of this family of proteins and may help with future computational biology determinations of function or binding partners.

1.14 Thesis Statement

Bacterial conjugation serves an important role in the transfer of genetic information in bacteria, including that of antibiotic resistance genes. The tra family of proteins, including TraT, are heavily involved with conjugation, but there is still much to
discover regarding their structure, function, and interactors. In this thesis, these aspects of TraT will be explored in multiple directions. Our previously developed TraT construct is further purified and characterized through FPLC Ion-Exchange Chromatography and Size-Exclusion Chromatography to improve purity and homogeneity of TraT and aid in downstream applications. Interactors are sought out through the use of non-site-specific crosslinking and pull down assays, to better understand TraT’s role in the cell. Stability and role of various aspects of TraT are tested over a range of values. The overarching aim of this work is to further our understanding of the functionality and structural organization of the TraT protein and by doing so, comprehend the process of bacterial conjugation in a more definite way.
CHAPTER 2: MATERIALS AND METHODS

2.1 Preparation of Growth Media

During the course of this research, both liquid medium and solid agar medium in the form of lysogeny broth (LB) were used. The standard recipe used was 25 g of LB powder per liter of solution, and in the case of solid media, 15 g of agar per liter was added as well for plate preparation. In the case where less than a liter of solution was needed, weight of constituents in grams was adjusted proportionally. To make liquid growth media, LB powder and deionized water were added to a glass flask and stirred until combined; the solution was then sterilized in an autoclave. To make solid agar plates, LB powder, agar, and deionized water were added to a glass flask and stirred until the LB had dissolved; the solution was then sterilized in an autoclave. The liquid agar would then be poured into plastic petri dishes and allowed to stand at room temperature for 2-3 days in order to completely dry condensation, and then were stored in plastic sleeves in a 4°C cold room. If antibiotics were needed in either type of media, the antibiotics were added to the media post-sterilization after the solution had cooled. Antibiotics were taken from 1000x antibiotic stock and added at a concentration of 1 µL per mL of solution.

2.2 Buffer Compositions

Nickel affinity chromatography used two buffers (A and B) as well as degassed diH2O and degassed 20% ethanol for rinsing and storage. Buffer A was the low-
imidazole buffer, and consisted of 10 mM Tris Base, 300 mM NaCl, and 10 mM imidazole. To make 1 liter of Buffer A, 1.21 g of Tris Base, 17.53 g of NaCl, and 0.68 g of imidazole were added to 800 mL of deionized water. The solution was mixed in a beaker on a magnetic stir plate and was brought to a pH of 7.8 with the addition of concentrated HCl, after which the volume was adjusted to 1 L by addition of deionized water. Buffer B was the high-imidazole buffer, and consisted of 10 mM Tris Base, 300 mM NaCl, and 250 mM imidazole. To make 500 mL of Buffer B, 0.61 g of Tris Base, 8.77 g of NaCl, and 8.51 g of imidazole were added to 300 mL of deionized water. The solution was mixed in a beaker on a magnetic stir plate and was brought to a pH of 7.8 with the addition of concentrated HCl, after which the volume was raised to 500 mL by addition of deionized water. Both buffers underwent vacuum filtration to filter and de-gas and were then stored in screw-capped stock bottles at room temperature. For use following Ni-affinity chromatography, no-imidazole dialysis buffer was made which consisted of 10 mM Tris Base and 300 mM NaCl. To make a final volume of 4 L, 4.85 g Tris Base and 70.13 g NaCl were added to 3 liters of deionized water and mixed on a magnetic stir plate. After the solutes had dissolved, the solution was brought to a pH 7.8 with the addition of concentrated HCl, after which the volume was raised to 4 L by addition of deionized water. The final solution was stored in a 4 L plastic beaker covered in foil in a 4°C cold room.

Tris-Glycine SDS PAGE gel running buffer stock was prepared at a 10X concentration. To make 1 L, 30.3 g of Tris Base, 144 g of glycine, and 10 g of SDS were added to 500 mL of deionized water in a beaker and stirred on a magnetic stir plate. The volume was then raised to 1 L by addition of deionized water. Before use, the 10X buffer
was diluted to 1X by adding (in a screw-cap bottle) 100 mL of 10X stock to 900 mL of deionized water and gently inverting so as to combine without foaming the SDS.

Ion-exchange chromatography used two buffers (A and B) as well as degassed diH2O and degassed 20% ethanol for rinsing and storage. Buffer A was the no salt buffer and consisted of 10 mM Tris base. To make 1 L of Buffer A, 1.2114 g of Tris base was added to 500 mL of deionized water. The solution was mixed in a beaker on a magnetic stir plate and was brought to a pH 7.8 with the addition of concentrated HCl; the volume was then adjusted to 1 L by the addition of deionized water. Buffer B was the high salt buffer and consisted of 10 mM Tris Base and 1 M NaCl. To make 500 mL of Buffer B, 0.6057 g of Tris Base and 29.22 g of NaCl were added to 300 mL of deionized water. The solution was mixed in a beaker on a magnetic stir plate and was brought to a pH of 7.8 with the addition of concentrated HCl, after which the volume was raised to 500 mL by addition of deionized water. The same buffer was also used at a pH of 8.0. Both buffers A and B underwent vacuum filtration to filter and de-gas and were then stored in screw-capped stock bottles at room temperature.

Size exclusion (gel filtration) chromatography used one buffer – the same dialysis buffer that was used after Ni-affinity chromatography.

2.3 LPS Extraction Protocol

A protocol for the extraction of LPS from the bacterial cell was adapted from a thesis by Matevž Rumpret (Rumpret, 2014). To begin, a single colony of your chosen bacterial strain was inoculated into 5 ml of liquid LB, along with the appropriate
antibiotics needed to keep the culture pure. The tubes were incubated at 37 °C with shaking at 160–180 rpm overnight. The bacterial strains chosen for this experiment were MG1655 and J53, common E. coli lab strains with rough, minimal LPS predicted to be present. After the cultures had grown overnight, 3 milliliters from each tube were pelleted by centrifugation at 14000 rpm for 5 min. The pellets were washed three times with 0.09% NaCl. The cells were then pelleted and resuspended in 125 μL of lysis buffer with SDS (Tris-HCl 60 mM, EDTA 1 mM, SDS 2%, final pH of 6.8) and were vortexed. The cells were boiled for five minutes and vortexed once more. The now-lysed cells were resuspended in 875 μL of lysis buffer without SDS (Tris-HCl 60 mM, EDTA 1 mM, final pH of 6.8), and 100 μg of proteinase K was added with a two hour 65 °C incubation following. The LPS extracts were stored at 4 °C until analysis.

2.4 LPS Biotinylation

LPS was biotinylated using a protocol adapted from Luk, et al. (Luk et al., 1995) for use in binding assays. To begin, 0.2 mL LPS O111 (5 mg/mL) stock and 0.8 mL 0.5M sodium acetate were combined in a microcentrifuge tube. 0.1 mL of freshly prepared 100 mM sodium metaperiodate was added and mixed by pipetting. The tube containing the LPS mixture was then covered in foil and allowed to oxidize on ice (0°C) for 30 minutes. The reaction was stopped by adding 2.2 mL of 1X glycerol quenching solution. Five minutes later, the solution was transferred to two Amicon® Ultra-4 Centrifugal Filter Devices and PBS was added to reach a volume of 4 mL. The tubes were spun at 4,000 g for 13 minutes (on average), with the decreasing volume being checked at intervals of 5, 3, and 3 minutes to ensure no product loss. Once an approximate volume of 400 μL was
reached, the solution was resuspended up to 4 mL with PBS again. This process was repeated for three cycles, ending with a final volume of 1 ml. To begin biotinylation, 0.1 mL of biotin-lc-hydrazide (50 mM) was added to 1 mL of the oxidized LPS sample, making sure to leave the remaining 1 mL of oxidized LPS unbiotinylated to act as a control. This mixture was incubated at room temperature gentle end to end rotation provided by the Thermo Scientific Tube Revolver for 2 hours. Post incubation, the buffer exchange step was repeated, ending again at a volume of 1 mL. The biotinylated LPS and oxidized control were stored at 4°C until further use.

2.5 Protein Expression Protocol

To begin the procedure, *E. coli* BL21(DE3) strain harboring a transformed pET28b plasmid vector with encoded recombinant TraT was freshly streaked from freezer stocks onto plates of solid LB agar containing 50 μg/mL kanamycin and then incubated overnight at 37°C. The next day, an isolated colony was added to 4 mL of autoclaved liquid LB along with 4 μL of kanamycin stock and grown overnight again, still at a temperature of 37°C but this time with a shake speed of 200 RPM for aeration of liquid culture. Scale of expression varied throughout the work, and thus differing volumes of overnight culture, liquid LB, and kanamycin were used. However, at least 500 mL was expressed in a 2 L flask at a time, so whether the final volume expressed across flasks was 500 mL or 4 L, 12.5 mL of overnight culture was added to 500 mL of sterile liquid LB along with 500 μL of kanamycin. The newly made flask cultures were grown to exponential phase, where an OD600 of 0.6-0.8 was desired. Once growth to the ideal OD600 was reached, 500 μL of isopropyl β-d-1-thiogalactopyranoside (IPTG) was
added to each flask in order to induce the production of TraT. The flask cultures were
grown for another three hours post IPTG addition, and then evenly aliquoted into either
250 mL or 1 L centrifuge bottles, depending on the total volume of culture grown. The
bottles were then spun in a Sorvall LYNX superspeed centrifuge (Thermo Scientific) for
15 minutes at 4°C, 10k relative centrifugal force (RCF). The supernatant was discarded,
and the remaining cell pellets were stored in a -20°C freezer until use.

2.6 SDS PAGE Gel Preparation and Protocol

Tris-Glycine SDS-PAGE gels were cast by hand for analytical use. Gels used
were 13% w/v for analysis of lipopolysaccharides and 12.5% w/v for analysis of proteins.
Reagent volume varied between percentage and number of gels cast. One 13% gel was
prepared via the following recipe. The running gel was made by adding 2.1 mL of
deionized water, 2.45 mL of 40% acrylamide (29:1) solution, 2.8 mL of 1 M Tris pH 8.9,
75 μL of 10% SDS, 7.5 μL of TEMED, and 50 μL of 10% APS to a 50 mL conical tube,
making sure to add the APS last and gently inverting to combine. The stacking gel was
made by adding 1.85 mL of deionized water, 312.5 μL of 40% acrylamide (29:1)
solution, 315 μL of 1 M Tris pH 6.8, 25 μL of 10% SDS, 5 μL TEMED and 17.5 μL of
10% APS to a 50 mL conical tube, making sure to add the APS last and gently inverting
to combine. One 12.5% gel was prepared via the following recipe. The running gel was
made by adding 2.2 mL of deionized water, 2.35 mL of 40% acrylamide (29:1) solution,
2.8 mL of 1 M Tris pH 8.9, 75 μL of 10% SDS, 7.5 μL of TEMED, and 50 μL of 10% APS to a 50 mL conical tube, making sure to add the APS last and gently inverting to
combine. The stacking gel was made by adding 1.95 mL of deionized water, 190 μL of
40% acrylamide (29:1) solution, 315 μL of 1 M Tris pH 6.8, 25 μL of 10% SDS, 5 μL TEMED and 17.5 μL of 10% APS to a 50 mL conical tube, making sure to add the APS last and gently inverting to combine. The casting protocol was the same no matter the percentage and follows thusly. After the 1.0 mm gel casting cassettes (BioRad) were properly cleaned and set up, the running gel solution was added to the cassette via 1,000 μL micropipette, with a few centimeters left at the top to save room for the stacking gel. Around 10 μL of EtOH was added to the top of each gel, and the gels were allowed to polymerize until the remaining solution in the 50 mL conical was solidified, usually around an hour. Once this state was reached, the EtOH was removed via tilting the cassette and absorbing the liquid with filter paper. The tops of the gels were rinsed with deionized water and the removal procedure was repeated. The stacking gel was then added to the top of the cassette, and plastic 15-well 1.0 mm combs were placed in between the glass plates. Once the stacking gel was completely polymerized – again judging from the left-over solution, around an hour – the combs were carefully removed, and the gel-containing glass plates were unsnapped from the cassette. Each gel was wrapped in a paper towel dampened with deionized water and then further wrapped in plastic wrap to prevent drying. The gels were stored in a lidded plastic container at 4°C until needed.

2.7 Chromatography Protocol

Three different types of chromatography were applied in this work: Nickel affinity chromatography, ion exchange chromatography, and size exclusion (gel filtration) chromatography. These processes were carried out through the fast protein
liquid chromatography (FPLC) method using the BioRad NGC Quest Liquid Chromatography System and protein expressed in large batch cultures. The procedures for each type had both similarities and differences. The entire protocol will be described using the specifications for Nickel affinity, and then individual differences between Ni-affinity, IEC, and SEC will be discussed. Initial preparatory steps include powering on the FLPC machine and computer and allowing the machine’s UV lamps to warm up for thirty minutes, as well as fast-tempering the Eppendorf 5430R fixed-rotor table-top centrifuge to 4°C. The system is stored in 20% ethanol in order to prevent contamination, and so must be rinsed with de-gassed deionized water – to start, the sample pump, system pumps (A and B), and Ni-affinity column were primed and rinsed with 10 mL of the aforementioned water. Next, system pump A and the sample pump were primed and rinsed with 10 mL of Buffer A (low imidazole), while system pump B and the Ni-column were rinsed with 10 mL Buffer B (high imidazole). At this point, the machine was ready for use. To prepare the sample, the frozen cell pellet created from the large-scale expression procedure was allowed to come to room temperature for easier resuspension, and then thoroughly but carefully resuspended in 5 mL of buffer A (low imidazole), making sure to cause as few bubbles as possible. The resuspended pellet was transferred to a 15 mL conical tube and placed into a beaker full of ice, and then sonicated on a Fisher Scientific sonicator (settings: amplitude of 51%, 30 second on/off intervals for 10 minutes total time). Post sonication, the 5 mL sample was evenly aliquoted into four microcentrifuge tubes, each containing a volume of 1.25 mL. These tubes were spun in the 4°C centrifuge for a total time of 10 minutes, 20k RCF. Resulting was a bilayer of sample – the soluble lysate portion (the supernatant) was pipetted off into a separate tube
and filtered through use of a 5cc syringe and 0.2 micron sterile filter to be used for purification, while the insoluble fraction (the pellet) of one of the tubes was resuspended in 1 mL buffer A (low imidazole). Twenty microliter aliquots were collected for analysis on SDS-PAGE gels from the phases of whole lysate, soluble lysate, and insoluble fraction. In later repetitions using high volumes of cell culture (4 L), a similarly scaled-up lysate protocol was utilized, involving use of the Fisher Scientific 550 Sonic Dismembrator (15 minutes total on time, with pulse on for 10 seconds and pulse off for 20 seconds) and then a spin in the Thermo Scientific Sorvall LYNX superspeed centrifuge at 17,000 RCF for 20 minutes. To run the Ni-affinity chromatography, an appropriate run method was chosen or created on the computer, and the sample pump tube was inserted into the conical tube containing the filtered soluble lysate sample. The sample volume in the method was set slightly higher than the actual volume of sample, and when the sample uptake was almost complete, buffer A (low imidazole) was added to the tube to ensure that all of the lysate was loaded.

2.7.1 Ion Exchange Chromatography

Ion exchange chromatography was run similarly to the Ni-affinity chromatography with a few notable differences. First, the sample used was the Ni-affinity purified TraT protein rather than a newly made lysate; the TraT protein buffer was changed to be the Buffer A (no salt) buffer rather than the dialysis buffer it was previously in to ensure binding. Buffers A (no salt) (Tris 10 mM, final pH 7.8) and B (high salt, 1 M) (Tris 10 mM, NaCl 1 M, final pH 7.8) for IEC were different than Buffers A (low imidazole) and B (high imidazole) for Ni-Affinity chromatography.
Different columns were used – Q and S. The manual load loop was used rather than the sample pump, so it was rinsed with appropriate buffers with a designated 1cc syringe and needle. The system pumps and column were rinsed similarly to what was previously discussed. It is also important to load the sample into the inject valve before starting the run and adjust the sample volume in the run parameters as needed.

2.7.2 Size Exclusion Chromatography

Size exclusion chromatography (SEC), also known as gel filtration, is a form of chromatography used to analyze or further purify a sample through separation based on size. Smaller particulate will become trapped in the resin beads of the matrix, eluting later, while larger particles will not be able to enter the beads and thus elute earlier, thereby separating the sample based on constituent size. This process runs similarly to the previously discussed chromatography methods, with some notable changes. There is only one buffer used, the same Tris buffer used for dialysis post-Nickel affinity, held at room temperature rather than 4°C. The columns used are much larger and are composed of a different matrix. SEC 70 column was used. The flow rate through the SEC columns needs to be lowered to 1 mL per minute, and it is important to load no more than 1% of the column volume; we loaded 200 μL of sample. The protein sample is loaded into a tubing loop. When programming the run method, the sample volume is always listed as higher than it is, 500 μL instead of 200 μL in this case, to allow buffer to flush the loading loop and thus load the complete fixed volume of the protein samples. When using gel filtration, it is important to make sure that the entire system is free from air bubbles and has a uniform buffer everywhere.
Figure 8: Calibration curve of known molecular weight standards. The equation of this line was used to estimate the molecular weights of SEC peaks.

Figure 9: FPLC chromatogram of known molecular weight standards used for creating the calibration curve for SEC70 column.

For preparing a calibration curve for SEC columns, BioRad Gel Filtration standards were used that contain thyroglobulin, gamma globulin, ovalbumin, myoglobin, and vitamin B12 with the known MW of 670 kDa, 158 kDa, 44 kDa, 17 kDa, 1.35 kDa, respectively (Figure 8, Figure 9). Elution volume of each standard’s peak was established and plotted against Log of molecular weight with consecutive linear fit in Microsoft
Excel to establish a linear equation. This equation was then used to estimate molecular weight of TraT peaks in separate runs at the same SEC column.

2.8 Dialysis and Protein Concentration Protocol

For changing the protein buffer, two different methods were used: dialysis into new buffer, and concentration/dilution protocol using ultrafiltration columns. To prep for dialysis, of the Ni-purified protein sample, 1 L of cold dialysis buffer was added to a 1 L beaker along with a magnetic stir bar. An appropriate amount of 12-14 kDa molecular weight cut-off (MWCO) dialysis tubing for the volume of Ni-purified protein sample (0.32 cm per mL sample) was cut from the roll and rinsed inside and out with deionized water. The tubing was then folded over itself and held closed on one end by a clamp. The protein sample was pipetted into the tubing, and then the top end of the tubing was folded and clamped shut. The tube was placed into the beaker of dialysis buffer, ensuring that the entire length of the tubing was submerged, and the beaker was placed on a magnetic stirring plate in the 4°C cold room overnight. On the next day, the now dialyzed protein sample was pipetted out of the tubing and into a conical tube. To concentrate, 500 μL of sample at a time were pipetted into an Amicon 10k molecular weight cut off microcentrifuge filter and spun at 14k rpm, 4°C for 10 minutes. Liquid that passed through the filter after each spin was discarded, the remaining protein was mixed by gentle pipetting, and more unconcentrated protein was added to reach the 500 μL mark again. This process was repeated until the volume of protein was reduced to a desired volume. The final concentrated protein was removed from the spin tubes and stored in a microcentrifuge tube at 4°C. For buffer exchange, this process of concentration was
followed by adding a larger volume of new buffer, and then concentrating the sample again. Usually 10-fold concentration and dilution steps were used, and the process was repeated at least twice to ensure full buffer change.

2.9 Bradford Assay for Measuring Protein Concentration

The Preparations of Standard and Assay Reagent protocol from Thermo Fisher Scientific was utilized to both make the dilution samples as well as to prepare the Bradford reagent itself. First, the Bradford reagent was aliquoted from the stock bottle into a conical tube and set on the lab bench until room temperature was reached. Next, a single vial of Albumin Standard (BSA) was diluted with deionized water into nine separate microcentrifuge tubes (Table 1). Standards were allowed to incubate with Bradford reagent for 10 minutes before measuring OD. To measure the concentration of protein in a particular sample or standard, a Thermo Scientific Genesys 50 spectrophotometer was used to measure absorbance at 595nm, with 1 mL of Bradford Reagent serving as the blank. Once the standard absorbances were measured, they were used along with the given concentrations to create the Bradford Standard Curve, which included a linear equation. For being used, the fit of the straight line should have had an $R^2$ value close to 1 (Figure 10).
When measuring an unknown TraT preparation, the sample was serially diluted in three-fold increments until a 595nm absorbance of samples was within the Standard Curve range (Figure 10). Sample was allowed to incubate with the Bradford reagent for 10 minutes before measuring OD.

2.10 Binding Assay Protocols

Three different forms of binding or pull-down assay were performed during the course of this research in order to search for potential interactors – streptavidin binding with LPS, streptavidin binding with TraT, and Ni-resin binding with TraT. To make the SL89 strain (Top10 E. coli) and SL88 strain (Top10F’ E. coli) whole cell lysates used in the first and third binding assay type mentioned above, overnight cultures of these strains were inoculated in either increments of ‘100 μL into 4 mL LB’ or ‘1.5 mL in 50 mL LB flasks’ and grown at 37°C with vigorous agitation until OD measurements determined exponential phase was reached (2 hours). The cells were pelleted at 14,000 rpm for 5
minutes and resuspended in 0.5 mL PBS. About 100 μL of 0.1mm glass disruption beads was measured into microcentrifuge tubes and each respective cell suspension was added to the appropriately labeled tube. The lid seam of the tube was parafilmed to ensure no solution loss and the tubes were shaken on the tabletop Fisher vortex at max speed (3000 rpm) for 5 minutes. The parafilm was then removed, and the tubes were spun in the microcentrifuge at 14,000 rpm for 10 minutes to pellet. The liquid lysate was carefully removed via pipette to a separate microcentrifuge tube and stored at 4°C until future use.

The protocol for LPS-Streptavidin binding is as follows. First, the bottle of M-280 Streptavidin was gently swirled to fully resuspend the iron beads. Fifty μL of suspended streptavidin was added to a microcentrifuge tube, and the tube was placed into the holder of a magnet. Once all of the iron was stuck to the magnet side of the tube, the supernatant was removed and the beads were washed three times with 1 mL of PBS, then resuspended with PBS to a final volume of 50 μL. A non-oxidated LPS control was made by combining 240 μL 5 mg/mL O111 stock and 360 μL PBS. Seven microcentrifuge tubes were labeled “LPS beads only”, “Beads with Top10”, “Beads with Top10F’”, “LPS beads with Top10”, “LPS beads with Top10F’”, “Top10 lysate”, and “Top10F’ lysate”; where “beads” referred to the presence of streptavidin beads, and “LPS beads” referred to the combination of streptavidin beads and biotinylated LPS. Ten μL of washed streptavidin beads were added to each tube as needed, and 200 μL of LPS (biotinylated or control) were added to appropriate tubes as well. The microcentrifuge tubes were incubated with gentle end to end rotation in the Thermo Scientific Tube Revolver for 30 minutes. Post incubation, the tubes were placed on the magnet for 3 minutes after which the supernatant was discarded and the beads were washed with PBS 3 times. Top10 or
Top10F’ whole cell lysates were added in volumes of 250 μL to appropriately labeled tubes, and the tubes were allowed to incubate again for 2 hours. The tubes were washed once more using the method mentioned previously, with a final resuspension volume of 10 μl. The samples were then used for SDS-PAGE analysis or stored at 4°C until future use.

For the second assay type involving streptavidin beads, LPS, and TraT, the procedure was largely the same as detailed above except for volumes and the samples created were “Beads with TraT1 only”, “LPS-Beads”, “LPS-Beads with TraT1”, and “LPS-Beads with TraT2; where ‘TraT1’ refers to a TraT concentration of 12.5 mg/mL and ‘TraT2’ is a 10-fold dilution of TraT1.

The protocol for the TraT-Ni-resin binding assay began by gently swirling the bottle of Nickel-NTA agarose resin to resuspend. 450 μL of Ni-NTA resin was pipetted into a microcentrifuge tube and spun in the 5430R centrifuge at 100 RCF, room temperature, for 1 minute. The supernatant was removed, 1 mL of Dialysis Buffer (no Imidazole) was added, and the resin was washed by gentle inversion. The microcentrifuge tube was spun again using the parameters listed above, and the supernatant was once more pipetted off. This wash step was completed a total of two times, then the buffer was removed. The Ni resin was resuspended in 450 μL of Dialysis Buffer (no Imidazole) and aliquoted in 100 μL increments into 4 new microcentrifuge tubes, labeled “A: TraT + Top10 Lysate + Resin”, “B: TraT + Top10F’ Lysate + Resin”, “C: Top10 Lysate + Resin + Buffer”, and “D: Top10F’ Lysate + Resin + Buffer”. The tubes were spun briefly, and the buffer was removed. To prepare the incubation tubes, 50 μL of TraT was added to tubes A and B and left to bind for 30 minutes on ice. Post
incubation, 400 μL of lysates and 50 μL of Dialysis Buffer (no Imidazole) were added to the appropriate tubes. All tubes were thoroughly mixed by inverting, and were incubated on ice for 15 minutes, with occasional mixing interspersed to keep the resin suspended. The tubes were spun using the parameters listed above, the supernatant was discarded, and the resin was washed three times with 1 mL of Buffer A (low imidazole buffer (10 mM)). To begin the elution step, the supernatant from the previous wash was discarded and 100 μL of Buffer B (250 mM (High Imidazole Buffer)) was used to wash the resin twice. Samples for SDS-PAGE were collected from each elution step, as well as from the first Buffer A wash. SDS-PAGE analysis was performed using the samples as well as reference TraT.

2.11 General Conjugation Assay Protocol

Conjugation assays were performed to test the effect of adding exogenous LPS and/or TraT to a liquid mating mixture. A few different variations to this protocol were run and will be mentioned at the end of this section, but the general protocol is as follows.

Overnight liquid cultures of both donor and recipient strains were made by adding isolated colonies of appropriate strains to 4 mL LB broth along with 4 μL of the antibiotic to which the strain holds resistance. This mixture was grown overnight at 37°C with shaking and aeration at 200 rpm. The next morning, 100 μL of each overnight culture was added to a separate fresh tube with 4 mL of liquid LB along with appropriate antibiotics and grown with shaking an aeration until exponential phase was reached, around 2 hours. Once this point was reached, the OD600 of both cultures were measured, and the cultures
were centrifuged (3000 rpm for 10 minutes at room temperature), supernatant removed, and cells resuspended in a calculated volume of LB broth to bring both cultures to an OD/mL of 2. The conjugation tubes were prepared by labeling falcon tubes so that there will be a tube for just donor and recipient cultures, donor + recipient + each experimental addition (LPS, TraT), and donor + recipient + the buffer solution in which the experimental additions reside. The donor cultures are added to the tubes, followed by the experimental additions and buffer. They are mixed by gentle pipetting and then allowed to sit for 5 minutes. Recipient cells are then added to the tubes in a 1:1 ration with the donor cells and combined by gentle pipetting. The conjugation tubes then incubate at room temperature for 1 hour. After this time, the conjugation tubes as well as both individual parents were subjected to 10-fold serial dilutions, from undiluted to $10^{-8}$. The dilutions were plated in 4 μL drops in triplicate onto appropriate antibiotic plates using a multichannel pipette – Azithromycin 100 or Tetracycline 12 for the parents, and both Az100 and Tet12 combined for the transconjugants. The plates were incubated at 37°C and checked for growth at 24 and 48 hours.

Colony growth for each dilution was counted and the conjugation efficiency (N) was measured using the formula below. The result was shown in mL/hour/CFU, where “Δt” is the time of incubation (1 hour) and “T”, “R”, and “D” are the average CFU/mL of the transconjugants, recipients, and donors (Lopatkin et al., 2016).

$$N = \frac{T}{(R)(D)(Δt)}$$

Error propagation for measuring conjugation efficiency was calculated using the following formula, where “E” is the conjugation efficiency and “StdD” is the standard
deviation of the three cell densities measured in CFU/mL for each transconjugant (t), donor (d), or recipient (r) in triplicate (Land, 2021):

\[ \text{Error} = (E)^*\text{Sqrt}((\text{StdD}(t)/T)^2+(\text{StdD}(d)/D)^2+(\text{StdD}(r)/R)^2) \]

As previously mentioned, variations on this protocol occurred – there were runs using the extracted LPS from J53, runs using the purchased O111 LPS, and runs using both O111 LPS as well as Ni-affinity purified TraT. The assays had the same general format as described above, the main change was the variable being tested, as well as a buffer change where needed for the control tube. Additionally, repeats were done involving timepoints, where the conjugation mixture was sampled and plated at timepoints of 30 minutes of conjugation, 1 hour, and 1.5 hours.

Figure 11: (A) Visualization of the conjugation assay protocol. (B) Visualization of serial dilution plating in experiment. Image created with Biorender.
2.12 Western Blot

Western blotting was performed to detect His-tagged TraT. The protocol begins with running two identical SDS-PAGE gels containing the samples. Once the run has finished, one gel is stained with Coomassie blue as usual for comparison, while the other gel is transferred via electrophoresis onto nitrocellulose membrane for use in the western blot. To accomplish this, the stacking gel is removed, then the running gel is added into a tray with Western Transfer Buffer (WTB) (Tris 25 mM, Glycine 192 mM, Methanol 20%, Final pH 8.3) and placed on a Fisher tabletop shaker and allowed to shake for 15 minutes. While this is incubating, the nitrocellulose membrane and two sheets of extra thick blot paper are cut to the size of the gel. The membrane is incubated in WTB for 10 minutes, and the blot paper is incubated in WTB until soaked through. Once these incubations have finished, the transfer sandwich is made as follows: bottom plate (anode), blot paper, membrane, gel, blot paper, top plate (cathode), safety cover. Air bubbles are gently removed by using a roller tool during the stacking process. The transfer is run at 15 volts for 45 minutes. The membrane is removed from the sandwich and added to a tray with 5% skim milk powder dissolved in Tris-Buffered Saline + Tween 20 (TTBS), where it is blocked for 1 hour at room temperature on the Fisher tabletop shaker. Once this time has elapsed, the 5% milk solution is removed and the primary antibody (mouse anti-His; diluted 1:5000 in 5% milk solution) is added, and the membrane is incubated overnight at 4°C with gentle shaking. The following day, the primary antibody solution is removed, and the membrane is washed 3 times for 15 minutes per wash in TTBS. The membrane is then incubated with the secondary antibody (goat anti-mouse horseradish peroxidase conjugated; 1:5000 diluted in 1x TTBS) for 1
hour at room temperature with gentle shaking. After this, the membrane is once again washed 3 times for 15 minutes per wash in TTBS, and then transferred into a different tray for addition of SuperSignal™ West Pico PLUS Chemiluminescent Substrate. Two milliliters of each part of the reagent (peroxide solution and enhancer solution) are added to the tray and mixed with the membrane, with a two-minute incubation of gentle shaking. The membrane is then imaged on the BioRad Chemidoc Imaging System set to chemiluminescent mode for the western results, and to colorimetric mode to image the ladder. The images are then compiled and labeled as needed for analysis.

2.13 Formaldehyde Crosslinking

Non-site-specific formaldehyde crosslinking was used to investigate oligomerization state and improve bait-prey binding in the pull down assays, in attempt to stabilize some interactions that might otherwise be disassembled during the assay. First, TraT was dialyzed into HEPES buffer so that the Tris would not quench the crosslinking reaction. A 4% stock concentration formaldehyde was added to the TraT at a concentration of 1%, along with 0.1 mM EDTA to protect against heavy metal effects. In trials with Trat only as well as with TraT + lysate prey, the mixture was allowed to incubate at room temperature for 15 minutes before formaldehyde was added to allow for interaction with the recombinant TraT. To ensure temperature reproducibility, all incubations took place in the Eppendorf ThermoMixer C heat block set to 25°C. Samples were taken at 5, 10, 15, 30, and 60 minutes initially to check for optimal crosslinking time, with 10 and 60 minutes chosen for further repeats. Once each timepoint was reached, the crosslinking of that sample was quenched by addition of the usual Tris
dialysis buffer. Samples were then either loaded and ran on an SDS-PAGE gel for analysis of the entire crosslinked reaction, or the mixture was separated by the pull down assay using Ni-affinity resin. For the crosslinked pull down samples, crosslinked TraT and lysate were used as negative controls.

2.14 TraT Crystallization

A protein crystallization assay for TraT was performed. A 96-well plate with large and small wells made for crystallization was loaded with 1 μL of 24mg/mL TraT in the small wells. The Hampton Research Crystal Screen I kit was used for the crystallization reagents; 200 μL of each remaining reagent was added to a large well. One μL of reagent was mixed with the protein aliquot in the small well, and the wells were covered by tape row by row to reduce evaporation of the samples. The finished plate was set on a shelf for approximately two weeks, and the results were viewed under a microscope.

2.15 Site Directed Mutagenesis of traT Gene

Site-directed mutagenesis was performed to alter the conserved functional motif of the TraT - NSAGA region (Figure 6). NSAGA count labeling as discussed in the introduction uses the mature form of the TraT protein to get their 116-120 numbers. However, when referencing the amino acid substitutions performed in this lab, the numbering will start from the 33rd amino acid (reflecting use of the truncated recombinant TraT33-244), which gives NSAGA counts of 106-110. In this work, a A110→Amber110 residue substitution and A110→ R110 substitution were performed,
using the second Alanine of NSAGA. For the two \textit{traT} mutations, a specific primer set was designed (Table 3) that was used in an amplification reaction with the wildtype \textit{traT} gene that was used to produce recombinant TraT truncation for purification (Figure 13). Wildtype plasmids were digested by DpnI and the mixture was transformed into NEB5alpha competent cells. Three colonies from each plate with DpnI-treated mixtures were isolated. Plasmids from the isolates were purified and the desired changes in the coding region were confirmed through Sanger sequencing using sequencing primers (Table 3). This prior work was performed by Nabin Ghimire.

In this work, the plasmids were transformed into BL21(DE3) \textit{E. coli} cells and used to test the expression and then purify the substituted TraT variants. For Amber stop codon suppression, the BL21(DE3)pET28-TraT$^{33\text{-}244}\text{His}^6$-A110Amber strain was co-transformed with suppressor plasmid pEVOL-pBpF (AddGene Plasmid #31190) suitable for expression of Amber-tRNA and tRNA synthetase for the unnatural amino acid, \textit{p}-benzoyl-l-phenylalanine (pBpa). Amber suppression within this system would result in expression of the recombinant A110pBpA variant of our TraT construct that contains pBpa photo-crosslinker in the position of the conserved alanine A110 (Figure 6, Figure 13).
CHAPTER 3: RESULTS

3.1 LPS Conjugation Assays

To review, it was theorized that addition of free LPS to a conjugative mating mixture would result in a noticeable lowering of transconjugants compared to controls, thinking that the free LPS would capture some of the donor pili, leaving fewer donor cells available to transfer their plasmids. However, results of the initial conjugation assays did not show the significant lowering of conjugative ability that was expected from previous literature. Growth to $10^{-3}$ or $10^{-4}$ with CT2 and growth to $10^{-3} – 10^{-5}$ with CT1 and controls were commonly observed, but results were often noisy and variable.

Figure 12: Conjugation efficiency for conjugation assays with added LPS.
When the overall average of results is considered, there is no statistically significant difference between any of the conditions tested to alter conjugation efficiency compared to addition of buffer; P-values were far from the significance threshold. There was a slight significance seen to the improved performance of CT2 over CT1, but sample sizes were relatively small.

3.2 Scaling Up and Improving Purification Protocol for Recombinant TraT Protein

The primary protein construct used in this work is a truncated form of the mature TraT protein. Referred to as TraT\textsuperscript{33-244}, this protein begins at the 33rd amino acid from the N-terminus; this differs from the native TraT produced in cells, which is cleaved at residue 22 (see Figure 6 for visual representation). Due to this truncation, recombinant TraT cannot be post-translationally modified and is not expected to be transported to the membrane or secreted outside of the cell. This construct was chosen during previous research trials due to its high degree of expression in a BL21(DE3) strain, and its greater level of solubility compared with other less truncated constructs tested (TraT\textsuperscript{1-244}His\textsubscript{6}, TraT\textsuperscript{22-244}His\textsubscript{6}, TraT\textsuperscript{25-244}His\textsubscript{6}); (Land, 2021). Based on initial \textit{in silico} models of TraT homologues, the truncated construct should still have intact folded domains; the truncation of TraT\textsuperscript{33-244} begins shortly before the core β-sheet. This protein is expressed in a BL21(DE3) \textit{E. coli} strain and contains a 6x Histidine tag attached to its C-terminal end to allow for purification via Nickel affinity chromatography.
This recombinant TraT\textsuperscript{33-244} protein has been chosen due to its higher expression and solubility (Figure 13). Due to truncation of the “post-translational modification” residues, we predict that no lipidation and secretion will be possible for the recombinant protein, and we presume its location to be within the \textit{E. coli} cytosol upon overexpression. Nevertheless, it is possible that lack of post-translational modifications and change in localization may result in lack of or reduction in usual interactions of this protein with membrane and possible protein interactors or binding partners. According to initial structural modeling (Figure 7), TraT\textsuperscript{33-244} should not have a strongly disrupted domain structure; however, without additional structural studies it is impossible to rule out the potential for this truncated construct to fold differently and thus function and bind distinctly from the native version of this protein.

3.3 Protein Purification

Nickel-affinity chromatography was used to purify the expressed TraT\textsuperscript{33-244}-His\textsubscript{6} protein from the whole cell lysate, obtained by sonication and clarified by centrifugation. This process resulted in traces with a single elution peak at 19\% buffer B – 40\% buffer B (high imidazole buffer). Height and width of the peak exhibited slight variations from run to run.
to run, depending on the concentration of protein in the sample and cell pellet mass. The
eexpression and purification processes were largely the same as in previous work. In prior
work, expression volumes of one liter or less were used (Land, 2021). To increase
available volume of purified protein, in this work protein was expressed in 4 L of culture
and therefore the lysis method was modified to accommodate the larger cell pellet
volume. Final protein concentration post-Nickel-affinity chromatography was slightly
variable between runs but averaged around 0.8 – 2.5 mg/mL. From 1 L of culture, ~4 g
of wet cell pellet was typically recovered. Through use of the scaled-up protocol, a
Nickel-affinity purification run resulted in an average chromatogram peak of around 500
mAU, and an isolation of 25 mg of TraT$^{33-244}$ protein per liter of culture on average. It
was noted that with switching to larger scale samples and thus using a different sonicator
and protocol, the overall concentration of recovered protein dropped, possibly due to
incomplete lysis of cells that has not yet been explicitly tested. SDS-PAGE gel analysis
of the Nickel-purified TraT construct showed the common presence of the same few
extra (non-25 kDa main) bands at ~50 kDa, ~37 kDa, and 10-15 kDa that were consistent
with presence of minor contaminants or degradation products (Figure 14).
Figure 14: Commonly seen extra banding at ~50 kDa (second image), ~37 kDa (both images) and 10-15 kDa (first image). These bands were initially thought to be contaminants or degradation. TraT stored in Tris buffer pH 7.8.

Due to this finding, after Nickel-affinity purification, further purification was desired for downstream applications such as various assays and structural studies (crystallization screens, cryoEM analysis, and antibody production all benefit from homogeneous recombinant protein population). It was decided that the protein should be run through both ion-exchange and size-exclusion chromatography, both for further purification as well as further characterization.
3.3.1 Fraction Collection Shift

As a note, there is a visible discrepancy between the peaks seen on the chromatogram and the collected fractions of each peak when run on SDS-PAGE gel. There appears to be a shift towards the right between the two versions, with sample collected from the “main peak” fraction appearing lighter than it should. This is due to the distance of tubing between the UV detector that gives the chromatogram trace and the fraction collector arm that dispenses the sample – the sample is detected before being dispensed, therefore there is a delay between peak detection and fraction collection. This finding is important to note for future purifications to allow for maximum protein recovery, as well as for ensuring accuracy if trying to isolate a particular oligomeric state.

3.3.2 Ion Exchange Chromatography

Ion exchange chromatography (IEC) was used to further purify the Nickel-affinity samples through separation based on charged surfaces. This further purification was to enable better results for further assays, where a homogenous, pure sample is required; the goal was to have a TraT sample where the extra banding was removed, and the sample presented as a single band at 25 kDa. Two different IEC columns were available for use – Q and S columns. These are both strong exchange columns, with the Q column being the anion exchanger and the S column being the cation exchanger (ENrich High-Resolution Ion Exchange Columns; BioRad). TraT was found to bind – to varying degrees – to both columns, and each run resulted in multiple peaks of varying sizes, regardless of column type (Figure 16, Figure 17). Most of the Ni-purified TraT\(^{33-244}\)His\(_6\) protein bound to the Q column. This is consistent with the predicted pI of this protein construct – 6.16. While the
mature untagged TraT should have a higher pI of 8.54, our recombinant TraT with TEV site and 6x-His-tag has a lower pI and should be negatively charged in the Tris buffer that is used for purification which has a pH of 7.8-8.

Figure 15: SDS-PAGE gel of IEC Q-column fractions. The right half of each peak appears more concentrated; this is due to the collection shift mentioned earlier. IEC run with buffer at pH 7.8.

Samples from the Q column peaks were run on SDS-PAGE gels to analyze composition, and all had the major band around the 25 kDa marker, implying that all peaks contained TraT (Figure 15). However, in several peaks faint low molecular weight banding could be seen under these darker bands of TraT$^{33-244}$His$_6$ indicating that Q column run alone was not efficient in increasing heterogeneity of the TraT preparations.
Figure 16: IEC trace showing both the protein peak (TraT) at 280 nm, as well as the unknown peak at 215 nm, typically used to measure absorbance of peptides or peptide bonds.

Figure 17: IEC trace showing the slight TraT binding to the S column. Most of the protein is in flowthrough.

As is seen when comparing the traces for Q and S columns, the Q column is the best choice for using with TraT, as indicated by the protein’s pI. Interestingly, an additional
peak was consistently seen at a different wavelength (215 nm) from what is typically used for protein absorbance (280 nm). This finding suggests the possibility of the presence of a potential interactor, and opens another avenue for further research to determine identity or cause of this occurrence.

3.3.3 Size Exclusion Chromatography

Size exclusion chromatography (SEC) separates molecules based on size, with larger molecules eluting first and smaller molecules eluting later. SEC was performed with the goal of analysis as well as further purification in mind; the expectation was for TraT to elute in a single sharp peak, with any possible contaminants eluting before or after. While a single sharp peak of TraT was expected, in reality, the trace was more complicated and showed multiple consistent, overlapping peaks (Figure 18). This finding was subjected to further investigation.
Figure 18: TraT\textsuperscript{33-244}\textsubscript{His\textsubscript{6}} protein exists as a mixture of oligomers. SEC Trace of TraT - note the main peak, shoulder, and small peaks to the left and right. Calibration curve of standards with known molecular weights is inset in the top right box. SEC70 column used. A 260/280 ratio of 0.4 indicated that DNA contamination was low.

Although pentamer formation is not frequently seen, the finding of the main oligomeric state as being pentameric is not without support. Previously, our full length TraT construct was thought to have an issue with expression, as even after many repeat trials, no induction in protein band of monomeric TraT was seen on SDS-PAGE after expression via IPTG induction (Figure 19). However, after the oligomerization of the truncated construct was found, those expression gels were re-examined and re-ran to see if the mature TraT was expressing in a different oligomeric state. It was found that the lanes containing expressed mature TraT showed a clear, strong band around 125 kDa that was not seen at such levels in the lanes containing protein that had not been expressed (Figure 19). It is unusual that the band is seen in a denaturing SDS-PAGE gel indicating an extremely stable complex, thus requiring further investigation to confirm.
When the main peak of soluble recombinant TraT was collected after SEC and run on a gel, the extra bands were still seen (Figure 23). Were the extra bands (consistently at ~50 and ~37 kDa) to just be contaminants, they should have not shown up post-SEC, unless the interaction was extremely strong. Additionally, if the extra bands were contaminants, they should not show up on a His-tag-specific Western blot. To test this, a sample was run on a Western blot to determine presence of His$_6$-labeled TraT (full length or C terminus) in extra banding, which was confirmed as seen in Figure 20 below.
Figure 20: Putative impurities bands are likely the derivatives of TraT protein. His-tag specific antibodies stain the additional bands observed on Coomassie stained gels with Ni-purified TraT preparations. That suggests that they contain His tag as part of TraT oligomers or degradation products. Prestained colorimetric ladder (Ladder Color) is added as pictured for this exact blot membrane and a part of this ladder is also stained by immunochemiluminescence (Ladder Chem).

Each peak seen in the SEC trace was collected, concentrated, and run on an SDS-PAGE gel and each peak – though in varying concentrations – ran at 25 kDa.

Calculations were performed to determine the oligomeric state of each peak, comparing sample elution volume to elution volume of previously run standards with known molecular weights and factoring in monomer molecular weight. From left to right on the trace, these calculations resulted in an oligomerization result of eight subunits for the earliest eluting peak, five subunits for the main peak, four subunits for the unresolved shoulder attached to the main peak, and two subunits for the rightmost, latest eluting peak.
To fully ensure that all peaks seen were indeed the same protein, freshly purified TraT was run on SEC, collected, and then all samples were concentrated to same OD for most accurate comparison. Due to the previously unknown collection shift, the dimeric peak was not collected, and the leftmost peak was too low a concentration to meet the others. However, the main peak and shoulder were able to be concentrated to the same level, and all lanes looked in agreement with each other.

3.4 Stability of the TraT Oligomers Under Broad Range of Salt Concentrations

As the TraT oligomerization we had observed presented itself as an issue in our search for interacting partners, we decided to attempt to isolate a single oligomeric state of TraT to improve homogeneity. Moreover, the TraT population heterogeneity is likely detrimental to the plans for structural characterization of this protein using crystallography or cryoEM studies. We tested whether increasing or reducing salt
concentration in the TraT buffer will shift the heterogenous population towards a more defined oligomer or monomeric population. An additional consideration to make salt testing useful is the conditions encountered in crystal screens. During the process of protein crystallization, the protein encounters a variety of different salt concentrations and buffers to find the optimal condition for crystal formation. However, the protein will not be stable in all salt conditions and is subject to degradation. As protein crystal formation is often a very long and laborious process of trial and error, it is reasonable to attempt to narrow down a few conditions so as to optimize the future crystal screen by removing conditions in which the protein has proven to no longer be stable.

To these ends, TraT was dialyzed overnight at 4°C into a range of buffers covering varying salt conditions to test stability of the protein, both through SDS-PAGE gels and on SEC traces to check for oligomerization changes. The solutions were Tris-buffered and varied only in their NaCl concentration; concentrations chosen were 0 mM NaCl, 150 mM NaCl, 300 mM NaCl, and 450 mM NaCl. Samples were run on an SDS-PAGE gel post-dialysis both before and after being run on SEC.
Figure 22: TraT in different salt concentration buffers. Protein was loaded after overnight dialysis into the individual buffers, as well as after SEC. Note: reduced concentration of 450 mM salt sample was due to human error, equivalent concentrations of the 450 mM salt condition can be seen in Figure 23.

When stained, samples for each salt concentration were virtually identical to each other as well as the “normal” salt concentration found in the standard buffer. Common extra banding was able to be seen before application on the SEC, and faintly to not at all afterwards. SEC traces of each salt condition lined up well, with only slight shifts partially attributed to different run orders seen.
Figure 23: Extra banding seen in differing lanes. Age-resultant degradation is also visible in more concentrated main peak samples.

Different peaks were isolated and run on an SDS-PAGE gel to see whether the extra banding separated with any particular peak. It was seen that the 50 kDa band tended to be found with the main peak, while the ~37 kDa band seemed to be found with the more leftmost peak.
Figure 24: Oligomerization calculations and different salt concentrations. Note: peaks were scaled arbitrarily to reduce visual distraction, as height of peak varied with concentration of protein loaded. Each run was performed with similar but not identical concentrations of protein. Calibration curve of standards with known molecular weights is inset in the top right box. SEC70 column used.

As seen in Figure 24 above, TraT oligomerization patterns appears to be largely the same – octamers, pentamers, tetramers, and dimers – in a broad range of salt concentrations ranging from no salt to 450 mM NaCl.

3.5 Formaldehyde Crosslinking

Non-specific crosslinking performed with formaldehyde showed banding at weights that agreed with the SEC data. Although there are high molecular weight aggregates and smudges in between bands (likely partially due to the formaldehyde), strong banding can be seen at 25 kDa, 50 kDa, and around 125 kDa with the samples that had been allowed to crosslink for the longer timeframes. Different timepoints were used to find the optimal crosslinking time; a longer incubation leads to more protein being
crosslinked, and the amount of crosslinking can become excessive, with high molecular weight aggregates forming and blocking potential results. Ten-minute and sixty-minute timepoints were chosen as showing good representation of lower and higher amounts of crosslinking. Formaldehyde crosslinking is reversible by boiling – with application of heat, the proteins fall out of aggregates and with enough time, back into their native state.

A minimal amount of boiling such as 5 minutes has shown to give a good balance between the extremes and clearer visibility of banding. Examples of a 5-minute boil, 30-minute boil, as well as no boiling are shown in Figure 25 below. Positive controls of TraT in two different buffers (the typically-used Tris buffer as well as the non-nucleophile HEPES buffer) are important to confirm the baseline of molecular weight and appearance of non-crosslinked TraT.

Figure 25: Non-specific formaldehyde self-crosslinking of TraT. Multiple timepoints and boil conditions are shown, along with control TraT preparations in two different buffers.
The image above clearly shows banding at just under 37 and at 50 kDa, as well as some higher molecular weight banding between 100-150 kDa and higher. The self-crosslinking was also run on a Western blot to enable better resolution of faint banding, resulting in images like Figure 26 shown below.

![Western Blot image of TraT formaldehyde self-crosslinking.](image)

Figure 26: Western Blot image of TraT formaldehyde self-crosslinking.

Non-specific formaldehyde crosslinking trials proved successful in self-crosslinking TraT, where the most concentrated bands formed agreed with the oligomerization peaks seen in the SEC chromatograms. The success of the developed assay shows promise for continued and improved future use to crosslink TraT and lysates to better capture any potential bonds.
3.6 Pull Down Assays

Pull down assays were used in this research for the purpose of identifying molecules that interact with TraT. It is based off of the assumption that whole lysate preparations will potentially contain molecules that should bind to TraT, which would then be purified out of solution using a Nickel resin slurry and visualized on SDS-PAGE gels. Regarding the lysate choices – Top10F' strain contains the F plasmid encoding for TraT and should express it, while Top10 does not. Following this procedure, the TraT band at 25 kDa was always seen for the elutions, commonly along with faint banding around the 37 and occasionally 50 kDa mark.

![Figure 27: Pull down assay with TraT and lysate. “No TraT” lanes are just lysate and Nickel-resin and should act as negative control with no binding. However, faint bands around 37-50 kDa are seen in these lanes, indicating that this result is most likely nonspecific binding.](image)

In all repeats of this assay, the results looked primarily the same. Although there were varying levels of TraT protein recovery, even when the recovery was high, no additional bands were seen save for the usual band around 37-50 kDa. Oddly, those consistent bands occasionally appeared slightly darker on the “no TraT” lanes. Rather than an exciting find, the cause of this banding is thought to be nonspecific binding due to its presence in lanes not including TraT, where it should not be binding to anything and should have just washed out, leaving a blank lane (Figure 27).

3.7 Crosslinking Lysates and TraT

After initial self-crosslinking trials were held, whole cell lysates of SL88, SL89, SL145, and TS83 (Table 2) were crosslinked with the TraT. This was performed to give a better chance at identifying interactors through ensuring proximity of interacting partners. General parameters were similar to the previous assays of self-crosslinking. Pull down assays were performed post-crosslinking to allow for visualization of potential interactors, but only slightly better results were seen when the bait and prey had been crosslinked first, with a little bit of fuzzy high molecular weight banding seen. There was strong nonspecific binding banding seen at ~50 kDa similarly to in Figure 27, however, it was interestingly only seen across all lysates in the control (no TraT, just lysate and resin) group, but not seen in the groups that had been crosslinked with TraT.
Figure 28: Pull down assay using TraT crosslinked with lysates. Results are still minimal. Some high molecular weight aggregation can be seen in the 60 minute crosslinking and no boil lanes, while faint TraT monomer recovery is visible across the 10 minute crosslinking lanes (NB - not boiled).

This protocol combination has potential to yield better results than the pull down assays alone, but initial trials still needed some ironing out to determine best conditions. TraT recovery amount was still a problem, but with the crosslinking, some of the usual monomer seen at 25 kDa would self-crosslink and be seen in higher molecular weight aggregates at the top of the gel. Boiling sample results in the high molecular weight constituents “falling out” and being slightly more visible throughout the gel, but the resulting bands are still very faint and fuzzy (likely due to the formaldehyde). A potential difficulty in this process is determining what may be crosslinked interactors versus what is just TraT self-crosslinking. Proper controls are essential for comparison to determine identities and differences.
3.8 TraT Amino Acid Substitutions and Site-specific Crosslinking

As previously discussed, single nucleotide changes in the NSAGA region have been shown to be involved in sequence specificity when determining whether conjugation will occur. It was desired to discover if changes in this region also affected other parameters such as oligomerization and serum resistance ability. To establish this goal, a TraT construct was created with a replacement of the second NSAGA Alanine (A) with an Arginine (R) (visualized by red “A” in Figure 13). This replacement was chosen due to the differences between the two amino acids - Alanine is small and nonpolar, while Arginine is large, polar, and amphipathic. This TraT\textsuperscript{A110R} construct was purified using the standard Nickel-affinity method and then run on SDS-PAGE gels, subjected to formaldehyde self-crosslinking, and run on the SEC to compare results to the standard TraT\textsuperscript{33-244}. Appearance was virtually identical on SDS-PAGE when run directly after purification, and the TraT\textsuperscript{A110R} construct performed comparably to the standard TraT\textsuperscript{33-244} when self-crosslinked, showing the usual strong banding around 37, 50, and 125 kDa. Interestingly, there were slightly less noticeable results around the 75 kDa mark than would be expected when compared to TraT\textsuperscript{A110R}’s SEC trace.
Figure 29: SDS-PAGE gel of formaldehyde self-crosslinking performed with TraT^{A110R}. Strongest concentrations seen agree with what was seen with the TraT^{33-244} self-crosslinking.

TraT^{A110R} was run on the SEC to compare the trace to TraT^{33-244} to confirm oligomerization changes more accurately.

Figure 30: A110R substitution in the conserved exclusion motif of TraT does not abolish oligomerization of this protein. SEC trace of wild-type TraT^{33-244}His_{6} compared to TraT^{A110R} variant. Slight shift is noticeable, but overall oligomerization remains similar.
Aside from a slight shift, the traces looked largely the same, as seen in Figure 30 above. Subunit calculations were completed for each $\text{TraT}^{\text{A110R}}$ peak seen and compared to the oligomerization data for the $\text{TraT}^{33-244}$; shifts were seen in the 8-subunit $\rightarrow$ 10-subunit far left peak, as well as a 4-subunit $\rightarrow$ 3-subunit shift for the shoulder.

In addition to chemical crosslinking with aldehydes, another somewhat more novel option is unnatural amino acid mutagenesis and subsequent crosslinking. Unnatural amino acid crosslinking (UAA) works through photo-activatable crosslinking - the use of UV light to form site-specific cross-linkages at the UAA. The beginning of work with this substituted protein was initiated by Kristiana Josifi. After transformation with the pBpa-containing plasmid, it was necessary to ensure proper incorporation, which was confirmed by visualization of the TraT on a western blot.

![Western Blot showing successful incorporation of pBpa](image)

Figure 31: Western Blot showing successful incorporation of pBpa. Were pBpa not incorporated, the protein would not be visible at the 25 kDa mark. Note: chemiluminescent ladder bands do not perfectly correspond with colorimetric ladder bands; image is labeled correctly.
Had the pBpa not been successfully incorporated, the protein would’ve been truncated via early termination to a degree where it would not have been visible in the usual manner due to lack of fully synthesized C-terminal His-tag.

These results show no extreme differences between the TraT\textsuperscript{A110R} substituted protein and the TraT\textsuperscript{33-244} recombinant protein we have been using thus far but do show slight shifts in oligomerization number for half of the peaks. Additionally, western blot analysis shows successful incorporation – while low concentration – of the unnatural amino acid pBpa into the TraT\textsuperscript{A110Amber} substituted protein, as evidenced by the protein showing up at the appropriate molecular weight marker instead of experiencing an early termination.
CHAPTER 4: DISCUSSION

4.1 LPS Conjugation Assay Findings

LPS conjugation assays did not show a dramatic lowering in conjugation efficiency upon the addition of free LPS to the conjugative mating mixture. There are multiple possible reasons for this outcome. Firstly, it is possible that the role of LPS in conjugation is simply not as significant in \textit{E. coli} or F plasmid models as what was seen in R64 \textit{Pseudomonas} models. Plasmid R64 belongs to Inc group I and MOB group P. As this different conjugative system than what is seen in IncF/Mob\textsubscript{F} plasmids and thus differs in function to a significant enough degree to warrant separate classification, it is not impossible that the initial binding partner for the donor pilus varies across the numerous types of conjugative systems. Although likely not the conjugative receptor, LPS is still involved in conjugation in some way. There is also a possibility that the varieties of LPS tested were not the best choice for the utilized systems. There are over 100 different O-antigen serotypes alone; with such great variety in mind, there is a potential that more effect would have been seen were a different serotype used. With so little yet confirmed regarding donor pilus docking across various conjugation systems and plasmid types, this is still a very interesting research direction that invites copious future study.

Two concentrations of LPS - “CT1” (0.5 mg/mL) and “CT2” (0.05 mg/mL) were used during the conjugation assays to test for effect of dose on conjugation ability, thinking that higher amounts of LPS added to the mixture would result in a more
conjugation-blocking activity. It may also be that in this case, “more” was actually “less” in terms of added LPS. Like other lipidated molecules, LPS is subject to what is known as the critical micelle concentration (CMC). Above this given concentration – thought to be around 14 microgram mL(-1) for LPS (Santos et al., 2003), the hydrophobic Lipid A tails join in a ring to form a micelle. This occurrence would result in far less free LPS available for binding with donor pili, skewing the results of the conjugation assay to appear more negative than is possible if the correct concentration for optimal free LPS was obtained. This theory is supported in part by the experimental results gathered that did show a fairly consistent, very slight decrease in conjugative efficiency with “CT2”, the lower (although still over the CMC) concentration of LPS. This result was confusing when considered by a standard dose-dependent, “more is more” viewpoint, but starts to make slightly more sense when viewed with the CMC in mind. In future studies of this topic, a wider range of LPS concentrations are recommended to be used, with multiple values above as well as markedly below the CMC.

4.2 TraT Substitution Protein Similarities and Significance

Both the TraT^{33-244} and the TraT^{A110R} constructs were run on the same column and the same volume of protein was loaded, although there were likely differences in concentration between the two samples. Shifts seen between traces may be partially due to instrumentation – slight elution shifts can occur depending on sample run order, initial concentration or load volume of sample, and other compounding factors. Often the first run on the SEC will be slightly different from subsequent runs during that day’s use of the FPLC machine. Although no extreme changes to oligomerization were seen, there
was noted two slight shifts in the far-left peak (eight subunits in TraT\textsuperscript{33-244} and ten subunits in TraT\textsuperscript{A110R}) and in the shoulder (four subunits in TraT\textsuperscript{33-244} and three subunits in TraT\textsuperscript{A110R}). Occasionally, the tetrameric shoulder of TraT\textsuperscript{33-244} will present as a trimer, but decamer formation has not previously been observed. This shows that, while slight, substituting the second Alanine of the NSAGA sequence specificity region with an Arginine did result in some manner of change in protein oligomerization behavior.

Ultimately, these TraT mutants show that the NSAGA region is likely not highly involved with oligomerization function and organization but may cause slight changes, and the pBpa incorporation confirmation is a good initial step for site specific crosslinking and hopeful better pull down findings as a result.

4.3 Pull Down Assays with and without Crosslinking

Despite the various alterations explored in the pull down assays, consistent banding indicating an interactor was not confirmed. Although there was often some degree of faint banding seen ranging from ~30 kDa up to around 75 kDa (most commonly near 37 and 50 kDa), these bands were considered to be nonspecific binding – the phenomenon in which prey molecules directly bind to the affinity resin rather than the ideal “bait binds to affinity resin, prey binds to bait” formula. These bands commonly showed up in the negative controls of “lysate + resin” as well as the experimental lanes. Elucidation of TraT interactors is far from complete, and there are multiple possible factors contributing to the results seen. As previously detailed, rough whole cell lysates were used as the source for cellular material used in crosslinking and pull down assays. Due to the position of TraT on the outside of the cell, it is reasonable to think that
potential interactors of this protein would also be found on the outside of the cell. However, with the use of whole cell lysates, it is possible that there is simply not a high enough concentration of outer membrane constituents when compared with total binding mixture volume to allow for results to be seen. Perhaps the lysate contains proteases that destroy possible binding partners before detection is possible. To test this hypothesis, it would be advisable to develop a protocol for purification of the cellular outer membrane fraction. This would ensure a high concentration of molecules that are more likely to actually interact with the protein of interest and reduce contaminants or potential complicating factors. Another avenue along this vein to explore would be to purify – or order, dependent on available funding – specific outer membrane proteins theorized to interact with TraT and directly test these in pull downs. OmpA would be a good candidate for this method due to its aforementioned history with TraT and bacteriophages, common availability to order, and is of additional interest due to its size at ~37 kDa, which coincides with the unexplained band that has been commonly seen with our purified TraT.

While this line of protocol change would surely help in producing more positive results, other changes are available for optimization as well. Throughout the course of performing these experiments, two complicating factors have been fairly consistent – low final protein yield after the pull down process, and high amounts of nonspecific binding. Protocol tweaks such as extra washes, increased spin length, and increasing TraT starting concentration were implemented to some success but did not fully solve the issue. The 6xHis-tag attached to the protein for purification purposes has affinity with Nickel resin, so a Nickel resin slurry is also used for the pull downs. However, Nickel resin is
notorious for allowing larger amounts of nonspecific binding than certain other affinity resin types. In future investigations of TraT interactors, it might be worthwhile to redesign the setup to allow for use of an alternate resin type that is less likely to allow for nonspecific binding in order to help alleviate this issue.

4.4 Oligomerization State

Literature suggests that the TraT protein in native, active form is not monomeric. Oligomerization is important for the functioning of this protein. In the course of investigating other facets of this protein, various factors were seen that led to the belief that TraT does indeed oligomerize, and the major oligomeric state is a pentamer. Supporting evidence was primarily seen in SEC traces, as well as being suggested by extra banding seen on gels. There was some initial uncertainty about the composition of each peak seen on the SEC traces, as one single sharp peak was expected. However, when each peak was collected separately, concentrated, and ran on an SDS-PAGE gel, it was seen that each peak ran at the TraT-expected mark of 25 kDa, and a Western Blot showed that each sample contained His-tag, and thus should be the desired protein. Knowing that some form of oligomerization was expected, it was thought that this phenomenon was likely the explanation for what was being seen. Aforementioned calculations labeled each peak as an octamer, pentamer, tetramer, and dimer respectively. Although it is biologically unusual to have pentamer formation as nature tends towards symmetry, this structural state would fit with calculative data (Harrison et al., 1992) theorizing the arrangement of TraT as potentially being a pore-like structure, as well as expression at 125 kDa seen in the mature form of the protein. Similar hints were seen in
both the formaldehyde self-crosslinking assays as well as typical SDS-PAGE gel visualization of the protein. During crosslinking, banding was seen that would fit into the calculated subunit range of the sample, particularly that of dimers, pentamers, and octamers.

More concretely, bands at 37 and 50 kDa was almost ubiquitously paired with the standard monomeric 25 kDa band seen for TraT. While the 50 kDa band could clearly be a dimeric form of the protein, the 37 kDa band poses a less clear definition. It could potentially have to do with the truncation of the protein construct being used, resulting in abnormal oligomerization behavior. This base cause might also be involved in something such as incomplete denaturation of a dimer, for example. There was an initial thought that the 37 kDa band could potentially be an extremely strongly bound interactor (OmpA runs around 37 kDa, for example) or a chaperone protein, but these theories seem less strong when it is recalled that this band shows up on a Western blot specific for His-tag, although perhaps the other molecule has a long enough string of available histidines to be detected by the anti-His antibodies. Whatever the cause, the consistent presence of these extra bands throughout chromatography procedures and the denaturing conditions of an SDS-PAGE gel show that the interaction and/or oligomerization is very strong. This poses an interesting avenue for future research with this protein.

4.5 Salt Stability and Purification for Downstream Applications

The consistency of each salt condition tested to the other new conditions as well as to the original buffer concentration show the protein to be stable in a variety of different salinities. This finding indicates a surprisingly stable protein complex
insensitive to drastic alterations in salt concentration. Additionally, the observation of the 37 and 50 kDa ‘extra banding’ tending to be more visible in different lanes when run on SDS-PAGE gel post-SEC may prove helpful in improving sample homogeneity. Stability is good news for future crystallization attempts as it gives more options to test against the protein of interest, but it is also important to note that the range tested is by no means comprehensive and higher salt concentrations are often encountered during crystal screens. Further testing with higher salt levels to determine the limit before the protein crashes would be an option to include in further optimization studies; testing a range of pH is advisable as well for observation of potential oligomerization shifts. Other advisable parameters to consider testing for optimization include temperature stability and stability in other buffer systems, to name a couple of notable options, with time as a compounding factor in all cases as well.

The observed salt stability also further confirms the strength of oligomerization exhibited by the extracellular domain of the TraT protein. When the SEC results are concentrated, sometimes the main TraT band at 25 kDa will become rather stretched and doubled; this is thought to be due to possible aggregation during the concentration process and is not a result of SEC itself. The usual extra banding being seen before SEC and much fainter or invisible after SEC is thought to be mainly an effect of concentration rather than purification, as the samples are always much more dilute after the run on the SEC. This is not to say further purification is not occurring, however. Although purity from lipid association (which would require a different molecular weight marker for SDS-PAGE if proven) or sugar derivatives are not yet confirmed, reduction in DNA contamination throughout the purification procedures can be seen through the low
260/280 ratios of 0.5-0.4 after IEC and SEC as compared to the ratio of the Nickel-affinity flowthrough of 1.08. After concentration post-SEC, the extra banding seems to settle into different lanes based off of which peak was collected. This is not thought to be simply due to concentrations, as it has been observed that the 37 kDa band is visible in a lane with lower overall TraT concentration, while the 50 kDa band is visible in a lane with higher overall TraT concentration. There is also low molecular weight banding seen, particularly in the more concentrated lanes. This is thought to most likely be protein degradation, as it is not seen when the protein is first purified but appears as the protein ages in storage. Older protein samples tend to have a larger variety of oligomerization states or aggregates/unresolved complexes, as well as degradation, than is typically seen in freshly purified protein samples. This is most clearly seen by presence and concentration of extra banding on SDS-PAGE gel. In future work, temperature and buffer used for storage will be modified and tested to determine ideal conditions for longer-term preservation of protein integrity.

Although further investigation needs to be performed in this area, it is possible that while still being self and not contaminant, the extra banding has something to do with a certain oligomeric state. Determining patterns behind how this process tends to occur could lead to a better method of sample homogenization and purification, which would have definite benefits for downstream applications in the future. Obviously, furthered purity and homogeneity of sample is necessary for increasing chances of a crystal forming, especially since TraT is an outer membrane protein, a group notoriously difficult to crystallize in the first place. This would also prove helpful in other future applications such as antibody production, cryoEM, or NMR. In order to send off a protein
to have antibodies produced against it for use in immunoassays such as Western blots and more, it is ideal that the protein has no artificially added tags attached to it or any contaminating factors in the sample. This is to ensure that all antibodies produced by the host animal are specific for the protein of interest, and there are no antibodies produced for any contaminating factor. Were this to occur, one could receive a false positive hit in the presence of the contaminating factor and the results data would be skewed and inaccurate. Due to the expense of the antibody production procedure, it would be ideal to cleave the His-tag from the TraT protein before antibody production to ensure most accurate results and prevent potential waste of funding. Initial trials to this end were performed with various modifications tested, and SDS-PAGE visualization showed what appeared to be a mixed population of standard and proteolyzed TraT. However, attempts to separate the sample with Nickel resin proved unsuccessful as each part of the sample tested still showed up when run on a Western blot against anti-His antibodies. A possible reason for this issue is that the His-tag might be partially inaccessible for cleavage due to the manner in which the protein folds. This could potentially explain a slight decrease in binding efficiency seen between the protein and the Nickel resin. More work will be done on this topic in the future; de- and renaturation of the protein may pose an avenue of exploration.
5.1 Conclusions

Based on the results of the experiments detailed above, it does not appear that LPS functions as the docking site for the donor pilus during F plasmid conjugation. While it was shown to be the docking site in *Pseudomonas* R64 conjugation, differing conjugative systems may have different pilus receptors; even if LPS doesn’t perform this key role for F-like plasmids, literature shows it still is involved in the process in some manner. Additionally, all conjugation efficiency experiments were performed with a concentration of LPS that was above the CMC, resulting in a much lower concentration of truly free LPS in solution than what was thought due to micelle formation. This could certainly have affected the results as well and made there appear to be less of an effect than what may be most accurate. Concentrations of LPS that are confirmed below the CMC should be used to confirm lack of effect. Given the multitude of different sorts of LPS, it is also possible that the LPS varieties tested were not the most effective serotypes for the plasmids and strains tested.

Although multiple conditions and lysed strains were tested, confirmable, non-self TraT interactors were not found during the binding assays. There are many possible avenues forward with this direction that have potential to glean better results. One such option is to give the bait TraT a more specific solution to bind with by using purified outer membrane fractions or purchased OmpA as bait rather than just whole cell lysate.
This would provide a much higher concentration in solution of the substances with the best chance of binding. Additionally, a different affinity resin system, or even a significantly higher concentration of TraT might prove helpful in ensuring that proper binding occurs, TraT recovery is high, and nonspecific binding is low. It is possible that the negative results were also in part to the truncated nature of our recombinant TraT construct, as it may not have behaved in a manner identical to native TraT found on the outside of a cell. A discovered factor that might have contributed to the negative results seen with the pull down assay is the finding of TraT’s strong oligomerization. Formation of high molecular weight oligomers that cannot be reliably separated by the usual SDS-PAGE gel may interfere with identification of binding partners.

Although negative results were obtained from the LPS trials and TraT pull down assays, useful new information was gleaned about TraT’s oligomerization state, stability under different conditions, and serum resistance ability. Additionally, protocols were developed for large scale expression, lysate prep, and purification, including further purification steps. Data regarding identity of extra “contamination” banding and degradation banding was uncovered, and further knowledge of the extent of sequence specificity region influence was obtained. TraT has been shown to form very strong oligomers, with the most prevalent state being pentameric; this oligomerization is very stable throughout various buffer conditions, remaining evident even under denaturing conditions and chromatography. A three-step chromatography series has been developed to enable further purification of TraT for downstream applications including structural studies. Cells containing plasmids encoding for TraT were shown to be much more highly protected against serum killing than cells not encoding for TraT (Lim et al., 2022).
Our recombinant TraT protein has also been shown to provide a partial protection against serum killing by 10-fold and greater levels when added to the serum-bacterial cell mixture \textit{in trans} (Lim et al., 2022). There is still much work to be done to understand this multifunctional protein, but the research performed in this thesis will prove to be helpful in advancing our knowledge of TraT in multiple directions, as well as opening up further interesting routes of discovery moving forward.

5.2 Future work

There are many possible avenues forward for future work with TraT. Experimentation can be done towards elucidating the structure of TraT, advancing the crosslinking and pull down assay optimization to continue searching for interactors to better understand the protein’s function, and further study of oligomerization and SEC data in general. It would be useful to run our protein on Native PAGE gels as they are not denaturing and should provide interesting information about the protein state and subunit interactions in native conditions. Other interesting areas of research include continuation of serum resistance studies, work integrating LPS and TraT research, as well as working to purify and characterize the native form of TraT alongside the previously used construct, to test a form more accurate to completely wild-type TraT as it exists in the cell and compare to previous results. Since the full length protein is able to be expressed and possibly processed, it is possible that a construct substituted with pBpa may be created for use with \textit{in vivo} site-specific crosslinking.

While one initial crystal screen was performed with TraT, all conditions were far from ideal, and no crystal formation was seen. Armed with a purer protein preparation
and better understanding of the tolerances and state of TraT, future runs should give more promising results. To give the crystallization assays the best chance possible, triple purification of the protein and concentration are ideal; further research on whether the common extra banding is attached to a particular oligomerization state and trials to separate the sample based on its oligomerization state will help produce a more homogenous sample for best results. Continued tolerance trials – stability at room temperature vs 4°C over time, for example – would also prove helpful to choosing the best parameters for performing the crystal screens. Additionally, trials on buffer and temperature stability would aid in finding the optimal storage conditions for the protein, resulting in having the protein stay fresh and undegraded for a longer amount of time and increasing the shear amount of protein available for use at any given time, enabling greater ease of high concentration.

To continue structural studies alongside crystallization screens, NMR or cryoEM may be investigated. There are advantages and disadvantages to each method of structural study. NMR requires high concentrations of highly pure protein samples, and more involved preparations and analysis as well as cost; it usually works best for smaller protein molecules. CryoEM, while typically lower resolution, is more suited for larger proteins and complexes with the estimated pentamer of TraT being at the lower end of cryoEM capabilities. Once the TraT protein can be reliably triple-purified (Nickel, Ion Exchange, and Size Exclusion chromatography steps), future goals include sending a sample to a cryoEM facility at the University of Alabama in Birmingham to acquire further information regarding the protein’s native oligomerization state and structure.
REFERENCES


Doorduijn, D. J., Bardoeel, B. W., Heesterbeek, D. A. C., Ruyken, M., Benn, G., Parsons, E. S., Hoogenboom, B. W., & Rooijakkers, S. H. M. (2020). Bacterial killing by complement requires direct anchoring of membrane attack complex precursor C5b-7. PLOS Pathogens, 16(6), e1008606. https://doi.org/10.1371/journal.ppat.1008606


APPENDIX A: SUPPLEMENTAL DATA

Table 1: Bradford assay BSA standard dilution preparations.

<table>
<thead>
<tr>
<th>Vial</th>
<th>Volume of Diluent (dH2O)</th>
<th>Volume and Source of BSA</th>
<th>Final BSA Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0</td>
<td>300 μL of stock</td>
<td>2000 μg/mL</td>
</tr>
<tr>
<td>B</td>
<td>125 μL</td>
<td>375 μL of stock</td>
<td>1500 μg/mL</td>
</tr>
<tr>
<td>C</td>
<td>325 μL</td>
<td>325 μL of stock</td>
<td>1000 μg/mL</td>
</tr>
<tr>
<td>D</td>
<td>175 μL</td>
<td>175 μL of Vial B</td>
<td>750 μg/mL</td>
</tr>
<tr>
<td>E</td>
<td>325 μL</td>
<td>325 μL of Vial C</td>
<td>500 μg/mL</td>
</tr>
<tr>
<td>F</td>
<td>325 μL</td>
<td>325 μL of Vial E</td>
<td>250 μg/mL</td>
</tr>
<tr>
<td>G</td>
<td>325 μL</td>
<td>325 μL of Vial F</td>
<td>125 μg/mL</td>
</tr>
<tr>
<td>H</td>
<td>400 μL</td>
<td>100 μL of Vial G</td>
<td>25 μg/mL</td>
</tr>
<tr>
<td>I</td>
<td>400 μL</td>
<td>0</td>
<td>0 μg/mL = Blank</td>
</tr>
</tbody>
</table>

Table 2: List of bacterial strains used.

<table>
<thead>
<tr>
<th>Strain Name</th>
<th>Description</th>
<th>Strain Name</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>SL88</td>
<td><em>E. coli</em> Top10F' Tetracycline Resistant</td>
<td>SL145</td>
<td><em>E. coli</em> pRK100 MG1655</td>
</tr>
<tr>
<td>SL89</td>
<td><em>E. coli</em> Top10</td>
<td>SL170</td>
<td><em>E. coli</em> NEB5alpha with A110Amber mutagenesis on plasmid pET28b TraT 33-244 Kanamycin Resistant</td>
</tr>
<tr>
<td>SL93</td>
<td><em>E. coli</em> DH5α pET28b TraT 33-244 Kanamycin Resistant</td>
<td>SL171</td>
<td><em>E. coli</em> NEB5alpha with A110R mutagenesis on plasmid pET28b TraT 33-244 Kanamycin Resistant</td>
</tr>
<tr>
<td>SL95</td>
<td><em>E. coli</em> BL21(DE3) with pET28b Plasmid Vector expressing TraT cleaved at position 33 Kanamycin Resistant</td>
<td>TS83</td>
<td><em>E. coli</em> HB101 with pRK100 Ampicillin &amp; Tetracycline Resistant</td>
</tr>
</tbody>
</table>
Table 3: Primer list for mutagenesis and sequencing.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Mutagenic primer for</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>oSL55_F</td>
<td>A120R</td>
<td>5'-ggccccacaccgcgtgctacgacggcagaattggaatt-3'</td>
</tr>
<tr>
<td>oSL56_R</td>
<td>A120R</td>
<td>5'-aattcccaattctgccgtgctacactcggtggtgggcc-3'</td>
</tr>
<tr>
<td>oSL57_F</td>
<td>A120amber</td>
<td>5'-caaggccccacaccgcgtcataacggcagaattggaattata-3'</td>
</tr>
<tr>
<td>oSL58_R</td>
<td>A120amber</td>
<td>5'-tataattcccaattctgccgtgctacactcggtggtgggcccttg-3'</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Function</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>oSL59</td>
<td>Sanger sequencing</td>
<td>5' ctaatggattgcaacttgtcag 3'</td>
</tr>
<tr>
<td>oSL60</td>
<td></td>
<td>5' tgattggccagtgtgc 3'</td>
</tr>
</tbody>
</table>