

CRYSTALLIZATION STUDIES OF THE TRAF PROTEIN FROM THE F PLASMID OF
ESCHERICHIA COLI

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ABSTRACT

Crystallization of suitable crystals for diffraction analysis is a major hurdle in structure-function studies of proteins using X-ray crystallography. This is especially true when the protein may be generally hydrophobic, membrane associated or contain regions of increased flexibility. The objective of the research outlined in this thesis was to determine a more amenable set of crystallization conditions for the protein TraF from the F plasmid for X-ray diffraction and structure solution. The F plasmid of *Escherichia coli* utilizes a conjugative type IV secretion system (T4SS) by which the transmission of genes in bacteria occurs contributing to the current issue of antibiotic resistance. The TraF protein of this system is a periplasmic protein with a dynamic hydrophobic N-terminal tail which interacts directly with TraH and a C-terminal thioredoxin-like domain. TraF is hypothesized to chaperone and interact with several other proteins of this system. Although its exact function remains unclear, TraF is essential in the F plasmid's T4SS as a lack of function abolishes gene transfer through this system. Modification of upstream conditions, including expression and purification of a GST-tagged variant of TraF, as well as modification of buffer conditions including the detergent NP-40 allowed for an increased solubility and concentration of the protein for crystallization trials. The traditional screening process with GST-TraF resulted in two conditions that showed birefringent structures which did not yield any better results when optimized. Optimization of the original reported TraF purification and crystallization conditions did double the protein concentration, however resulted in a lack of miscibility between the protein stock and the reservoir solution during crystallization. Furthermore, a head-to-head crystallization screening was conducted incorporating 5-amino-2,4,6-triiodoisophthalic (I3C) in the crystallization drop to advance structure solution using experimental phasing methods. The head-to-head experiment led to multiple crystallization conditions that should be optimized and further assessed while validating the novel process of incorporating I3C in the screen formation process.

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LIST OF ABBREVIATIONS

Amp - Ampicillin
ATP - Adenosine Triphosphate
CAT - Chloramphenicol Acetyltransferase
B3C - 5-amino-2,4,6-tribromoisophthalic
Da - Daltons
DNA - Deoxyribonucleic Acid
E. coli - *Escherichia coli*
GST - Glutathione S Transferase
HEPES 4-(2-hydroxyethyl)-1- piperazineethanesulfonic acid
HGT - Horizontal Gene Transfer
His - Histidine
I3C - 5-amino-2,4,6-triiodoisophthalic
IPTG - Isopropyl- β -D-thiogalactopyranoside
LB - Luria Bertani
MAD - Multi-Wavelength Anomalous Dispersion
Mdr - Multi-drug Resistant
Mpf - Mating Pair Formation
Mps - Mating Pair Stabilization
MW - Molecular Weight
MWCO - Molecular Weight Cut Off
NaCl - Sodium Chloride
NP-40 - Nonidet P-40
SAD - Single-Wavelength Anomalous Dispersion
SDS- PAGE - Sodium dodecyl sulphate polyacrylamide gel electrophoresis
T4SS -Type IV Secretion System
Ti - Tumour inducing
Tra - Transfer
Tris - Tris(hydroxymethyl)aminomethane
Vir - Virulence

CHAPTER 1 – INTRODUCTION

1.1 Protein Crystallization and Analysis

Modern crystallography originated from Max Von Laue exposing a blue copper sulfate pentahydrate crystal to X-rays and describe the images observed (Friedrich et al., 2013). It wasn't until Sir William Henry Bragg and Sir William Lawrence Bragg analyzed Laue's results mathematically that Lawrence Bragg defined the angle of diffraction with $n\lambda = 2d\sin\theta$ - aptly termed Bragg's Law - where θ represents the angle of diffraction, λ the wavelength and d the interplanar settings in the crystal lattice (W. L. Bragg, 1929). His father went on to create the world's first X-ray spectrometer (W. H. Bragg & Bragg, 1913). These contributions to science led Laue to a Nobel Prize in 1914 and the Braggs to Nobel prize in 1915 (Jaskolski et al., 2014). Following this, J.D. Bernal and Dorothy Crowfoot-Hodgkin imaged the first protein, pepsin, in 1934 (Bernal & Crowfoot, 1934). Dorothy Hodgkin continued to make exceptional discoveries in macromolecular biology including the structure of penicillin, insulin and Vitamin B₁₂ for which she won the 1964 Nobel prize in chemistry for (Baker et al., 1988; Hodgkin et al., 1957; Robinson, 2011). Max Perutz pioneered the technique of isomorphous replacement to ascertain the structures of myoglobin and haemoglobin (D. W. Green et al., 1954). The overall goal of the research presented in this thesis is to determine the structure of TraF from *E. coli* through crystallization.

Crystallization occurs when a protein is in an ideal condition allowing it to be in the nucleation zone which allows for crystal formation as shown in Figure 1.2 (McPherson & Gavira, 2014). Protein crystal formation requires interactions that are highly directional, specific and organized in a manner that is appropriate for three-dimensional crystal lattice formation. The three

stages of crystal growth are nucleation, crystal growth and cessation of growth which is common to all molecules (Figure 1.1) (Russo Krauss et al., 2013).

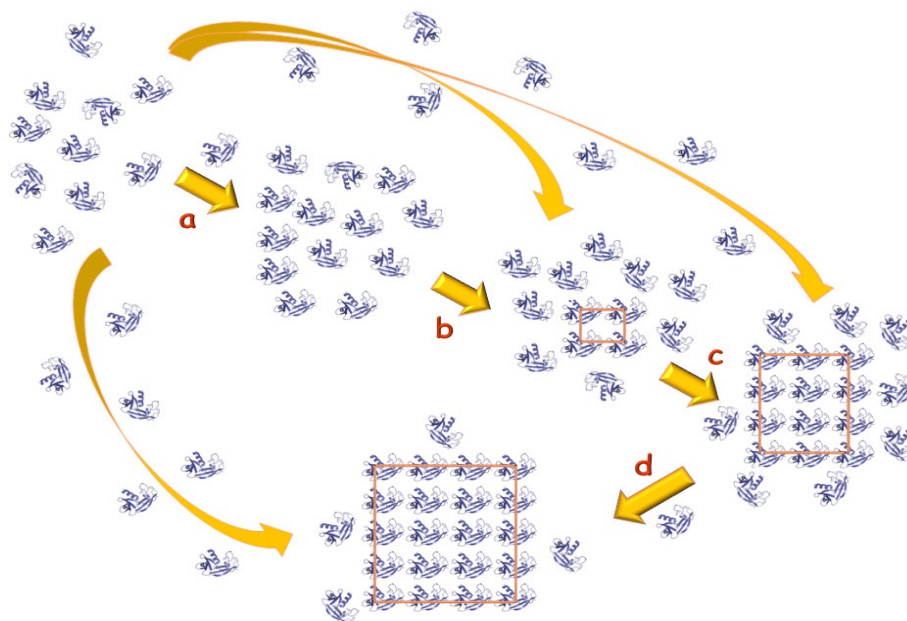


Figure 1.1: A fluid aggregate model of crystallization. As concentration increase supersaturation promotes molecule association beginning as large, disordered aggregates. **A)** molecules within the core of aggregates reorient themselves to form stronger geometric interactions. **B)** This stabilizes the aggregate core which increase allowing for the creation of nucleus. **C)** This nucleus ultimately results in a crystal. **D)** The crystal increases in size by absorbing free molecules through the crystal surface incorporating it into the lattice. Adapted from (Russo Krauss et al., 2013).

The general affect between the salt concentration and protein concentration for protein crystallization can be visualized in a phase diagram (Figure 1.2). Ideally nucleation occurs in the labile form commencing the first stage of the crystal formation (McPherson & Gavira, 2014). Crystal growth can occur in both labile and metastable zones of supersaturation, with growth

cessation of growth eventually occurring once the surrounding protein concentration decreases into the stable zone (Russo Krauss et al., 2013).

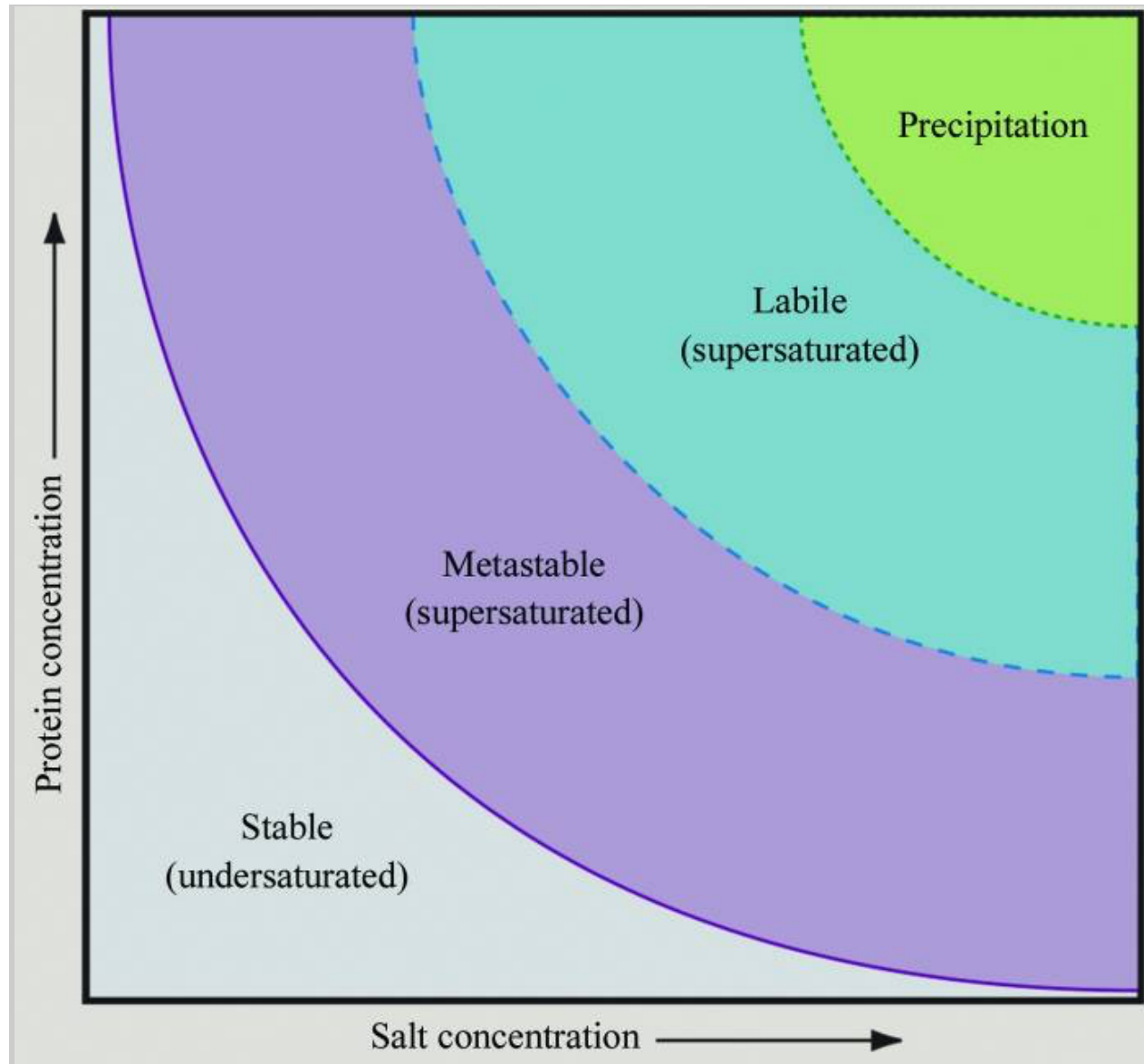


Figure 1.2: Shows the gradient of conditions from stable and undersaturation to precipitated and over saturation, with supersaturation separated into two segments. In the metastable zone nuclei can form crystals but nucleation will not occur (McPherson & Gavira, 2014). In the labile zone nucleation can occur, nuclei can also grow to form crystals. Image adapted from (McPherson & Gavira, 2014).

Crystallization conditions can depend on many factors (Table 1.1) such as sample concentration, temperature, pH value, precipitant, buffer, additive, detergent, physical fields, pressure etc. (Russo Krauss et al., 2013). The importance of gaining a crystal is undercut by the fact that as of yet, there is no process that guarantees a crystal (Russo Krauss et al., 2013). Sample purity and homogeneity play a key role in the crystallization process, with a common method of assessment for proteins being running the sample on sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Table 1.1: Parameters Affecting the Crystallization Process

Physical factors	Chemical factors	Biochemical factors
Temperature	Precipitant type	Sample purity
Pressure	Precipitant concentration	Sample homogeneity
Gravity	pH	Sequence modifications
Magnetic fields	Buffer type	Posttranslational modifications
Electric fields	Ionic strength	Chemical modifications
Dielectric properties	Sample concentration	Aggregation
Viscosity	Metal ions	Proteolysis
Vibrations and sound	Polyions	Sample pI
Time	Detergents	Ligands, co-factors, inhibitors
Equilibration rate	Heavy metals	
Nucleants	Small molecule impurities	
Methodology	Crosslinkers	
Surface of crystallization device	Reagent source	
Sample handling	Reagent formulation	

Adapted from (Russo Krauss et al., 2013)

Given all the parameters that can affect crystallization, the process is a relatively empirical method and relies on numerous trials performed with commercially available screens with each

screen assessing 96 separate conditions (McPherson & Gavira, 2014). The screens use the vapour diffusion method shown in Figure 1.3.

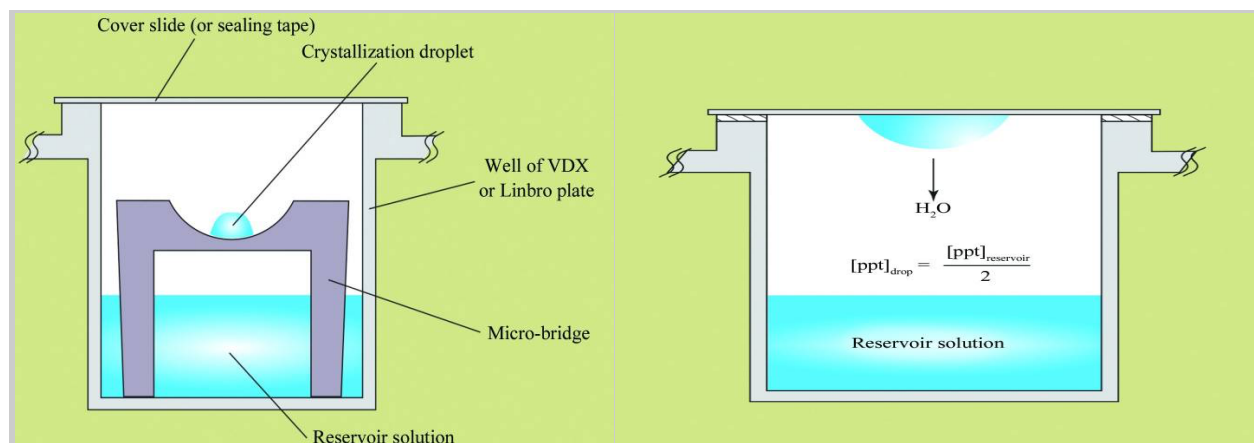


Figure 1.3: Shows two main methods of crystallization. On the left is the sitting drop vapour-diffusion method used in crystal screens, on the right is the hanging drop vapour-diffusion. Image adapted from (McPherson & Gavira, 2014).

Generally speaking, several outcomes can be seen when examining the droplets, with clear droplets indicating an undersaturated condition (Figure 1.4). Birefringence of the contents in each droplet is also assessed. This done with a polarizing filter on a microscope, allowing for polarized light to pass through the sample. Polarized light passing through a birefringent sample resolves into a faster travelling wave and a slower travelling wave which results in a phase difference that turns linearly polarized light into elliptically polarized light (Echalier et al., 2004). The result of this change is a fluorescent like image as seen in the microcrystals in Figure 1.4. Spherulites are circular crystals that can be acicular in nature, unfortunately due to the spherical nature these crystals can not be diffracted (Luft et al., 2011). In comparison crystals that can be diffracted are relatively large in size with hard straight edges. Microcrystals are generally birefringent in nature and can seem slightly granular at times, these are essentially exceptionally small crystals indicating that crystallization has occurred but with an abundance of nucleation sites (Luft et al., 2011). Liquid-Liquid phase separation may be a good indicator of crystal growth since the surface are

where the phase separation occurs may have higher chance of nucleation. The screens are monitored daily with any conditions showing some form promising results such as phase separation, spherulites, microcrystals or even crystals to be optimized.

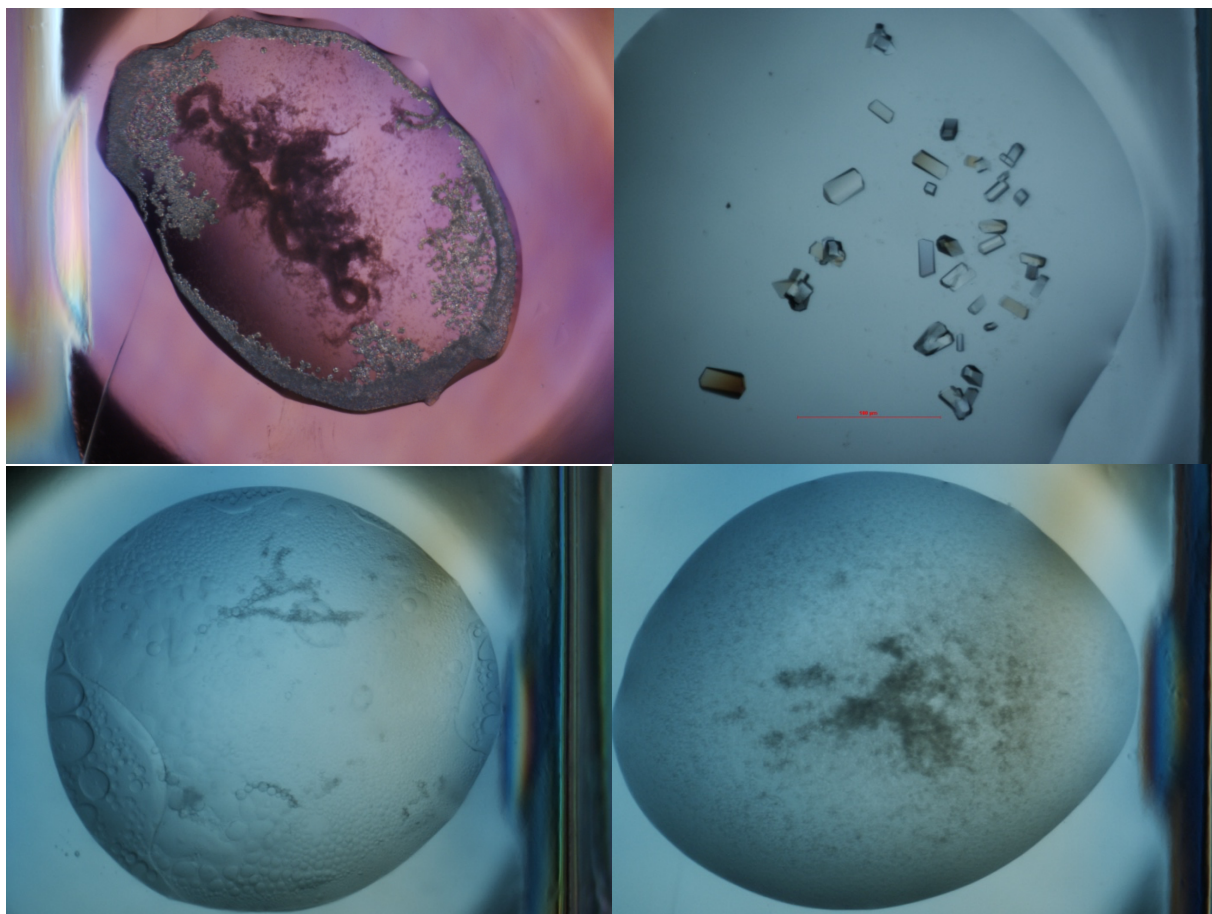


Figure 1.4: The top left droplet contains birefringent microcrystals surrounding amorphous precipitate. The top right droplet contains relatively large straight edge crystals where the red bar indicates a measurement of 100 μM . The bottom left droplet indicates liquid-liquid phase separation while the bottom right contains light granular precipitate.

To further increase probability of obtaining crystals that can be diffracted, microseeding may also be performed (McPherson & Gavira, 2014). This circumvents the need to create nucleation as you are already incorporating nuclei into each drop. To achieve this a seed stock is creating by vortexing already obtained crystals/microcrystals with reservoir solution and a small

Teflon bead. Afterwards this stock is diluted to avoid incorporating to many potential nuclei and then a small volume of this seed stock is incorporated into each drop during plating.

Optimization is mainly performed in 24-well plates using the hanging drop method (Figure 1.3). Optimization is performed by taking an identified condition of interest (Table 1.1) and adjusting the components slightly such as gradient in protein concentration, precipitant or pH across the 24 wells. This process of screening and optimizing is repeated until a single crystal large enough to diffract is formed.

Crystallographic data is obtained from crystals by the use of an X-ray diffractometer. The resultant diffraction patterns are processed to obtain diffraction coordinates and measured intensities, which can then be used to solve the structure factor. Ultimately, an X-ray diffraction experiment is the computation of the electron density function $\rho(x, y, z)$ according to the Fourier synthesis of equation $\rho(x,y,z)=1/V\sum h\sum k\sum l F_{hkl}e^{-2\pi i(hx+ky+lz)}$ where h,k,l and x,y,z represent reciprocal indices and real space coordinates, respectively; the structure factor (F_{hkl}) is a complex number that can be represented as $|F_{hkl}|e^{i\phi_{hkl}}$ (Sherwood & Cooper, 2010b). This requires knowledge of both the magnitudes ($|F_{hkl}|$) and the phase angles (ϕ_{hkl}) (Sherwood & Cooper, 2010b). Unfortunately, the X-ray diffraction experiments, which collect intensities of the reflections, only derive the magnitudes and not the corresponding phases. Since diffraction can occur in a reflective (opposite) manner Friedel's law states that the intensities of h,k,l and $-h,-k,-l$ (known as a Friedel pair) are equal which results in a positive number while ϕ and $-\phi$ being opposite cancel each other out (Friedel, 1913). Unfortunately, that simplifies $e^{-2\pi i(hx+ky+lz)}$ to $e^{-2\pi i(0)}$ with anything to the power of zero resulting in one and truncating our electron density function $\rho(x, y, z)$ to $1/V\sum h\sum k\sum l F_{hkl}$ leaving us unable to solve the phase yet needing to do so in

order to ascertain a structure (Sherwood & Cooper, 2010b). This in essence is known as the phase problem which can be illustrated with an argand diagram (Figure 1.5).

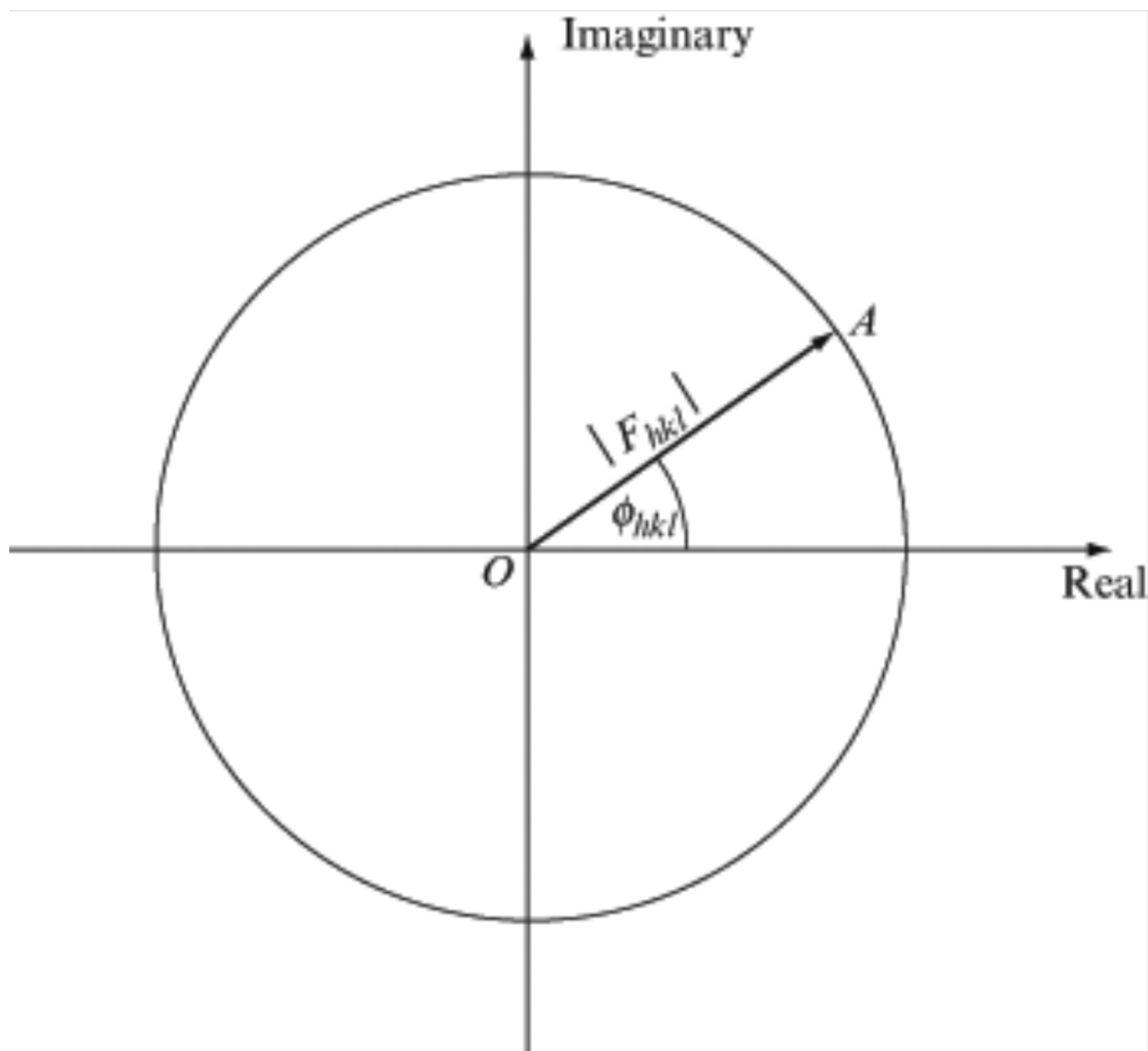


Figure 1.5: The phase problem. The Argand diagram represents the structure factor F_{hkl} with a vector of length $|F_{hkl}|$ and making an angle ϕ_{hkl} with the real axis. If only the magnitude $|F_{hkl}|$ is known, then the structure factor can be any vector from origin O to the circle. Adapted from (Sherwood & Cooper, 2010b)

There are three main factors that contribute to the current inability of finding the phase angle. The first being that X-rays travel at 10^{18} Hz meaning that in order to monitor the phase wave meaning that the instrument must be able to make changes in the order of 10^{-18} s which would be

extremely fast and beyond current technological capabilities (Sherwood & Cooper, 2010b). The second reason is that to accurately measure phase the distance between a diffracted wavelength and a reference wavelength must be known for this to be achieved a ruler capable of measuring a distance smaller than the size of an X-ray wavelength would be required, which would require further technological advancement (Sherwood & Cooper, 2010b). The last reason stems from the very nature by which X-ray diffraction experiments are conducted. All present X-ray sources utilize X-ray from an incoherent source, meaning that no two X-rays are propagated with a well-defined phase relationship. For the phase to be determined the X-rays would need to come from a coherent source which emits radiation with highly correlated phase relationships (Sherwood & Cooper, 2010b). It should be kept in mind that all three problems are due to the limitations of the current technology used and not based on the physics involved in X-ray crystallography, indicating that the phase problem can be resolved without any other methods in the future.

A common method for solving the phase problem is molecular replacement, this allows structures to be solved using already solved structures from the protein databank (PDB) (Beck et al., 2010; Sherwood & Cooper, 2010a). While there are over 180,000 structures in the PDB, if there are no homologous proteins based on sequence identity to the target protein of interest, molecular replacement is not a viable option for structure solution.

The phase problem can also be resolved experimentally in several ways. The first is isomorphous replacement which involves crystallization of the protein with a heavier atom in complex, normally a metal of some sort, or complexes such as 5-amino-2,4,6-triiodoisophthalic (I3C) or 5-amino-2,4,6-tribromoisophthalic (B3C) (Figure 1.6). This allows for distinct patterns of electron energy throughout the crystal from the heavy atom which can be used to solve the phase problem. Crystals soaked with such heavy atoms can also be used to perform single-wavelength

anomalous dispersion (SAD) and multi-wavelength anomalous dispersion (MAD) (Beck et al., 2010) experiments to determine phases.

Recently a new method incorporating I3C during buffer preparation has been published (Truong et al., 2021). This has been found to not only to increase the chance of crystallization of proteins but also means no extra experiments or preparation is required to solve for the structure since I3C is already incorporated into any crystals and utilizing SAD would resolve the phase problem. To assess whether similar results are seen with proteins that are typically difficult to crystallize, TraF from the F plasmid of *Escherichia Coli* was utilized. The F plasmid is key component in bacterial cell function and attributes to the spread of antibiotic resistance.

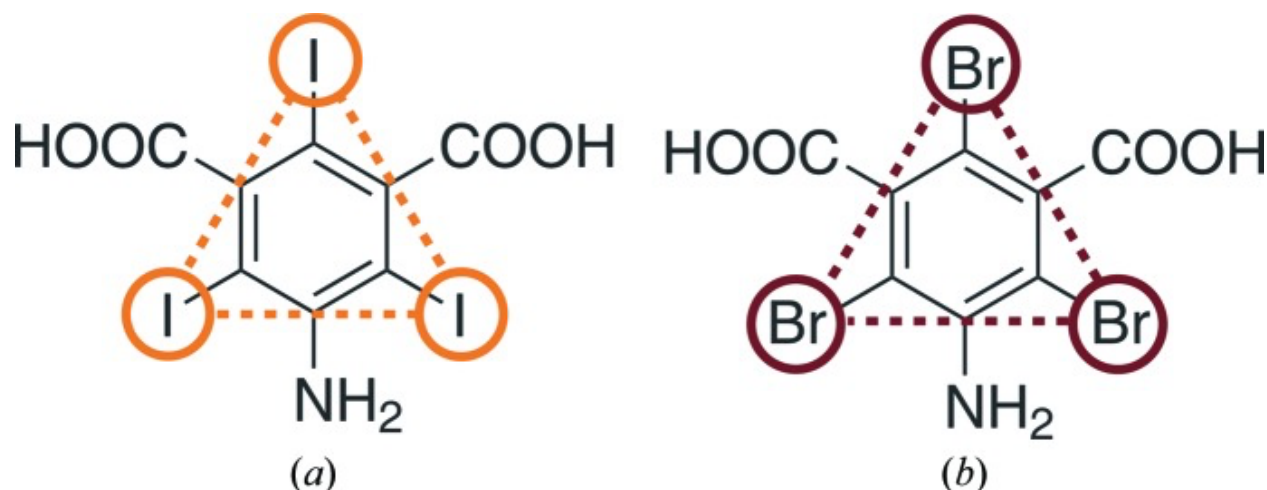


Figure 1.6: A) The chemical structure of 5-amino-2,4,6-triiodoisophthalic (I3C), the iodine atoms create an equilateral triangle with each side being 6.0 angstroms in length. **B)** The chemical structure of 5-amino-2,4,6-tribromoisophthalic (B3C), the bromine atoms create an equilateral triangle with each side being 5.65 angstroms in length. Adapted from (Beck et al., 2010).

1.2 Significance of Antibiotic Resistance

Bacteria are incredible organisms that have survived for over 3.5 billion years on earth, adapting to various harsh conditions such as toxic environments, massive fluctuations in temperature and lethal compounds from other microbes (Bennett, 2008). It comes as no surprise then that antibiotic resistance is becoming a more pressing issue with each passing year. Over 23,000 deaths are caused by antibiotic resistant bacteria yearly in the U.S.A. alone, and it's estimated that by 2050 more deaths will be attributed to antibiotic resistant bacteria than current deaths caused by cancer (Chang et al., 2015; Singh, 2017). It is becoming increasingly challenging to address the issues posed by bacterial outbreaks due to their ability to acquire drug resistances through adaptation.

To understand the origin of drug resistances it is best to do discuss the drugs in question. The first antibiotic, penicillin, was identified by Sir Alexander Fleming in 1929, earning him a Nobel prize just 16 years later in 1945 (Browne et al., 2020; Fleming, 1929). Even during his acceptance speech Sir Fleming warned the world of penicillin resistance in bacteria, which proved a true concern in 1948 when a penicillin resistant strain of *Staphylococcus* was declared a global pandemic (Browne et al., 2020.,). Between 1950 and 1970 over 20 new classes of antibiotics were released (Figure 1.1). After the 1970s, drug discovery started to decline while there have been many “new” antibiotics they fall into the “follow on” or more commonly referred to “me too” drug category (Browne et al., 2020). These are drugs that are based of pre-existing antibiotics with small changes in functional groups which means that bacteria resistant to the related drug are preadapted or can quickly become adapted to these antibiotics as well (Browne et al., 2020). The inevitable

occurred when a woman succumbed to a strain of *Klebsiella pneumoniae* that proved to be resistant to all current classes of antibiotics (McCarthy, 2017).

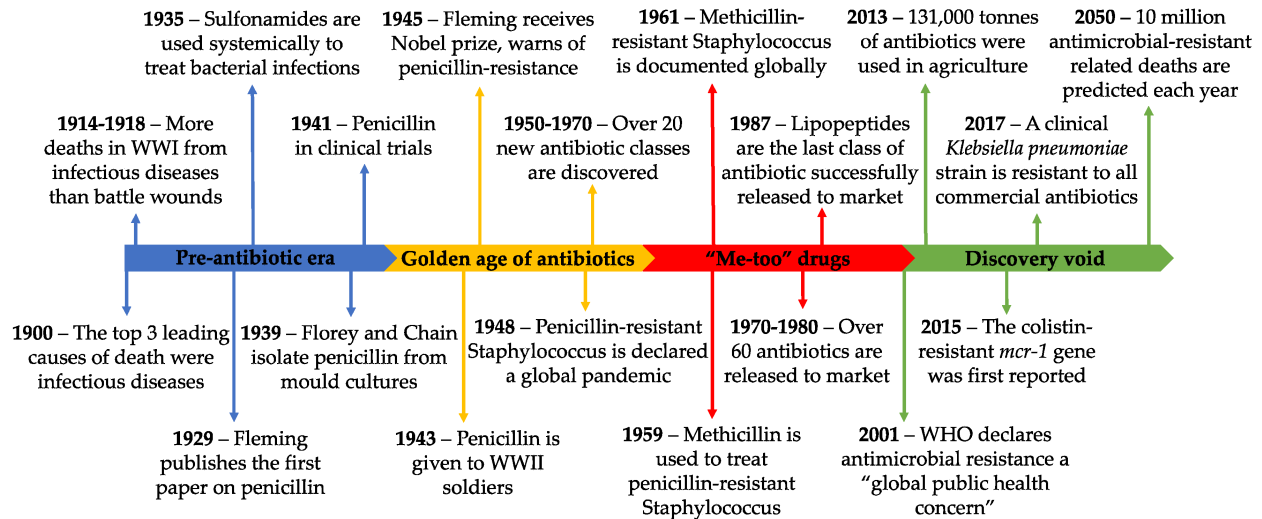


Figure 1.7: A timeline of key events regarding antibiotics and antibiotic resistant bacteria. The discovery of penicillin lead to a prevalent amount of antibiotics discovered from the 1950s to the 1970s with the last new class of antibiotics discovered in 1987. In 2017 a strain of bacteria was discovered to be resistant to all commercially available antibiotics. Adapted from (Browne et al., 2020).

Multi-drug resistance (Mdr) is classified as bacterial pathogens resistant to three or more classes of antibiotics (Thomson & Bonomo, 2005). These species are growing globally due in part to an overuse of antibiotics in agriculture, hospital-derived infections and antibiotics that have been over-prescribed to patients (Chang et al., 2015; Pelat et al., 2015). Evolutionarily speaking, Mdr can be traced back to certain plasmids adequately termed resistance (R) plasmids (Davies, 1994). Generally, resistance is conferred through transposable elements that allow for site specific recombination of resistance genes which, through the use of various genetic transfer and exchange mechanisms, are acquired by pathogenic bacteria (George, 1996). Unfortunately, treating bacteria with antibiotics can result in dire consequences with the antibiotic eliminating non-resistant cells

in the population whilst selecting for the resistant cells allowing for the proliferation and spread of the resistant bacteria (George, 1996). Today, Mdr is a commonplace term used to describe intrinsic mechanisms of antibiotic resistance from genes that constitute part of the genome of a cell (George, 1996).

Antibiotic resistance is simply a form of adaptation to the surrounding environment for bacteria, which in essence is something that has happened for over 3 billion years across many different environments (Bennett, 2008). Most adaptations result in structural changes to the antibiotic target, such as mutations in 23S rRNA that inhibit the antibiotic linezolid from binding to 23S rRNA rendering it ineffective (Browne et al., 2020; Long & Vester, 2012). Furthermore, gene sequences that target the antibiotic specifically such as bacteria that now express β -lactamase enzymes which directly hydrolyse β -lactam class antibiotics (including penicillin) rendering them ineffective (Browne et al., 2020; Egorov et al., 2018). It is hypothesized that a significant portion of Mdr is conferred through horizontal gene transfer (HGT) with roughly 20% of the *Escherichia coli* genome has been modified over time through HGT (Browne et al., 2020; Lawrence & Ochman, 1997). These genes of antibiotic resistance are conserved and selected for utilizing DNA replication through binary fission and HGT to transmit DNA throughout the bacterial colonies (Allen et al., 2010). HGT allows for the proliferation of advantageous genes that code for factors such as altered drug targets, drug efflux pumps and other virulence drug factors independently of cell replication (Figure 1.8) (Sultan et al., 2018).

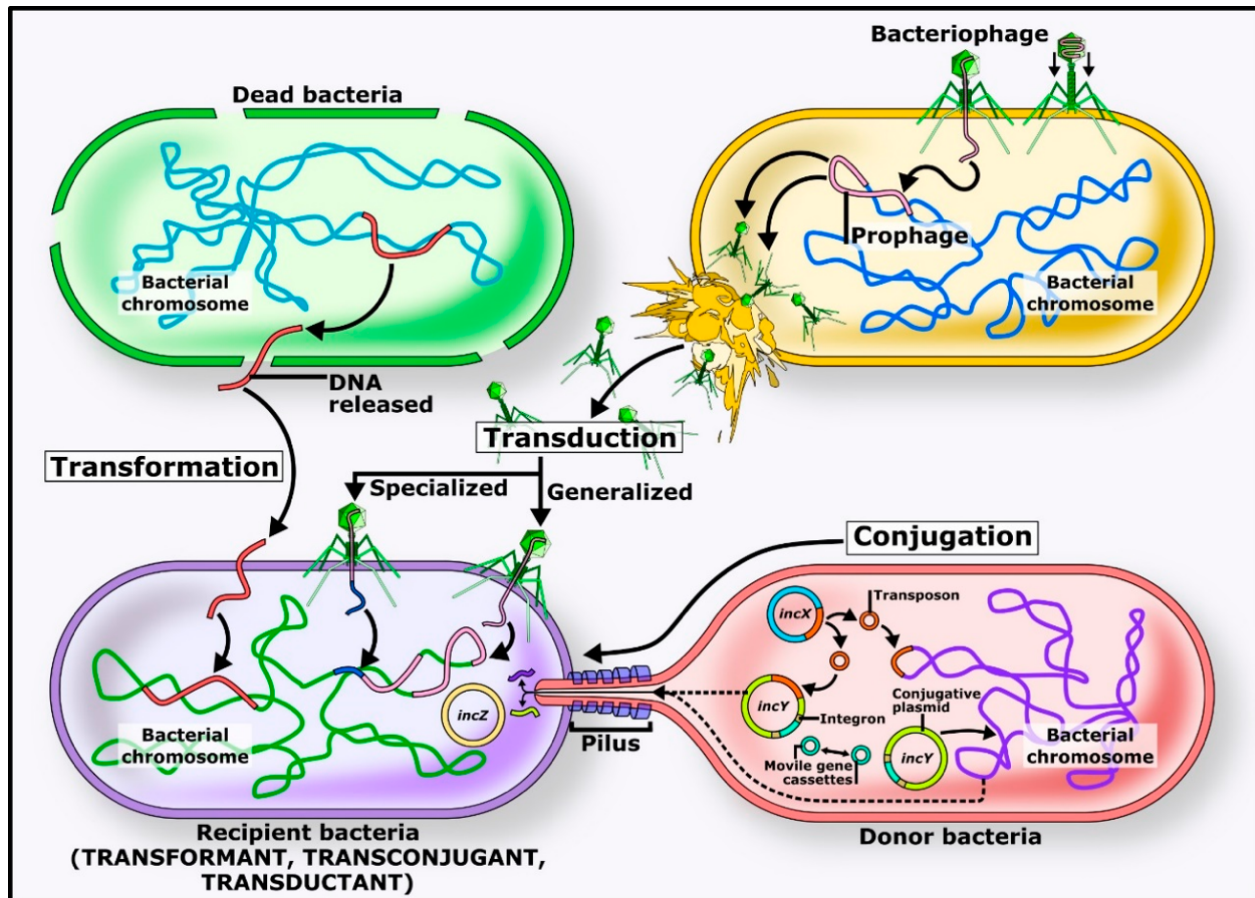


Figure 1.8: Shows various methods of HGT which can occur through (a) transformation of DNA from dead bacteria, (b) transduction utilizing bacteriophages and (c) conjugation from donor to recipient. Adapted from (Bello-López et al., 2019)

As the frequency of Mdr gram-positive and gram-negative bacteria increases, targeting cell secretions systems becomes of greater importance as an alternative to conventional antibiotic treatments (Baron & Coombes, 2007; Browne et al., 2020). Rather than traditional antibiotics that attempt to kill the bacteria outright and have led to a worldwide concern of Mdr bacteria, novel drugs could inhibit the process by which bacteria transfer Mdr to one another and excrete virulence factors in the host organism thereby allowing the host to degrade the pathogenic bacteria (Figure 1.9) (Baron, 2010).

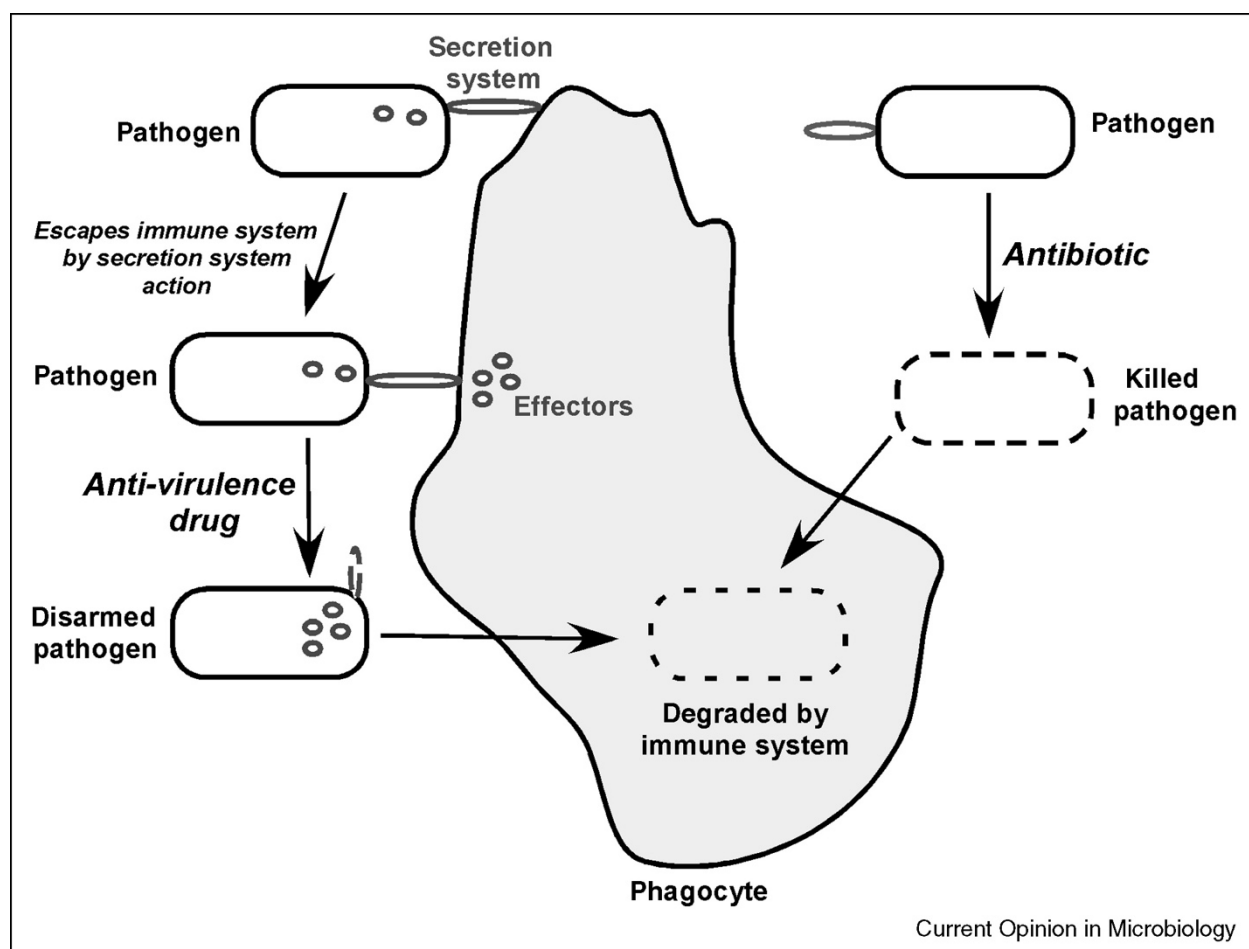


Figure 1.9: Comparing the standard method of treating pathogenic bacteria with more novel methods that render the pathogen benign enough to be degraded by the host's immune system. Adapted from (Baron, 2010).

1.2 Bacterial Secretion Systems

Bacteria transport macromolecules between cellular membranes through specialized secretion systems, which number I through IX (Figure 1.3) (Abby et al., 2016; E. R. Green & Mecsas, 2016). Bacterial secretion systems are necessary to transfer virulence factors to host cells and required for pathogenicity which makes them a key target for antivirulence drug development (Baron, 2012). The Type VIII and IX secretion systems are the latest types to be uncovered (Abby et al., 2016). Type I, III, IV and VI secretion systems use a one-step process allowing macromolecules to pass from within the inner membrane to the through the outer membrane and

past the membrane of the host cell utilizing a secretion tube (Figure 1.10) (Bocian-Ostrzycka et al., 2017). While Type II, V, VII, VIII and IX secretion systems utilize a two-step process where either secretin (Sec) or twin arginine transportation (Tat) allows macromolecules through the inner membrane and then the respective secretion system excretes the macromolecule past the outer membrane. Type I secretion systems are conserved through most gram-negative bacteria and closely resemble a family of ATP-binding cassette transporters that export toxins and antibiotics out of the cell (Thomas et al., 2014). Type II secretion systems are more complicated, conserved in most gram-negative bacterial systems and utilized to transport folded proteins (Korotkov et al., 2012). Depending on the bacterial system type II secretion systems transport multiple substrates or can be limited to just one substrate (Cianciotto, 2005). Type III secretion systems are conserved through many gram-negative bacteria and are described as having a “needle and syringe” structure also referred to as “injectosomes” (Büttner, 2012; E. R. Green & Mecsas, 2016). Type IV secretion systems can transfer various macromolecules across the hosts cell membrane including single proteins, protein-protein and DNA-protein complexes while being ancestrally related to bacterial DNA conjugations systems (Cascales & Christie, 2003). Type V secretion systems are unique in comparison to other secretion systems since they do not secrete other substrates from the cell but substrates from their own secretion system (Leyton et al., 2012; Pohlner et al., 1987). Type VI secretion systems are a relatively novel discovery and require further study to fully understand all functions but does seem to mediate interbacterial function (Mougous, 2006; Schwarz et al., 2010). Type VII secretion systems are unlike aforementioned secretion systems since they normally reside in gram-positive bacteria and transport virulence factor from the WXG100 protein family. (Freudl, 2013). The protein family is named after their conserved amino acids sequence of W-X-G (where X is any amino acid) which are roughly 100 amino acids in length. Type VIII secretion

systems involve the transport of extracellular proteinaceous fibers known as Curli (Barnhart & Chapman, 2006; Dueholm et al., 2012; Olsén et al., 1989). Type IX Secretion systems are a novel discovery that have been noted in *Bacteroidetes* found in the digestive tract of living organisms and *Porphyromonas gingivalis* a key pathogen involved in periodontitis (Grenier & Dang La, 2011; Lasica et al., 2017; Sato et al., 2013).

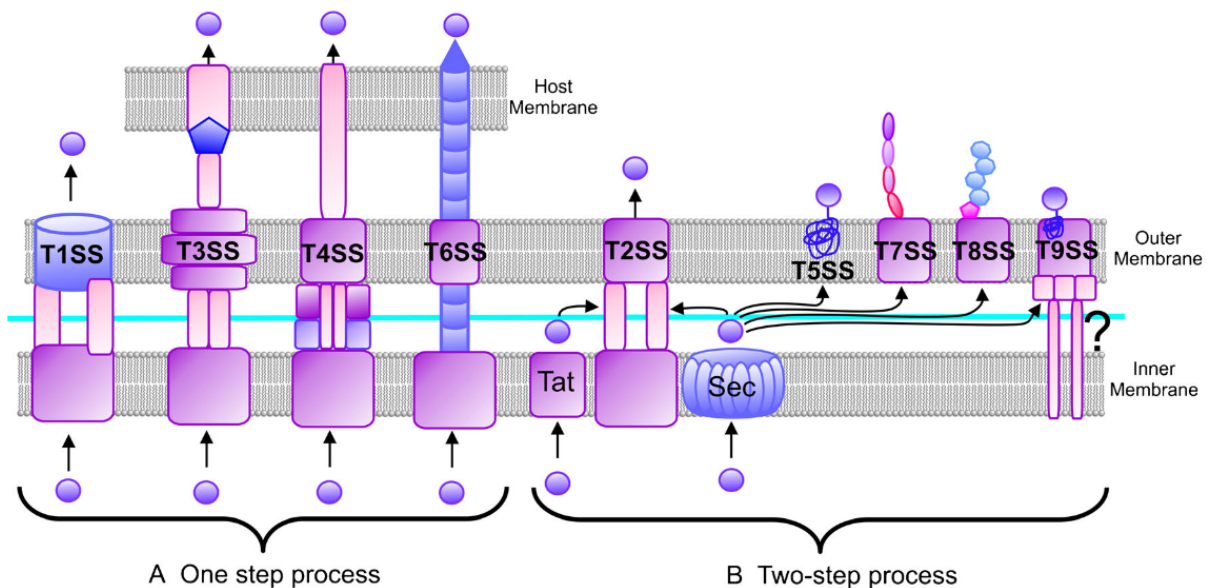


Figure 1.10: The various bacterial secretion systems grouped by being one step or two-step process. One-step secretion systems allow for direct transport of macromolecules from the inner membrane of the donor cell past the outer membrane and past membrane of the host cell through a secretion channel. Two-step systems first require the use of secretin (Sec) or twin arginine transportation (Tat) to move macromolecules across the inner membrane and then transported past the outer membrane utilizing the secretion system. Adapted from (Bocian-Ostrzycka et al., 2017)

Type III secretion systems were the first target for novel drug development since they are key virulence factors in gram-negative plant and animal pathogens (Moraes et al., 2008; Mota & Cornelis, 2005). The first published paper utilized a cell-based assay to assess for inhibited transcription of genes in the type III secretion system in *Yersinia pseudotuberculosis* that occurs

when the type III transcription system is inhibited (Allaoui et al., 1995; Kauppi et al., 2003). After screening for a chemical library of 9,400 molecules were screened for any effect 3 were shown to have an impact (Kauppi et al., 2003). These molecules belonged to the family of salicylaldehyde hydrazones, and were the first example of potential secretion system targeting compounds. Another study screened 20,000 molecules for inhibited type III secretion activity in *E. coli* which resulted in a halogenated salicylaldehyde providing the greatest inhibition followed by 3 salicylideneanilines (Gauthier et al., 2005). This indicates that inhibition with this family of compounds is reserved across other pathogens. Further structural studies verified that inhibition was achieved by inhibiting the type III secretion substrates and not by directly inhibiting gene transcription of the type III secretion system (Nordfelth et al., 2005).

Type IV secretions systems (T4SSs) are also a target for drug development since they can transfer macromolecular virulence factors and genetic information. The VirB8 protein from *Brucella* is an essential protein involved in that bacteria's T4SS (Paschos et al., 2006). Utilizing structural Cryo-EM data of VirB8, potential inhibitors were designed and selected based off the active site and were screened against VirB8 for binding activity, which inhibits VirB8 interaction with other proteins in the T4SS (Smith et al., 2012; Terradot et al., 2005). This resulted in an inhibitor known as B8I-2 (Smith et al., 2012). Similar experiments were conducted with the VirB8-like protein TraE which is a necessary protein for the T4SS utilized by the plasmid pKM101 with similar results showing not only conserved function but conserved protein interaction sites across different T4SS (Casu et al., 2017). Further insight into T4SSs systems and their method of function may lead to novel drugs to combat Mdr.

For over 75 years since the birth of modern molecular biology, T4SSs have been studied starting with a description of a bacterial conjugation system (Tatum & Lederberg, 1947). T4SSs

are relatively diverse with two sub classes of T4SSs, IVA based of effector transport systems which include Vir (virulent) protein and homologs (such as Cag proteins (cytotoxin-associated genes)) and IVB for conjugative protein systems which include Tra homologs (Table 1.2) (Christie & Vogel, 2000).

Table 1.2: Similarities Between Several Type IV Secretion Systems*

(PROPOSED) FUNCTION / HOMOLOGY	T4SS SYSTEM				
	EFFECTOR TRANSPORT		CONJUGATION		
	Ti	Cag	F	R27	pKM101
Pilin	VirB2	CagC	TraA	TrhA	TraM
Cyclase				TrhP	
Acetylase			TraX		
Lysozyme	VirB1	Cag γ	Orf169	Orf130	TraL
Pore	VirB3	CagE	TraL	TrhL	TraA
Secretion	VirB4	CagE	TraC	TrhC	TraB
Pore	VirB5	CagL	TraE	TrhE	TraC
Pore	VirB6	CagW	TraG	TrhG	TraD
Lipoprotein	VirB7	CagT	TraV	TrhV	TraN
Pore	VirB8	CagV			TraE
Secretion/pore	VirB9	CagX	TraK	TrhK	TraO
Pore	VirB10	CagY	TraB	TrhB	TraF
Secretion	VirB11	Cag α			TraG
Pore			TraF	TrhF	
Pore			TraH	TrhH	
Pore			TraW	TrhW	
Pore			TrbC	TrhW	
Pore			TraU	TrhU	
Adhesin			TraN	TrhN	
Relaxase			TraI	TraI	TraI
Transport			TraD	TraG	TraJ

* The TFSSs are categorized into two main categories effector transport (as seen in the Ti (tumour inducing) and Cag and Conjugation (F, R27 and pKM101). This table illustrates the homologies of protein from various T4SSs. Adapted from (Shala et al., 2016).

These genes of antibiotic resistance are conserved and selected for utilizing DNA replication through binary fission and horizontal gene transfer (HGT) to transmit DNA throughout the bacterial colonies (Allen et al., 2010). HGT allows for the proliferation of advantageous genes that

code for factors such as altered drug targets, drug efflux pumps and other virulence drug factors independently of cell replication (Sultan et al., 2018).

Type IV secretion systems (T4SS) are the conjugative HRT method used to transfer antibiotic resistant factors and other factors of interest in a donor-recipient fashion (Bennett, 2008). T4SS are conserved throughout most gram-negative bacteria and even various gram-positive bacteria (Wallden et al., 2010). Since T4SSs seem to be the method by which antibiotic resistance proliferates within colonies of pathogens, ascertaining the exact function and structure should of key components in this system could lead to novel methods of inhibition and the digression of antibiotic resistant bacteria.

Conjugative transfer occurs in 5 main steps through a plasmid driven process (Anthony et al., 1994). The first step involves a conjugative pilus of the donor cell interacting with the cell surface of the recipient cell (Figure 1.5). Once attached to the recipient cell the T4SS retracts the pilus allowing the donor and recipient cell to come into close proximity to each other. Pilus extension and retraction is a dynamic process that occurs asynchronously from other pili on the cell surface (Clarke et al., 2008). This active process allows the cell to identify any potential recipient cells.

Once in close proximity the two cells form a mating pair. A mating pair formation (Mpf) complex is formed from T4SSs proteins that allow for the transfer of plasmid DNA by transferring single stranded DNA (ssDNA) through ATP-dependent process. This complex stabilizes the transfer from external forces but is not required for transfer as it still occurs but with reduced efficiency (Manning et al., 1981). Once transfer has completed and a complimentary strand is synthesized the two cells separate, resulting in two donor cells (Anthony et al., 1994).

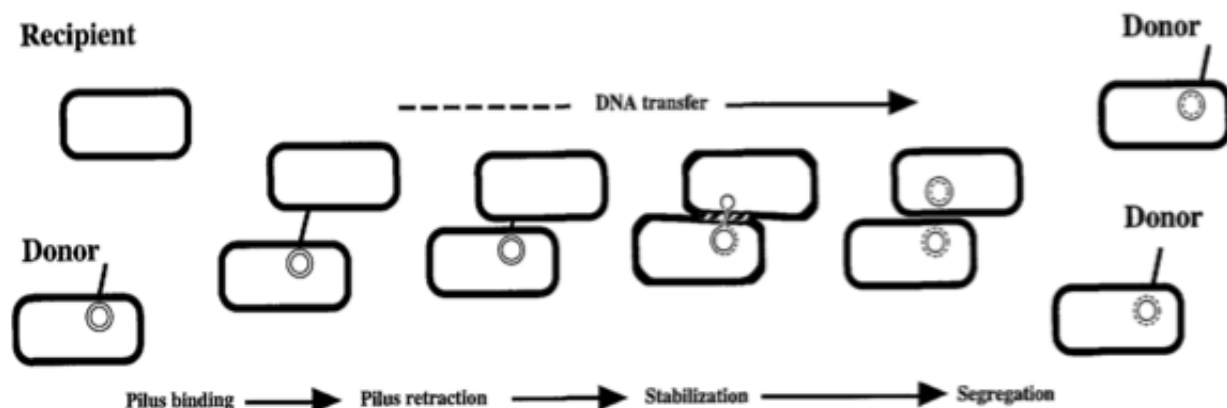


Figure 1.11: The tip of the pilus from the F⁺ donor cell initiates contact with the recipient cell. The F pilin retracts drawing in the recipient cell into close proximity with the F⁺ donor. Mating pair formation occurs aiding DNA transfer from the F⁺ donor to recipient. Once complete, the cells separate leaving both cells as F⁺ donors. Adapted from (Firth et al., 1999).

As the significance behind T4SSs becomes more prevalent so does the importance of structural studies on these systems. Cryo-electron microscopy (Cryo-EM) and crystallographic studies have been utilized to elucidate the core complex structure of pKM101 which is a homolog of IncN homolog of the F plasmid (Chandran et al., 2009; Fronzes et al., 2009). The Cryo-EM structural data revealed that in the core complex TraF_{KM101} (TraB_F/Virb10 homolog) co-purifies with TraN_{KM101} (TraV_F/VirB7) and TraO_{KM101} (TraK_F/VirB9) resulting in a 1.1 MegaDalton (MDa) complex (Fronzes et al., 2009). This trimer of TraF-N-O enables substrate passage by forming a membrane bound tetradecameric complex.

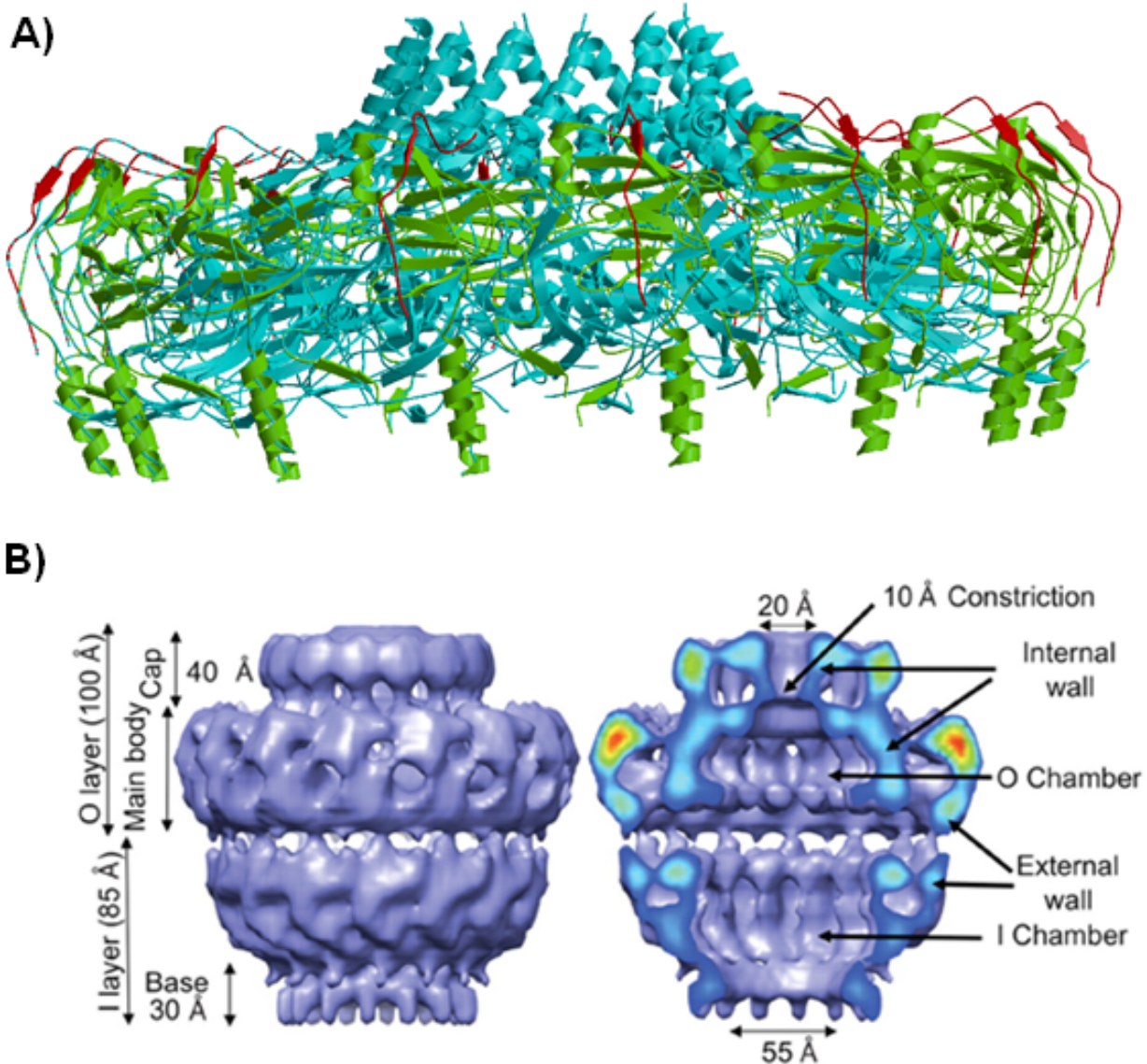


Figure 1.12: The core complex of pMK101 T4SS. **A)** The structure of the TraF (cyan)-N (red)-O (green) tetradecamer (Chandran et al., 2009). **B)** The outer layer (O) and inner layer (I) of T4SS core complex reconstructed utilizing Cryo-EM. On the left is the full complex, while the right is a model highlighting the inner dimensions. Adapted from (Shala et al., 2016)

The core complex consists of an inner (I) and outer (O) membrane which has a width and height of 185 nm (Figure 1.5B) (Fronzes et al., 2009). The outer membrane consists of two regions a cap that is 11 nm in diameter and 4 nm high and the main body which has a diameter of 185 nm and is 60 nm tall (Fronzes et al., 2009). The cap is formed by the $\alpha 2$ and $\alpha 3$ helices of

TraF_{KM101}. The cap has 2 nm hole that tapers down to 1 nm at the main body with a 5.5 nm opening at the bottom of the inner membrane allowing for the assembly of the conjugative pilus and the transfer of DNA from donor cell to recipient cell.

Recently key components of the F-like T4SS of the conjugative pED208, an IncFV homolog has been revealed through Cryo-EM (Figure 1.7) (Costa et al., 2016; Hu et al., 2019). The pilus is made of composed of TraA subunits in 5 distinct helical strands, with each strand composed of 12.8 subunits per turn (Costa et al., 2016). The width of the pilus is 8.7 nm with a cavity that is 2.8 nm to allow for the transfer of DNA (Costa et al., 2016). The core complex was also resolved with Cryo-EM, identifying the ATPase TraC dimers which assemble as a hexamer at the inner membrane where it regulates substrate transfer and F pilus conjugation (Hu et al., 2019).

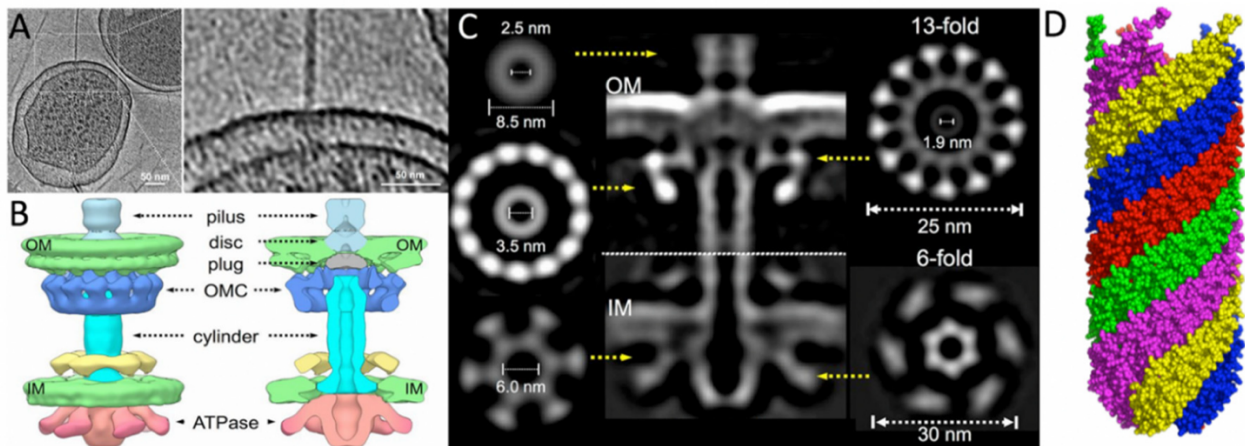


Figure 1.13: Cryo-EM of all major segments of the F-like conjugative T4SS from pED208. **A-C)** Cryo-EM of the T4SS machinery spanning from the inner membrane to outer complex (OMC). The TraC dimers that assemble as a hexamer are shown in pink (Hu et al., 2019). **D)** Cryo-EM structure of the pED208 pilus, with 5 helical structures each of which are individually coloured (Costa et al., 2016). Image adapted from (Bragagnolo et al., 2020).

There are several conjugation systems that follow the F-type conjugation system, accordingly grouped as the F-like systems (Datta, 1975; Willetts & Skurray, 1980). These plasmids are further subdivided based on their incompatibility (Inc) to coexist in the same host which originally was categorized and limited to 7 groups (IncFI to IncFVII) but further expanded to include other Inc groups such as IncHI, -T and -J (Lawley et al., 2003; Willetts & Skurray, 1980). All F-like conjugation plasmids construct long, flexible pili that allow them to establish mating in liquid culture and are associated with phage f1 (Datta, 1975).

1.2 *E. Coli* F Plasmid

The F plasmid of *E. Coli* was the first to be examined for genetic transfer and is still the cornerstone of modern bacterial conjugation research (Tatum & Lederberg, 1947). The IncFI plasmid is roughly 100 kb and take roughly 5 minutes to transfer from donor to recipient resulting in a recircularization of the transferred DNA strand and complimentary DNA synthesis of the single stranded plasmid on the old and new donor cells (Frost et al., 1994). All the DNA sequences required to express the proteins required for conjugative transfer are located in the transfer region of the F plasmid which is roughly 33.3 kb long (Figure 1.14).

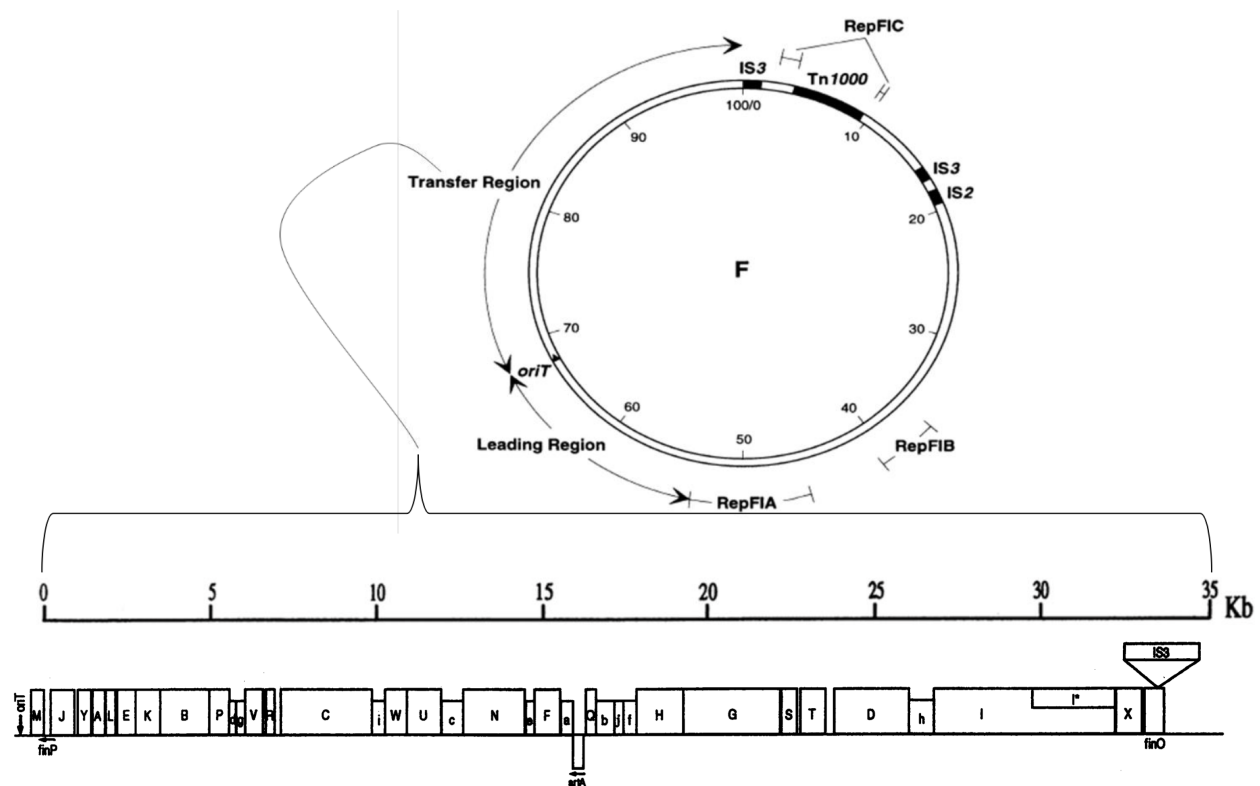


Figure 1.14: F Plasmid Structure. The top shows the F plasmid in its 100 kb entirety with approximate positions of important genetic markers. The origin of transfer (*oriT*) is where the plasmid is nicked by *TraI* allowing for the transfer of ssDNA in a clockwise direction. Insertion sites 2 and 2 (IS2 & IS3) are simple transposable elements while *Tn1000* is a complex transposable element all of which are indicated by black boxes (Roberts et al., 2008). RepFIC is a non-functional replicon while RepFI and B serve as origins of replication (Villa et al., 2010). The 33.3 kb transfer region is shown below the plasmid. It contains antisense *artA* and *finP* sequences along with all *tra* and *trb* gene sequences (Frost et al., 1994). I* sequence represents the region of *TraI* that is responsible for DNA transfer activity (Matson & Ragonese, 2005). Adapted from (Firth et al., 1996; Frost et al., 1994).

F Plasmid conjunctive transfer occurs through an F-like T4SS utilizing F pili which involves several key proteins spanning across the inner and outer membranes of the cell in gram-negative bacteria (Figure 1.15 and Table 1.3) (Fronzes, R., Christie, P. J., & Waksman, 2009). These proteins assemble conjugative pili to extend and bind to a neighboring cell and retract to bring it closer and exchange genetic information (Schröder & Lanka, 2005). The F plasmid assembly has *Tra* proteins which are homologous of *Vir* proteins in other T4FSs such as N, P, W,

X (Lawley et al., 2003). TraC allows for the extraction and assembly of TraA monomers to form the pilus through an ATP dependant manner (Kerr & Christie, 2010). This process is aided by other chaperone proteins such as TraL TraE, TraK and TraG (Costa et al., 2016). Once the pilus is attached to the neighboring cell retraction occurs through subunits TraH, TraF and TrbI interacting with TraW and TrbC (Arutyunov & Frost, 2013; Lawley et al., 2003; Shala-Lawrence et al., 2018). Retraction is also mediated with other proteins such as TrbB which is homologous to TraF (Figure 1.15). TraF (along with TraH -U and -W) seems to associate with the outer membrane alongside a hypothesized interaction with TraV that anchors the periplasmic complex to the core complex of the outer membrane (Bragagnolo et al., 2020).

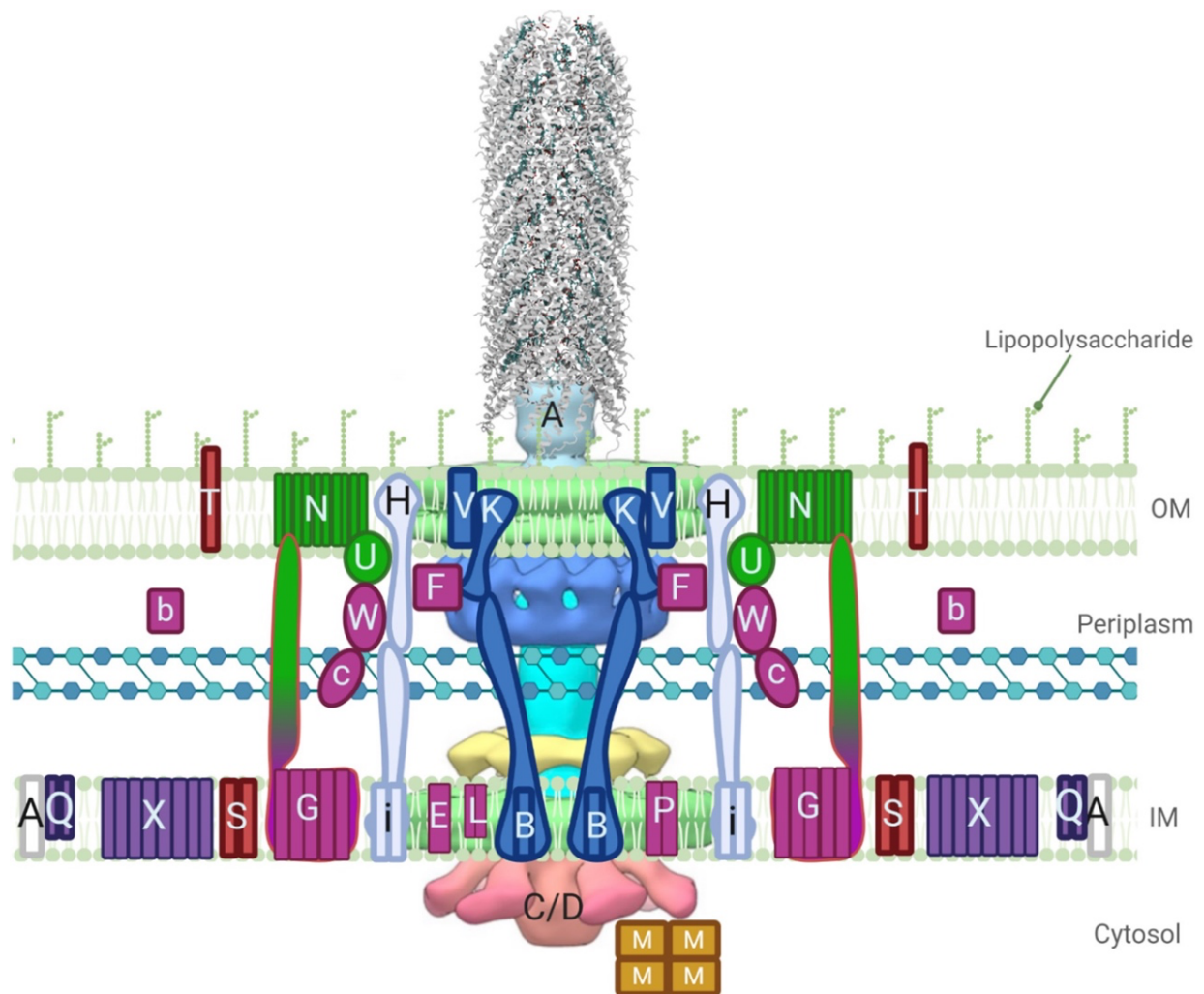


Figure 1.15: A model of the F-like T4SS showing the various complexes spanning both membranes. Tra proteins are capitalized while Trb proteins are lowercase. Adapted from (Bragagnolo et al., 2020).

Table 1.3: Proteins from The F Plasmid T4SS With Predicted Structural Dynamics*

Function	Protein	Localization	Size (aa)	% Disordered	Highest Confidence Model		
					Sequences	% Identity	PDB
Transcription Regulation	FinO	Cytoplasm	186	42%	33–184	100%	1DVO
	TraJ	Cytoplasm	229	21%	15–128	100%	4KQD
	TraR	Cytoplasm	73	19%	4–73	100%	5W1S
ProPilin Maturation	TraA	IM, ECM	70	20%	21–41	29%	2L8S
	TraQ	IM	94	22%	32–64	46%	3MP7
	TraX	IM	248	13%	28–37	24%	2KP6
T4SS Core Proteins	TraB	IM, Periplasm	475	44%	203–412	14%	6GYB
	TraK	OM, Periplasm	242	34%	26–107	8%	6GYB
	TraV	IM	171	61%	25–36	42%	5V8K
Pilus Assembly/Extension	TraP	IM	196	24%	57–118	18%	2V4J
	TraE	IM	188	11%	93–182	16%	5KPE
	TraL	IM	91	13%	18–47	30%	3GEB
	TraC	Cytoplasm, IM	875	15%	448–819	18%	4AG5
	TraW	Periplasm	210	16%	100–171	20%	2FU3
	TrbC	Periplasm	212	29%	103–190	13%	1HYU
	TraF	Periplasm	247	17%	146–256	19%	6GC1
	TrbB	Periplasm	181	31%	33–175	19%	2HYX
Pilus Retraction	TraH	OM, Periplasm	458	25%	90–144	24%	6SZ9
	TrbI	IM, Periplasm	128	23%	47–120	81%	1U2M
Mating Pair Stabilization	TraN	OM, Periplasm	602	25%	127–408	98%	4XBM
	TraG	IM, Periplasm	938	43%	774–930	93%	3CWG
	TraU	Periplasm	330	26%	31–39	26%	3TOW
Relaxase	TraI	Cytoplasm	1756	20%	1–1473	98%	5N8O
Relaxosome Accessory	TraY	Cytoplasm	131	25%	1–51	25%	1U9P
	TraM	Cytoplasm	127	33%	2–121	39%	3ON0
	TraD	Cytoplasm, IM	717	29%	145–574	30%	1E9R
Surface Exclusion	TraT	OM, ECM	244	34%	52–98	15%	3BF2
Entry Exclusion	TraS	IM	173	8%	22–35	38%	2M4V

* Proteins involved in the F plasmid T4SS. Shown is the percentage of disordered content based on secondary structure predictions and highest confidence models as determined through sequence homology of structures deposited in the PDB. Based on structural predictions in Phyre2 (Kelley et al., 2015). Adapted from (Bragagnolo et al., 2020).

Both TraF and TrbB contain thioredoxin-like domains but with TraF lacks the key C-X-X-C catalytic sequence (Where X is any amino acid residue but with the second X usually an aromatic

or hydrophobic residue) (Arutyunov et al., 2010). TrbB helps mediate pili retraction but is not necessary for successful conjugation as loss of function mutations in TrbB still result in successful F plasmid conjugation (Elton et al., 2005). TrbB is hypothesized to mediate disulphide bond formation since it contains a thioredoxin fold and C-P-Y-C sequence like dsbC and dsbG which are disulphide bond isomerases (Ren et al., 2009). Disulphide bonds are generally located in outer membrane and periplasmic proteins and while they can form spontaneously they are generally mediated

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F_TrB -----
pED208_TrB -----
F_TraF MKEKVMKGMKMNKALLPLLLCCFIFPASGKDAGWQWYNEKINPKKENKPVPAAPRQE---PDIMQKLA
pED208_TraF MRCIAGALALMMAASLHAAPEDVLAPEYAREEGWQWYNP--SAEEAEPEPVSPTLQERLASMTPEQKK

F_TrB --MSLTKSLLEFTLLLSAAAVQASTRDEIERLWN-----PQGMATQPAQPAAGTSARTAKP-APRWFR
pED208_TrB MLITALRTAILMAAMLGCLQAGTDDIRALEAGKTRGSAPAMDALSLENRATPTTDRAAQDTSPVWYT
F_TraF ALQTATKRALYEAILYPGVDNFVKYFRLQNYWAQQAG-LFTMSARKAMLAHPELDYNLQYSHYNGTVRNQ
pED208_TraF VLQQATREALDTAILYPSAENFRRFMTLQNFWDRTAT-DFTQTAKLARLKYPELDYNVKRSHYNGTVEAR

F_TrB LSNGRQVN-----LADWKVVLFMQGHCPYCHQFDPVLKQLAQQYGFSSVFSYTLDGQGDATFPEALPVP
pED208_TrB LSDGRKVN-----LQDWKVVLFMQSTCQYCRQFAPVLKAFSQQTGLDVFPISLDGKGDAEFPDVLPAT
F_TraF LAADQAQQRQAIKLAHEYGIMFFYRGQDPIDGQLAQVINGFRDYGLSVIPVSVVDGVINPLLPDSRTDQ
pED208_TraF LTEKKVQNAVISQVAQRYGLFFFYRGNNAVDNLMAGVIRAFCEDRGISLMAVSVDGKLSDDLQPSRPDS

F_TrB PDVMQTFPPN-IPVATPTTFLVNVNTLEALPLLQGATDAAGFMARVDTVLQMYGGKKGAK-----
pED208_TrB PDVMVEYFQSGLPVATPTTFLTNVNTMETWPLLQGAETGEFRSRLDEVLRLMALDRQSGKSIPLNAQGQQ
F_TraF GQAQRLGVKY-----FPAMMLVDPKQGSVRPLSYGFI SQDDLAKQFLNVS EDFKPNF-----
pED208_TraF GQAEKMRVTH-----FPATFLVDPKTHQWQPLAWGFMSHDDLDRQMVSVLTHFAPDY-----

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Figure 1.16: Homologies in sequence between TraF and TrbB. Sequences of TraF and TrbB from the F plasmid and the IncFV pED208 plasmid.

TraF is a 26 kilodalton (kDa) periplasmic protein that is required for conjugative plasmid transfer and the formation of sex pili with deletion mutations resulting in the elimination of conjugation (Lento et al., 2016). Loss of function mutations allow for the pilus formation but inhibit pilus extension past the cell surface which prevents conjugation (Anthony et al., 1999; Wu et al., 1988). TraF has a CTD (Glu127-Leu247) that has a thioredoxin-like fold while the NTD is a hydrophobic motile region that interacts directly with TraH (Figure 1.18) (Harris & Silverman, 2004; Lento et al., 2016). Based on Phyre2 predictions it is predicted to have at least three α -helices

bordered by a four-strand β -sheet. Originally it was hypothesized that TraF assisted with disulfide bond formation since it has a thioredoxin like region, but this does not seem to be the case as it does not have the C-X-X-C active site (Audette et al., 2004).

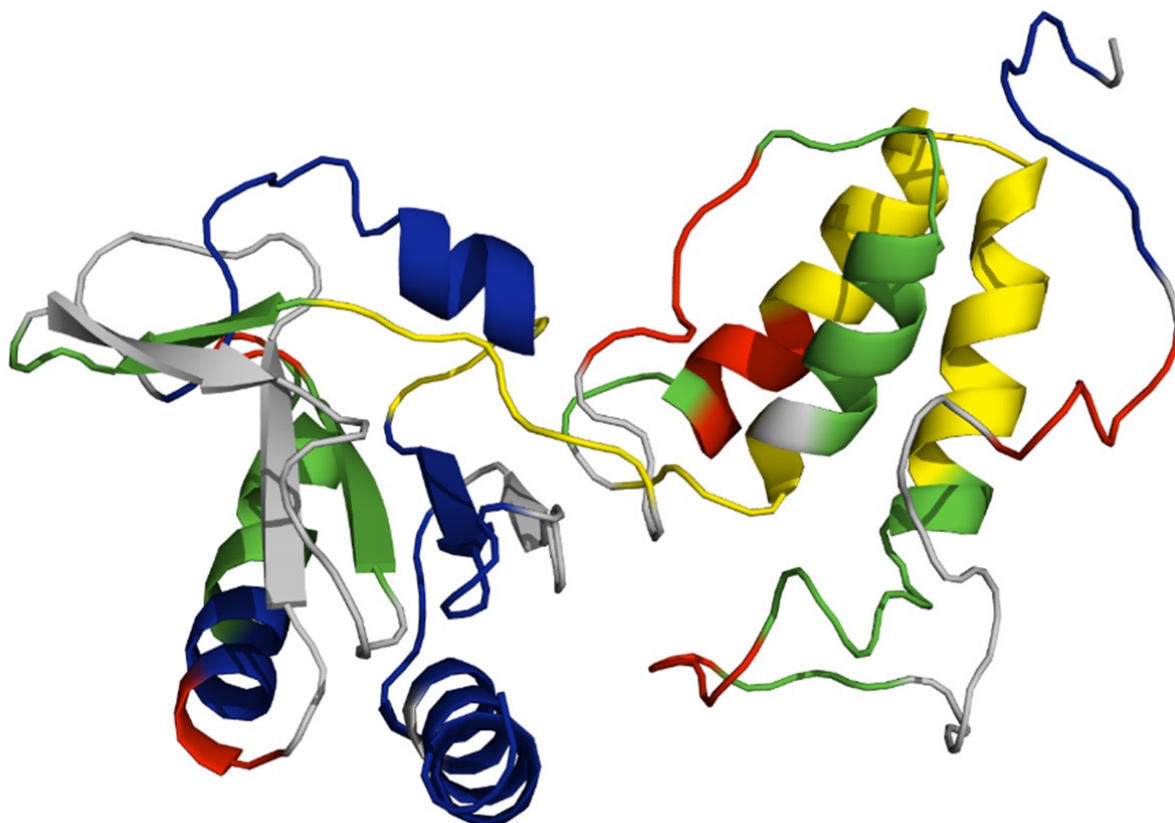


Figure 1.17: Predicted structure of TraF, coloured based on hydrogen-deuterium exchange (HDX) of main-chain amide hydrogens by time resolved HDX mass spectroscopy (Lento et al., 2016). Rates of exchange are coloured as follows: Red = high, yellow = moderate-high, green = moderate-low and blue = low. Adapted from (Bragagnolo et al., 2020).

TrbB is a 20 kDa TraF homolog that contains a thioredoxin fold but unlike TraF also conserves the thioredoxin C-X-X-C motif (Elton et al., 2005). TrbB helps mediate pili retraction but is not necessary for successful conjugation as loss of function mutations in TrbB still result in successful F plasmid conjugation (Elton et al., 2005). TrbB is hypothesized to mediate disulphide bond formation since it contains a thioredoxin fold and C-P-Y-C sequence like the disulfide

isomerases DsbC and DsbG (Ren et al., 2009). Disulphide bonds are generally located in outer membrane and periplasmic proteins and while they can form spontaneously they are generally mediated *in vivo* by thiol-disulfide exchange enzymes like DsbC and DsbG (Elton et al., 2005). TrbB seems to function similarly to DsbC as conjugation is not negatively affected when cells expressed had a null mutant in one or the other but mating efficiency was reduced 10-fold in cells that contained null mutants of both DsbC and TrbB. Many F-like systems encode at least one Dsb homolog containing the C-X-X-C motif (such as TrbB) which implies a necessity for increased isomerase activity for adequate mating efficiency (Elton et al., 2005). Furthermore, in other F-like systems that encode TraF with the C-X-X-C motif TrbB is not transcribed (Elton et al., 2005).

TraH is a cysteine rich protein 458 amino acids (aa) long processed to 433 amino acids with a coiled-coil domain utilized for oligomerization and other protein-protein interactions (Lawley et al., 2003). This domain is hypothesized to mediate the interaction complex TraF, -W, -U, TrbB and -I, directly contacting TraF, TrbI and the mating pair stabilization protein TraU (Harris & Silverman, 2004). Furthermore, there are three N-terminal hydrophobic domains of roughly 20 amino acids each allowing for TraF -U, and W being bound to TraH as a complex in the outer membrane (Arutyunov et al., 2010). Mutational assays indicate proper TraH activity relies heavily on disulphide bond formation from DsbA and isomerization by TrbB. Conserved sequences from Gly193-Leu226 allow for interaction with TrbI and while having a walker motif in this region, TraH does not exhibit any NTPase activity that is exhibited by other proteins with this motif (Arutyunov et al., 2010; Harris & Silverman, 2004).

A yeast-two hybrid essay was performed that revealed an interaction group of TraH, -F, -W, -U, TrbB and I (Figure 1.18) (Harris & Silverman, 2004). This complex was between the inner and outer membrane and TraH serves as the core with the most connections, directly attaching to TraF, TraU and TrbI. TraH was used as a bait plasmid to reveal that TraF binds to the 315-458 aa C-terminal region region of TraH.

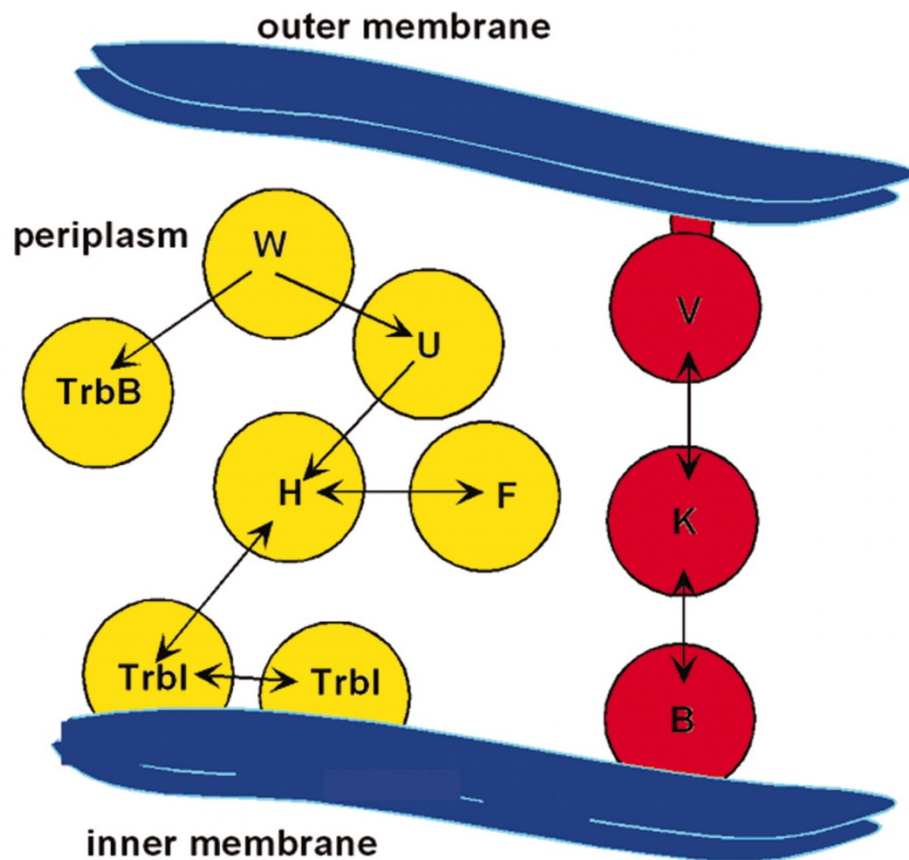


Figure 1.18: A 6 protein Tra complex required for F plasmid conjugation, utilizing yeast-two hybrid essay to reveal interacting proteins. Arrows are used to indicate interacting proteins with the arrowhead leading to the prey. Adapted from (Harris & Silverman, 2004).

1.4 Research Goals and Objectives

The goal of the following research is to assess various modern crystallization techniques by attempting to ascertain a crystal of protein that in the past has been relatively difficult to crystallize, in particular TraF. This would also expand on the research completed by Audette and his colleagues in 2004 resulting in a crystal that diffracted at 2.3 Angstroms but could not provide a structure solution (Audette et al., 2004). Based on previous research conducted it seems that TraF's hydrophobic tail is of concern in regards to crystallization which is also corroborated by mass spectrometry (Lento et al., 2016). This problem is being addressed with a few new approaches. Crystallization of a TraF-fusion protein will enable molecular replacement using the fusion partner and allow for structure solution leading to a high resolution structure of TraF. The second was utilizing acid (I3C) in a novel approach as noted by Truong and his colleagues (Truong et al., 2021). This method incorporates I3C into the stock protein solution and utilizes a seed bead stock made of previously grown microcrystals which is incorporated into the protein droplet when making optimization plates or crystal screens. This should allow for more favourable conditions (and crystals) for conditions that already have microcrystals. Along with more conditions that allow for crystal growth as seen in Figure 1.19. Another approach is further optimization of the crystal condition found by Audette and colleagues in 2004.

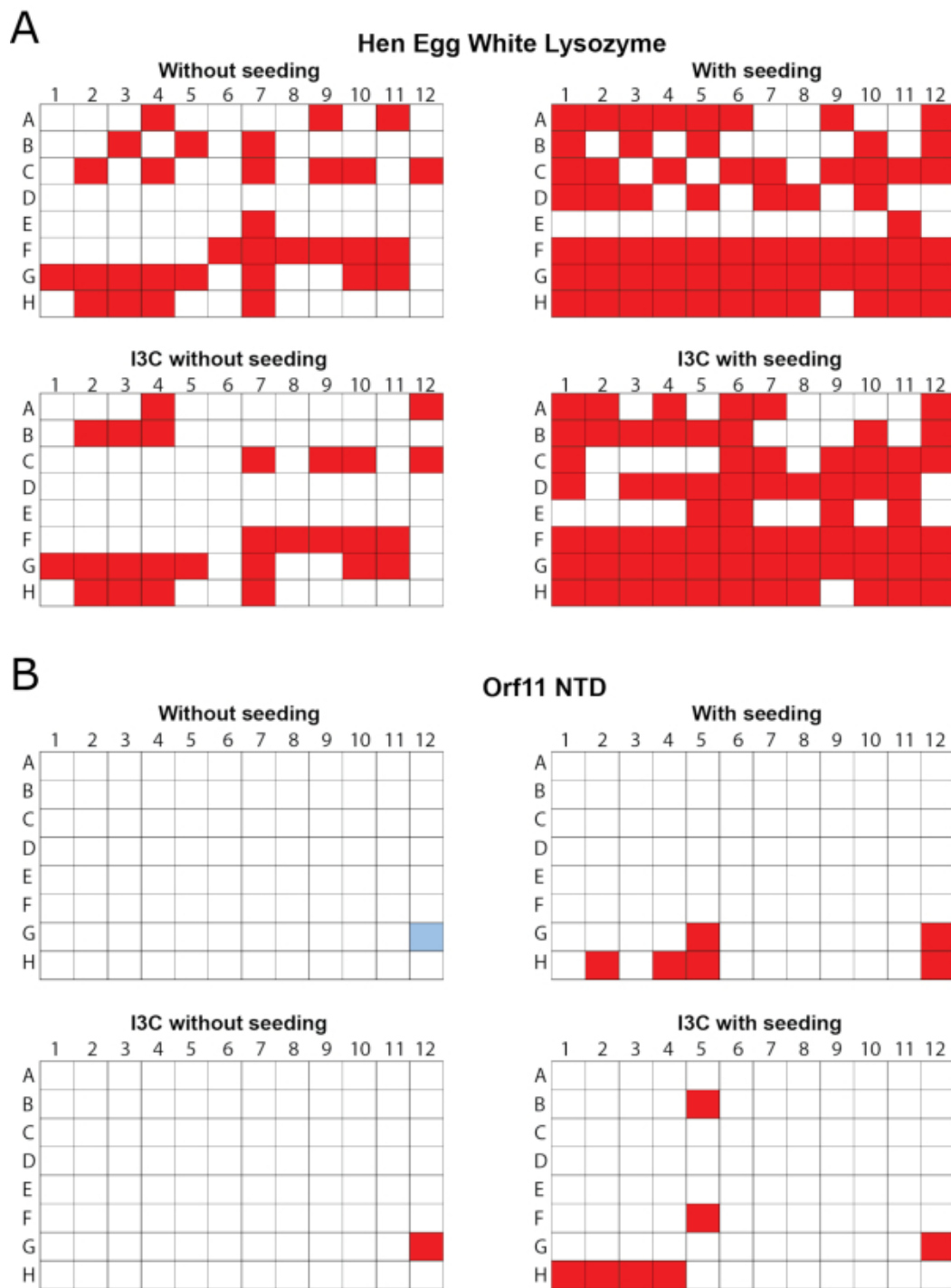


Figure 1.19: A) Screens of Hen egg lysozyme comparing a standard screen, a microseeded screen, a screen with I3C and a microseeded I3C screen. **B)** Orf11 lysin N-terminal domain (Orf-NTD) from bacteriophage P68 comparing a standard screen, a microseeded screen, a screen with I3C and a microseeded I3C screen. Red indicates wells with crystals. Blue indicates the well that was used to make Orf-NTD seedstocks. Adapted from (Truong et al., 2021).

CHAPTER 2 – MATERIALS AND METHODS

2.1 Expression and Purification of GST-TraF

2.1.1 Expression of TraF

Fifty mL of Luria broth (LB) along with 50 µg/mL of ampicillin (Amp) was inoculated with BL21 *E. coli* containing pGEX-6P2 vectors inserted with GST-TraF and shaken overnight at 200 rotations per minute (rpm) incubated at 37 °C. Ten milliliters of overnight culture was used to inoculate 900 ml of (Terrific Broth) TB containing 50 µg/mL of Amp and 10mm glucose in 2L expression flasks and incubated at 37 °C with shaking at 200 rpm until mid-log growth as per OD600nm. A 1% ethanol shock was performed to promote assembly of chaperone proteins (Chhetri et al., 2015). GST-TraF expression was induced with a final concentration of 1mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) for 16 hours at 18 °C and 200 rpm. afterwards cells were pelleted at 6000 x g for 20 minutes at 4 °C.

2.1.2 Sonication of GST-TraF

Cells were resuspended in 40 mL of loading buffer (containing 20 mM Tris(hydroxymethyl)aminomethane (Tris), 1mM ethylenediaminetetraacetic acid (EDTA), 150 mM of NaCl at a PH of 7.4) along with phenylmethylsulphonyl fluoride (PMSF), a protease inhibitor, and then sonicated using a Fisher Model 500 dismembrator. Sonication was performed for 10 minutes alternating every 15 seconds between the on cycle and the off cycle at an amplitude of 30%. The lysate was separated from cellular debris through centrifugation at 35,000 x g for 30 min at 4°C after which the supernatant, which contained the cytosolic fractions (and hence the protein of interest) was collected.

2.1.3 Glutathione affinity Chromatography of GST-TraF

Glutathione affinity chromatography was performed utilizing a XK16 column packed with glutathione Sepharose fast flow resin using an Äkta Purifier 10S FPLC (GE Healthcare). The lysate was loaded onto the column at 1 mL/min and washed with 10 column volumes of loading buffer. The bound protein was eluted with 75% Loading Buffer and 25% Elution Buffer (100 mM glutathione, 50 mM Tris at a pH 8.0) in 4 mL fractions. Samples containing protein of interest were analysed using Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS PAGE).

2.1.4 SDS PAGE analysis of purification fractions

Samples were mixed 3:1 with 4x SDS PAGE loading dye (160mM Tris pH 6.8, 4.0% SDS, 20% glycerol, 0.0012% Bromophenol blue, 20% β -mercaptoethanol) and boiled at 100°C for 10 minutes. Ten microliters of each sample were loaded onto a gel with a separation of 12.5% acrylamide and a stacking of 4% acrylamide and underwent electrophoresis for 40 minutes at 220 volts. The gel was stained with a Coomassie solution (50% methanol, 10% glacial acetic acid, 0.1% Coomassie Brilliant Blue R250) and then destained to visualize protein bands.

2.1.5 Buffer Exchange and Protein Quantification

Samples with a relatively high ratio of GST-TraF compared to impurities were pooled together into a spectra pore dialysis tube with an 8 kilodalton (kDa) molecular weight cut off (MWCO). The tube was placed in 4L of buffer (10 mM Tris, 50 mM NaCl at a pH of 7.5) and

dialyzed by spinning on a magnetic stirrer at 4°C for 8 hours. After which the container was replaced with fresh buffer and dialyzed for another 4 hours, this process was repeated with fresh buffer. The dialyzed protein sample was aliquoted into a 30 kDa MWCO concentrator and spun at 4,000 x g at 4°C a higher concentration of sample.

Protein concentration was quantified with a Bicinchoninic Acid Assay (BCA) as per protocol from the Pierce BCA Protein Assay Kit (Thermo Scientific). After making various dilutions, 12.5 µL of each was mixed with 237.5 µL of working reagents and loaded on a 96 well plate. After incubating for 30 minutes at 37°C a Synergy H4 Hybrid plate reader was used to obtain the OD₅₆₂ to ascertain protein concentration.

2.2 Screening and Optimization for Crystallization Conditions of GST-TraF

2.2.1 Screening of Crystallization Conditions

Numerous 96-reservoir sitting well crystallization plates were loaded with 70 µL of premade buffers from a deep-well block. From each reservoir 1 µL of buffer was pipetted into each corresponding well. Then 1 µL of GST-TraF at varying concentrations was pipetted into each well. The plate was sealed with tape to allow for vapour diffusion. Plates were kept undisturbed at 19°C and examined periodically utilizing a stage microscope with a cross-polarizer. The buffers were screening kits purchased from Anatrace (MCSG -1, -2, -3, -4, PUREPEG and TOP 96) and Qiagen (JCSG+).

2.2.2 Optimization of Favourable Conditions

Conditions that were deemed favourable to yield GST-TraF crystals were optimized with 24-well hanging drop plates. Optimization consisted of altering salt concentration, precipitant content, drop size, protein content and pH of the buffer. Each well consisted of 1 mL of mother liquor in the reservoir. A 1 μ L droplet mother liquor droplet was placed onto a siliconized coverslip with 1 μ L of protein added to the mother liquor droplet. The edges of the 24 well plate was greased with vacuum grease and the coverslip was inverted over the corresponding well and sealed. This allowed for a sealed reservoir with the drop hanging above the mother liquor. These plates were also left to incubate undisturbed at 19 °C while examined periodically under the microscope.

2.2.3 On Column Cleavage of GST-TraF and Optimization of TraF Crystal Conditions

To reassess conditions of the original crystal reported in 2004 (Audette et al., 2004), GST-TraF was cleaved with on column cleavage. Buffer was exchanged from 10 mM Tris, 50 mM NaCl at a pH of 8.0 to 0.5% NP-40, 10 mM Tris and 50 mM NaCl at a pH of 7.5 utilizing a 30 kDa MWCO concentrator spun at 4,000 x g at 4°C. Once protein concentration was assessed GST-TraF was loaded onto glutathione Sepharose gravity column. Cleavage was performed with 1 unit of PreScission protease (GE Healthcare) for every 0.1 mg of protein loaded on the column which was left rotating overnight at 4°C. Cleaved TraF was eluted with buffer and concentrated with a 10 kDa MWCO spun at 4,000 x g at 4°C. Optimization plates based off the 2004 paper were also conducted (Audette et al., 2004).

2.3 Head-to-Head Between Lysozyme and GST-TraF Utilizing I3C

2.3.1 Preparation I3C Spiked Protein Stocks

To conduct a head-to-head (H2H) trial, a 20 mg/mL stock of lysozyme was prepared by adding 20 mg of lyophilized lysozyme (Sigma-Aldrich) to 1 mL of buffer (10 mM Tris, 50 mM NaCl at a pH of 7.5). A 0.5 M stock of I3C was produced by combining 120 mg of I3C with 200 μ L of 2 M Lithium hydroxide (LiOH) which was heated to 60 °C and vortexed until completely dissolved. A final volume of 400 μ L was achieved with ddH₂O. Then two separate aliquots of buffer (10 mM Tris, 50 mM NaCl at a pH of 7.5) were spiked with the I3C stock so that one buffer had a final concentration of 10 mM I3C while the other had final concentration of 30 mM I3C. The spiked buffer with 10 mM I3C was mixed 1:1 ratio by volume with lysozyme stock yielding a 10 mg/mL solution of lysozyme with 5 mM I3C while the same was done with the buffer containing 30 mM I3C and 10 mg/mL GST-TraF stock (in 10 mM Tris, 50 mM NaCl at a pH of 7.5) to yield a 5 mg/mL GST-TraF stock with 15 mM I3C.

2.3.2 Preparation of Seeds Stocks and I3C Blanks

Seed stocks were made by looping a 200 μ m³ lysozyme crystal and placing in 1 μ L drop of mother liquor which was then pipetted into a 1.5 mL microfuge tube with 999 μ L of mother liquor and crushed by vortexing with a Hampton seed bead for 3 minutes. After 1 μ L of solution was pipetted into numerous 1.5 mL microfuge tubes with 99 μ L of reservoir solution which were vortexed and frozen. For reproducibility a new seed stock was taken and thawed on ice for use whenever a new plate was made for the H2H trial. To create blanks the buffer used for lysozyme

and GST-TraF (10 mM Tris, 50 mM NaCl at a pH of 7.5) was spiked to a final concentration of 5 mM I3C (for blanking the lysozyme screens) and 15 mM I3C (for blanking the GST-TraF screens).

2.3.3 Crystal Screen Preparation and Setup

The screens used were MCSG -1, -2, -3 and -4 (Anatrace). Screening plates were set up in a similar manner as noted above except with the second well of each reservoir containing 1 μ L of corresponding blank solution (buffer with 5mM I3C for lysozyme plates and buffer with 15 mM I3C for GST-TraF plates) and 1 μ L of mother liquor. This yielded 4 plates (MCSG -1, -2, -3 and -4) of lysozyme with I3C along with the blank and 4 plates of GST-TraF with I3C along with the blank. Plates were also made utilizing the seed stock to microseed each well. These plates were set up in the same manner but with the addition of 0.1 μ L of seedstock added to each well (including the blank wells). Each plate was left to incubate undisturbed at 19 °C and observed for at least 21 days.

2.4 X-ray Diffraction of Crystals

Diffraction of crystals was performed utilizing a on a Rigaku MicroMax-007 HF rotating anode X-ray generator with a Saturn944+ CCD detector. Any potential GST-TraF crystals were looped with MiTeGen loops sized accordingly to the crystal. Crystals were soaked in cryoprotectant consisting of reservoir solution with an increased amount of ethylene glycol as needed. The crystal was flash frozen with liquid nitrogen and set up on the system. Ten seconds

of screening was performed with X-ray exposure that collected 4 images at 60.0mm crystal-to-detector distance with angles from 0° - 2° and 70° - 72° .

CHAPTER 3 – RESULTS AND DISCUSSION

3.1 Expression and Purification of GST-TraF

Purification of the protein from expressions over 16 hours at 18°C yielded relatively pure fractions GST-TraF as shown in the purification scheme and SDS PAGE gel of the purification in Figure 3.1. The chromatogram indicates the column is sufficiently washed when the UV reading at 280nm returns to baseline (0 mAU). Furthermore, a very tall, thin and sharp peak is shown at the elution step. There are what seems to be minute amounts of cleaved GST and TraF observed below 30 kDa when analyzed with SDS-PAGE. This was further purified/concentrated with a 30 kDa MWCO concentrator.

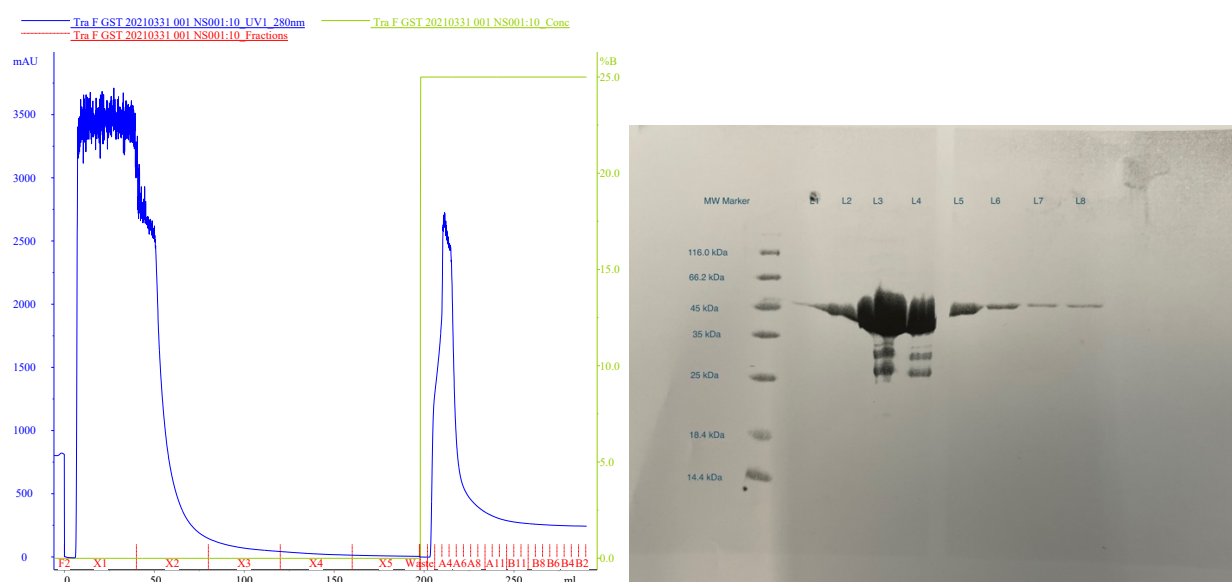


Figure 3.1: Optimized affinity purification of GST-TraF (left), along with a 12.5% SDS PAGE gel analysis (right). Samples for the gel were taken corresponding to the 280 nm peak shown after the wash step once elution began. Each sample was prepared with SDS-PAGE dye and heated to 100°C for 5 minutes. 15 µL of each sample was loaded onto 12.5% SDS-PAGE gel run at 200 volts for 55 minutes. Lanes 3 and 4 show a heavy blot of GST-TraF followed by smaller bands of GST and then TraF.

3.2 Initial Crystal Screens of GST-TraF

There was some progress made with initial the screening trials of GST-TraF. A Top96 screen using 10mg/ml of GST-TraF in 10 mM tris at a pH of 7.5 showed a potential hit in a condition containing 0.2 M potassium sodium tartrate, 0.1M sodium citrate at a pH of 5.6 and 2M ammonium sulfate but was not reproducible through optimization plates or identical screens. Instead, GST-TraF was buffer exchanged into in 0.1M sodium citrate at a pH of 5.6 and crystal trials of Top96 and JCSG+ were done yielding microcrystals in a TOP96 well with reservoir solution of 0.02 M Calcium chloride (CaCl), 0.1 M sodium acetate at a pH of 4.6 and 30% 2-Methyl-2,4-pentanediol (MPD) shown in Figure 3.2. Optimization plates were made varying the pH from 4.2-4.8 and the MPD from 24%-34% giving various wells structures that resemble those seen in the original screen but not any closer to that of a crystal. A JCSG+ screen reservoir containing 0.16 M calcium acetate 0.08 M sodium cacodylate and 14.4% PEG 8000 also led to results like those in Figure 3.2. Optimization varying calcium acetate between 0.120 -0.2M and between 4.2%-26.4% PEG 8000 yielded the same results within a matter of hours but became fully precipitated by days end. Due to the difficulties encountered with crystallizing GST-TraF through initial crystal screens, reassessing the original conditions of the TraF crystal as conducted by Audette and his colleagues in 2004 was attempted (Audette et al., 2004).

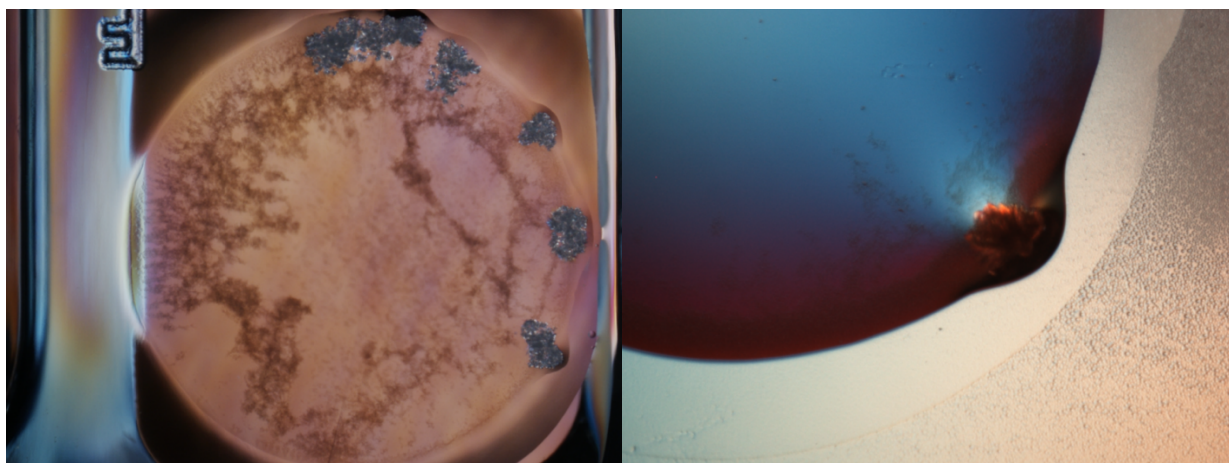


Figure 3.2: On the left crystalline birefringent formations in 0.02 Calcium chloride (CaCl), 0.1M sodium acetate at a pH of 4.6 and 30% 2-Methyl-2,4-pentanediol. On the right is an optimization plate with a similar crystalline structure formed in an optimization of 28% MPD and a pH pf 4.6 in 0.02M CaCl and 0.1M Sodium Acetate using 10mg/ml GST-TraF in 0.1M sodium citrate at a pH of 5.6.

3.3 Attempted Optimization of TraF Crystal Conditions

A key factor in maintaining the solubility of TraF cleaved from the GST tag was the use of Nonidet P-40 (Audette et al., 2004), referred to as NP-40. Increasing the amount of NP-40 in the protein solution from 0.01% to 0.5% allowed TraF to be concentrated to 2.45 mg/mL compared to the original 1.2 mg/mL before precipitating from solution. The main objective was to create a larger crystal by increasing protein in the protein stock that could then be more effectively soaked with a heavy atom derivative for phasing. While this increase in TraF concentration was achieved, the optimization plates performed seemed to have brought to light another problem. The increased NP-40 appears to have made created miscibility issues between the reservoir solution and the protein. Initially it was perceived that all the droplets had undergone phase separation, which normally indicates a favourable condition in crystallography. Unfortunately, even after 8 weeks the same the droplets have not changed in appearance as shown in Figure 3.3.

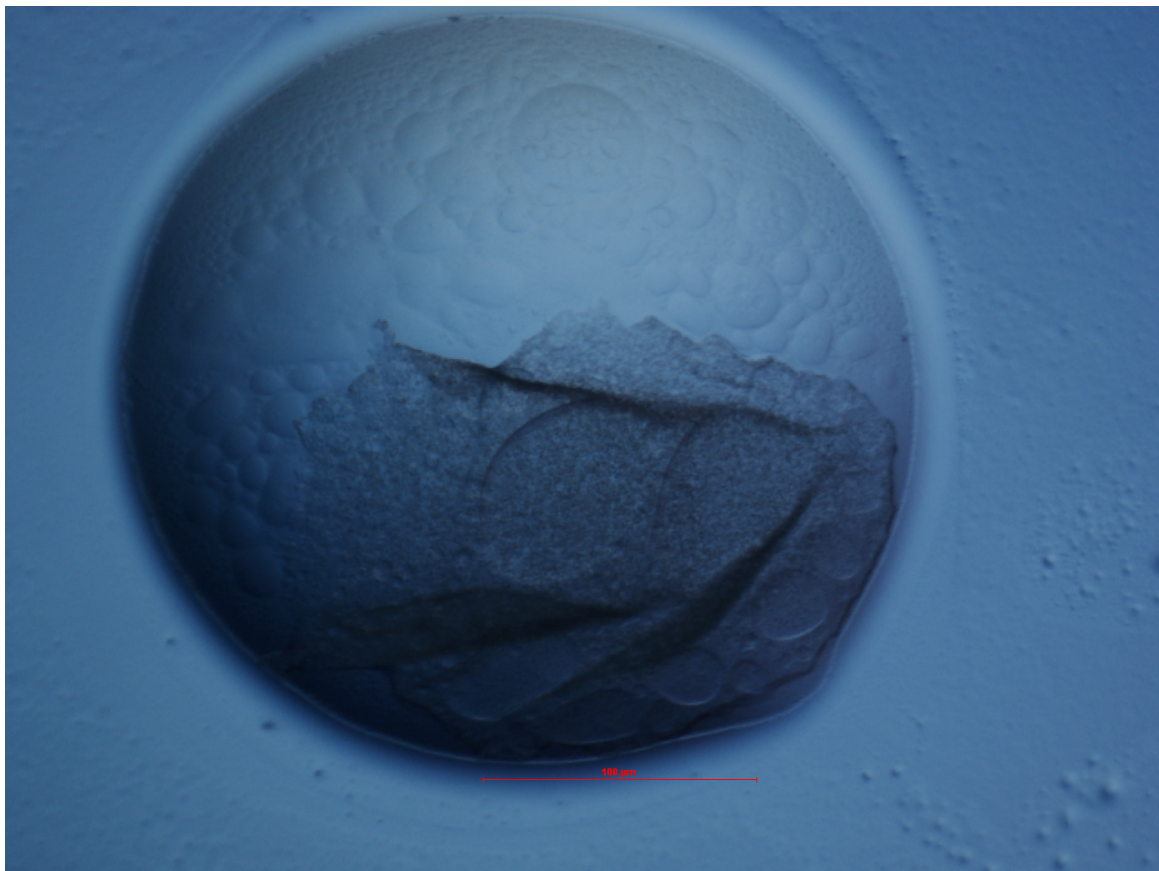


Figure 3.3: A droplet with 1 μL of 2.45 mg/mL of TraF in 0.5% NP-40 10 mM Tris and 50 mM NaCl at a pH pf 7.5 in 1 μL of 25% PEG 3350, 100 mM HEPES at a pH of 7.3 and 200 mM of diammonium citrate from an optimization plate 90 days after being plated. The red bar below the drop indicates 100 μm .

3.4 Initial Findings with I3C

Initially GST-TraF was assessed to see if the method advised by Truong and colleagues (2021) would work for GST-TraF (Truong et al., 2021). It was determined that a GST-TraF stock solution (between 4-10 mg/mL) could be spiked with I3C to final concentration of 15-40 mM. Initial microseeded crystal screens with Top96, MCSG-1, -2 and -3 were shown to give some results. Spherulites were observed to form in a well that contained a mother liquor 0.1 M Tris at

a PH of 8.5 and 0.3 M magnesium formate with 4 mg/mL of GST-TraF in 9.5 mM Tris 48 mM NaCl and 20 mM I3C (Figure 3.4).

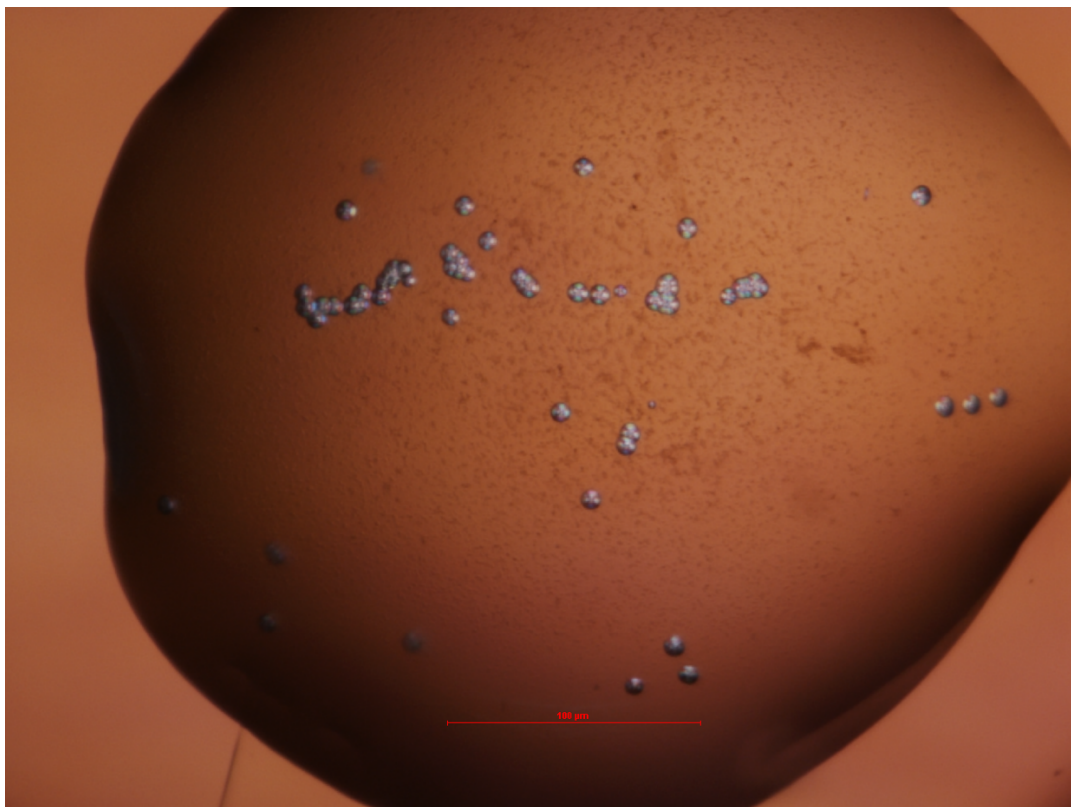


Figure 3.4: Small birefringent spherulites can be seen in a well droplet containing .1 M Tris at a PH of 8.5 and 0.3 M magnesium formate with 4 mg/mL of GST-TraF in 9.5 mM Tris 48 mM NaCl and 20 mM I3C. The red bar on the drop indicates 100 μ M.

Furthermore, there is a small crystal seen in a Top96 screen of 20% PEG 3350 and 0.2 M sodium fluoride, 0.1 μ L of seed stock along with a protein concentration of 2 mg/mL of TraF in 9.5 mM Tris 48 mM NaCl and 40 mM I3C. X-ray diffraction was attempted but yielded no conclusive results.

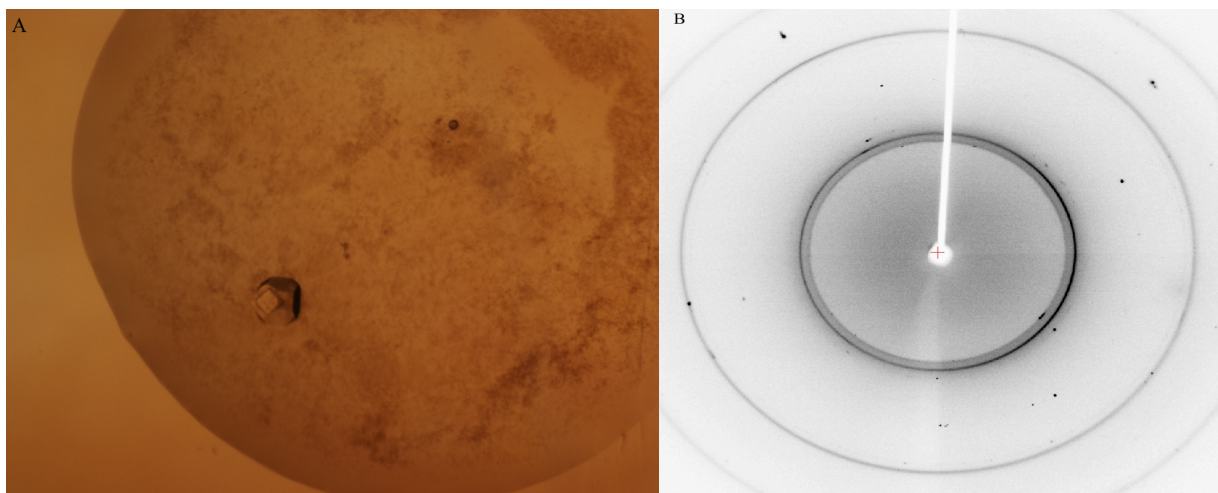


Figure 3.5: A) a small crystal in in a Top96 screen of 20% PEG 3350 and 0.2 M sodium fluoride, 0.1 μ L of seed stock along with a protein concentration of 2 mg/mL of GST-TraF in 9.5 mM Tris 48 mM NaCl and 40 mM I3C. B) An image of X-Ray diffraction of the crystal showing ice rings.

3.5 Head-to-Head I3C Comparison Between Lysozyme and GST-TraF

Figure 3.6 shows the result of unseeded lysozyme plates after 21 days. Out of the 396 wells 57 of them developed crystals while a majority did not. This does indicate that for future trials the lysozyme concentration can be increased to induce nucleation in more wells.

The microseeded lysozyme crystal trials had marginally more conditions with crystals, with 68 wells that had crystals compared to the 57 seen with unseeded plates. Another result of note is that a lot of the conditions that lead to crystals in the microseeded screens were different than those of the unseeded screens. Furthermore, of the conditions that have crystals in the microseeded screens 13 are conditions that also have phase separation which doesn't occur in any of the unseeded screens.

L	1	2	3	4	5	6	7	8	9	10	11	12
A	Red	Red		Red	Red							
B									Green			Red
C								Purple				
D						Light Blue			Red			
E	Light Blue				Light Blue	Light Blue				Red		
F		Red									Red	
G					Light Blue		Red					
H				Red		Light Blue	Light Blue	Red		Red	Red	Red

L	2	1	2	3	4	5	6	7	8	9	10	11	12
A	Red				Light Blue				Red	Red	Light Blue	Light Blue	Red
B					Light Blue		Red	Light Blue	Light Blue		Red		Red
C	Red	Red			Red		Red				Light Blue		
D	Light Blue	Light Blue	Light Blue			Light Blue			Red				Light Blue
E	Red	Red			Light Blue					Light Blue	Light Blue		
F						Red	Light Blue						Light Blue
G			Light Blue	Light Blue							Red	Red	
H					Red					Light Blue		Light Blue	

L	3	1	2	3	4	5	6	7	8	9	10	11	12
A	Light Blue	Light Blue	Light Blue				Light Blue		Red	Red			
B	Light Blue	Light Blue	Red	Red			Light Blue						
C										Red			
D			Light Blue	Light Blue	Light Blue		Red			Light Blue			
E						Light Blue				Red	Light Blue		Light Blue
F	Light Blue				Light Blue		Light Blue						
G						Light Blue	Light Blue						
H		Red										Red	

L	4	1	2	3	4	5	6	7	8	9	10	11	12
A								Light Blue	Red				
B	Light Blue										Red		
C	Red		Light Blue	Light Blue	Red				Light Blue	Red			
D										Purple			
E										Light Blue			Light Blue
F	Red									Red	Red	Red	
G										Red	Light Blue	Red	
H	Red			Red	Red					Light Blue			

Figure 3.6: Crystal screens performed with a lysozyme stock at 10 mg/mL of lysozyme in 10 mM Tris, 50 mM NaCl and 5 mM I3C. Screening was done against commercial screens MCSG-1 (L1), MCSG-2 (L2), MCSG-3 (L3) and MCSG-4 (L4). Plates were visualized manually, and results are colored as follows at 21 days post-setup: red represents drops with crystals, light blue represents light precipitate, green is precipitate with crystals and purple represents phase separation.

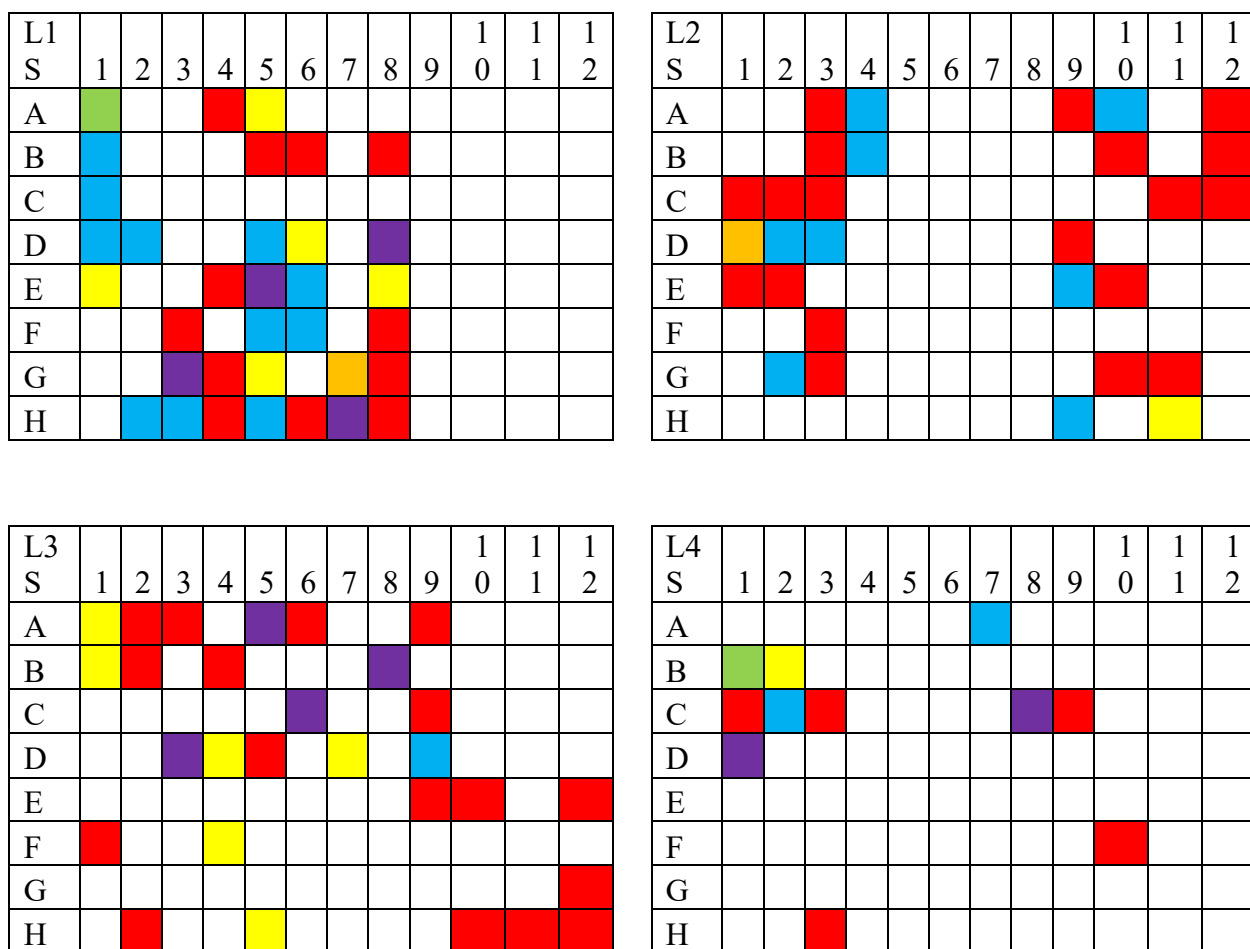


Figure 3.7: Crystal screens performed with a lysozyme stock at 10 mg/mL of lysozyme in 10 mM Tris, 50 mM NaCl and 5 mM I3C which involved being microseeded with a lysozyme seedstock. Screening was done against commercial screens MCSG-1 (L1M), MCSG-2 (L2M), MCSG-3 (L3M) and MCSG-4 (L4M). Plates were visualized manually, and results are colored as follows at 21 days post-setup: red represents drops with crystals, light blue is light precipitate, green is precipitate with crystals, orange represents light precipitate with phase separation, purple represents phase separation and yellow represents phase separation with crystal formation.

In addition, all the crystals observed had ideal characteristics for diffraction. As shown in Figure 3.8, they are large with hard edges and are birefringent in nature. Furthermore, each well that contains crystals has several of them in each drop.

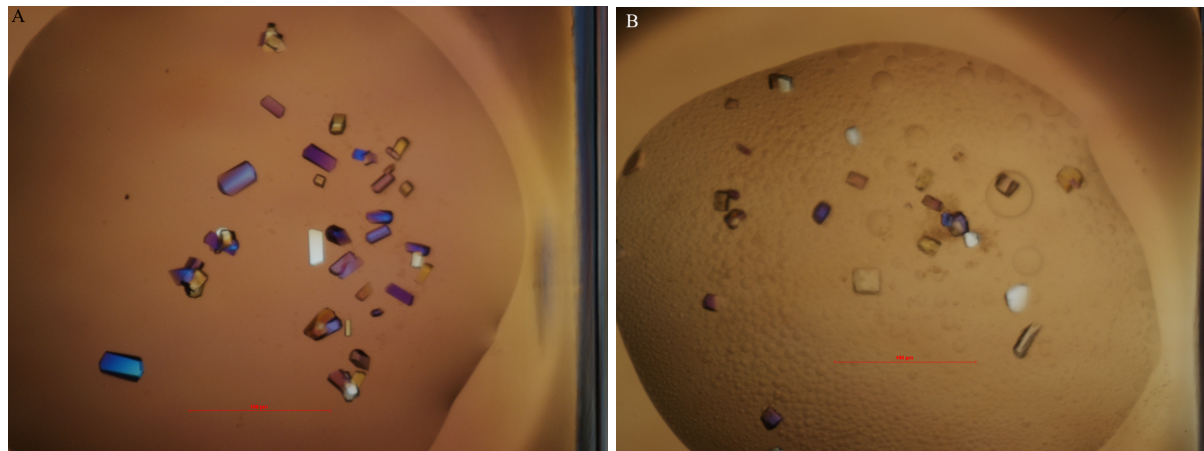


Figure 3.8: Crystals observed after 21 days from a droplet containing 1 μ L 10 mg/mL of lysozyme in 10 mM Tris, 50 mM NaCl and 5 mM I3C with 0.1 μ L of seedstock combined with 1 μ L of reservoir solution. A) Birefringent crystals observed in a drop with a reservoir solution 3.5 M potassium formate at a pH of 7.0. B) Birefringent crystals observed in a drop with a reservoir solution of 0.1 HEPES at a PH of 7.5 and 2 M ammonium sulphate. The red bar on the drop indicates 100 μ M.

Comparatively, most of the wells for the crystal screens with GST-TraF contained light precipitate with very few clear wells. Furthermore, there are also many wells that have heavy precipitate. There are 9 wells across all four unseeded screens that show crystal growth after 21 days. There are 17 wells that show phase separation while most of the wells having light precipitate that was not amorphous which or generally good indicators of potential crystal growth compared to clear or heavily precipitated wells.

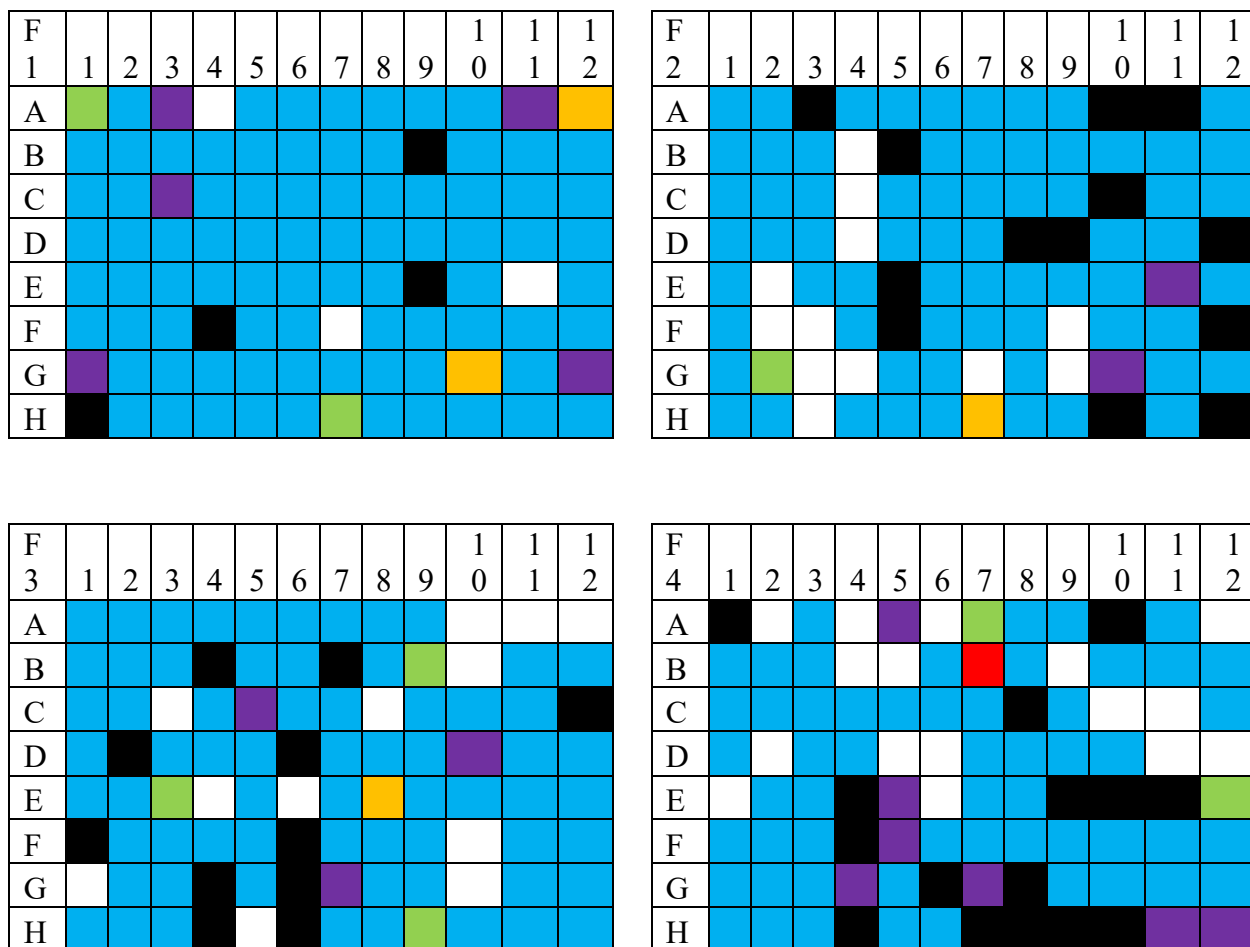


Figure 3.9: Crystal screens performed with a GST-TraF stock at 5 mg/mL in 10 mM Tris, 49 mM NaCl and 15 mM I3C. Screening was done against commercial screens MCSG-1 (F1), MCSG-2 (F2), MCSG-3 (F3) and MCSG-4 (F4). Plates were visualized manually, and results are colored as follows at 21 days post-setup: red represents drops with crystals, light blue is light precipitate, green is precipitate with crystals, black represents heavy precipitate and purple represents phase separation.

For the microseeded GST-TraF screens it is observed that there are far less wells with heavy precipitate and far more wells that are altogether clear (Figure 3.10). It is worth noting that the microseed conditions yielded only 1 well with a crystal. This, along with fact that there are more wells that are completely clear may indicate that for microseeded conditions protein concentration may need to be increased to give greater results in screening. There are some unique

results from the microseeded plates with 3 wells showing birefringent microcrystals/particulates and one well condition containing spherulites.

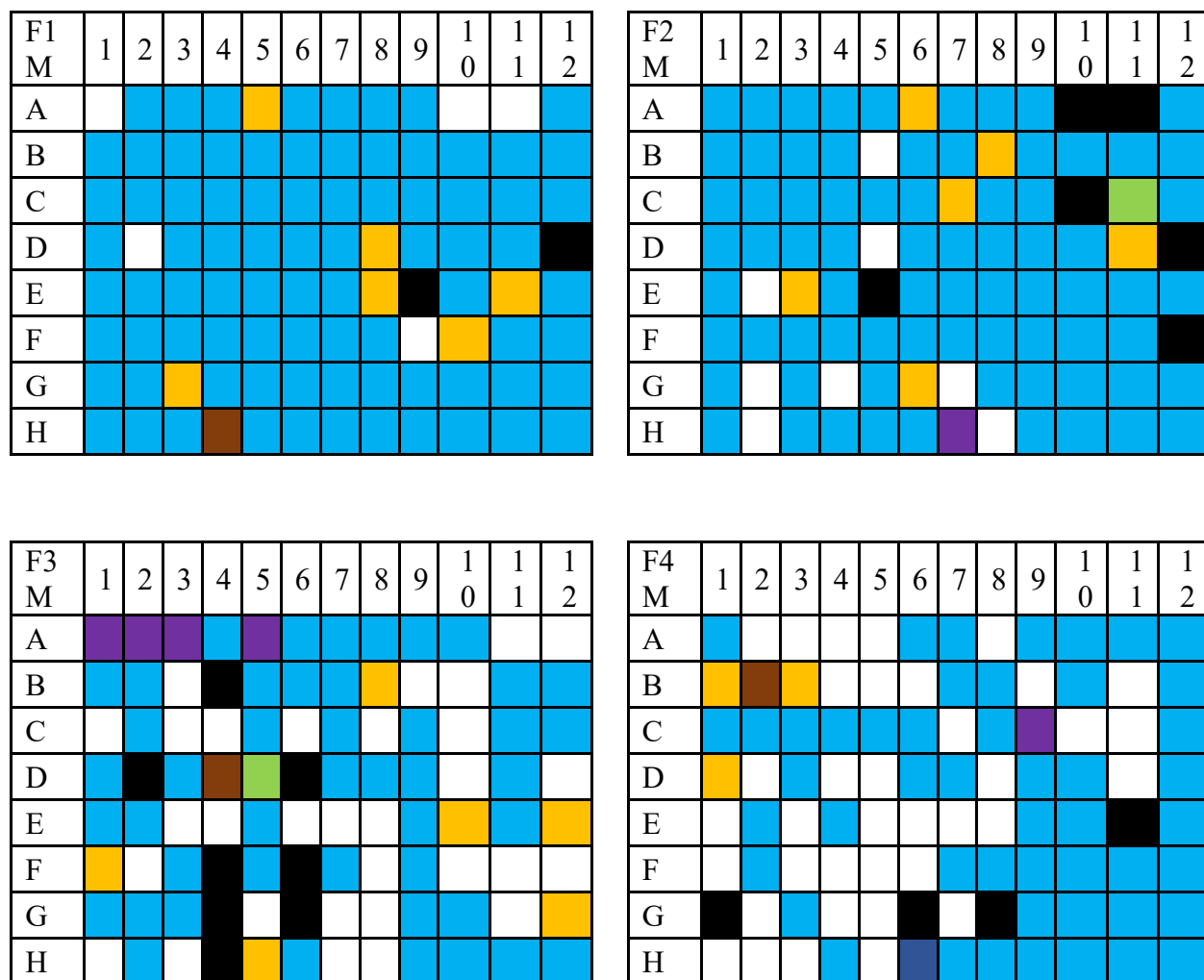


Figure 3.10: Crystal screens performed with a GST-TraF stock at 5 mg/mL in 10 mM Tris, 49 mM NaCl and 15 mM I3C with a lysozyme seedstock. Screening was done against commercial screens MCSG-1 (F1M), MCSG-2 (F2M), MCSG-3 (F3M) and MCSG-4 (F4M). Plates were visualized manually, and results are colored as follows at 21 days post-setup: brown represents wells with birefringent microcrystals/particulates, light blue is light precipitate, green is precipitate with crystals, black represents heavy precipitate, dark blue represents wells with microcrystals and purple represents phase separation.

Unfortunately, none of the crystal conditions for GST-TraF yielded a crystal large enough or with enough hard edges to diffract (Figure 3.11).

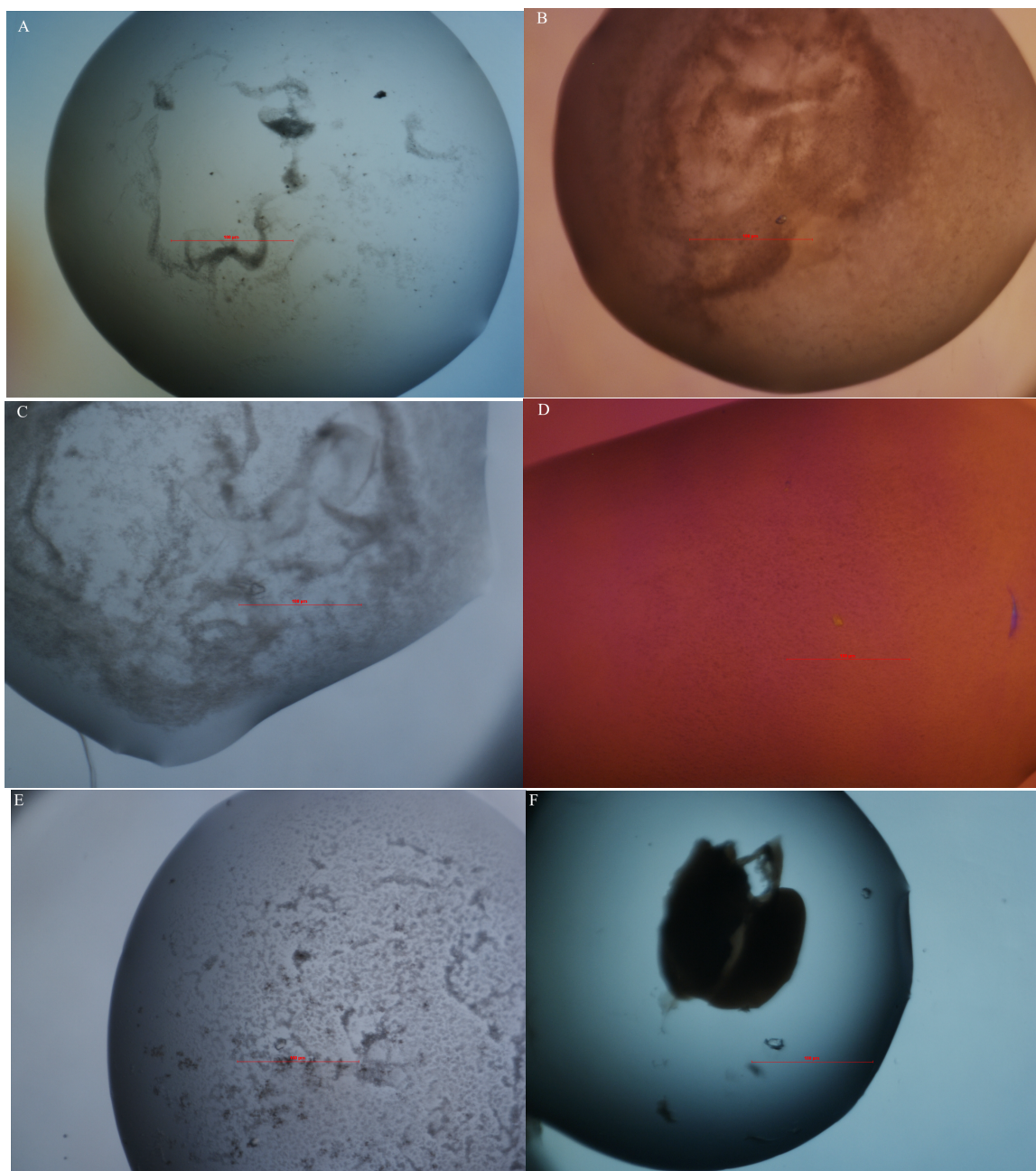


Figure 3.11: These are the most clearly defined crystals of the GST-TraF screens performed with a 1 μ L of GST-TraF at 5 mg/mL in 10 mM Tris, 49 mM NaCl and 15 mM I3C combined with 1 μ L of reservoir solution. **A)** A crystal under precipitate in a well with a mother liquor of 0.1 HEPES (pH 7.5) and 20% PEG 8000. **B)** A slightly birefringent crystal under precipitate in a well with a mother liquor of 0.1 Bis-Tris (pH 5.5) and 2 M ammonium sulphate. **C)** A small crystal in light precipitate with a mother liquor of 0.1 Tris (pH 8.5) and 2.4 M ammonium phosphate dibasic. **D)** A birefringent crystal in well with a mother liquor of 0.1 Tris (pH 8.5) and 1 M ammonium phosphate dibasic. **E)** A small crystal in precipitate in a well with a mother liquor of 0.1 M sodium acetate, 0.1 M MES (pH 6.5) and 20% PEG 2000 MME. **F)** A Crystal next to precipitate in a well with a mother liquor of 0.2 M zinc acetate, 0.1 imidazole (pH 8.0) and 2.5 sodium fluoride. The red bar on drop indicates 100 μ m increment.

The crystals pictured are too small for diffraction at least after 21 days. They should be observed for a longer period to assess whether they become large enough for diffraction. Furthermore, there also several other conditions that should be monitored with birefringent conditions (Figure 3.12).

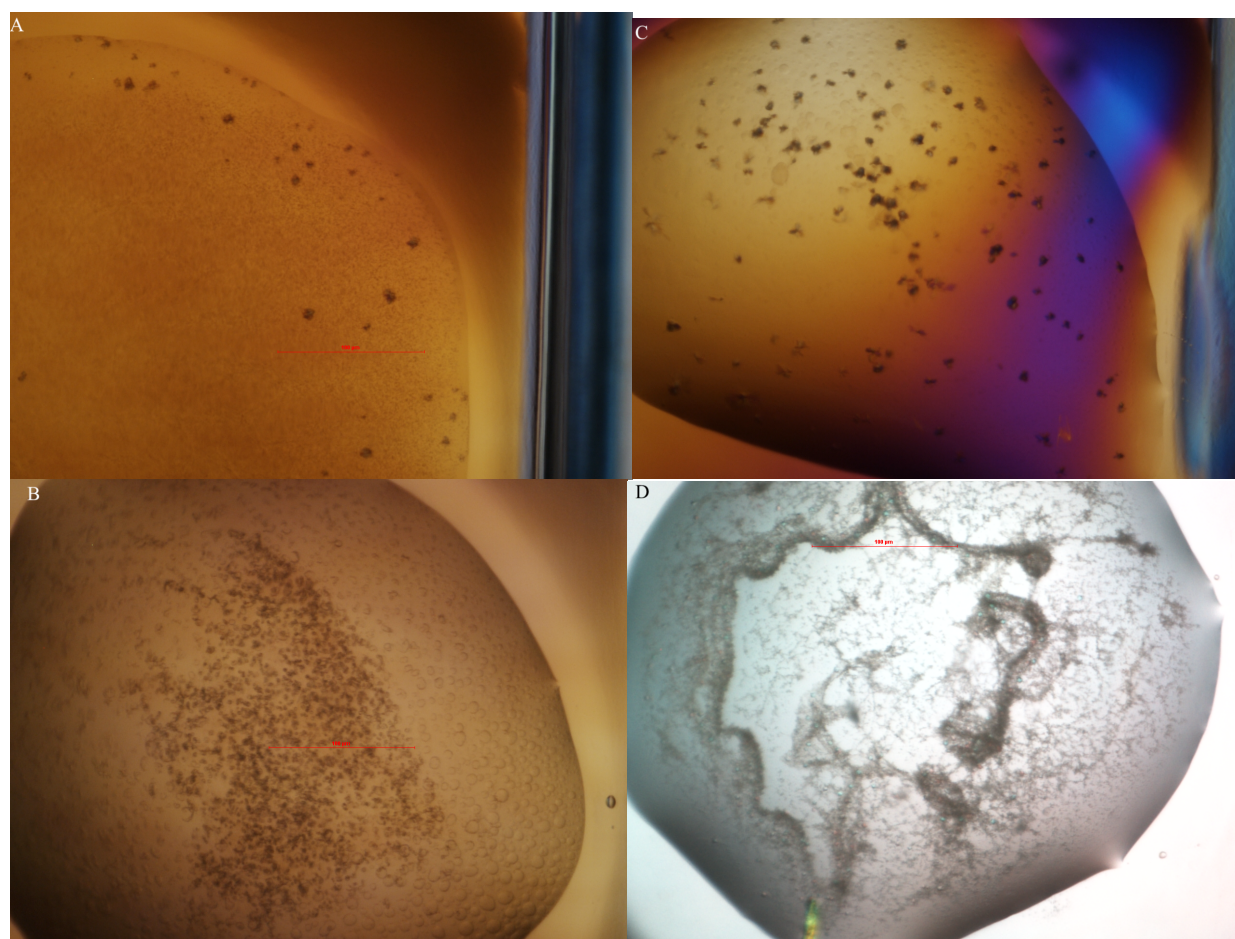


Figure 3.12: GST-TraF screens performed with a 1 μL of GST-TraF at 5 mg/mL in 10 mM Tris, 49 mM NaCl and 15 mM I3C with 0.1 μL lysozyme seedstock combined with 1 μL of reservoir solution. **A)** Birefringent particles in a drop with a mother liquor of 0.1 sodium citrate (pH 5.6), 20% PEG and 20% isopropanol. **B)** Birefringent particles 0.1 M Imidazole (pH 8.0) and 1 M sodium citrate **C)** Birefringent microcrystals in Bis-Tris Propane (pH of 7.0) and 1.2 M potassium sodium tartrate. **D)** Small birefringent spherulites can be seen 0.5 lithium sulphate and 2% PEG 8000. The red bar on drop indicates 100 μm .

CHAPTER 4 - CONCLUSIONS AND FUTURE WORK

The ultimate goal of the research was to ascertain a crystal structure of TraF from which to forward the structure-function analysis of the protein. However, none of the results from the research conducted thus far have led to a structure at the time of writing. This being said, progress has been made to move forward on several key factors. First, soluble protein concentration has increased for TraF from 1.2 mg/mL to 2.45 mg/mL which other crystallization attempts and NMR potentially viable options (Audette et al., 2004). Furthermore GST-TraF can be concentrated upwards of 20 mg/mL. In addition, the I3C H2H has yielded crystals which in time may grow large enough to be diffracted.

Overall, the H2H results appear to identify some favourable conditions by utilizing I3C in the crystal screen process. The lysozyme screens had a majority of clear wells indicating that the stock concentration 10mg/mL may be too low. The GST-TraF wells that grew crystals (Figure 3.9 and 3.10) may lead to crystals that are large enough to be diffracted. Furthermore, there are many wells that have light precipitate or phase separation both of which are conditions that may yield crystals in the future. The screens should continue to be observed while the favourable conditions already noted (Figure 3.9 and 3.10) should be optimized and assessed for crystal growth.

In general, there seems to be an innate difficulty with the crystallization of TraF, be it on its own or tagged. This appears to stem from TraF's hydrophobic N-terminal domain, which was also corroborated by Lento and colleagues in 2016 (Lento et al., 2016). Another approach to crystallization would be to express N-terminal deletion mutants which may allow for easier crystallization. Deletion mutants have been made just recently but due to time constraints expression, optimization and crystal screens have yet to be performed. A key feature and mechanism of TraF may revolve around the hydrophobic tail as TraF interacts directly with

intermembrane protein TraH, therefore this approach should be taken with caution (Bragagnolo et al., 2020). The original crystal conditions from 2004 should also be reassessed by adding a minimal amount of I3C (a final concentration of 5 mM) while keeping every other condition that led to the crystal unchanged (Truong et al., 2021; Audette et al., 2004). This would allow for the phase data needed to be present because of the I3C and if successful may lead to a crystal structure. Thermofluor stability assays can also be performed with and without the use of I3C to potentially find more favourable buffer conditions for crystallization.

There are also other experiments that can, and should, be performed. The polydispersity of the protein sample should be further analyzed using size exclusion chromatography paired with the multi-angle light scattering (MALS) to verify the protein sample and insure nothing unaccounted for such as dimerization has occurred. Unfortunately, the hardware to run this experiment was unavailable until the writing of this thesis began. For other sources of structural data Nuclear Magnetic Resonance (NMR) may be utilized. As more novel methods and approaches become more common place Cryo-electron microscopy may also prove to be a tangible experiment. While initially limited larger protein structures advancements have allowed structures for proteins as small as 43 kDa to be solved (Zhang et al., 2020).

In addition, as technological improvements have occurred there has also been an increased prevalence in computer software that predicts protein structure based off sequence alone, most recently noted with Alphafold (Jumper et al., 2021). This could be used as important supplemental information. It may very accurately predict most protein structures but there are a few caveats with TraF that may or may not prove limiting. The first is that most programs give the C-Terminal region a thioredoxin-like structure due to its thioredoxin-like domain, yet TraF is devoid of the

key motif that allows for thioredoxin-like function. Furthermore, TraF has a highly motile hydrophobic tail the structure of which may be highly dependent on external factors such as the conditions of the periplasmic region and the hypothesized interaction with TraH (Harris & Silverman, 2004; Lento et al., 2016). This being said with the improvements in artificial algorithm software it may end up being an accurate model, unfortunately without any high-resolution structural data obtained as of yet there is no way to corroborate this.

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