DEVELOPMENT OF CHIMERIC TYPE IV SECRETION SYSTEMS FOR TRANSFER OF HETEROLOGOUS SUBSTRATES ACROSS THE GRAM-NEGATIVE CELL ENVELOPE

Trista M. Berry

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DEVELOPMENT OF CHIMERIC TYPE IV SECRETION SYSTEMS FOR TRANSFER OF HETEROLOGOUS SUBSTRATES ACROSS THE GRAM-NEGATIVE CELL ENVELOPE

by

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DEVELOPMENT OF CHIMERIC TYPE IV SECRETION SYSTEMS FOR TRANSFER OF
HETEROLOGOUS SUBSTRATES ACROSS THE GRAM-NEGATIVE CELL ENVELOPE

A
THESIS

Presented to the Faculty of
The University of Texas Health Science Center at Houston
and
The University of Texas MD Anderson Cancer Center
Graduate School of Biomedical Sciences
in Partial Fulfillment of the Requirements
for the Degree of

MASTER OF SCIENCE

by
Trista M. Berry, B.S.
Houston, Texas
August 2014
Acknowledgements

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Finally, I want to express my gratitude to my family and friends for your persistent support and reassurance. I could not have made it this far without the encouragement of my husband, Adam, our two amazing children, and my parents who taught me to never give up in the face of opposition. For all that I have done and all that I will do, to God be the glory.
Many bacteria use Type IV Secretion Systems (T4SSs) to aid in pathogenesis by translocating virulence factors across the cell envelope and into eukaryotic cells. These systems are structurally and functionally diverse, but are often compared to the archetypal VirB/VirD4 T4SS of *Agrobacterium tumefaciens*. This system is composed of the VirD4 type IV coupling protein (T4CP) and 11 VirB subunits (VirB1-11) that assemble as the secretion channel and an extracellular pilus. The T4CP is an inner membrane ATPase that interacts with T4SS substrates and the secretion channel, and is thought to link substrates with the secretion channel and possibly energize transfer through the channel lumen. In this thesis, I sought to adapt T4SSs in the surrogate hosts *A. tumefaciens* and *Escherichia coli* for use in identification of novel T4SS effector proteins from genetically-intractable Rickettsial species.

I first constructed chimeric T4SSs in *A. tumefaciens* by substituting native VirD4 with Rickettsial VirD4 homologs. However, I was unable to demonstrate transfer of the promiscuous IncQ plasmid pML122 or known *A. tumefaciens* effector proteins. I next tested the *E. coli* pKM101-encoded T4SS, which is known to transfer DNA substrates, for the capacity to deliver heterologous protein substrates to *E. coli* recipients. Using the Cre-recombinase reporter assay for translocation (CRAfT), I showed that pKM101 translocates effector proteins from *A. tumefaciens* and two Rickettsial species, *Anaplasma phagocytophilum* and *Wolbachia pipientis*. I next created chimeric T4CPs by joining the transmembrane domain (TMD) of pKM101-encoded TraJ with the soluble domains (SDs) of VirD4 homologs from *A. tumefaciens* and the Rickettsial species. I showed that all of these chimeric systems translocate protein substrates, although less efficiently than the native
pKM101 T4SS. Finally, I demonstrated that a variable C-terminal extension (CTE) that is present on the *A. tumefaciens* and Rickettsial T4CPs plays a modulatory role for secretion of different protein substrates. My findings showed for the first time that a T4SS encoded by an *E. coli* conjugative plasmid is capable of translocating a variety of protein substrates from phylogenetically diverse alphaproteobacterial species, including *A. tumefaciens*, *A. phagocytophilum*, and *W. pipientis*. 
Table of Contents

Acknowledgements........................................................................................................................................iii
Table of Contents........................................................................................................................................vi
List of Illustrations........................................................................................................................................ix
List of Tables................................................................................................................................................x
Abbreviations..............................................................................................................................................xii

Chapter 1. Introduction to Type IV Secretion Systems and the VirD4 Coupling Protein ...... 1

Introduction..................................................................................................................................................2
Type 4 secretion systems are diverse and multi-functional.................................................................2
VirD4: The substrate receptor.................................................................................................................4
Chimeric T4SS as a study tool..................................................................................................................10
Significance................................................................................................................................................11

Chapter 2. Materials and Methods.........................................................................................................13

Bacterial strains and growth/induction conditions..................................................................................14
Plasmid constructions.............................................................................................................................19
Construction of virD4 deletion strains and chimeric virD4 plasmids..................................................24
Construction of Cre fusion plasmids.....................................................................................................27
Protein analysis by western blot...........................................................................................................28
Conjugation assays................................................................................................................................28
Virulence assays.....................................................................................................................................29
CRAFT (Cre-recombinase Reporter Assay for Translocation)............................................................30

Chapter 3. Chimeric T4SS in Agrobacterium and Functional Characterization of the VirD4Al C-terminal Extension.....................................................................................................................32

Introduction............................................................................................................................................33
Results......................................................................................................................................................37
Rickettsial VirD4 homologs do not complement a virD4 mutant for substrate transfer ..................................................................................................................37

Evidence for expression of virD4 homologs in A. tumefaciens A348 ………………41

The C-terminal extension (CTE) of VirD4At is important for virulence but not for IncQ transfer ..............................................................................................................................43

Discussion ..................................................................................................................46

Transfer of effector molecules using VirD4 homologs in the Agrobacterium type IV secretion system ...........................................................................................................46

The CTE of VirD4At is required for translocation of some, but not all, substrates ………47

Chapter 4. The Type IV Secretion System of Escherichia coli pKM101 and Related Chimeric Systems Mediate Translocation of Heterologous Protein Substrates ………50

Introduction ..................................................................................................................51

Results ..........................................................................................................................52

The pKM101 conjugative T4SS transfers non-cognate proteins ………..52

trans-expression of traJ confers elevated substrate transfer ........................................63

Do heterologous T4CPs functionally substitute for TraJ in mediating DNA transfer through the pKM101 transfer system? ….................................................................68

Do heterologous T4CPs functionally substitute for TraJ in mediating protein transfer through the pKM101 transfer system? .................................................................71

Discussion ..................................................................................................................73

The TraJ T4CP is essential and rating limiting for pKM101 and IncQ plasmid transfer .........................................................................................................................73

Protein translocation through the conjugative T4SS of pKM101: A novel finding ………73

A chimeric T4SS composed of homologous T4CPs and the pKM101 encoded mating channel ..................................................................................................................78
Chapter 5. The Development of Chimeric Coupling Proteins in an Effort to Enhance Substrate Transfer Through a Surrogate T4SS

Introduction .................................................................80

Results .............................................................................83

DNA substrate transfer using chimeric coupling proteins ................83

Protein effectors are translocated by the pKM101 system via a chimeric coupling protein .................................................................85

The T4CP CTE modulates effector translocation ......................90

Discussion ........................................................................91

Chimeric coupling proteins retain the ability to translocate a variety of substrates in a surrogate T4SS .....................................................91

The roles of the AAD and CTE in type IV secretion ....................93

Chapter 6: Summary and Perspectives ........................................96

Summary ...........................................................................97

Comparison of effector translocation through the native and chimeric pKM101 systems .................................................................100

Future Experiments .............................................................101

What motifs and/or residues of the T4CP are required for substrate transfer? .................................................................101

How is the CTE contributing to substrate secretion? .................102

References ........................................................................104

Vita .................................................................................113
List of Illustrations

Figure 1.1 DNA transfer through the A. tumefaciens VirB/VirD4 type IV secretion system….3
Figure 1.2 VirD4 coupling protein (T4CP) domain structure............................................7
Figure 1.3 Sequence alignment of the all-alpha domains (AAD’s) of VirD4 homologs........8
Figure 1.4 Sequence alignment of the C-terminal extensions (CTE’s) of VirD4 homologs.....9
Figure 3.1 Diversity of T4SSs in Alphaproteobacteria.........................................................35
Figure 3.2 Tests for complementation of the A. tumefaciens ΔvirD4 mutation by virD4 genes from Rickettsial species.....................................................................................................................39
Figure 3.3 Tests for negative dominant effects of virD4 genes from Rickettsial species expressed in wild-type A. tumefaciens strain A348 .................................................................42
Figure 3.4 Contribution of the C-terminal extension of A. tumefaciens VirD4 to substrate transfer........................................................................................................................................45
Figure 4.1 Interbacterial protein translocation by the pKM101 T4SS.........................56
Figure 4.2 Detection of Cre-effector fusion proteins in E. coli cells .................................57
Figure 4.3 Translocation of heterologous T4SS substrates through the pKM101 transfer system........................................................................................................................................61
Figure 4.4 Effects of a pKM101 traJ deletion and complementation with heterologous virD4 genes on DNA substrate transfer..................................................................................................................65
Figure 4.5 Transfer of heterologous T4SS substrates through the E. coli pKM101 transfer system........................................................................................................................................66
Figure 4.6. Effects of trans expressed traJ and virD4 homologs on wild type pKM101 self-transfer ........................................................................................................................................70
Figure 4.7 Cre-mediated transfer of Anaplasma effector protein Ats-1 through pKM101-based chimeric T4SSs..................................................................................................................72
Figure 4.8 Analysis of the effector C-terminal tails................................................................76
Figure 5.1 Schematic of chimeric coupling proteins ................................................................. 82

Figure 5.2 Conjugative transfer of pKM101 and the IncQ plasmid pML122 between *E. coli* cells ........................................................................................................................................ 84

Figure 5.3. Protein translocation between *E. coli* cells mediated by T4SSs composed of the pKM101-encoded transfer channel and the Tra::VirD4At T4CP ........................................... 87

Figure 5.4. Interbacterial protein transfer using chimeric coupling proteins with the soluble domain from *A. phagocytophilum* .............................................................................................................. 88

Figure 5.5 Interbacterial protein transfer using chimeric coupling proteins containing the soluble domain from *W. pipientis* .............................................................................................................. 89
List of Tables

Table 2.1 Bacterial strains and plasmids.................................................................15
Table 2.2 Oligonucleotides used for T4CP and effector constructions........................21
## Abbreviations

(in alphabetical order)

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>AAD</td>
<td>All-alpha domain</td>
</tr>
<tr>
<td>Cam</td>
<td>Chloramphenicol</td>
</tr>
<tr>
<td>Co-IP</td>
<td>Co-immunoprecipitation</td>
</tr>
<tr>
<td>CRAFT</td>
<td>Cre recombinase reporter assay for translocation</td>
</tr>
<tr>
<td>Crb</td>
<td>Carbenicillin</td>
</tr>
<tr>
<td>CTD</td>
<td>C-terminal domain</td>
</tr>
<tr>
<td>CTE</td>
<td>C-terminal extension</td>
</tr>
<tr>
<td>Dtr</td>
<td>DNA transfer and replication</td>
</tr>
<tr>
<td>FA</td>
<td>Formaldehyde</td>
</tr>
<tr>
<td>Gen</td>
<td>Gentamicin</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione S-transferase</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>Kan</td>
<td>Kanamycin</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani media</td>
</tr>
<tr>
<td>Mpf</td>
<td>Mating pair formation</td>
</tr>
<tr>
<td>NBD</td>
<td>Nucleotide binding domain</td>
</tr>
<tr>
<td>OD</td>
<td>Optical Density</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>Rif</td>
<td>Rifampicin</td>
</tr>
<tr>
<td>SD</td>
<td>Soluble domain</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>Spc</td>
<td>Spectinomycin</td>
</tr>
<tr>
<td>T4CP</td>
<td>Type IV coupling protein</td>
</tr>
<tr>
<td>T4SS</td>
<td>Type IV secretion system</td>
</tr>
<tr>
<td>T-DNA</td>
<td>Transfer DNA</td>
</tr>
<tr>
<td>Tet</td>
<td>Tetracycline</td>
</tr>
<tr>
<td>TMD</td>
<td>Transmembrane domain</td>
</tr>
<tr>
<td>TrIP</td>
<td>Transfer-DNA immunoprecipitation</td>
</tr>
<tr>
<td>VirD4&lt;sub&gt;Ap&lt;/sub&gt;</td>
<td>VirD4 from <em>A. phagocytophilum</em></td>
</tr>
<tr>
<td>VirD4&lt;sub&gt;At&lt;/sub&gt;</td>
<td>VirD4 from <em>A. tumefaciens</em></td>
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<tr>
<td>VirD4&lt;sub&gt;Rr&lt;/sub&gt;</td>
<td>VirD4 from <em>R. rickettsii</em></td>
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<tr>
<td>VirD4&lt;sub&gt;Wp&lt;/sub&gt;</td>
<td>VirD4 from <em>W. pipientis</em></td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
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Chapter 1.  Introduction to Type IV Secretion Systems and the VirD4 Coupling Protein
Introduction

Type 4 secretion systems are diverse and multi-functional

Many Gram-negative and Gram-positive bacteria use Type IV secretion systems (T4SSs) to translocate virulence factors across the cell membrane(s) and into target cells to aid in pathogenesis [1-3]. T4SSs are typically separated into three main subfamilies: (a) conjugation systems that translocate DNA substrates between cells in a contact dependent manner; (b) effector translocation systems that deliver protein substrates directly into target cells; and (c) DNA uptake and release systems, known to move DNA to or from the extracellular environment [1, 3, 4]. However, functionality of a specific system is not always exclusive to one subfamily. For instance, *Agrobacterium tumefaciens*, of the class Alphaproteobacteria, uses the prototypical VirB/VirD4 conjugative T4SS to deliver both transfer DNA (T-DNA) and protein substrates (e.g., VirE2) into plant cells [4, 5] thereby making it both a conjugative system and an effector translocation system. This T4SS-mediated transfer of both T-DNA and protein effectors from *A. tumefaciens* causes the formation of tumors on the plant, known as crown gall disease [3, 4].

T4SSs are diverse structurally and in the types of substrates they secrete. In *A. tumefaciens*, the *virB* operon encodes all the subunits for assembly of the T4SS, including the channel subunits VirB6-VirB10; the pilus subunits VirB2 and VirB5; and three ATPases VirB4, VirB11 and VirD4 (Figure 1.1) [6]. Most T4SSs employed by Gram-negative bacteria are composed of homologs of the VirB and VirD4 subunits, but there are many evolutionary adaptations as well, resulting in T4SSs composed of additional subunits of unrelated ancestries to the VirB/VirD4 subunits [7]. In Gram-positive bacteria, the T4SSs appear to be minimized in the sense that they are composed principally of homologs or orthologs of *A. tumefaciens* VirD4, VirB4, VirB1, VirB3, VirB6, and VirB8 [2].
Figure 1.1 DNA transfer through the *A. tumefaciens* VirB/VirD4 Type IV Secretion System.

The schematic shows the subcellular localizations of the VirD4 substrate receptor or T4CP and two other ATPases VirB4 and VirB11, the channel subunits VirB6-VirB10, and the pilus subunits VirB2 and VirB5. Also shown is the path of the DNA substrate, indicating a primary interaction with the VirD4 coupling protein, followed by shuttling to the VirB11 ATPase, prior to entering the secretion channel. OM, outer membrane, IM, inner membrane.
VirD4: The substrate receptor

In 2004, our lab used an assay termed transfer-DNA immunoprecipitation (TrIP) to experimentally describe the pathway of substrate secretion in A. tumefaciens [8]. Briefly, by formaldehyde (FA) cross-linking proteins to the DNA substrate, followed by immunoprecipitation of the Vir proteins, we identified T4SS subunits that formed an FA-crosslinkable contact with DNA as it exited the cell by PCR amplification. In order to describe the pathway of substrate secretion, mutants lacking single virB or virD4 genes were analyzed by TrIP; such mutations blocked DNA transfer at specific stages during translocation. Using this methodology, it was established that VirD4 initially recruits the relaxasome/T-DNA complex to the T4SS apparatus (Figure 1.1). From here, the substrate is transferred to the VirB11 ATPase, then VirB6/B8 at the inner membrane, and finally through a channel composed of the VirB2 and VirB9 subunits for passage through the periplasm and outer membrane.

Much of the work in this thesis is focused on the type IV coupling protein (T4CP) from A. tumefaciens, VirD4, and its homologs, including TraJ from the IncN plasmid pKM101 and the VirD4-like proteins from some Rickettsial species. In order to delineate the different VirD4-like proteins, I will indicate the species of origin as a subscript (VirD4At, A. tumefaciens; VirD4Ap, Anaplasma phagocytophilum; VirD4Wp, Wolbachia pipientis; and VirD4Rr, Rickettsia rickettsii). VirD4 is an inner membrane ATPase that interacts with T4SS substrates and is thought to energize the transfer of the substrate through the lumen of the channel created by the other Vir proteins [1]. VirD4 is termed a coupling protein (or T4CP) because it links the relaxosome, formed by the DNA transfer and replication (Dtr) proteins, with the translocation channel and pilus, formed by the mating pair formation (Mpf) proteins [1, 9]. It is suggested that the T4CP interaction with the Mpf proteins occurs via transmembrane domains at the inner membrane, while recruitment and binding of the relaxosome and associated Dtr proteins occurs via one or more domains comprising the
soluble portion of the T4CP [9, 10]. VirD4-like coupling proteins are associated with most T4SSs; those of interest to my thesis project include VirD4At, the Rickettsial T4CPs mentioned above, and T4CPs encoded by *E. coli* conjugative plasmids including TrwB<sub>R388</sub> [11], TraD<sub>F</sub> [12], and TraJ<sub>pkM101</sub> [10].

T4CPs generally consist of an N-terminal transmembrane domain (TMD), a nucleotide binding domain (NBD), the all-alpha domain (AAD), and often a C-terminal extension (CTE) (Figure 1.2) [11]. The T4CP from *E. coli* plasmid R388, TrwB, is the structural paradigm for all T4CPs. A soluble variant of TrwB has been crystallized as a homohexamer with a central channel of approximately 20 Å in diameter [11, 13]. The overall structure of TrwB, is that of an F<sub>r</sub>-F<sub>O</sub> ATPase-like ball-stem with the TMD having an integral role in hexamer formation in addition to anchoring the T4CP within the inner membrane [11, 13]. The TMD of the TrwB monomer consists of at least two transmembrane helices that span the inner membrane [1], and is thought to be responsible for interaction of the T4CP with the channel subunits [1, 11].

The NBD includes Walker A and B nucleoside triphosphate binding site motifs characteristic of ATPases, and shares both structural and sequence similarities with *E. coli* FtsK and *Bacillus subtilis* SpoIIIIE [14]. FtsK and SpoIIIIE are hexameric double-stranded (ds) DNA translocases that bind and translocate chromosomal DNA during cell division or sporulation. This movement of DNA is thought to occur through the central channel of the hexameric ring and has led to proposals that the T4CPs may translocate DNA substrates across the inner membrane in a similar manner [14, 15]. The AAD is a seven-helix bundle positioned at the cytoplasmic end of the T4CP hexamer and bears structural similarity to the XerD site-specific recombinase [11, 16]. Both its relative location in the T4CP and its structural homology to a DNA-binding protein has lead to the suggestion that the AAD participates in substrate recognition and delivery to the transfer channel.
The specific interface for T4CP-substrate interaction has yet to be determined.

While the AAD might be involved in substrate docking, it is noteworthy that the AAD’s of VirD4 homologs are generally closely related among different species of the alphaproteobacteria, e.g., *A. tumefaciens* and Rickettsial spp. (Figure 1.3) [1], suggesting that this domain alone does not mediate substrate specificity to cognate T4SSs. To better define the domains contributing to T4SS substrate specificity, we constructed various T4CP domain deletions and substitutions. Specifically, a postdoctoral fellow in the Christie lab, Dr. N. Whitaker, determined that deletion of the AAD of pKM101-encoded TraJ abolished plasmid transfer, indicating that this AAD may have a role in DNA substrate interactions (See Chapter 5). Additionally, I determined that the C-terminal extension (CTE), which is carried by a subset of the T4CPs, is highly variable (Figure 1.4) [1]. In Chapters 3 and 5, I present data indicating that deletion of the CTE’s from T4CPs from *A. tumefaciens* Rickettsial homologs alters translocation efficiencies of some protein substrates.
Figure 1.2  VirD4 coupling protein (T4CP) domain structure

A. Schematic representation of the domain organization of A. tumefaciens VirD4. Numbers depict residues marking the domain boundaries along the length of the protein. TMD, transmembrane domain; AAD, all alpha domain; NBD, nucleotide binding domain; CTE, C-terminal extension.

B. Topology schematic of TrwB modified from Gomis-Ruth, et al. 2002. This figure was modified to identify domains, but TrwB lacks a C-terminal extension found in VirD4 and other T4CPs. Permission for use granted by the American Society for Biochemistry and Molecular Biology.
Figure 1.3  Sequence alignment of the all-alpha domains (AAD's) of VirD4 homologs

AAD sequence alignment from different alphaproteobacteria under study in this thesis.

VirD4At, *A. tumefaciens* residues 201-346; VirD4Ap, *A. phagocytophilum* residues 218-351;


Alignment shows high sequence conservation amongst the AAD’s, especially those of the Rickettsial homologs. Red residues, high consensus (90%); blue residues, low consensus (50%).
Figure 1.4  Sequence alignment of the C-terminal extensions (CTE’s) of VirD4 homologs

CTE sequence alignment from different alphaproteobacteria under study in this thesis.

VirD4At, *A. tumefaciens* residues 552-656; VirD4Ap, *A. phagocytophilum* residues 573-740;

Alignment shows almost no sequence conservation, high variability in domain length, and
over-representation of acidic residues. Blue residues, low consensus (50%).
Chimeric T4SS as a study tool

*A. tumefaciens* is phylogenetically closely related to Rickettsial species, including those of *Anaplasma*, *Wolbachia*, and *Rickettsia* genera [1, 7, 17]. In Rickettsiales, much of the *A. tumefaciens* VirB/VirD4 T4SS is conserved, including many of the VirB1-VirB11 channel subunit proteins and the VirD4 T4CP [1, 17]. These Rickettsial T4SSs translocate effector proteins into host cells to cause a variety of animal and human diseases, making the study of these organisms medically important [1]. However, functional characterization of Rickettsial T4SSs and the effectors translocated during infection have been hindered by difficulties in cultivation of Rickettsial species in the lab and their genetic intractability.

Various approaches have been taken to identify novel effectors whose translocation contributes to the virulence of species that are difficult to genetically manipulate, e.g., intracellular pathogens [18, 19]. Production of candidate effectors in surrogate hosts followed by tests for T4SS translocation has supplied evidence for translocation of candidate effectors of intracellular bacteria, as summarized by Alvarez-Martinez and Christie [1]. For example, *A. phagocytophilum* AnkA was identified as a possible effector by demonstrating its translocation through the *A. tumefaciens* VirB/D4 T4SS [20]. In this case, AnkA was fused to the Cre recombinase and the fusion protein was shown to translocate through the *A. tumefaciens* VirB/VirD4 T4SS into plant cells. Translocation was detected by engineering plant cells to carry a GFP reporter gene that is interrupted by a DNA segment flanked by two lox sites. Translocation of Cre-AnkA to plants resulted in Cre-mediated excision of the lox cassette, allowing production of GFP in the plant. Similar surrogate systems have been used to identify possible T4SS substrates of *Coxiella burnetii* [21], and *Anaplasma marginale* [22].

Others have attempted using chimeric T4SSs to demonstrate transfer of non-cognate substrates [9, 23]. These chimeric T4SSs produce a heterologous substrate receptor along with the native VirB channel complex and have been shown to retain the
capacity to translocate at least some substrates [1]. For example, a chimeric T4SS in plasmid R388, producing the T4CP from RP4, was shown to functionally complement for transfer of the mobilizable plasmid RSF1010 [23]. In this case, the authors found that the native T4CP, TrwB, could complement an R388 trwB- strain of *Escherichia coli* for translocation of the plasmid RSF1010. Additionally, the T4CP from RP4, TraG, was able to complement the same strain at wild-type levels for RSF1010 transfer, but not for R388 self-transfer. This demonstrated that such chimeric T4SSs are limited in their functionality.

**Significance**

The current approaches being used to identify novel effectors using native hosts or surrogate systems are often complicated by difficulties in genetic manipulation of the host species or inefficiencies in effector recognition by surrogate systems [20-22]. The overarching aim of this thesis project was to determine the capacity of two model and easily manipulated T4SSs, one from *A. tumefaciens* and the second from *E. coli*, to serve as surrogate hosts for translocation of candidate T4SS effectors of Rickettsial spp. A second goal was to define the importance of the VirD4 CTE’s for substrate transfer. Although the work presented utilizes known or previously identified substrates, the methods used should allow for high-throughput screens to identify novel substrates.

In chapter 3, I present results of studies exploring the capacity of genes encoding Rickettsial VirD4-like proteins to complement an *A. tumefaciens* virD4 null mutation for transfer of *A. tumefaciens* effectors and a mobilizable IncQ plasmid, pML122. The Rickettsial virD4 genes do not complement for translocation of *A. tumefaciens* effectors, but their expression in an otherwise wild-type background confers dominant negative phenotypes suggestive of disruptive interactions with other T4SS components. I also defined the importance of VirD4’s CTE for substrate transfer in *A. tumefaciens*. 
In chapter 4, I explore use of the Tra T4SS encoded by *E. coli* plasmid pKM101 as a surrogate system for translocating substrates shown or postulated to be translocated through *A. tumefaciens* or Rickettsial spp. T4SSs. I collaborated with another student, Jay Gordon, to create a nonpolar *traJ* deletion mutant using recombineering. Using this deletion mutant, I demonstrated that while genes encoding Rickettsial VirD4 homologs do not complement the Δ*traJ* mutation for pKM101 self-transfer or for transfer of IncQ, they do complement for transfer of some protein substrates. Additionally, I was able to establish the ability of the wild-type pKM101 T4SS to translocate non-cognate substrates. These discoveries are highly important, as this is the first demonstration of protein transfer in the pKM101 system.

Finally, in chapter 5, I explore the functionality of chimeric T4CPs in mediating substrate transfer through the pKM101 transfer system. These chimeric T4CPs are composed of the TMD from TraJ and the soluble domain (SD) of the VirD4 homologs. The hypothesis under investigation was that TraJ’s TMD would mediate a productive interaction with pKM101’s T4SS channel, and the SD would recruit heterologous substrates for delivery through the channel. Since the native pKM101 system was found to translocate heterologous protein substrates, the goal of this line of work was to optimize translocation efficiencies. I determined that some chimeric VirD4 proteins support protein transfer, and I explored the roles of the CTE and AAD in mediating substrate transfer using the chimeric systems.
Chapter 2. Materials and Methods
Bacterial strains and growth/induction conditions

All strains used are listed in Table 2.1. *A. tumefaciens* A348 and *E. coli* DH5α and MS411 served as the wild-type strains for these studies [24-26]. Strain Mx355, a derivative of A348 with a transposon insertion in *virD4* [27], served as a *virD4* null mutant. Plasmid constructions were carried out with *E. coli* DH5α, as described below. Growth conditions of *A. tumefaciens* cells have been previously described [28]. Briefly, strains are grown on MG/L plates (Luria Bertani (LB) media supplemented with mannitol and glutamate) for 2 days at 28 °C [28, 29]. Colonies were inoculated into MG/L broth and grown overnight at room temperature while shaking. Appropriate antibiotics were added for maintenance of plasmids in the following concentrations: carbenicillin (100 µg/ml), kanamycin (100 µg/ml), gentamicin (100 µg/ml), spectinomycin (200-400 µg/ml) [24, 30, 31]. For induction of *vir* genes, cultures of *A. tumefaciens* strains were grown overnight with antibiotic selection, and then 1 ml of the culture was pelleted and resuspended in ABIM (minimal media supplemented with the inducer, acetosyringone (100 µM)) [32]. Cells were incubated for 16-18 hours with shaking at room temperature.

*E. coli* strains were grown as previously described [25, 33]. Briefly, strains were grown on LB plates overnight at 37 °C. Colonies were inoculated into LB broth and grown overnight at 37 °C while shaking. Appropriate antibiotics were added in the following concentrations unless noted otherwise: carbenicillin (100 µg/ml), kanamycin (50 µg/ml), gentamicin (20 µg/ml), spectinomycin (100 µg/ml), tetracycline (20 µg/ml), chloramphenicol (20 µg/ml) [24, 34]. For arabinose induction of genes encoding Cre-effector fusion proteins, 50 µl of overnight culture was inoculated into 5 ml LB broth and grown to mid-log phase (OD$_{600}$ ~0.4-0.6). Optical density readings were taken on a Beckman DU 530 UV/Vis spectrophotometer. Cells were induced by addition of 0.2% arabinose and incubation with shaking for 2-4 hrs. Cultures were normalized by OD$_{600}$ and 1 ml of cells was used for protein analysis.
### Table 2.1 Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristic(s)</th>
<th>Source</th>
</tr>
</thead>
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<tr>
<td><strong>E. coli strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5α</td>
<td>F- φ80lacZΔM15 Δ(lacZYA-argF)U169 recA1 endA1 hsdR17(r-, mk+) phoA supE44 thi-1 gyrA96 relA1 λ-</td>
<td>Gibco-BRL/Invitrogen</td>
</tr>
<tr>
<td>WM1650</td>
<td>CAG18477; Tet linked to priA(+)</td>
<td>[35]</td>
</tr>
<tr>
<td>MS411</td>
<td>ilvG rfb-50 thi</td>
<td>[33]</td>
</tr>
<tr>
<td>CSH26Cm::LTL</td>
<td>Tet', CSH26 galK::cat::loxP-Tet-loxP; Cam' after Cre mediated excision at loxP sites</td>
<td>[33]</td>
</tr>
<tr>
<td><strong>A. tumefaciens strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A348</td>
<td>A. tumefaciens containing octopine-type Ti plasmid pTiA6NC</td>
<td>[26]</td>
</tr>
<tr>
<td>A348-Spc'</td>
<td>Spc'; derivative of A348</td>
<td>[36]</td>
</tr>
<tr>
<td>Mx355</td>
<td>A348 derivative virD4::Tn3HoHo1</td>
<td>[27]</td>
</tr>
<tr>
<td><strong>Vector plasmids</strong></td>
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<td></td>
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<tr>
<td>pBSKS</td>
<td>Crb'; cloning vector</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pBSK</td>
<td>Crb'; cloning vector</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pBSIKS*</td>
<td>Crb'; cloning vector</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pBSIKS*Ndel</td>
<td>Crb'; cloning vector containing Ndel restriction site at the translational start site of lacZ</td>
<td>[37]</td>
</tr>
<tr>
<td>pBBR1MCS2-GenR</td>
<td>Gen'; broad-host-range cloning vector</td>
<td>[38]</td>
</tr>
<tr>
<td>pXZ151</td>
<td>Kan'; broad-host-range IncP derivative of pSW172 encoding a Kan' cassette</td>
<td>[39]</td>
</tr>
<tr>
<td>pML122ΔKm</td>
<td>Gen'; mobilizable IncQ derivative (RSF1010)</td>
<td>[40]</td>
</tr>
<tr>
<td>pUC4K</td>
<td>Kan'; source of cassette conferring Kan'</td>
<td>Amersham</td>
</tr>
<tr>
<td>pHIP45Ω</td>
<td>Spc'; source of cassette conferring Spc'</td>
<td>[41]</td>
</tr>
<tr>
<td>pBAD24</td>
<td>Crb'; pBR322/ColE1 expression vector inducible with arabinose</td>
<td>[42]</td>
</tr>
<tr>
<td>pBAD24-Kan'</td>
<td>Kan', Crb'; gene conferring Kan' from pUC4K inserted in the Crb' gene of pBAD24</td>
<td>This Study</td>
</tr>
<tr>
<td>Plasmid</td>
<td>Description</td>
<td>Notes</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------------------------------------------------------------------------------------------</td>
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</tr>
<tr>
<td>pKM101</td>
<td>Crb&lt;sup&gt;+&lt;/sup&gt;; broad-host-range IncN plasmid derived from R46</td>
<td>[43]</td>
</tr>
<tr>
<td>pKM101-Spc&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Spc&lt;sup&gt;+&lt;/sup&gt;, Crb&lt;sup&gt;+&lt;/sup&gt;; pKM101 with the cassette conferring Spc&lt;sup&gt;+&lt;/sup&gt; from pH45Ω inserted into the Crb&lt;sup&gt;+&lt;/sup&gt; gene at EcoRI</td>
<td>This Study</td>
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<tr>
<td>pKM101&lt;sup&gt;Δ&lt;/sup&gt;traJ</td>
<td>Spc&lt;sup&gt;+&lt;/sup&gt;, Crb&lt;sup&gt;+&lt;/sup&gt;; pKM101-Spc&lt;sup&gt;+&lt;/sup&gt; containing a clean deletion of traJ</td>
<td>This Study</td>
</tr>
<tr>
<td>pBAD33</td>
<td>Cam&lt;sup&gt;+&lt;/sup&gt;; pACYC184/p15A expression plasmid inducible with arabinose</td>
<td>[42]</td>
</tr>
<tr>
<td>pBAD33-Cre</td>
<td>Cam&lt;sup&gt;+&lt;/sup&gt;; pBAD33 expressing Cre recombinase from an arabinose inducible promoter</td>
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</table>

**A. tumefaciens**

**Expression Plasmids**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
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<tbody>
<tr>
<td>pPC914KS&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Crb&lt;sup&gt;+&lt;/sup&gt;; pBSIIKS&lt;sup&gt;+&lt;/sup&gt; derivative expressing P&lt;sub&gt;virB&lt;/sub&gt;-virB1; expression vector when substituting other genes for virB1</td>
<td>[44]</td>
</tr>
<tr>
<td>pXZ27</td>
<td>Crb&lt;sup&gt;+&lt;/sup&gt;; pBSIIKS&lt;sup&gt;+&lt;/sup&gt;Ndel expressing P&lt;sub&gt;virB&lt;/sub&gt;-virE2; expression vector when substituting other genes for virE2</td>
<td>[39]</td>
</tr>
<tr>
<td>pZZ11</td>
<td>Crb&lt;sup&gt;+&lt;/sup&gt;; pPC914KS&lt;sup&gt;+&lt;/sup&gt; expressing P&lt;sub&gt;virB&lt;/sub&gt;-GST</td>
<td>[37]</td>
</tr>
<tr>
<td>pKA9</td>
<td>Crb&lt;sup&gt;+&lt;/sup&gt;; pBSIIKS&lt;sup&gt;+&lt;/sup&gt; expressing P&lt;sub&gt;virB&lt;/sub&gt;-virD4&lt;sub&gt;At&lt;/sub&gt;</td>
<td>[45]</td>
</tr>
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<td>pTB7</td>
<td>Crb&lt;sup&gt;+&lt;/sup&gt;; pPC914KS&lt;sup&gt;+&lt;/sup&gt; expressing P&lt;sub&gt;virB&lt;/sub&gt;-virD4&lt;sub&gt;Ap&lt;/sub&gt;</td>
<td>This Study</td>
</tr>
<tr>
<td>pTB12</td>
<td>Crb&lt;sup&gt;+&lt;/sup&gt;; pPC914KS&lt;sup&gt;+&lt;/sup&gt; expressing P&lt;sub&gt;virB&lt;/sub&gt;-virD4&lt;sub&gt;Wp&lt;/sub&gt;</td>
<td>This Study</td>
</tr>
<tr>
<td>pTB19</td>
<td>Crb&lt;sup&gt;+&lt;/sup&gt;, Kan&lt;sup&gt;+&lt;/sup&gt;; pTB12 ligated to pXZ151 for expression in A. tumefaciens</td>
<td>This Study</td>
</tr>
<tr>
<td>pTB20</td>
<td>Crb&lt;sup&gt;+&lt;/sup&gt;, Kan&lt;sup&gt;+&lt;/sup&gt;; pTB7 ligated to pXZ151 for expression in A. tumefaciens</td>
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<td>pTB22</td>
<td>Crb&lt;sup&gt;+&lt;/sup&gt;, Kan&lt;sup&gt;+&lt;/sup&gt;; pKA9 ligated to pXZ151 for expression in A. tumefaciens</td>
<td>This Study</td>
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<tr>
<td>pTB37</td>
<td>Crb&lt;sup&gt;+&lt;/sup&gt;; pXZ27 expressing P&lt;sub&gt;virB&lt;/sub&gt;-virD4&lt;sub&gt;At&lt;/sub&gt;Δ553</td>
<td>This Study</td>
</tr>
<tr>
<td>pTB46</td>
<td>Crb&lt;sup&gt;+&lt;/sup&gt;, Kan&lt;sup&gt;+&lt;/sup&gt;; pTB37 ligated to pXZ151 for expression in A. tumefaciens</td>
<td>This Study</td>
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**E. coli**

**Expression Plasmids**

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<th>Notes</th>
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<td>pTB25</td>
<td>Kan&lt;sup&gt;+&lt;/sup&gt;; pBAD24-Kan&lt;sup&gt;+&lt;/sup&gt; expressing virD4&lt;sub&gt;Wp&lt;/sub&gt; from pTB12</td>
<td>This Study</td>
</tr>
<tr>
<td>pTB26</td>
<td>Kan&lt;sup&gt;+&lt;/sup&gt;; pBAD24-Kan&lt;sup&gt;+&lt;/sup&gt; expressing traJ from pKM101</td>
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</tr>
<tr>
<td></td>
<td>Description</td>
<td>Source</td>
</tr>
<tr>
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<tr>
<td>pTB47</td>
<td>Kan'; pBAD24-Kan' expressing virD4&lt;sub&gt;Ap&lt;/sub&gt; from pKA9</td>
<td>This Study</td>
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<tr>
<td>pTB48</td>
<td>Kan'; pBAD24-Kan' expressing virD4&lt;sub&gt;Ap&lt;/sub&gt; from pTB7</td>
<td>This Study</td>
</tr>
<tr>
<td>pTB49</td>
<td>Crb'; pBAD24 expressing virD4&lt;sub&gt;Rr&lt;/sub&gt;</td>
<td>This Study</td>
</tr>
<tr>
<td>pNW5</td>
<td>Crb'; pBSI1KS+Ndel expressing virD4&lt;sub&gt;At&lt;/sub&gt; from pKA9</td>
<td>This Study</td>
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<tr>
<td>pNW7</td>
<td>Crb'; traJΔAAD, containing a deletion of the all-alpha domain, cloned into pBAD24</td>
<td>This Study</td>
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<td></td>
<td><strong>Chimeric virD4 plasmids</strong></td>
<td></td>
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<tr>
<td>pTB38</td>
<td>Kan'; traJ::virD4&lt;sub&gt;Ap&lt;/sub&gt; cloned into pMK-RQ as provided by Invitrogen</td>
<td>This Study, Invitrogen</td>
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<td>pTB39</td>
<td>Crb'; pBAD24 expressing traJ::virD4&lt;sub&gt;Ap&lt;/sub&gt; from pTB38</td>
<td>This Study</td>
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<tr>
<td>pTB50</td>
<td>Crb'; pBAD24 expressing traJ::virD4&lt;sub&gt;Ap&lt;/sub&gt;Δ574</td>
<td>This Study</td>
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<tr>
<td>pTB51</td>
<td>Crb'; pBAD24 expressing traJ::virD4&lt;sub&gt;Wp&lt;/sub&gt;</td>
<td>This Study</td>
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<tr>
<td>pTB52</td>
<td>Crb'; pBAD24 expressing traJ::virD4&lt;sub&gt;Wp&lt;/sub&gt;Δ574</td>
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<tr>
<td>pNW1</td>
<td>Crb'; transmembrane domain of traJ cloned into pBSI1KS*</td>
<td>This Study</td>
</tr>
<tr>
<td>pNW2</td>
<td>Crb'; soluble domain of virD4&lt;sub&gt;At&lt;/sub&gt; cloned into pNW1</td>
<td>This Study</td>
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<tr>
<td>pNW4</td>
<td>Crb'; virD4&lt;sub&gt;At&lt;/sub&gt;Δ553 containing a C-terminal deletion at residue 553 cloned into pNW1 generating traJ::virD4&lt;sub&gt;At&lt;/sub&gt;Δ553</td>
<td>This Study</td>
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<tr>
<td>pNW6</td>
<td>Crb'; virD4&lt;sub&gt;At&lt;/sub&gt; SDΔAAD containing a deletion of the all-alpha domain cloned into pNW1 generating traJ::virD4&lt;sub&gt;At&lt;/sub&gt;ΔAAD</td>
<td>This Study</td>
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<td>pNW10</td>
<td>Crb'; traJ::virD4&lt;sub&gt;At&lt;/sub&gt; with the AAD of traJ cloned into pBSI1KS*</td>
<td>This Study</td>
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<tr>
<td>pNW11</td>
<td>Crb'; traJ with the AAD of virD4&lt;sub&gt;At&lt;/sub&gt; cloned into pBAD24</td>
<td>This Study</td>
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<tr>
<td></td>
<td><strong>Effector Plasmids</strong></td>
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<tr>
<td>pTB30</td>
<td>Crb'; putative <em>Anaplasma</em> effector Aph_0111 cloned into pBSK</td>
<td>This Study; J. Carlyon¹</td>
</tr>
<tr>
<td>pTB40</td>
<td>Cam'; ats-1 cloned into pBAD33-Cre</td>
<td>This Study; J. Carlyon¹</td>
</tr>
<tr>
<td>pTB41</td>
<td>Cam'; Aph_0111 from pTB30 subcloned into pBAD33-Cre</td>
<td>This Study</td>
</tr>
<tr>
<td>pTB42</td>
<td>Cam¹; ats-1 CTD cloned into pBAD33-Cre</td>
<td>This Study; J. Carlyon¹</td>
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<tr>
<td>pTB43</td>
<td>Cam¹; putative Wolbachia effector WD0636 cloned into pBAD33-Cre</td>
<td>This Study; I. Newton²</td>
</tr>
<tr>
<td>pTB44</td>
<td>Cam¹; putative Wolbachia effector WD0811 cloned into pBAD33-Cre</td>
<td>This Study; I. Newton²</td>
</tr>
<tr>
<td>pTB45</td>
<td>Cam¹; putative Wolbachia effector WD0830 cloned into pBAD33-Cre</td>
<td>This Study; I. Newton²</td>
</tr>
<tr>
<td>pTB53</td>
<td>Cam¹; virE3 cloned into pBAD33-Cre after PCR amplification of the Ti plasmid</td>
<td>This Study</td>
</tr>
<tr>
<td>pTB54</td>
<td>Cam¹; virF cloned into pBAD33-Cre after PCR amplification of the Ti plasmid</td>
<td>This Study</td>
</tr>
<tr>
<td>pTB55</td>
<td>Cam¹; virE2 from pXZ27 subcloned into pBAD33-Cre</td>
<td>This Study</td>
</tr>
</tbody>
</table>

¹ Anaplamsa phagocytophilum genomic DNA provided by Jason Carlyon at Virginia Commonwealth University; ² Wolbachia pipientis putative effector genes provided by Irene Newton at Indiana University, all were received in pENTR-D/TOPO vectors and PCR amplified for cloning.
Plasmid constructions

Plasmids used in this study are listed in Table 2.1 and primers used for PCR are listed in Table 2.2. The vector plasmid pBAD24-Kan’ was made by isolation of a gene conferring kanamycin resistance as a HincII fragment from pUC4K and insertion into the single Scal restriction site within pBAD24 [42]. The conjugative plasmid pKM101-Spc’ was made by isolation of a gene conferring spectinomycin resistance as an EcoRI fragment from pH45Ω and insertion into similarly digested pKM101. The resulting plasmid is Spc’ and Crb⁸ and retains all of its conjugative abilities.

Plasmid pTB7 expresses the A. phagocytophilum virD4 homolog (virD4_Ap) from the P_virB promoter. virD4_Ap was PCR amplified from pBT-virD4 [46] using the primers 5’-AGTCGTCA\_CATATGC\_G\_CATAGTTCCAATCAT-3’ and 5’-TTAGTG\_CTCGA\_G\_CTACTTTAGTCTTCC-3’. The PCR product was digested with NdeI and Xhol and ligated to similarly digested CoIE1 plasmid, pPC914KS’ [44]. Plasmid pTB12 expresses the W. pipiens virD4 homolog (virD4_Wp) from the P_virB promoter. virD4_Wp was PCR amplified using pCR-VirD4 as a template (provided by Katrin Gentil; Bonn, Germany) and primers 5’-TAAGCGAT\_CCATGG\_G\_TCATAGC-3’ and 5’-GCTAGCT\_CG\_GGTACC\_TTACTTTCC\_3’. The PCR product was digested with NcoI and KpnI, and the resulting fragment was ligated into similarly digested pPC914KS’. Plasmid pTB26 expresses the pKM101 virD4 ortholog (traJ) from the P_BAD promoter. traJ was PCR amplified using pKM101 as a template and primers 5’-CAGTAGCC\_CAT\_GG\_G\_CGATAGAGAAAGA-3’ and 5’-ACAATT\_GGTACC\_TCAGATCTCCCTCAG-3’. The PCR product was digested with NcoI and Kpnl, and the resulting fragment was ligated into similarly digested pBAD24-Kan’. Plasmid pTB37 expresses virD4_AaΔ553, which encodes A. tumefaciens VirD4 deleted of its C-terminal 104 residues, from the P_virB promoter. virD4_AaΔ553 was PCR amplified using plasmid pKA9 [45] as a template and primers 5’-CGGTGA\_CAT\_G\_G\_AT\_T\_CCAGAA\_A\_A\_A\_G\_A-3’ and 5’-ACAATT\_GGTACC\_TCAGATCTCCCTCAG-3’.
and 5′-CTATTAGGTACCTCAGGGCTCAGGCAGAGA-3′. The PCR product was digested with Ndel and KpnI, and the resulting fragment was ligated into similarly digested pXZ27 [39], replacing the virE2 gene. Plasmid pTB49 expresses the R. rickettsii virD4 homolog (virD4Rr) from the P_{BAD} promoter. virD4Rr was PCR amplified from genomic DNA sent from the Betsy Kleba lab using the primers 5′-GTGCCATGGCATAAGATCTTTAAAG-3′ and 5′-ATTCTCGAGTTACTCTATTTTCCGG-3′. The PCR product was digested with Ncol and Xhol, and the resulting fragment was ligated into pBAD24 digested with Ncol and Sall. All plasmid constructs were confirmed by PCR, digestion analysis, and sequencing.

A postdoctoral fellow in the Christie lab, Dr. N. Whitaker, created the following plasmid constructs. Plasmid pNW5 expresses virD4_{At} from the P_{lac} promoter. virD4_{At} was PCR amplified using plasmid pKA9 as a template and primers 5′-CGGTGAACATATGACCGCTAGCTCAGTCAGAGAAGAGGC-3′ and 5′-GTTCTCGAGTCATTCCGCAGGCTGTGACC-3′ (Table 2.2). The PCR product was digested with Ndel and Xhol and the resulting fragment was ligated into similarly digested pBSIIKS′Ndel [37]. Plasmid pNW7 expresses traJΔAAD from the P_{BAD} promoter. The AAD of TraJ (amino acids 186-298) was deleted using overlapping PCR [47, 48]. The gene sequences encoding residues 1-185 and 299-509 of traJ were PCR amplified using the primers 5′-CCATGGACGATAGAGAAAGAGGC-3′ and 5′-GCAGGGAAAAATTTTCAGTGCTGCCC-3′, and 5′-TGATAAAGCCTGAAATTTCCTGCTGACTGAGGC-3′ and 5′-GGTACCTCAGATCTCCCTCCTGTTCAA-3′, respectively. The two PCR products were used as templates for overlapping PCR with outside primers (5′-CCATGGACGATAGAGAAAGAGGC-3′ and 5′-GGTACCTCAGATCTCCCTCCTGTTCAA-3′). The final PCR product was digested with Ncol and KpnI, and the resulting fragment was ligated into similarly digested pBAD24. Plasmids were confirmed with sequencing.
Table 2.2 Oligonucleotides used for T4CP and effector constructions

<table>
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<th>VirD4 Homologs</th>
<th>5'-AGTCGTCATATG'CATAGTCCAAATCAT-3'</th>
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<tr>
<td></td>
<td>5'-TTAGTGCTCGAG'CTACTTTTAGTCTTCC-3'</td>
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<td>VirD4WP</td>
<td>5'-TAAGCGATCACCATGG'GTCATAGC-3'</td>
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<td>5'-GCTAGTCGGGTACC'TTACTTTCCC-3'</td>
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<td>VirD4RR</td>
<td>5'-GTGCCATGG'CATAAGATACTTTAAAG-3'</td>
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<td></td>
<td>5'-ATTCTCGAG'TTACTCATTATTTCCCAG-3'</td>
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<td>VirD4APΔ553</td>
<td>5'-CGGTGAACATATG'AATTCCAGCAA-3'</td>
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<td>5'-CTATTAGGTACC'TCAGGGTCAGGCAGA-3'</td>
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<tr>
<td>VirD4APΔ574 Reverse</td>
<td>5'-AGTTCTCGAG'C'TACTTTTCAGGATCGTACCG-3'</td>
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<tr>
<td>VirD4At Reverse (pNW5)</td>
<td>5'-GTTCTCGAG'TCATTTCGCAGGCTGTGCCG-3'</td>
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<tr>
<td>TraJ</td>
<td>5'-CAGTAGCCATGG'ACGATAGAGAAAGA-3'</td>
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<td></td>
<td>5'-ACAATTGGTACC'TCAGATCTCCCTCACG-3'</td>
</tr>
<tr>
<td>TraJΔAAD (1-185)</td>
<td>5'-CCATGG'ACGATAGAGAAAGAGGC-3'</td>
</tr>
<tr>
<td></td>
<td>5'-GCAGGAAAAATTTTCAGTGCGTTTATCATAGG-3'</td>
</tr>
<tr>
<td>TraJΔAAD (299-509)</td>
<td>5'-TGATAAAACGCACTGAAAATTTCTCGTGACTGGC-3'</td>
</tr>
<tr>
<td></td>
<td>5'-GGTACC'TCAGATCTCCCTCAGTTCAA-3'</td>
</tr>
<tr>
<td>Chimeric VirD4's</td>
<td></td>
</tr>
<tr>
<td>TraJ::VirD4APΔ574*</td>
<td>5'-TTCCGGGTCATAAGGTTCTTG-3'</td>
</tr>
<tr>
<td></td>
<td>5'-TAAATTCGTGGTGGTGTTGAAGG-3'</td>
</tr>
<tr>
<td>TraJ::VirD4WP**</td>
<td>5'-GATTATTCTTCTCTATAATGATAAAAGCGATCAGACC CCAAC-3'</td>
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<tr>
<td></td>
<td>5'-GCTTTATCATTATAGAAAGAATAATCGAGTGCGGCC-3'</td>
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<tr>
<td></td>
<td>5'-GTATCTCGAG'TTACTTTCCATTACTTTTGTTTATCACC ATCATCCTTCATC-3'</td>
</tr>
<tr>
<td>TraJ::VirD4WPΔ574**</td>
<td>5'-CTATCTCGAG'TTATGGGTCAATGGCTCTGTTGATAGGTCACAATGTC-3'</td>
</tr>
<tr>
<td>TraJ TMD (1-75)</td>
<td>5'-CATAACATGG'ACGATAGAGAAAGAGGCTTAGCATTTTATTTG-3'</td>
</tr>
<tr>
<td></td>
<td>5'-GTATCTCGAG'TTATATCATATG'ATAAATGATAAAAGC-3'</td>
</tr>
<tr>
<td>Gene</td>
<td>Forward Primer</td>
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</tr>
<tr>
<td>VirD4&lt;sub&gt;At&lt;/sub&gt; SDΔ553</td>
<td>5'- CATATATATACATATG&lt;sub&gt;1&lt;/sub&gt;AATCAGAAGCATCACGGGACGG-3'</td>
</tr>
<tr>
<td>TraJ::VirD4&lt;sub&gt;At&lt;/sub&gt; (1-190)</td>
<td>5'-TCATGAT&lt;sup&gt;4&lt;/sup&gt;ATGGACGATAGAGAAAGAGG-3'</td>
</tr>
<tr>
<td>TraJ AAD (186-298)</td>
<td>5'-TCCCCCCTAGATTCTGGCTGGGTGTTTTTCAATGAAA-3'</td>
</tr>
<tr>
<td>VirD4&lt;sub&gt;At&lt;/sub&gt; (347-656)</td>
<td>5'-AAAATGCGGGAAAGTAAAGAACCTGCATTATCTTTGT GTCAGTC-3'</td>
</tr>
<tr>
<td>TraJ (1-185)**&lt;sup&gt;3&lt;/sup&gt;</td>
<td>5'- ATGAGTCTTCCGCTCTTCAGTGCGTTTATCATAGGCG-3'</td>
</tr>
<tr>
<td>VirD4&lt;sub&gt;At&lt;/sub&gt; AAD (132-346)</td>
<td>5'-GATAAAGCGCAGTAAGAGGAACCTGCATTATCTTTGT TCC-3'</td>
</tr>
<tr>
<td>TraJ (299-509)**&lt;sup&gt;3&lt;/sup&gt;</td>
<td>5'- TTACGATCTCCGGAGGAAATTATTTCCCTGCGTGACTGCG TT-3'</td>
</tr>
<tr>
<td><strong>Effector genes</strong></td>
<td></td>
</tr>
<tr>
<td>Ats-1</td>
<td>5'-GTGCTCCATATG&lt;sub&gt;1&lt;/sub&gt;CTAATAAGAAGAATTCG-3'</td>
</tr>
<tr>
<td>Ats-1 CTD Forward</td>
<td>5'-GTACTTTCATATG&lt;sub&gt;1&lt;/sub&gt;GAACGCATTTTCTCATTG-3'</td>
</tr>
<tr>
<td>Aph_0111</td>
<td>5'-GGACTGCATATG&lt;sub&gt;1&lt;/sub&gt;TCAATCGATTGT-3'</td>
</tr>
<tr>
<td>WD0636</td>
<td>5'-GCGGACGGCATATG&lt;sub&gt;1&lt;/sub&gt;AGTAAAAAGAAAAAGAG-3'</td>
</tr>
<tr>
<td>WD0811</td>
<td>5'-GTCCCATATG&lt;sub&gt;1&lt;/sub&gt;ATGATATCCTCAAATTCT-3'</td>
</tr>
<tr>
<td>WD0830</td>
<td>5'-TCGTCATATG&lt;sub&gt;1&lt;/sub&gt;AAACAAAGGAGATAAG-3'</td>
</tr>
<tr>
<td>VirE3</td>
<td>5'-GATGCATATG&lt;sub&gt;1&lt;/sub&gt;GTGAGCCTACGAAG</td>
</tr>
<tr>
<td>VirF</td>
<td>5'-GCACCATATG&lt;sub&gt;1&lt;/sub&gt;AGAAATTCGAGTTTGC</td>
</tr>
<tr>
<td>Other primers</td>
<td>Cre</td>
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<td>-----------------------</td>
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<tr>
<td></td>
<td>5'-GATAGAGCTCGAGGAGGTATTCCACCATGTCCATTTACTGACCCTACACACAAAAATTTGC-3'</td>
</tr>
<tr>
<td></td>
<td>5'-CATGGTACCATATATCATATG1ATCGCCATCTTCCAGCAGGC-3'</td>
</tr>
</tbody>
</table>

Underlined restriction sites are as follows: ¹NdeI (CATATG), ²XhoI (CTCGAG), ³Ncol (CCATGG), ⁴KpnI (GGTACC), ⁵SacI (GAGCTC), ⁶BspHI (TCATGA)

*TraJ::VirD4ApΔC was constructed via inverse PCR by insertion of a stop codon.

**Chimeras TraJ::VirD4Ap and TraJ::VirD4ApΔ574 were constructed by overlapping PCR using the TraJ forward primer. Internal and reverse primers are specified.

***The TraJ+VirD4ApAAD chimera was constructed using the same forward primer as TraJ TMD and the same reverse primer as TraJ.
Construction of *virD4* deletion strains and chimeric *virD4* plasmids

The *virD4* null strain Mx355 contains an IncP plasmid, pPH1JI [27, 49], which encodes resistance to gentamicin, chloramphenicol, spectinomycin, and streptomycin. Mx355 was cured of pPH1JI as follows. ColE1 plasmids containing VirD4 homologs behind a *P*<sub>VirB</sub> promoter (Table 2.1) were ligated to another IncP plasmid, pXZ151 [39]. The resulting co-integrates were introduced to Mx355 cells by electroporation [50] and transformants were plated on high concentrations of kanamycin (300 µl/ml). Colonies were consecutively streaked for isolation on kanamycin plates for two overnight growth cycles, after which colonies were patched on gentamicin (100 µg/ml) to test for sensitivity as an indicator of pPH1JI curing.

A *traJ* deletion in pKM101-Spc<sup>+</sup> (Table 2.1) was obtained by recombineering [51-53]. pKM101-Spc<sup>+</sup> was conjugatively transferred into *E. coli* strain HME45 [51, 53], which contains a defective λ prophage that can be temperature induced to activate Red recombination. In this system, the λ Red subunits Exo and Beta use single strand annealing to generate recombinants. The Exo subunit is a 5’-3’ exonuclease that is thought to bind the end of a dsDNA fragment during replication, while the Beta subunit is a ssDNA binding protein that promotes annealing between complementary ssDNA. Briefly, upon degradation of dsDNA by Exo, Beta binds 3’ ssDNA overhangs. Introduction of the complementary sequence (e.g. PCR product) allows Beta to anneal the two complementary stands to generate recombinants.

A linear DNA fragment containing a cassette conferring Kan<sup>+</sup> from the plasmid pUC4K, flanked by Ncol restriction sites, was PCR amplified using primers 5’-CTGGGAACCAAAAAGGAGCGCTGACCATGGGTGGGTAACGCCAGGGTTTTCC-3’ and 5’-TGGCGGGTAATCGTGTTATATCAACCATGGCACACAGGAAACAGCTATGACCATGATTAC-3’, bearing 25 bp of sequence homology to the regions upstream and downstream from...
traJ, respectively (Table 2.2). The purified PCR product was electroporated into HME45 cells containing pKM101-Spc' after temperature induction and Kan' recombinants were selected. pKM101-Spc' is a multicopy plasmid, and recombination does not occur on all copies within a single cell. Therefore, it was necessary to isolate a strain carrying only the traJ-deleted plasmid. For this, Kan' colonies were sequentially grown in LB broth containing kanamycin (200 µg/ml) for 4 days prior to plating for isolated colonies on LB plates containing kanamycin (200 µg/ml). The kan' cassette was deleted by Ncol digestion and religation of the pKM101-Spc' plasmid. The traJ deletion was confirmed by absence of a PCR product corresponding to traJ using primers 5'-CAGTAGCCATGGACGATAGAGAAAGA-3' and 5'-ACAATTGGGTACCTCAGATCTCCTCAG-3' (Table 2.2).

Plasmids pTB51 and pTB52 express the traJ::virD4وفق و traJ::virD4وفقΔ574 chimeric T4CP's, respectively, from the P_{BAD} promoter. These plasmids were created by overlapping PCR [47, 48] with the gene fragments corresponding to the TraJ TMD and the respective SD fragments of VirD4وفق. PCR products were digested with Ncol and Kpnl, and the resulting fragments were ligated into similarly digested pBAD24[42] or pBAD24-Kan'. Plasmid pTB38 encodes traJ::virD4وفق within the vector plasmid pMK-RQ; the traJ::virD4وفق gene fragment was synthesized and codon optimized for use in E. coli by Invitrogen. Plasmid pTB39 expresses traJ::virD4وفق from the P_{BAD} promoter. The traJ::virD4وفق gene fragment was obtained as an Ncol/Kpnl fragment from pTB38, and inserted into similarly digested pBAD24. Plasmid pTB50 expresses traJ::virD4وفقΔ574 which encodes the TraJ::VirD4 chimeric T4CP deleted of C-terminal residues 574-740. This plasmid was generated by inserting a stop codon at codon 574 of pTB39 by inverse PCR with primers 5'-TTCCGGGTCAAGGTTCTTG-3' and 5'-TAAATCGTGGGTTGAAGG-3' (Table 2.2).

Plasmid pNW1 expresses coding sequence for the TraJ TMD from the P_{lac} promoter. It was constructed by Dr. N. Whitaker by amplification of coding sequence for the TraJ TMD
from a pKM101 template using primers 5’-
CATACCATGGACGATAGAGAAAGGCTTAGCATTTTTATTG-3’ and 5’-
GTATCTCGAGTATATATCATATGATAATGATAAAAGCGATCAGACGCACCAAC-3’ (Table 2.2). The PCR product was digested with NcoI and XhoI, and the resulting fragment was ligated into similarly digested pBSIIKS⁺ [32]. Plasmid pNW2 expresses the traJ::virD4₅₅₃ chimeric T4CP from the Plac promoter. It was constructed by inserting coding sequence for the SD of VirD4₅₅₃ obtained as an Ndel/XhoI fragment from pKA38 [45], into similarly digested pNW1. Plasmid pNW4 expresses traJ::virD4₅₅₃Δ553 from the Plac promoter. The sequence encoding the VirD4₅₅₃ SD without residues 553-656 was PCR amplified using pKA38 as a template and primers 5’-
CATATATACATATGAATCAGAAGCATCAGCGGACGG-3’ and 5’-
GTTCTCGAGTCAAGGGTGCGGTCTCAGGCG-3’ (Table 2.2). The PCR product was digested with Ndel and XhoI and the resulting fragment was ligated to similarly digested pNW1. Plasmid pNW6 expresses traJ::virD4₅₅₃ΔAAD from the Plac promoter. The sequence encoding the VirD4₅₅₃ SDΔAAD, obtained as an Ndel/XhoI fragment from plasmid pCM39, was inserted into similarly digested pNW1.

Plasmid pNW10 expresses traJ::virD4₅₅₃ with AADtraJ from the Plac promoter. The sequences encoding amino acids 1-190 of the TraJ::VirD4₅₅₃ chimera expressed by pNW2, the AAD domain of TraJ (amino acids 186-298), and amino acids 347-656 of VirD4₅₅₃ were PCR amplified using primers 5’-TCATGAATGGACGATAGAGAAAGG-3’ and 5’-
GAAAAACACCCAGCCAGAATCTAGGGGGGAAAACCTTGAAAAAC-3’, 5’-
TCCCCCCTAGTCTGGGTGGGTGGTTTTTCAATGAAA-3’ and 5’-
AATGCAGGTCTTCTACCTCCGGCATTTTTCAATGTG-3’, and 5’-
AAAATGCCGGAAGTAAGACCTGCATTTATCTTTGTGCAGTC-3’ and 5’-
CTCGAGTCATTTCGAGGGCTGGTGCCGGGTGC-3’ (Table 2.2). Overlapping PCR was used to amplify the three PCR products together as a single product with outside primers
from the first and third reactions. The final PCR product was digested with BspHI and XhoI and the resulting fragment was ligated into similarly digested pBSIIKS. Plasmid pNW11, expresses traJ with $\text{AAD}_{\text{VirD4 At}}$ from the $P_{\text{BAD}}$ promoter. The sequences encoding TraJ amino acids 1-185, VirD4$_{\text{At}}$ AAD (amino acids 132-346), and the amino acids 299-509 of TraJ were PCR amplified using primers 5’-CATACCATGGACGATAGAAAAGGGCTTTAGCATTTTTATTTG-3’ and 5’-ATGAGTCTTCCGCTTTCAGTGCTTTATCATAGGCG-3’, 5’-GATAAAACGCACTGAAGCGGAAGACTTGTAGTTACATCC-3’ and 5’-CACGCAGGAAAAATTCTCCGGAGATCGTAAACGGAA-3’, and 5’-TTACGATCTCCGGAGGAATTTCCTCCGAGGACTGTTACATAACC-3’ and 5’-ACAATTGTACCTCAGATCTCCCTCAG-3’ (Table 2.2). Overlapping PCR was used to amplify the three PCR products together as a single product with outside primers from the first and third reactions. The final PCR product was digested with Ncol and KpnI and the resulting fragment was ligated into similarly digested pBAD24. All chimeric constructs were verified by sequencing.

**Construction of Cre fusion plasmids**

The plasmid pBAD33-Cre was prepared by PCR amplification of the cre insert, using pZD96 as a template and the primers 5’-

GATAGAGCTAGAGGTATTACCATGTCCAATTTACTGACCATCACACAAAATTTCGC-3’ and 5’-CATGGTACCTATATATCATATGATCGCCTCTTCCAGCAGGC-3’ (Table 2.2). PCR products incorporated a SacI site followed by the Shine-Dalgarno sequence (AGG AGG) at the 5’ end and Ndel and KpnI sites at the 3’ end. The two restriction sites at the 3’ end allows for the creation of Cre-effector fusion proteins. The PCR product was digested with SacI/KpnI and the resulting fragment was ligated to similarly digested pBAD33 [42]. Known or putative effector genes were PCR amplified or isolated as described in Table 2.1. The DNA fragments were digested with Ndel/KpnI or Ndel/XhoI, and the resulting fragments
were ligated to similarly digested pBAD33-Cre to create translational fusions. All constructs were verified by restriction digestion analysis and sequencing.

**Protein analysis by western blot**

To assay for the accumulation of Cre-effector fusion proteins, arabinose induced cells were harvested at mid-log phase and normalized to equivalent optical densities (OD$_{600}$), re-suspended in Laemmli’s buffer (50 mM Tris-HCl, 4% SDS, 20% glycerol, 20 mM dithiothreitol, 1% β-mercaptoethanol, 0.1% bromophenol blue, pH 6.8), and boiled for 10 minutes. The boiled cell lysates were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as previously described [54], with modifications. Proteins were transferred to polyvinylidene fluoride (PVDF) membranes and incubated with anti-Cre antibodies (Novus Biologicals) overnight. Western blots were developed with goat anti-mouse antibodies conjugated to horseradish peroxidase (HRP; New England Biolabs), and visualized by chemiluminescence (Thermo Scientific).

**Conjugation assays**

*A. tumefaciens* and *E. coli* donor strains were assayed for conjugative DNA transfer through the VirB/VirD4 and pKM101-encoded T4SSs as follows [33, 36, 55]. The promiscuous, mobilizable IncQ plasmid pML122ΔKm [40, 55] served as a DNA substrate. For *A. tumefaciens* conjugative matings, pML122ΔKm was introduced by electroporation [56] together with the ColE1/pXZ151 co-integrates described above into wild-type strain A348 or the Mx355 (*virD4*) mutant. These strains functioned as donors with A348-Spc’ as a recipient in mating experiments [36, 40]. Conjugative matings were carried out as previously described [55]. Briefly, *A. tumefaciens* strains were induced 16-18 hours at room temperature, as described above. Cells were mixed in a 1:5 ratio of donors to recipients and spotted onto sterile nitrocellulose filters placed on ABIM agar plates. Suspensions of
donors only and recipient only were also spotted onto filters to serve as controls. Plates were incubated for 4-5 days at 18 °C. Cells were re-suspended in 1x PBS and serial dilutions were prepared. Dilutions were spread on antibiotic containing MG/L plates selecting for donors, recipient, and transconjugants. Frequency of transfer was calculated as the number of transconjugants per donor cell. Mating experiments were carried out at least three times in duplicate, and results are reported as mean frequency of transfer with standard deviations indicated.

*E. coli* conjugation assays were carried out as previously described [57], with strains DH5α and WM1650 serving as the donor and recipients, respectively. Briefly, strains were grown overnight with antibiotic selection in LB broth at 37 °C. Cells were then diluted 1:10 in fresh LB with the addition of 0.2% arabinose or 200 µM IPTG as appropriate for plasmid induction, and grown at 37 °C for 1 hour. Donors and recipients were mixed in a 1:1 ratio and centrifuged for 1 min. Cells were re-suspended in 20 µl of the supernatant and spotted onto sterile filters placed on LB induction plates. Suspensions of donors alone and the recipient alone were prepared simultaneously as controls. After overnight incubation at 37 °C, cells were re-suspended in 1x PBS and serial dilutions were plated on LB plates selecting for donors, recipient, and transconjugants. Frequency of transfer was calculated as the number of transconjugants per donor. All mating experiments were performed at least three times in duplicate or triplicate, and results are reported as the mean frequency of transfer with standard deviations indicated.

**Virulence assays**

*A. tumefaciens* strains were assayed for T-DNA and effector protein transfer using a tumor formation assay on *Kalanchoe daigremontiana* leaves [24, 39]. Wild-type A348 and avirulent strain Mx355 (*virD4*) served as positive and negative controls, respectively. Briefly, bacterial strains were freshly streaked on MG/L agar plates with the appropriate
antibiotics and grown at room temperature for 2 days. Plant leaves were wounded by scratching with a sterile toothpick and immediately inoculated with cells from the 2-day plates. All strains were inoculated onto at least 5 different leaves and virulence was assessed after 6-8 weeks.

**CRAfT (Cre-recombinase Reporter Assay for Translocation)**

The Cre fusion reporter [5] was used to assay for the translocation of known or putative effector proteins from *E. coli* MS411 donor cells into the recipient *E. coli* strain CHS26Cm::LTL, which contains a *loxP*-TetR-*loxP* cassette interrupting a *cat* gene on the bacterial chromosome [33]. Plasmid pBBR1MCS2-GenR was introduced into the recipient strain for additional antibiotic selection of recombinants. Cre-mediated excision in the recipient strain results in colonies exhibiting a CamR, GenR, TetS phenotype. This recipient strain produced higher than desired levels of background growth (i.e. growth on Cre-only controls). Several experiments were repeated with another recipient generated by plating 100 ml of CHS26Cm::LTL liquid cell culture on LB plates containing 50 µg/ml of rifampicin. After growing at 37 °C overnight, RifR colonies were selected and made competent. The non-mobilizable plasmid pUC4K, encoding resistance to carbenicillin and kanamycin, was introduced to the RifR recipient to produce CHS26Cm::LTL,rifR(pUC4K). The latter recipient generally confirmed the results observed with the former recipient, while diminishing the background growth. Results are reported using the original CHS26CM::LTL(pBBR1MCS2-GenR) recipient unless otherwise noted.

**CRAfT** was performed similarly to the conjugation assay described above. Briefly, strains were grown overnight in LB broth with antibiotic selection at 30 °C. Cells were diluted 1:10 into fresh LB containing 0.2% arabinose or 200 µM IPTG as appropriate for plasmid induction, and grown at 30 °C for 1 hour. Cell suspensions were normalized by OD600. Donor and recipient cells were mixed in a 1:1 ratio and centrifuged for 1 min. Cell
pellets were re-suspended in 20 µl of the supernatant and spotted onto sterile nitrocellulose filters on LB induction plates. Concurrently, donors alone and recipient alone were prepared as controls. Plates were incubated overnight at 30 °C. Cells were re-suspended in 1x PBS and serially diluted. Donors, recipient, and recombinants were selected for on LB agar plates containing the appropriate antibiotics. Frequency of recombination was calculated as the number of recombinants per donor. All CRAfT experiments were performed at least three times in duplicate, and results are reported as the mean frequency of recombination with standard deviations.
Chapter 3. Chimeric T4SS in *Agrobacterium* and Functional Characterization of the VirD4ΔC C-terminal Extension
Introduction

The *A. tumefaciens* VirB/VirD4 T4SS is comprised of a channel that directs oncogenic T-DNA and effector proteins across the cell envelope and into plant cells during infection [6, 58]. Previous studies from our lab have shown that the VirD4 T4CP functions as a receptor for the DNA and protein substrates of the VirB/VirD4 T4SS [8, 59, 60]. *A. tumefaciens* is a member of the Alphaproteobacteria and is closely related phylogenetically to Rickettsial species (Figure 3.1A). Members of the Rickettsiales are intracellular pathogens and they also carry *virB/virD4*-like genes in their genomes (Figure 3.1B). Therefore, the Rickettsia spp. are postulated to assemble T4SSs resembling the *A. tumefaciens* VirB/VirD4 T4SS for the purpose of delivering effector proteins into the mammalian host during infection. However, studies of Rickettsial pathogens are currently hampered by the inability to grow these pathogens in laboratory media and also because they are genetically intractable.

Here, I sought to test whether T4CP’s from three Rickettsial species, *A. phagocytophilum, R. rickettsii* Sheila Smith strain, and *W. pipientis*, could functionally substitute for the *A. tumefaciens* VirD4 subunit to mediate translocation of DNA or protein substrates. The overarching goal was to use *A. tumefaciens* as a surrogate host and chimeric T4SSs composed of *A. tumefaciens* VirB subunits and Rickettsial VirD4 subunits, first, to gain evidence that the Rickettsial VirD4 subunits are functional, and second, to identify novel protein substrates that are translocated through Rickettsial VirB/VirD4 T4SSs.

This line of study gains support from previous work showing that substitutions of VirD4 subunits with closely related homologs enable assembly of functional T4SS’s [9, 61]. Here, to test whether Rickettsial VirD4 homologs could form a chimeric T4SS in *A. tumefaciens*, I assayed for the capacity of *virD4* genes from *A. phagocytophilum* (*virD4*Ap), *R. rickettsii* (*virD4*Rr), and *W. pipientis* (*virD4*Wp) to complement the *virD4*At null mutation. The initial complementation tests assayed for i) translocation of the T-DNA and protein
effectors to plant cells using a virulence assay and ii) translocation of the promiscuous, mobilizable IncQ plasmid to *A. tumefaciens* recipients. I also assayed for the capacity of Rickettsial *virD4* genes to exert negative dominance when expressed in an otherwise wild-type *A. tumefaciens* background.

Finally, as discussed earlier, *VirD4*$_{At}$ and other T4CPs, including those from *A. phagocytophilum* and *W. pipientis*, carry C-terminal extensions compared with T4CPs such as pKM101-encoded TraJ or the structural prototype for the T4CP family, *E. coli* *TrwB*$_{R388}$. These CTEs are typically variable with an overall negative charge, and I sought to test whether the *A. tumefaciens* *VirD4* T4CP requires its CTE for function. I was unable to demonstrate that the chimeric T4SSs functioned to translocate substrates, however, I gained genetic evidence for poisoning interactions between the Rickettsial T4CPs and the *A. tumefaciens* *VirB* system. I also demonstrated that *VirD4*$_{At}$’s CTE is required for translocation of T-DNA and the *VirE2* effector protein to plant cells, but dispensable for translocation of the promiscuous IncQ plasmid to Agrobacterial recipients.
Figure 3.1 Diversity of T4SSs in Alphaproteobacteria

A. Phylogenetic tree indicating the close relationship between *A. tumefaciens* and Rickettsial species. This image is modified from Beninati, et al. 2004 to indicate species presented in this thesis [62]. Permission for use was granted by the American Society for Microbiology.

B. Comparison of T4SSs between species of the Rickettsiales and other bacterial species. This image was modified from Gillespie, et al. 2010 to highlight the species presented in this thesis [17]. Permission for use was granted by the American Society of Microbiology. At, *A. tumefaciens*; Ec, *E. coli*; Rt, *Rickettsia* sp.; Wp, *Wolbachia pipientis*; Ap, *A. phagocytophilum*.
Results

Rickettsial VirD4 homologs do not complement a virD4 mutant for substrate transfer

To test the ability of Rickettsial VirD4 homologs to functionally substitute for A. tumefaciens VirD4, I first cloned the virD4 genes from plasmids obtained from the labs of Katrin Gentil and Yasuko Rikihisa, as described above. For expression in A. tumefaciens, I cloned each of the genes downstream of the acetylsyringone-inducible virB promoter (P_{virB}) on a ColE1 plasmid. I then ligated the ColE1-based virD4 expression plasmids to an IncP plasmid, which replicates in A. tumefaciens. I introduced the virD4 expression plasmids into the virD4 mutant strain Mx355 or the wild-type strain A348 to assay for functionality or negative dominance, respectively. I confirmed the presence of the plasmids and virD4 genes of interest in the different A. tumefaciens strains by PCR amplification with the primers originally used to clone the genes. It is important to note that much of this work using the plasmid containing virD4_{Rr} yielded negative results and it was later discovered that the construct provided to our lab contained a deletion of ~100 bp. This construct, and any data generated, has been left out of this thesis. Several attempts to generate the correct construct failed, although I was able to clone virD4_{Rr} behind a P_{BAD} promoter for use in E. coli as discussed in chapter 4.

I first assayed for the capacity of Mx355 carrying the virD4-expression plasmids to translocate the A. tumefaciens T-DNA and effector proteins to plants, as monitored by virulence on Kalanchoe leaves. As shown in Figure 3.2, A. tumefaciens strains A348 (WT) and Mx355 (ΔvirD4) expressing virD4_{At} incited tumors within 6-8 weeks on plant leaves, although the latter strain incited tumors that were reproducibly smaller than WT A348. By contrast, Mx355 expressing the Rickettsial virD4_{Ap}, or virD4_{WP} genes failed to incite virulence on plants.
Both A348 and Mx355 expressing \( \text{virD4}_{\text{At}} \) mobilize the transfer of the nonself-transmissible IncQ plasmid pML122 into agrobacterial recipients. I introduced pML122 into the above strains to assay for the capacity of the Rickettsial \( \text{virD4} \) genes to mobilize IncQ transfer. As shown in Figure 3.2B, A348 and Mx355 expressing \( \text{virD4}_{\text{At}} \) mobilized IncQ plasmid transfer at frequencies of \( \sim 10^{-4} \) transconjugants per donor (Tc's/D). By contrast, Mx355 expressing the Rickettsial \( \text{virD4} \) genes failed to mobilize transfer of the IncQ plasmid. Taken together, these findings suggest that the Rickettsial \( \text{virD4} \) genes do not functionally substitute for \( \text{virD4}_{\text{At}} \) for transfer of \( \text{A. tumefaciens} \) DNA or protein substrates.

Protein levels of the VirD4 homologs could not be assessed at this time, as antibodies were not available and previous attempts to attach an N- or C-terminal tag to VirD4_{At} have rendered it non-functional. Polyclonal antibodies against VirD4_{At} were tested for cross-reactivity with the VirD4 homologs, however detection of the homologs by western blot was not successful. Therefore, it is unknown if lack of complementation by the Rickettsial \( \text{virD4} \) genes is due to problems in gene expression, instabilities of the synthesized T4CPs, or the inabilities of the T4CPs to recognize the \( \text{A. tumefaciens} \) substrates.
Figure 3.2 Tests for complementation of the *A. tumefaciens* Δ*virD4* mutation by *virD4* genes from Rickettsial species.

A. T-DNA and effector protein transfer as monitored by virulence on wounded *Kalanchoe* leaves. Representative leaf showing tumor induction of wild-type (WT) strain A348, Δ*virD4* mutant Mx355, and Mx355 expressing *virD4At*, *virD4Ap*, or *virD4Wp*.

B. DNA substrate transfer frequency and virulence data. Conjugative transfer frequency of the IncQ plasmid, pML122, is presented as transconjugants per donor cell (black bars) with standard deviations indicated. Virulence on plant leaves (white bars), as measured on a scale of three pluses (as incited by the WT strain), two and one pluses for attenuated virulence, minus sign, avirulent. * IncQ plasmid transfer is statistically significant when compared to WT (P<0.0001)
Evidence for expression of *virD4* homologs in *A. tumefaciens* A348

To examine the possibility that the Rickettsial VirD4 T4CPs interact nonproductively with the *A. tumefaciens* VirB channel or VirB/VirD4 substrates, I tested whether A348 strains carrying the *virD4* expression plasmids displayed altered substrate transfer as monitored by plant virulence assays and IncQ plasmid mobilization. As shown in Figure 3.3, A348 carrying a plasmid expressing *virD4*<sub>At</sub> displayed a slight reduction in virulence as shown by slightly smaller tumors than WT A348. A348 carrying the *virD4*<sub>Wp</sub> expression plasmid also showed a slight attenuation in virulence. However, A348 carrying the *virD4*<sub>Ap</sub> expression plasmid was highly attenuated for virulence, consistent with the idea that VirD4<sub>Ap</sub> somehow poisons substrate engagement with or transfer through the VirB/VirD4 T4SS.

A348 strains carrying the *virD4* expression plasmids were also assayed for mobilization of the IncQ plasmid pML122. As shown in Figure 3.3, trans-expression of all of the *virD4* homologs including *virD4*<sub>At</sub> resulted in a slight reduction of ~1 order of magnitude in IncQ plasmid transfer compared with WT A348, although this was not a statistically significant decrease from wild-type transfer. All of the trans-expressed *virD4* genes were from an IncP replicon, whose copy number is estimated at ~5 times that of the Ti plasmid, which carries the *virB* and *virD4* genes as well as the oncogenic T-DNA and effector genes. The observed dominant negative effects accompanying trans-expression of the *virD4* genes on virulence and IncQ plasmid transfer could arise from overproduction of the VirD4 T4CPs. The overproduced T4CPs might interact nonproductively with the VirB machinery, creating nonfunctional secretion channels, or they might sequester DNA or protein substrates and prevent their engagement with the native VirB/VirD4 machine.
Figure 3.3 Tests for dominant negative effects of virD4 genes from Rickettsial species expressed in wild-type A. tumefaciens strain A348

DNA substrate transfer frequency and virulence data. Conjugative transfer frequency of the IncQ plasmid, pML122, is presented as transconjugants per donor cell (black bars) with standard deviations indicated. Virulence on plant leaves (white bars), as measured on a scale of three pluses (as incited by the WT strain), two and one pluses for attenuated virulence, minus sign, avirulent. WT, A348; WT strain expressing the virD4 genes indicated. Expression of virD4 genes in trans did not cause a statistically significant decrease in IncQ transfer compared to wild-type (P>0.05).
The C-terminal extension (CTE) of VirD4At is important for virulence but not for IncQ transfer

The VirD4 family members share generally high sequence conservation among their nucleotide-binding domains, but are considerably more variable among their TMD’s, AAD’s, and CTE’s. The TMD’s are implicated in mediating interactions with transfer channels [9], and their sequence variation could be attributable to an evolved specificity in interaction with the cognate T4SS channel. The variations in the AAD’s and/or CTE’s however, might be important for evolved specificity in engagement of effector proteins with cognate T4SS channels. As mentioned above, AAD’s within the Rickettsial species are quite conserved, suggesting that if this domain contributes to substrate docking, substrates are likely very similar between these species.

Sequence analysis of several T4SSs revealed that T4SSs dedicated to translocation of protein effectors generally possess CTE’s of variable length and amino acid composition; whereas systems dedicated to conjugative DNA transfer typically lack CTE’s. Among the Rickettsial homologs in this study, the VirD4 subunits possess variable C-terminal domains from 21-167 residues that are highly enriched in acidic residues (Figure 1.4), e.g., VirD4Ap CTE has 47% Glu/Asp residues. In A. tumefaciens, the VirB/VirD4 T4SS functions both in DNA and protein transfer. In this system, the CTE is 104 residues with 16% being acidic. In well-characterized E. coli conjugative plasmids, R388 and pKM101, the VirD4 orthologs lack CTE’s altogether.

Given these findings, I hypothesized that the C-termini of the VirD4 subunits contribute to substrate specificity either by a) acting as a docking point for effectors prior to translocation or b) blocking DNA translocation thereby only allowing protein substrates to be translocated. To test this hypothesis, I created a virD4 allele encoding VirD4AtΔ553, which is deleted of its CTE, and assayed for functional complementation of a virD4 null mutation. As shown in Figure 3.4, Mx355 producing full length VirD4At incites formation of plant tumors.
and transfers the IncQ plasmid at WT frequencies, whereas Mx355 producing VirD4₅₅₃₅₅₃ was avirulent on plants but displayed near wild-type levels of IncQ plasmid transfer. These findings suggest that the CTE is necessary for VirD4 to interact productively with A. tumefaciens virulence factors, but is dispensable for interaction and translocation of the promiscuous IncQ plasmid. Further studies of VirD4’s CTE are described in Chapter 5.
Figure 3.4. Contribution of the C-terminal extension of *A. tumefaciens* VirD4 to substrate transfer

DNA substrate transfer frequency and virulence data. Conjugative transfer frequency of the IncQ plasmid, pML122, is presented as transconjugants per donor cell (black bars) with standard deviations indicated. Virulence on plant leaves (white bars), as measured on a scale of three pluses (as incited by the WT strain), two and one pluses for attenuated virulence, minus sign, avirulent. WT, A348; ΔvirD4 mutant, Mx355; virD4 expressing wild-type virD4 or virD4Δ553. * IncQ plasmid transfer is statistically significant when compared to WT (P<0.0001)
Discussion

Transfer of effector molecules using VirD4 homologs in the *Agrobacterium* type IV secretion system

The use of chimeric and surrogate T4SSs has been established in a variety of contexts [9, 20, 22, 23]. This study was aimed at determining whether VirD4 homologs assemble with the *A. tumefaciens* VirB subunits to form functional chimeric T4SSs. I expressed *virD4* genes from two Rickettsial homologs, *A. phagocytophilum* and *W. pipientis*, in a *virD4*-mutant background and determined that they were unable to form a functional chimeric T4SS, at least with respect to the translocation of *A. tumefaciens* effectors or the promiscuous IncQ plasmid pML122. This is not entirely surprising, as I was assaying for translocation of non-cognate substrates, and at this time there is no evidence that Rickettsial species are capable of interbacterial conjugation or mobilization of IncQ plasmids.

I also expressed the Rickettsial *virD4* genes in the *A. tumefaciens* strain A348 to genetically test for dominant effects of these genes over *virD4*<sub>At</sub>. I determined that trans-expression of the wild-type *virD4*<sub>At</sub> gene as well as the *virD4*<sub>Ap</sub> and *virD4*<sub>Wp</sub> genes conferred negative dominance both with respect to plant tumorigenesis and IncQ plasmid transfer, although the latter was found not to be statistically significant. To account for negative consequence of *virD4*<sub>At</sub> trans-expression, I propose that synthesis of VirD4 from the higher copy number IncP replicon compared with the Ti plasmid results in elevated copies of the T4CP which interferes with assembly of functional VirB/VirD4 T4SSs or binds available substrates preventing their interaction with functional VirB/VirD4 T4SSs. The finding that the Rickettsial *virD4* genes, in particular *virD4*<sub>Ap</sub>, also exerted negative dominance, suggests first that the Rickettsial T4CPs are synthesized and second that they similarly ‘poison’ substrate transfer through the VirB/VirD4 T4SS.
At this time, it is not known how the \textit{trans}-expressed \textit{virD4} genes negatively affect substrate transfer through the VirB/VirD4 T4SS. It is possible that expression of the Rickettsial \textit{virD4} genes from a single copy plasmid or integration into the Ti plasmid would enable functional complementation of the \textit{virD4\textsubscript{At}} mutation with respect to translocation of the \textit{A. tumefaciens} effectors or IncQ plasmids. It is also possible that the Rickettsial VirD4 T4CPs in fact do interact productively with the \textit{A. tumefaciens} VirB channel subunits, but only for translocation of Rickettsial substrates. Although this remains to be tested, such studies using \textit{A. tumefaciens} as a surrogate host are complicated because of the difficulty of genetic manipulation of this bacterium compared to other commonly used model bacterial species such as \textit{E. coli}. In Chapter 5, I present results of my studies using \textit{E. coli} as a surrogate host in which I demonstrate functionality of chimeric VirD4 T4CPs composed of a TMD from an \textit{E. coli} plasmid-encoded T4CP and SDs from \textit{A. tumefaciens} or Rickettsial T4CPs. These studies demonstrate a proof-of-principle for the use of chimeric T4CPs, and warrant future investigations testing whether native or chimeric Rickettsial T4CPs can mediate translocation of Rickettsial substrates through the \textit{A. tumefaciens} VirB channel.

\textbf{The CTE of \textit{VirD4\textsubscript{At}} is required for translocation of some, but not all, substrates}

There is already experimental support for the idea that T4CP CTEs contribute to substrate engagement. In the \textit{E. coli} F-plasmid transfer system, the TraD T4CP carries a CTE and this CTE has been shown to bind the TraM accessory factor [12]. TraM is an essential component, along with the TraI relaxase, of the relaxosome that assembles at the F-plasmid origin-of-transfer (\textit{oriT}) sequence. Consequently, the TraD CTE-TraM interaction serves to physically couple the relaxosome with the transfer channel prior to plasmid transfer. In addition, although the F-plasmid transfer system mobilizes promiscuous IncQ plasmids, it does so only at very low frequencies of $<10^{-6}$ Tc's/D, whereas this T4SS transfers the F-plasmid at high frequencies exceeding $10^{1}$ Tc/D. Deletion of TraD's CTE,
however, reverses this pattern, so that the T4SS instead translocates the F-plasmid at low frequencies and the IncQ plasmids at comparatively high frequencies. Therefore, TraD’s CTE confers substrate specific docking of the F-plasmid substrate and blocks IncQ plasmid transfer.

In this study, phenotypic analysis of the VirD4ΔΔ553 derivative deleted of the CTE confirmed the importance of the CTE for translocation of A. tumefaciens substrates required for plant virulence. Yet, this construct supported IncQ plasmid transfer to recipient agrobacteria at near wild-type levels, indicating that the CTE is not required for recruitment or translocation of the IncQ plasmid. These findings support a general model in which the promiscuous IncQ plasmids have evolved to interact with domains or motifs that are conserved among members of the T4CP superfamily, e.g., possibly the NBD or AAD, whereas the CTE contributes to engagement of substrates that are dedicated for translocation through a given T4SS. In Chapter 6, I further analyze the effect of deleting VirD4’s CTE on DNA and protein transfer using an E. coli chimeric T4SS, and results further support the idea that the CTE functions as a substrate specificity domain.

After completing these studies with A. tumefaciens as a surrogate host, I determined that the best strategy for increased translocation efficiency would be to assay for translocation of native substrates for each of the different VirD4 homologs. Additionally, I wanted to develop a surrogate host system that was more amenable to genetic manipulation, and a system in which substrate transfer could be assessed more rapidly and quantitatively than with the A. tumefaciens virulence assay. It is also noteworthy that in A. tumefaciens, mobilization of IncQ plasmids through the VirB/VirD4 system occurs at low frequencies (e.g., the frequency of IncQ plasmid transfer is $10^{-4}$ Tc’s/D after a five-day mating). By contrast, E. coli conjugation systems such as the pKM101-encoded system which bears strong overall similarity to the A. tumefaciens VirB/VirD4 system, transfers IncQ plasmids and other plasmid substrates at considerably higher frequencies in 1 - 2 hour
matings. For these reasons, I focused the remainder of my studies on development of *E. coli* as a surrogate host to characterize the functionality of chimeric T4SSs and monitor translocation of heterologous T4SS effector proteins.
Chapter 4. The Type IV Secretion System of *Escherichia coli* pKM101 and Related Chimeric Systems Mediate Translocation of Heterologous Protein Substrates
Introduction

The broad-host range conjugative IncN plasmid pKM101, a derivative of R46, has been studied extensively as an efficient and easily manipulated T4SS [43]. This plasmid conjugatively transfers from *E. coli* donors to recipients in 1 h filter matings at efficiencies \( \geq 10^{-1} \) transconjugants per donor (Tc’s/D) [43]. By contrast, the *A. tumefaciens* VirB/VirD4 system delivers IncQ plasmid substrates to agrobacterial recipients at frequencies of \( 10^{-4} - 10^{-5} \) Tc’s/D in 4-5 day filter matings (Figure 3.2). The highly efficient pKM101 transfer system, coupled with the ease of genetic manipulation of *E. coli*, makes this an ideal system for detailed mechanistic studies of type IV secretion in Gram-negative bacteria.

Transposon mutational analyses in the 1980’s identified the regions of pKM101 required for conjugative DNA transfer [43, 63]. More recently, the plasmid was sequenced, enabling precise definition of the transfer (*tra*) genes and their putative functions [64]. One *tra* gene cluster, closely resembling the *A. tumefaciens virB* genes both in composition and gene order, codes for the transfer channel. The second cluster, resembling the *A. tumefaciens virD* operon, codes for the TraJ T4CP and the Dtr processing factors.

While the pKM101 T4SS efficiently transfers pKM101 and IncQ plasmid substrates to *E. coli* recipient cells, no studies have yet examined whether this system also translocates protein substrates. Here, I tested whether the native pKM101 system mediates transfer of known protein substrates of the *A. tumefaciens* VirB/VirD4 T4SS as well as known or putative substrates of Rickettsial VirB T4SSs. The *A. tumefaciens* substrates included three well-characterized effectors, VirE2, VirE3, and VirF, which are delivered to plant cells during the course of infection. The Rickettsial substrates included *A. phagocytophilum* Ats-1, which is translocated into mammalian cells during infection, and several other proteins whose T4SS-mediated translocation has been postulated on the basis of sequence composition or phenotypes detected from production of the Rickettsial proteins in yeast.
A second goal of these studies was to test whether chimeric systems derived from the pKM101 T4SS function to mediate transfer of DNA or protein substrates. Specifically, I substituted the T4CPs from *A. tumefaciens* or Rickettsial T4SSs for pKM101-encoded TraJ and tested whether the resulting chimeric systems would translocate the IncQ plasmid or the respective *A. tumefaciens* or Rickettsial protein substrates. Earlier studies established a precedent for the functionality of chimeric T4SSs composed of a T4CP from one system and the channel subunits from a second [23, 61]. For example, substitution of the R388 T4CP TrwB, with TraG from plasmid RP4, was shown to restore mobilization of RSF1010 by a mutant of R388 defective for substrate transfer [23]. It is noteworthy that each of these chimeric systems was assayed only for their capacity to translocate DNA substrates. My studies were the first to test whether chimeric conjugation machines are capable of translocating protein substrates.

**Results**

**The pKM101 conjugative T4SS transfers non-cognate proteins**

I assayed for the capacity of the pKM101 T4SS to translocate heterologous protein substrates with the Cre recombinase Reporter Assay for Translocation (CRAFT). This assay has been used previously to demonstrate effector protein translocation through the *A. tumefaciens* VirB/VirD4 T4SS to yeast or plant cells, and the *Legionella pneumophila* Dot/Icm T4SS to other bacterial cells [33, 65, 66]. As shown in Figure 4.1, I expressed genes encoding the Cre-effector fusions from an arabinose inducible P\textsubscript{BAD} promoter in *E.coli* donor strain MS411. The recipient, CSH26Cm::LTL (pBBR1MCS2-Gen'), contains the cat gene, interrupted by a *tet* gene that is flanked by *loxP* sites. Upon translocation of the Cre-effector fusion protein into the recipient cell, Cre recombination at the *loxP* sites excises the *tet* gene, resulting in a Cam\textsuperscript{r}, Tet\textsuperscript{r} phenotype. In these studies, recombinants were routinely
tested for Tet\textsuperscript{S} by patching the colonies onto an LB plate containing 20 µg/ml of tetracycline. On numerous occasions, colonies displayed a partial Tet\textsuperscript{S} phenotype where individual colonies would arise within the patch, as opposed to solid growth of the patch, which would indicate Tet\textsuperscript{R}. We postulate that this partial sensitivity is due to Cre-mediated recombination occurring during a point in cell division where two alleles are present within the cell and only one allele is recombined.

Donor cells expressed genes for the Cre-effector fusion from pBAD33, which is also Cam\textsuperscript{R}. Therefore, to discriminate recipients in which the lox cassette had been excised from donor cells, it was necessary to introduce additional resistance markers into the recipient. Initially, I used the broad-host range vector, pBBR1MCS2-Gen\textsuperscript{R}, in the recipient and selected for Cre-effector protein transfer by plating the mating mix on chloramphenicol and gentamicin. However, this approach proved problematic for the following reason. In these matings, donor cells potentially translocate not only the Cre-effector protein, but also pKM101 at high efficiencies, to recipient cells. Once in the recipient cell, pKM101 elaborates a T4SS, which I found to be capable of mobilizing the transfer of pBBR1MCS2-Gen\textsuperscript{R} back into donor cells, thus enabling donor cells to grow on Cam- and Gen-containing plates.

pBBR1MCS2-Gen\textsuperscript{R} has a mobilization region from an IncP plasmid and no studies to date have shown that pKM101 is capable of recognizing and translocating a mobile element with an IncP origin-of-transfer [67, 68]. This discovery, while interesting, necessitated introduction of alternative resistance markers into the recipient strain for selection of lox cassette excisants. A suitable recipient was generated through selection of a rifampin resistant (Rif\textsuperscript{R}) recipient by plating dense cell cultures on LB plates containing rifampicin followed by introduction of the non-mobilizable plasmid pUC4K, which codes for resistance to carbenicillin and kanamycin. The Kan\textsuperscript{R}/Crb\textsuperscript{R}/Rif\textsuperscript{R} strains were tested for their capacity to function as recipients for uptake of pKM101-Spc\textsuperscript{R} as well pML122. While the use of the Rif\textsuperscript{R}
recipient did not change the overall conclusions drawn from the experiments, it did provide less background and therefore cleaner data.

I constructed plasmids expressing genes for the Cre-effector fusion proteins from the P_{BAD} promoter, and a plasmid encoding Cre alone as a control. As mentioned above, I tested for Cre translocation mediated by effectors from \textit{A. tumefaciens} (VirE2, VirE3, VirF) and \textit{A. phagocytophilum} (Ats-1, Ats-1 CTD – the C-terminal 100 residues of Ats-1 that presumptively carries the C-terminal signal sequence). Transfer of the \textit{A. tumefaciens} effectors has been shown by CRAfT [5, 65], and Ats-1 was initially identified as a VirD4_{Ap} binding partner with a bacterial two-hybrid system and subsequently identified in the cytoplasms of infected mammalian cells [46]. I tested for the ability of the C-terminus of Ats-1 to mediate Cre translocation because it bears features such as clusters of basic amino acids and a net positive charge identified among translocation signals carried by the \textit{A. tumefaciens} T4SS effectors [46]. Additionally, I tested for Cre translocation by fusion to \textit{A. phagocytophilum} Aph_0111. Aph_0111 is a putative substrate provided by our collaborator, Dr. Jason Carlyon, and is annotated as an uridylate kinase (personal communication). I also assayed for translocation of several \textit{Wolbachia} putative effectors provided by a second collaborator, Dr. Irene Newton (personal communication). These effectors were identified in a high-throughput screen for proteins that induce phenotypic changes such as cell death or morphological changes when produced in yeast cells. The candidate effectors include the ankyrin repeat containing protein, WD0636, and two annotated hypothetical proteins WD0811 and WD0830.

I first tested for production of the Cre-effector proteins in \textit{E. coli} donor cells. Cells were induced for synthesis of the Cre-effector proteins as described in the Materials and Methods, and total cellular proteins were analyzed for accumulation of the fusion proteins by SDS-PAGE, western transfer, and development of the immunoblots with commercially available anti-Cre antibodies (Novus Biologicals). As shown in Figure 4.2, most of the
fusion proteins were detected in the cell lysates, with the exception of WD0830. Several of
the fusion proteins migrated aberrantly in protein gels, as judged from their predicted
molecular sizes. This could be due to intrinsically stable folds or extended structures that
impeded mobility of the fusion protein through the polyacrylamide gels. Regardless, by
immunostaining, I gained evidence that most of the Cre-effector fusions accumulated to
detectable levels upon arabinose induction of the *E. coli* host cells.
Figure 4.1 Interbacterial protein translocation by the pKM101 T4SS

The Cre-Recombinase Assay for Translocation (CRAfT) was used to monitor interbacterial protein transfer. Translocation of the Cre-effector fusion protein, from a donor bacterial cell containing the pKM101 encoded T4SS is measured by excision of a gene conferring tetracycline resistance (Tet<sup>r</sup>) and a transcriptional terminator (term) that is flanked by loxP sites on the chromosome of *E. coli* recipient strain CSH26Cm::LTL. Bacteria harboring the intact reporter are Tet<sup>r</sup> and Cam<sup>s</sup>. Cre-mediated recombination at the loxP sites allows full read-through of the cat gene, resulting in a Tet<sup>s</sup>, Cam<sup>r</sup> phenotype. Plasmids shown are pKM101, pBAD33-Cre-X, where X is a translocated effector protein; and pBBR1MCS2-Gen<sup>r</sup> (included in the recipient to aid in selection of recombinants)
Figure 4.2 Detection of Cre-effector fusion proteins in *E. coli* cells

Western blot analysis of Cre-effector fusion proteins produced in *E. coli* donor cells without and with induction using 0.2% arabinose were detected by immunostaining of blots with anti-Cre antibodies and anti-HRP secondary antibodies as described in the Materials and Methods. Putative fusion proteins are shown by white arrows and Cre is indicated by a black arrow.

A. Detection of Cre only and Cre fused to the *A. tumefaciens* effector proteins shown.

B. Detection of Cre fused to the putative *A. phagocytophilum* effectors indicated. CTD, Cre fused to the C-terminal domain (CTD) of Ats-1 comprised of the last 100 residues; the gene encoding the putative Aph_0111 effector was provided by Jason Carlyon.

C. Detection of Cre fused to putative *Wolbachia* effector proteins; the WD effector genes were provided by Irene Newton.
Interestingly, all of the tested Cre-effector fusion proteins were translocated to recipient cells at detectable frequencies, as monitored by CRAfT (Figure 4.3). Effectors Ats-1 CTD and Aph_0111 transferred below the threshold with the original recipient (Figure 4.3A), but transferred into the Rifrecipient above the Cre-alone control at statistically significant levels (Figure 4.3B). This further emphasizes the superiority of the Rifrecipient in accurately distinguishing genuine effector translocation. Translocation required arabinose induction of the Cre-effector proteins and, furthermore, the donor strains and the recipient strain failed to form colonies on plates selective for the recombinants. The recipient-only control was especially important, since it has been reported that lox cassettes can spontaneously excise as a result of homologous recombination. I did observe that sequential subculturing of the recipient strain, CSH26Cm::LTL, from a frozen stock, occasionally yielded Cam’, Tet’ colonies, presumably as a result of homologous recombination at the lox sites. However, by routinely growing recipients freshly from the -80°C stock, growth of the recipient-only on plates selective for recombinants was rarely detected.

As reported previously for other T4SSs [33, 65], I observed Cre translocation at very low frequencies through the pKM101 T4SS independent of fusion to an effector protein. However, Cre transfer was detected only in a few of the many repetitions of these experiments, and in these cases only at frequencies in the range of \( \leq 10^{-7} \) Recombinants per donor (Rc’s/D) when using the original recipient and \( \sim 10^{-8} \) Rc/D when using the Rifrecipient. This low frequency of transfer establishes a threshold above which translocation of Cre was considered to be mediated by the fused effector protein. Finally, as discussed below, I collaborated with another graduate student, Jay Gordon, to generate a nonpolar traJ deletion in pKM101. As expected, donor cells carrying pKM101ΔtraJ failed to conjugatively transfer DNA substrates, and also failed to translocate the Cre-effector
proteins to recipient cells at detectable frequencies (Figure 4.3A). These findings established the requirement for an intact pKM101-encoded T4SS for translocation.

The tested effector proteins mediated Cre transfer at frequencies ranging from $1.65 \times 10^{-6}$ to $8 \times 10^{-9}$ recombinants per donor (Rc's/D) (Figure 4.3B). The *A. phagocytophilum* effector Ats-1 mediated Cre transfer at the highest observed frequency of $1.65 \times 10^{-6}$ Rc's/D, the *A. tumefaciens* effector VirE2 mediated Cre transfer at the lowest observed frequency, and most of the other tested effectors mediated Cre transfer at frequencies of $10^{-7} – 10^{-8}$ Rc's/D. This variation in transfer frequency could reflect biologically relevant differences in translocation efficiencies of the different effectors, or be attributable to differences in stabilities of the fusion proteins or their capacities to engage productively with the heterologous pKM101-encoded T4SS. Additionally, it is important to note that Nathan Rosenthal performed CRAfT experiments using the Rifr recipient, whereas I performed the experiments with the original recipient. While Dr. Christie or myself closely supervised Mr. Rosenthal and verified his data, any deviations from protocol may have further added to variance within and between experiments.
Figure 4.3 Translocation of heterologous T4SS substrates through the pKM101 transfer system.

Known and putative effector genes were fused to cre, and pKM101 dependent protein translocation into an E. coli recipient was determined by monitoring Cre-mediated excision of a Tet’ gene as described in the Materials and Methods.

A. Translocation of Cre-effector fusion proteins into recipient CSH26Cm::LTL containing the plasmid pBBR1MCS2-Gen’. Black bars, transfer by wild type pKM101 with mean excision frequency and standard deviations shown; White bar, transfer by pKM101ΔtraJ. Experiments were performed at least 3 times. The dashed line indicates the threshold of Cre-only background transfer. ** Excision frequency is statistically significant from the Cre-only control (P<0.0001) * Excision frequency is statistically significant from the Cre-only control (P<0.05)

B. Translocation of Cre-effector fusion proteins by the wild-type pKM101 system into the recipient CSH26Cm::LTL, rif’(pUC4K). Experiments were performed at least 3 times. Cre alone transfer was undetectable using this recipient due to elimination of any retroactive transfer. ** Excision frequency is statistically significant from the Cre-only control (P<0.0001)
**trans-expression of traJ confers elevated substrate transfer**

As mentioned above, to confirm that the pKM101 T4SS was responsible for mediating protein translocation, I constructed and tested the effect of a *traJ* deletion on substrate transfer. *traJ* encodes the T4CP, which functions to link substrates with the pKM101 transfer channel. In collaboration with another graduate student, Jay Gordon, a precisely deletion of *traJ* was generated by recombineering [51, 53]. This technique exploits the homologous Red recombination system of bacteriophage λ, which can catalyze recombination between a linear DNA fragment and a sequence of interest with as few as 25 bp’s of homology. Once the *traJ* deletion mutation (Δ*traJ*) was confirmed, I first assayed for effects on DNA substrate transfer. As shown in Figure 4.4, the pKM101Δ*traJ* donor strain failed to conjugatively transfer the mutant plasmid and an isogenic strain also carrying the IncQ plasmid, pML122, similarly failed to mobilize IncQ plasmid transfer at detectable frequencies. To ensure that the Δ*traJ* mutation did not exert polar effects on expression of downstream genes, I expressed the wild-type *traJ* gene from the P_{BAD} promoter carried on plasmid pBAD24-Kan’r. The complemented donor strains induced for *traJ* expression efficiently transferred both the pKM101Δ*traJ* and pML122 plasmids, whereas uninduced cells transferred these plasmids at detectable but very low frequencies (data not shown).

Recently, Dr. N. Whitaker showed that arabinose induction was required for detection of His-tagged TraJ in membrane extracts of these donor strains by western blotting and immunostaining with anti-His antibodies, and that presence of the N-terminal His-tag does not hinder transfer of the pKM101 derivative or pML122 upon induction (data not shown).

Taken together, the above findings establish that synthesis of TraJ is essential for plasmid transfer through the pKM101 T4SS. Interestingly, arabinose-induction of *traJ* from pBAD24-Kan’r consistently yielded transfer of both pKM101Δ*traJ* and pML122 at frequencies of ~1 order of magnitude higher than observed with wild-type pKM101 (Figure 4.4). While I was unable to directly compare steady-state levels of TraJ produced from the P_{BAD} promoter
and its native promoter, I suspect that synthesis from the strong P_{BAD} promoter yielded higher amounts of TraJ than from pKM101. If so, synthesis of the TraJ substrate receptor for the pKM101 T4SS might be a rate-limiting step in the process of conjugative DNA transfer.

I next tested whether trans-expression of traJ in a pKM101ΔtraJ donor strain supported transfer of the Cre-effector fusion proteins. Interestingly, several of the fusion proteins transferred at considerably higher levels than observed with the native pKM101 system (Figure 4.5). Most strikingly, donor strains translocated the WD0811 and WD0830 fusion proteins at frequencies nearly $10^{-5}$ and $10^{-4}$ Rc’s/D, respectively, as monitored by CRAfT. Donor strains producing the other fusion proteins also exhibited elevated transfer frequencies compared with the wild-type system. Additionally, when Nathan Rosenthal used the Rif$^\text{r}$ recipient in recent experiments, translocation of most effectors was detected at approximately $10^{-6}$ to $10^{-4}$ Rc’s/D with transfer of Cre alone reduced to $1.0 \times 10^{-8}$ Rc’s/D. Of further interest, translocation of Cre alone occurred at a frequency slightly above that observed with the wild-type system with both recipients. Nevertheless, the transfer frequency of Cre alone remained considerably less than those of the Cre-effector fusion proteins. Based on these findings, I propose that trans-expression of traJ from the strong P_{BAD} promoter on multicopy pBAD24-Kan$^\text{r}$ confers elevated transfer of both DNA and protein substrates through the pKM101 transfer channel.

Finally, it should be noted that although I detected translocation of the Cre-VirE2 fusion protein, previous work has shown that VirE2 requires its secretion chaperone VirE1 for translocation through the A. tumefaciens VirB/VirD4 T4SS. VirE1 is not required for substrate docking with VirD4 but instead prevents VirE2 from self-aggregating. In future studies, it will be of interest to determine if VirE1 coproduction confers Cre-VirE2 translocation in the pKM101 system at higher frequencies than observed in the present studies.
Figure 4.4  Effects of a pKM101 traJ deletion and complementation with heterologous
virD4 genes on DNA substrate transfer.

* Substrate transfer was statistically significant from wild-type.

E. coli donor cells carrying pKM101 or pKM101ΔtraJ lacking or expressing the genes
indicated were tested for pKM101 transfer and mobilization of the IncQ plasmid, pML122, to
E. coli recipients. Transfer frequencies are reported as transconjugants per donor.
Experiments were repeated at least 2 times in duplicate and the mean transfer frequencies
with standard deviations are shown. WT, pKM101; ΔtraJ, pKM101 deleted of traJ, and
ΔtraJ expressing traJ and virD4 homologs. * Substrate transfer was statistically significant
from wild-type.
**Figure 4.5 Transfer of heterologous T4SS substrates through the *E. coli* pKM101 transfer system.** Protein transfer was performed as described in *Materials and Methods* by using *E. coli* containing pKM101ΔtraJ and plasmids expressing cre fusions and traJ. Cre only was used as a negative control, with the dashed line indicating the threshold of Cre background transfer.

A. Translocation of Cre-effector fusion proteins into recipient CSH26Cm::LTL containing the plasmid pBBR1MCS2-Gen’. Experiments were repeated at least 2 times in duplicate with the mean excision frequency and standard deviations shown.

* Excision frequency is statistically significant from the Cre alone control (P<0.05)

B. Translocation of Cre-effector fusion proteins by the pKM101 system expressing traJ from the multicopy plasmid, pBAD24-Kan’, into the recipient CSH26Cm::LTL, rif’(pUC4K). Experiments were repeated at least 2 times. ** Excision frequency is statistically significant from the Cre alone control (P<0.0001)
Do heterologous T4CPs functionally substitute for TraJ in mediating DNA transfer through the pKM101 transfer system?

In Chapter 3, I determined that the Rickettsia VirD4 homologs did not functionally substitute for VirD4A1 in A. *tumefaciens*. Although these T4CPs might be unstable or fail to interact productively with the *A. tumefaciens* VirB channel, it is also possible that the *A. tumefaciens* VirB/VirD4 T4SS does not mediate transfer of substrates to other agrobacteria at frequencies sufficient for detecting functionality of the swapped T4CPs. By contrast, the highly efficient pKM101 system, supporting plasmid transfer at frequencies exceeding 1 Tc/D, might be sufficient for detection of low-frequency substrate transfer. To test this idea, I asked whether the VirD4 T4CPs from *A. tumefaciens* or the Rickettsial species functionally substituted for TraJ.

I expressed the *virD4* genes from *A. tumefaciens* and *W. pipientis* from a *P_{BAD}* promoter carried on pBAD24 in donor strains carrying pKM101Δ*traJ*. Perhaps not surprisingly, I was not able to detect complementation of the Δ*traJ* mutation as monitored by transfer of pKM101Δ*traJ* (Figure 4.4). I next tested for the capacity of isogenic donors for mobilization of the IncQ plasmid pML122, but again I was unable to detect plasmid transfer (Figure 4.4). It is important to note that, although there is no evidence for IncQ plasmid transfer by the native T4SSs carried by *W. pipientis*, the *A. tumefaciens* VirB/VirD4 T4SS mobilizes the transfer of the IncQ plasmid to agrobacterial recipients. In view of these findings, I envision two possibilities for the failure of the heterologous T4CPs to substitute for TraJ. First, these T4CPs might be unstable in the *E. coli* donor cells. Second, they might fail to interact productively with the pKM101-encoded transfer channel. I examine the second possibility in more detail in Chapter 5 of this thesis. To address the first possibility, Dr. N. Whitaker is currently adding His-tags to the heterologous T4CPs to evaluate their steady-state abundance in arabinose-induced donor cells by western blot analysis.
In view of my finding above that *trans*-expression of the Rickettsial T4CPs in wild-type *A. tumefaciens* conferred diminished substrate transfer through the VirB/VirD4 T4SS, I tested for poisoning interactions of the heterologous T4CP on functionality of the native pKM101 T4SS. Interestingly, production of VirD4 subunits from *A. tumefaciens* or the Rickettsial species did not abrogate transfer of pKM101ΔtraJ to recipients (Figure 4.6). These findings are consistent with the proposal that the heterologous T4CPs fail to interact with the pKM101 T4SS.
Figure 4.6. **Effects of trans-expressed traJ and virD4 homologs on wild type pKM101 self-transfer**

*E. coli* donor cells carrying pKM101 and expressing the genes indicated were tested for pKM101 transfer to *E. coli* recipients. Transfer frequencies are reported as transconjugants per donor. Experiments were repeated at least 3 times in duplicate and the mean transfer frequencies with standard deviations are shown. WT, pKM101 lacking (−) or expressing the *virD4* homologs shown.
Do heterologous T4CPs functionally substitute for TraJ in mediating protein transfer through the pKM101 transfer system?

To further assess the functionality of the chimeric T4SSs, I assayed for translocation of effector proteins. Specifically, I tested for translocation of the Cre::Ats-1 fusion protein by donor strains producing the VirD4 T4CPs from A. tumefaciens and the Rickettsial homologs. Ats-1 was chosen as a test substrate because it consistently transferred at high levels without much variation, whereas other Cre-effector fusions showed greater inconsistency between experiments. The chimeric T4SSs were also assayed for transfer of Cre alone as a control.

As shown in Figure 4.7, pKM101ΔtraJ-carrying donor cells engineered to produce the VirD4_{Ap} T4CP transferred the Cre::Ats-1 fusion protein at frequencies only slightly lower than the isogenic donors producing native TraJ. This donor did not translocate Cre alone at detectable frequencies. The VirD4_{Ap} T4CP is thus capable of engaging productively with the pKM101-encoded T4SS to mediate translocation of the cognate Ats-1 effector to E. coli recipient cells. These findings established that Ats-1 engages productively with two heterologous T4CPs, TraJ and VirD4_{Ap}, in E. coli. I next tested whether the donor cells producing other T4CPs, including those from A. tumefaciens, W. pipientis, and R. rickettsii mediated Ats-1 transfer (Figure 4.7). These donors failed to transfer the Ats-1 fusion protein at detectable levels.
Figure 4.7 Cre-mediated transfer of *Anaplasma* effector protein Ats-1 through pKM101-based chimeric T4SSs

Transfer of *Anaplasma* T4SS substrate Ats-1 between bacterial cells. Ats-1 Transfer of Cre fused to Ats-1 by donor *E. coli* cells carrying pKM101ΔtraJ and expressing *virD4* genes from *A. tumefaciens* and *Rickettsial* species was monitored by Cre-mediated excision of a Tet^R^ gene as described in Materials and Methods. Cre-only was used as a negative control. Experiments were repeated at least 2 times with mean excision frequencies and standard deviations shown. * Excision frequency was statistically significant from the Cre-only control (P<0.0001)
Discussion

The TraJ T4CP is essential and rating limiting for pKM101 and IncQ plasmid transfer.

The conjugative plasmid pKM101 has long been known to transfer itself between *E. coli* cells via a T4SS similar to that of the VirB/VirD4 system of *A. tumefaciens*. This transfer is mediated by interactions of VirD4-like TraJ, with the relaxasome components [43, 64, 69]. This was confirmed with the creation of a clean deletion mutant of *traJ* in pKM101, which was found to completely abolish conjugal transfer of the plasmid. Importantly, this mutant could be complemented with *trans*-expressed *traJ*, indicating the deletion had no polar effects on the remainder of the Tra operon. In addition to conjugative self-transfer, I determined that the T4SS of pKM101 also mobilizes the IncQ plasmid, pML122. Even with lower frequency of transfer at $10^{-2}$-$10^{-3}$ Tc/D for an overnight mating, this is a novel substrate of this system. Furthermore, I showed that transfer of IncQ is TraJ-dependent, since IncQ transfer was also completely abolished in pKM101ΔtraJ.

Protein translocation through the conjugative T4SS of pKM101: a novel finding

The T4SS encoded on plasmid pKM101 has been sequenced in its entirely [64], and to date no proteins have been shown to be translocated through this system exclusive of relaxasome components in complex with DNA. Although the interactions between coupling proteins and relaxasome components have been shown to be very system specific [9], interactions with protein effectors seem to be less specific as shown by transfer of heterologous proteins through the pKM101 system. Based on these findings, it is possible that the T4CPs of the T4SSs recognize general features of protein substrates such as C-terminal hydrophobic tails or clusters of positively charged residues, or internal motifs of unspecified sequence composition or secondary structure.
Previous studies have identified potential signal sequences in the C-terminal 50 residues of some effectors. Specifically, Vergunst, et al. demonstrated in 2000 that the C-terminal 37 residues of VirF in *A. tumefaciens* were sufficient for translocation into recipient cells indicating the possible presence of a transport signal [5]. Further comparison with the amino acid sequence of the effector VirE2, revealed a conserved R-P-R motif in this region. Another study showed that the C-terminal 50 residues of VirE2 and VirE3 were sufficient for effective transfer using the CRAfT assay [65], providing further support of a C-terminal transport signal. Later studies of these C-terminal regions identified the C-terminal 19 amino acids to be adequate for transfer of VirF, and identified a consensus sequence of R-X(7)-R-X-R-X-R-X(n)> upon comparison to other effectors [66].

Examination of the C-terminal regions of the effectors presented in this thesis indicated that the R-P-R or R-X(7)-R-X-R-X-R-X(n)> signal sequences previously identified were not present in the *Anaplasma* or *Wolbachia* effectors (Figure 4.8A). Previous examination of the Ats-1 C-terminal 20 residues revealed a greater abundance of positively charged residues relative to the remainder of the protein [46]. Indeed, examination of the C-terminal 30 residues yielded a net positive charge of +1.2 at pH 7.0 for Ats-1, while Aph_0111, WD0636, and WD0830 all had net charges of -0.1. WD0811 yielded a net positive charge of +2.2 in this region (Figure 4.8B).

While positively charged residues in the C-termini have been shown to be important for substrate recognition, other factors must also be necessary. As demonstrated above, expression of Ats-1 CTD yielded consistently less transfer than full-length Ats-1 through the native pKM101 system, suggesting that other domains may be necessary for efficient transfer through this heterologous system. Similarly, WD0636 and WD0830 transferred at rates similar to effectors carrying charged C-termini, indicating these effectors are using another means of recognition. As shown above, sequence alignment and analysis of the C-
terminal 30 residues does not reveal any conserved sequence or motif among these proteins.

In Chapter 3, I hypothesized that the CTE of VirD4At played a role in recognition of effector proteins, since deletion of the CTE allowed transfer of IncQ but abolished plant tumorigenesis. Additionally, I suggested that conjugation T4SSs such as pKM101 do not have a CTE because they translocate DNA but not protein substrates. However, the finding that TraJ is able to transfer heterologous proteins in the absence of a CTE, indicates that translocation of these proteins is not dependent on interaction with a C-terminal extension. It is possible that CTE’s carried by other T4CPs play a modulatory role, such as mediating transfer of specific substrates, enhancing transfer of some substrates by promoting substrate-T4CP contacts or coordinating the timing of transfer of numerous substrates through a T4SS. The function of the CTE is addressed further in Chapter 5.
Figure 4.8 Analysis of the effector C-terminal tails

A. Sequence alignment of the C-terminal 30 residues of the *A. tumefaciens* effectors VirE2, VirE3, and VirF indicating a conserved R-P-R motif (top), and the Anaplasma and Wolbachia effectors presented in this thesis (below). Alignment of the Rickettsial effectors show the R-P-R or R-X(7)-R-X-R-X-R-X-X)n> signal sequences previously identified are not present in these effectors.

B. C-terminal 20 residues of the *A. tumefaciens* and Rickettsial effectors. Positively charged residues are identified in bold print, while hydrophobic residues are underlined. Overall charge and pI of the protein fragment are indicated.
A

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<td>WD0830</td>
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A chimeric T4SS composed of homologous T4CPs and the pKM101 encoded mating channel

It has been shown that T4CPs are able to form stable interactions with channel subunits from non-cognate T4SS [9], and that they are able to interact with and transfer non-cognate protein effectors into target cells [22]. Therefore, we wanted to explore the use of pKM101 as a chimeric T4SS for the transfer of DNA and protein substrates. Genes encoding full-length coupling proteins from either *A. tumefaciens* or *Wolbachia* were expressed in *E. coli* containing pKM101ΔtraJ. These homologous T4CPs failed to support transfer of pKM101 or the IncQ plasmid. As mentioned above, pKM101 is not a natural substrate for the *A. tumefaciens* or *Wolbachia* T4SSs, although the *A. tumefaciens* system does transfer the IncQ plasmid to other agrobacteria. The lack of transfer of any DNA substrate by the chimeric T4SSs could be due to instabilities of the T4CPs in *E. coli*, or a failure to engage properly with the pKM101-encoded secretion channel. Recall that in the *A. tumefaciens* VirB/VirD4 system, the Rickettsial T4CP genes conferred negative dominance when expressed in wild-type strain A348, suggesting that the T4CPs somehow poison substrate transfer through the VirB/VirD4 T4SS. In the *E. coli* pKM101 system, expression of the heterologous T4CPs did not exert negative dominance. From these experiments, no conclusions can be drawn regarding the capacity of the heterologous T4CPs to engage productively or nonproductively with the pKM101 system or its DNA substrates. In the next chapter, however, I describe results of experiments showing that the heterologous T4CPs in fact can function to mediate substrate transfer when their N-terminal transmembrane domains are substituted with that of TraJ.
Chapter 5. The Development of Chimeric Coupling Proteins in an Effort to Enhance Substrate Transfer Through a Surrogate T4SS
Introduction

The Tra type IV secretion system of pKM101 translocates DNA substrates including pKM101 and mobilizable IncQ plasmids to other bacterial cells through a process requiring direct cell-cell contact [43]. This transfer system consists of a secretion channel and the type IV coupling protein (T4CP) termed TraJ, the latter of which physically joins secretion substrates to the transfer channel. Previous studies have shown that some T4CPs can be substituted for another and mediate transfer of IncQ plasmids through the heterologous T4CP [9, 23]. As reported in Chapter 4, substitution of A. phagocytophilum VirD4 for pKM101-encoded TraJ supported translocation of the effector protein Ats-1 through the pKM101 secretion channel. However, I was unable to create chimeric systems capable of mediating DNA transfer by substituting Rickettsial T4CPs for VirD4 in the A. tumefaciens system or heterologous T4CPs for TraJ in the E. coli pKM101 system.

As mentioned earlier, the T4CPs are composed of several distinct domains, the N-terminal transmembrane domain (TMD), nucleotide binding domain (NBD), all-alpha domain (AAD), and in some cases a C-terminal extension (CTE). There is some evidence that the TMDs mediate interactions of T4CPs with one or more subunits of the transfer channel, whereas one or more of the other domains mediate interactions with secretion substrates [9, 12, 23, 45, 70-73]. I thus hypothesized that the failure of the Rickettsial and A. tumefaciens T4CPs to mediate substrate transfer through the pKM101 translocation system is due to the inability of the respective TMDs to engage productively with the pKM101 channel subunits. To test this hypothesis, I asked whether chimeric coupling proteins composed of the TMD of TraJ and the remaining domains from the A. tumefaciens or Rickettsial T4CPs would mediate substrate transfer (Figure 5.1).

Overall, this line of study was intended to: i) test whether the TMD mediates the T4CP-channel interaction, ii) enhance the efficiency of heterologous protein substrate transfer through the pKM101 transfer system over that achieved with the native pKM101
system, and iii) test the importance of the C-terminal domain of *A. tumefaciens* VirD4 for recognition of *A. tumefaciens* protein substrates. My work also was intended to generate the molecular tools necessary for longer-term studies aimed at testing whether chimeric systems can be used to identify novel protein substrates of the Rickettsial VirB/VirD4 T4SSs that fail to translocate through the native pKM101 system.
Figure 5.1 Schematic of chimeric coupling proteins

Diagram illustrating the domain organization of the different chimeras. The TMD of TraJ is represented on the left in yellow, with the various soluble domains on the right. The numbers represent the amino acid residue at the beginning or end of the domain. The different domains are identified at the top: TMD, trans-membrane domain; AAD, all-alpha domain; NBD, nucleotide binding domain; CTE, C-terminal extension.
Results

**DNA substrate transfer using chimeric coupling proteins**

Chimeric proteins were constructed by combining the TMD of TraJ with the SD of another VirD4-like coupling protein. Dr. N. Whitaker created two chimeras by joining the TraJ TMD with the *A. tumefaciens* VirD4 soluble domain (SD). Plasmid pNW2 expresses a gene encoding TraJ::VirD4\textsubscript{At} and pNW4 expresses a gene encoding TraJ::VirD4\textsubscript{At}Δ553, which is deleted of VirD4’s C-terminal extension (Table 2.1, Figure 5.1). Upon expression of both chimeric genes in pKM101ΔtraJ, conjugative transfer of pKM101 was not detected (Figure 5.2). However, we did observe mobilization of the IncQ plasmid at frequencies slightly below those observed for IncQ plasmid transfer by wild type pKM101. Dr. Whitaker also made a TraJ::VirD4\textsubscript{At} chimera deleted of its AAD (TraJ::VirD4\textsubscript{At}ΔAAD; pNW6), and this chimera was not able to translocate pKM101 or the IncQ plasmid. These findings establish that the TraJ::VirD4\textsubscript{At} chimera is fully functional for translocation of the promiscuous IncQ plasmid. Furthermore, VirD4\textsubscript{At}’s SD is not capable of engaging or delivering the pKM101 substrate through the translocation channel. Finally, as reported with the *A. tumefaciens* system, VirD4\textsubscript{At}’s CTE is not important for IncQ plasmid transfer to recipient bacteria. By contrast, the AAD is essential for all plasmid substrate transfer.

I followed this up by creating chimeric coupling proteins containing the SDs of Rickettsial homologs (Table 2.1, Figure 5.1). TraJ::VirD4\textsubscript{Ap} (pTB39) and TraJ::VirD4\textsubscript{Wp} (pTB51) were tested for their ability to mediate pKM101 and IncQ plasmid transfer through the VirB channel. As shown in Figure 5.2, neither chimera supported DNA substrate transfer, indicating that the SDs of these chimeric proteins do not engage productively with or deliver the DNA substrates to the transfer channel. Another possibility is these chimeric proteins are unstable in *E. coli.*
Figure 5.2  Conjugative transfer of pKM101 and the IncQ plasmid pML122 between *E. coli* cells

Conjugative transfer mediated by T4SSs composed of the pKM101-encoded transfer channel and *traJ* or chimeric coupling proteins. Deletion of the all-alpha domain (AAD; residues 186-298) of *TraJ* or the chimeras show abolished substrate transfer. Experiments were repeated at least 2 times; results are reported as mean frequency of transfer with standard deviations indicated. WT, pKM101; Δ*traJ*, pKM101 deleted of *traJ*; pKM101Δ*traJ* expressing *traJ*, *traJAAD*, or the indicated chimeras. * Substrate transfer is statistically significant from wild-type (P<0.0001)
Protein effectors are translocated by the pKM101 system via a chimeric coupling protein

Next, I tested whether the chimeric T4CPs mediate transfer of effector proteins. E. coli strain MS411 carrying the Cre-effector expression plasmids and pKM101ΔtraJ served as donors and CSH26Cm:LTL(pBBR1MCS2-Gen') served as the recipient. As shown in Figure 5.3, donor strains producing the TraJ::VirD4At chimeric coupling protein translocated the A. tumefaciens effector VirF at frequencies of $3 \times 10^{-8}$ Rc's/D, whereas the isogenic Cre-only donor failed to translocate Cre at detectable frequencies. Donor strains producing the TraJ::VirD4At chimera failed to translocate Cre::VirE2 or Cre::VirE3 at detectable levels. However, in more recent work, Nathan Rosenthal in the Christie lab showed that Cre-VirE2 was translocated at $\sim 10^{-6}$ Rc's/D when the secretion chaperone VirE1 was coproduced in the donor cells (data not shown). These findings are consistent with previous reports showing that VirE1 is essential for translocation of VirE2 through the A. tumefaciens VirB/VirD4 T4SS.

The above findings prompted further tests of chimeric T4CPs composed of TraJ's TMD and SDs from A. phagocytophilum and W. pipientis. I was interested in testing whether codon optimization of the SD from A. phagocytophilum VirD4 enabled substrate transfer, and to this end I sent the TraJ::VirD4Ap chimera to Invitrogen for codon optimization and artificial synthesis using their GeneArt® technology. I then generated plasmids expressing genes for the TraJ::VirD4Ap chimeric T4CP as well as this T4CP deleted of its C-terminal extension designated as TraJ::VirD4ApΔ574. Donor strains carrying pKM101ΔtraJ, pTB39 which produces TraJ::VirD4Ap, and plasmids producing the Rickettsial effector fusion proteins were assayed for protein transfer by CRAfT. Interestingly, I was able to detect translocation of several of the Cre-effector fusions (Figure 5.4). The Cre::Ats-1 and Cre::Aph_0111 fusion proteins were translocated at the highest frequencies of $\sim 2 \times 10^{-7}$ Rc's/D. The Cre::Ats-1 CTD and Cre::WD0811 fusion proteins were delivered to recipients
at frequencies of $\sim 3 \times 10^{-8}$ Rc's/D. Cre joined to another putative Wolbachia effector, WD0636, was translocated but at frequencies only slightly above background levels observed with Cre only. Recent experiments using the Rif' recipient have shown that the Anaplasma effectors all transfer at approximately $10^{-7}$ Rc's/D, while the Wolbachia effector WD0811 has transferred at frequencies approaching $10^{-6}$ Rc's/D (data not shown). Additional experiments are needed to test transfer of the other substrates using this recipient.

I also tested for transfer of the Wolbachia effectors by donor cells producing the TraJ::VirD4$_{wp}$ chimeric protein (Figure 5.5). Transfer with this chimeric system was rather weak using the original recipient with Ats-1, WD0636, and WD0811 transferring at levels only slightly above Cre alone. Interestingly, this chimeric system supported a much more robust excision frequency with the Rif' recipient, where background is nearly undetectable. This system was able to transfer Cre::Ats-1 and Cre::Ats-1 CTD and frequencies of $\sim 10^{-6}$ and Cre fused to the three Wolbachia effectors at frequencies between $10^{-7}$ and $10^{-6}$ Rc's/D (data not shown). Again, further experiments are required to test the remaining effectors with the Rif' recipient.
Figure 5.3. Protein translocation between *E. coli* cells mediated by T4SSs composed of the pKM101-encoded transfer channel and the Tra::VirD4$_{At}$ T4CP.

*A. tumefaciens* effectors of the VirB/VirD4 T4SS were fused to Cre and tested for translocation to recipient cells carrying a lox-*tet*'-lox cassette, as described in Materials and Methods. Donor strains produced TraJ::VirD4$_{At}$ (black bars) or TraJ::VirD4$_{At}$Δ553 (white bars). Experiments were repeated at least 2 times and results are reported as mean frequency of Cre-mediated excision, with standard deviations indicated. * Excision frequency is statistically significant compared to the Cre-only control (P<0.0001) ** Excision frequency is statistically significant with deletion of the CTE (P<0.0001)
Figure 5.4. Interbacterial protein transfer using chimeric coupling proteins with the soluble domain from *A. phagocytophilum*

T4SSs composed of the pKM101-encoded transfer channel and the Tra::VirD4Ap T4CP were assayed for translocation of Cre fused to putative T4SS effectors from Rickettsial species. Donor strains produced TraJ::VirD4Ap (black bars) or TraJ::VirD4WpΔ574 (white bars). Experiments were repeated at least 2 times, and results are reported as mean frequency of Cre-mediated excision, with standard deviations indicated. ND, experiments were not done.

* Excision frequency is statistically significant compared to the Cre-only control (P<0.05)  
** Excision frequency is statistically significant with deletion of the CTE (P<0.05)
Figure 5.5  Interbacterial protein transfer using chimeric coupling proteins containing the soluble domain from *W. pipientis*

T4SSs composed of the pKM101-encoded transfer channel and the Tra::VirD4<sub>wp</sub> T4CP were assayed for translocation of Cre fused to putative T4SS effectors from Rickettsial species. Donor strains produced TraJ::VirD4<sub>wp</sub> (black bars) or TraJ::VirD4<sub>wp</sub>Δ574 (white bars). Experiments were repeated at least 3 times, and results are reported as mean frequency of Cre-mediated excision, with standard deviations indicated. * Excision frequency is statistically significant compared to the Cre-only control (P<0.0001) ** Excision frequency is statistically significant with deletion of the CTE (P<0.0001)
The T4CP CTE modulates effector translocation

As shown earlier in this thesis, in *A. tumefaciens* the VirD4_AT deleted of its CTE did not alter translocation of the IncQ plasmid to agrobacterial recipients but abolished T-DNA transfer to plants. Similarly, in *E. coli*, the TraJ::VirD4_AT and TraJ::VirD4_ATΔCTE chimeras supported IncQ plasmid transfer but not pKM101 through the pKM101 transfer channel. These findings established that VirD4’s CTE contributes to translocation of DNA substrates native to the cognate T4SS, but not to the promiscuous IncQ plasmids. I next tested whether the CTE of VirD4_AT contributes to effector protein transfer. Interestingly, although I did not see any effect of deleting the CTE on translocation of VirE2 or VirE3, translocation of VirF was dramatically enhanced (Figure 5.3). This suggests that the CTE of VirD4_AT plays an inhibitory role in VirF translocation.

CTE deletions of *Anaplasma* and *Wolbachia* chimeras, TraJ::VirD4_ApΔ574 and TraJ::VirD4_WpΔ574, were also examined for their ability to translocate the Cre-effector fusions. Donor cells producing the TraJ::VirD4_ApΔ574 chimera translocated the Ats-1 and Ats-1 CTD effectors at higher frequencies than donors producing the full-length chimera (Figure 5.4). However, translocation of Aph_0111 was abolished, suggesting that the CTE is important for translocation of this putative effector. Donor cells producing the TraJ::VirD4_WpΔ574 chimera failed to translocate the *A. phagocytophilum* effectors and also the *Wolbachia* putative effectors, with the exception of WD0811, which transferred at approximately the same frequency as the system composed of the TraJ::VirD4_Wp chimera (Figure 5.5). Experiments are still being completed to examine the effects of CTE deletion using the Rif' recipient.

Taken together, results of these studies establish that the CTE of *A. tumefaciens* VirD4 contributes to translocation of cognate T-DNA substrate, and possibly effector proteins, to plant cells. However, the CTE is not important for IncQ plasmid in either *A. tumefaciens* or *E. coli*, and in *E. coli* VirD4_AT’s CTE also negatively affects translocation of
the protein effector VirF. The CTE’s of the A. phagocytophilum and W. pipientis effectors modulate - either positively or negatively - translocation of effector proteins. Of particular interest, VirD4Ap’s CTE disrupts Ats-1 transfer, but is essential for translocation of Aph_0111. VirD4Wp’s CTE was shown to be required for transfer of Ats-1 and dispensable for transfer of WD0811, however interpretation of these results were inconclusive due to overall low excision frequencies.

Discussion

Chimeric coupling proteins retain the ability to translocate a variety of substrates in a surrogate T4SS

Although previous studies showed that chimeric T4SSs composed of a T4CP from one system and the channel subunits from another are functional [9, 20, 22], my work has shown for the first time that T4SSs composed of chimeric coupling proteins are functional. The goal of these studies was to determine whether such chimeric systems would support translocation of cognate and non-cognate substrates with higher sensitivity and higher efficiency than observed for the native pKM101 system. If such a system could be designed, it would greatly improve our ability to identify and study substrates from pathogens that are known to contain T4SSs but are difficult to grow or genetically manipulate. Additionally, such systems will allow for further detailed studies aimed at identifying specific T4CP domains of functional importance as well as motifs or domains required for substrate-T4CP engagement. In the previous chapters, I demonstrated that the pKM101 T4SS transfers known and putative substrates, including an IncQ plasmid, the A. phagocytophilum effector Ats-1, and other effectors from Wolbachia and A. tumefaciens. Here, I showed that various chimeric T4CPs supported DNA or effector protein transfer,
although not at levels higher than native pKM101 system or the pKM101 system presumptively overproducing the TraJ T4CP.

Fusion of TraJ’s TMD with the SD of VirD4At resulted in a chimera that supported transfer of the IncQ plasmid and the A. tumefaciens effector VirF. It is noteworthy that this system translocated the IncQ plasmid at frequencies comparable to native pKM101, yet it failed to translocate pKM101 at detectable frequencies. This suggests that VirD4At’s SD contains specific sequence information or a structural fold necessary for productive engagement with the IncQ plasmid substrate. By contrast, this SD lacks the interaction domain necessary for engagement with the pKM101 substrate. My finding that the TraJ::VirD4At chimera supported VirF translocation established that VirD4At’s SD also carries the motif necessary for engagement with this substrate.

VirF is not known to require a secretion chaperone for translocation through the VirB/VirD4 T4SS. By contrast, VirE2 requires its chaperone VirE1 and it appears that my inability to detect VirE2 translocation was because the donor strain used in my studies did not produce VirE1. Nathan Rosenthal, a Rice University undergraduate student, demonstrated that VirE1 coproduction with Cre-VirE2 supported transfer of the fusion protein (data not shown). Together, these findings established that the TraJ::VirD4At chimera supports transfer of both chaperone-independent and -dependent effector proteins. The A. tumefaciens VirB/VirD4 T4SS translocates T-DNA and effector proteins to yeast cells, as well as to plant cells as a part of the infection process. It will be interesting in future studies to test whether the E. coli pKM101 system with the TraJ::VirD4At chimera also supports transfer of T-DNA substrates to E. coli or eukaryotic recipient cells.

I was also able to demonstrate translocation of Cre-effector fusion proteins through pKM101 systems relying on the TraJ::VirD4Ap and TraJ::VirD4Wp chimeras. The former supported transfer of the A. phagocytophilum effectors in addition to WD0636 and WD0811 from Wolbachia, whereas the latter was more rigid in its capacity to bind and mediate
translocation of most of the *A. phagocytophilum* and *Wolbachia* effectors. With the exception of their variable CTE’s, the VirD4\textsubscript{Ap} and VirD4\textsubscript{Wp} SD’s are highly similar in overall sequence composition (78% identity). As discussed further below, these observations suggest that the CTE’s of these T4CPs may play important roles in substrate selection and translocation modulation.

**The roles of the AAD and CTE in type IV secretion.**

An important goal of these experiments was to assign functional importance to the T4CP domains. For example, although the native T4CPs from *A. tumefaciens* and *Wolbachia* did not mediate translocation of any tested substrates, the corresponding chimeric proteins composed of the TraJ TMD joined to the SD’s of these T4CPs supported translocation of at least some substrates. These findings strongly indicate that the TMD of TraJ is critical for productive interactions with the rest of the pKM101 transfer machinery. This finding is consistent with a previous study in which mutations in the TMD of R388-encoded TrwB abolished the capacity of this T4CP to mediate DNA transfer and also disrupted an interaction with the VirB10-like subunit TrwE [9].

The functionality of the chimeric T4CPs further suggested that SD’s of the T4CP confer both substrate binding and specificity. The SD’s of *A. tumefaciens*, *A. phagocytophilum*, and *W. pipientis* are composed of three smaller domains, the NBD’s, AAD’s, and CTE’s. Here we have shown that the AAD’s of both TraJ and VirD4\textsubscript{At} are required for translocation of DNA substrates in the pKM101 system. Dr. N. Whitaker has expanded this study to show this domain is also required for translocation of protein substrates in the pKM101 system, as well as DNA and protein substrates in the *A. tumefaciens* system (data not shown). He has also shown that the AAD domain of VirD4\textsubscript{At} binds DNA and also interacts with VirE2. These findings suggest that the AAD’s of T4CPs play a role in substrate binding and translocation. Interestingly, however, the AAD’s of VirD4
proteins from *A. phagocytophilum* and *W. pipientis* are highly similar, suggesting that these domains probably are not solely responsible for mediating binding of the different effectors translocated through these Rickettsial species. However, the CTE’s of these proteins as well as the CTE of *A. tumefaciens*, are highly variable in sequence composition and overall charge. In view of these observations, I tested a hypothesis that the T4CP CTEs contribute to substrate transfer.

TraJ::VirD4Δ₅₃₃ was the only chimeric T4CP of those tested that supported DNA transfer through the pKM101 transfer system (Figure 5.2). Deletion of VirD4’s CTE had no effect on IncQ plasmid transfer, establishing that this domain is not important for transfer of this promiscuous plasmid. This conclusion is further supported by the fact that pKM101-encoded TraJ lacks a CTE and yet transfers the IncQ plasmid at frequencies similar to that of donor cells producing TraJ::VirD4Δ₅₃₃.

In general, deletions of the CTEs associated with VirD4Δ₅₇₄, VirD4Δ₅₇₄, and VirD4Wp had a range of effects on effector protein translocation. For example, donor cells producing TraJ::VirD4Δ₅₃₃ transferred Cre-VirF at higher frequencies than donors producing TraJ::VirD4Δ₅₃₃ (Figure 5.3). Similarly, cells producing the VirD4Ap chimera deleted of its CTE translocated the Ats-1 and Ats-1 CTD effectors at a higher frequency than cells producing the full-length chimera. In view of my finding that *trans*-expression of *traJ* in a pKM101ΔtraJ donor conferred elevated DNA transfer frequencies compared to the native pKM101 system, it is formally possible that the CTEs deletions enhanced stabilities of the T4CPs and that the higher amounts of the chimeric T4CPs correlated with higher transfer frequencies. I suspect this is not the case, however, because strains producing the VirD4Ap and VirD4Wp chimeras with CTE deletions did not always confer elevated effector protein translocation. For example, TraJ::VirD4Δ₅₇₄ did not support translocation of the Aph_0111 putative effector at detectable levels. Additionally, TraJ::VirD4WpΔ₅₇₄ translocated WD0811 at similar levels to the full-length chimera. My data suggest, therefore, that the CTEs modulate translocation
of effector proteins in different ways, in some cases enhancing transfer while in other cases impeding or completely blocking transfer. Additional experiments are being done to explore the effects of CTE deletions using the Rif\(^r\) recipient. It will be especially interesting to see these effects with TraJ::VirD4\(_{Wp}\)\(\Delta 574\) since transfer frequencies seems to be generally higher using this recipient and any effects should be more obvious.

To reconcile these diverse findings, it is interesting that the CTE’s of the T4CPs under study varied in length and overall negative charge. CTE’s with these physical properties could be important for binding of some substrates, but dispensable for binding of others. Also, it is important to note that, although the \(E.\ coli\) pKM101 system in general appears to be a useful surrogate for monitoring transfer of heterologous T4SS effectors, it also has several limitations. \(E.\ coli\) is a different genetic context than \(A.\ tumefaciens\), \(A.\ phagocytophilum\) or \(W.\ pipientis\), and therefore might lack important adaptors or chaperones necessary for proper presentation of the effectors to the T4CP. The absence of these auxiliary factors could affect how the CTE participates in substrate engagement.

Finally, it is remarkable that the VirD4\(_{Ap}\) and VirD4\(_{Wp}\) chimeric T4CP CTEs are very long (166 and 102 residues, respectively) and highly-negatively charged. It is possible that the long CTEs adopt a structural fold within the T4CP that modulates the binding of some effector proteins that form charge-based contacts with the T4CP. In this context, it is interesting that the VirD4\(_{Ap}\) and VirD4\(_{Wp}\) chimeras did not support transfer of the mobilizable IncQ plasmid. Conceivably, the CTE’s of these proteins block engagement of the T4CP with DNA substrates by establishing charge-based interactions with a binding interface of the T4CP that alternatively could bind DNA. If so, the CTE’s would essentially function as a DNA mimic. In the natural \(A.\ phagocytophilum\) and \(W.\ pipientis\) hosts, the CTE’s might function as substrate specificity determinants by selectively blocking DNA translocation while promoting delivery of a repertoire of effector proteins into the mammalian cytosol to aid in infection.
Chapter 6: Summary and Perspectives
Summary

Type IV secretion systems are large complex machines, composed of a dynamic arrangement of 11 VirB proteins along with the coupling protein, VirD4 [1, 4]. These machines assemble across cell membranes to form an expansive secretion channel for the transport of macromolecules across the cell envelope and into the extracellular environment or other cells [1]. This thesis is focused on enhancing our understanding of substrate specificity and recruitment to the T4S channel, through interactions with the VirD4-like coupling proteins, and development of a chimeric translocation system. I hypothesized that transfer of non-cognate effector substrates could be observed through a chimeric T4SS, and that the CTE of VirD4-like coupling proteins are functionally important for the transfer of protein effectors.

In chapter 3, I demonstrated that, while the A. tumefaciens T4SS did not serve as an efficient chimeric system for transfer of A. tumefaciens effectors or the IncQ plasmid, homologous T4CPs seemed to be interacting with components of the T4SS, as indicated by a “poisoning” phenotype. This was measured through a reduction of virulence and IncQ transfer when homologous VirD4 proteins were produced in a wild-type strain, which also produced the native coupling protein. While we were unable to confirm expression of the homologous coupling proteins, this experiment provided evidence that the proteins were produced in sufficient amounts to cause a disruption in substrate transfer.

Functional analysis of the CTE of VirD4At in A. tumefaciens implied that the CTE plays a critical role in the transfer of some substrates required for plant virulence, but was dispensable for the RSF1010 derived IncQ plasmid, pML122. This is comparable to a previous study that showed the TraD coupling protein, from the F-plasmid, carries a CTE that is required for F-transfer but inhibitory for RSF1010 transfer [12]. Taken together with the later evidence provided in chapter 5, showing variable effects of CTE deletion on chimeric T4CPs, it appears that the CTE may have an operative role in transfer of the
conjugative Ti-plasmid and a possible modulatory role in transfer of protein effectors. Given the variable effects observed with deletion of the CTE on effector transfer, further studies are needed to elucidate the exact function of the CTE in protein transfer and any important residues, motifs, or structural folds required for interaction.

Chapter 4 examines the capacity of the Tra T4SS, encoded on the *E. coli* plasmid pKM101, to serve as a surrogate system for translocating heterologous substrates. Here I presented data identifying the IncQ plasmid, pML122, as a novel substrate, in addition to protein substrates from *A. tumefaciens* and Rickettsial homologs. Cre recombinase based assays have been widely used for demonstrating transfer of effectors through a T4SS [20, 25, 33]. In this study, I used the Cre-recombinase Reporter Assay for Translocation (CRAfT) to demonstrate that pKM101 could effectively translocate Cre-effector fusion proteins into a recipient cell. This was very surprising, as pKM101 had not been examined for the ability to transfer effector proteins previously. Additionally, I confirmed these proteins were being transferred in a manner contingent on the presence of TraJ, as a ΔtraJ mutant showed undetectable transfer of all protein substrates. Interestingly, presumed overexpression of traJ caused variable increases in transfer of several proteins, especially the putative *Wolbachia* effector WD0830 whose expression was undetectable on western blot. However, use of an improved recipient strain, CHS26Cm::LTL.rif^r(pUC4K), showed less variation in transfer levels.

Also in chapter 4, I examined the use of heterologous T4CPs and a chimeric T4SS in *E. coli*. As opposed to previous studies indicating T4CPs are able to interact productively with non-cognate Mpf proteins [9] or effector proteins[22], I was unable to show functional substitution of heterologous T4CPs for TraJ in mediating DNA transfer, nor did any of the heterologous T4CPs produce a “poisoning” phenotype in a wild-type pKM101 background. Additionally, most of the T4CPs tested were unable to functionally substitute for TraJ in
mediating protein transfer, with the exception of VirD4\textsubscript{Ap}, which was able to transfer Ats-1 at levels similar to those of strains producing TraJ.

In Chapter 5 I focused on trying to enhance substrate transfer through the use of chimeric coupling proteins, containing the TMD of TraJ and the SD of the different VirD4 homologs [9]. These chimeric coupling proteins were designed based on bioinformatic examination of homology between the protein sequences. Therefore, it is possible that the junction points identified may not be optimal for these chimeras. Further bioinformatics analysis and experimentation are necessary to determine the ideal junctures for these chimeric proteins, conceivably resulting in more robust transfer.

Based on the data presented, I was able to establish that the TraJ::VirD4\textsubscript{At} chimera is functional for IncQ and VirF substrate transfer, at frequencies of $\sim 10^{-4}$ Tc\textsubscript{s}/D and $\sim 10^{-8}$ Rc\textsubscript{s}/D, respectfully. Additionally, I tested several domain deletions of TraJ and the TraJ::VirD4\textsubscript{At} chimera, prepared by Dr. N. Whitaker, to assess the contributions of the AAD in substrate transfer, and found that the AAD was required for transfer of both pKM101 and IncQ. This supports a model that the AAD is involved with the processing of substrates for transfer [11]. Furthermore, I determined that chimeras made with the VirD4\textsubscript{Ap} and VirD4\textsubscript{Wp} soluble domains were similarly functional for protein translocation, although neither proved functional for DNA substrate transfer.

Finally, I developed C-terminal deletions in the chimeric T4CPs presented above, to assess the functional role of the CTE in substrate transfer. While the CTE deletion of TraJ::VirD4\textsubscript{At} indicated an inhibitory role for the transfer of VirF, similar to Ats-1 with the CTE deletion of TraJ::VirD4\textsubscript{Ap}, the CTE of TraJ::VirD4\textsubscript{Ap} appears to be required for transfer of Aph\_0111. Similarly, previous studies have indicated the CTE of TraD provides increased specificity and efficiency with regards to F-plasmid transfer [72]. Furthermore, deletion of the CTE from TraJ::VirD4\textsubscript{Wp} had no effect on transfer of WD0811, indicating this effector is likely
interacting with another region within the VirD4<sub>WP</sub> SD. This assortment of results suggests the CTE may play a modulatory role for the transfer of some, but not all, effector proteins.

The studies presented here were aimed at establishing a ‘proof-of-principle’ for the use of surrogate or chimeric T4SS’s to identify novel Rickettsial effectors, and to refine our knowledge of the molecular basis underlying the VirD4 receptor – substrate docking reaction. Based on the data presented in this thesis, I was able to demonstrate that wild-type pKM101 serves as an adequate surrogate T4SS for transport of non-cognate protein effectors. Additionally, I determined that a chimeric T4SS in pKM101 was achievable through the use of chimeric coupling proteins. Lastly, I provided additional evidence that the CTE of VirD4-like proteins are functionally relevant for transfer of substrates. However, the functionality of this domain appears to be T4CP and substrate specific.

**Comparison of effector translocation through the native and chimeric pKM101 systems**

Surrogate T4SSs are currently being used in *A. tumefaciens* and *Legionella pneumophila* to identify novel effector substrates from intracellular pathogens known to produce a T4SS [20-22]. However, these systems are complicated by prolonged growth cycles and, in the case of *L. pneumophila*, requirements of intracellular growth. In this study, I presented data showing the native pKM101 system serves as a suitable surrogate for translocation of effector proteins from three different bacteria with frequencies of transfer ranging from ~10<sup>-6</sup> to 10<sup>-8</sup> Rc’s/D. Furthermore, presumable overexpression of the T4CP, TraJ, from the multicopy plasmid pBAD24-Kan’ enhances this transfer to nearly 10<sup>-4</sup> Rc’s/D in some cases.

In an effort to improve efficiency of this system, chimeric T4CPs were produced along with the pKM101<sub>ΔtraJ</sub> T4SS. While I did not achieve the enhanced efficiency I had hypothesized, I demonstrated that chimeric T4SSs were functional for protein translocation.
and, in some cases, DNA transfer. Additionally, the results obtained with chimeric T4CPs strongly suggest that the trans-membrane domain of the T4CP is necessary and sufficient for interacting with the rest of the translocation channel. This is in agreement with previous studies that suggest the TMD interacts with the VirB10-like protein of the secretion channel [9] and the secretion channel as a whole [74]. The use of these chimeric coupling proteins in the pKM101 T4SS will allow further investigation into the roles of the AAD and CTE in substrate transfer, as well as identification of the interacting channel subunit.

**Future experiments**

Experiments in the future should be centered on optimizing the efficiency of the heterologous or chimeric T4SS. This would include i) reducing background transfer of Cre only and, ii) determining the optimal junctions for chimeric proteins. Through use of the improved CRAfT recipient CHS26Cm::LTL,rif(pUC4K), I have already eliminated non-specific background, which might have given rise to aberrantly high frequencies of Cre translocation and translocation efficiencies of other substrates. Members of the Christie lab are continuing to test the native pKM101 system and the pKM101 T4SS producing chimeric T4CPs. Additionally, two unanswered questions are being explored: i) What motifs and/or residues of the T4CP are required for substrate transfer, and ii) what is the role of the CTE in effector translocation?

**What motifs and/or residues of the T4CP are required for substrate transfer?**

To further advance our understanding of the coupling protein – substrate interaction, and perhaps to help build a more effective chimeric T4SS, it would be beneficial to identify the exact binding interfaces. The data present in this thesis indicates this interaction is substrate specific. That is, different substrates appear to be dependent on different domains of the T4CP for transfer. For example, in *A. tumefaciens*, presence of the VirD4_A CTE is
required for the transfer of the Ti-plasmid and/or effector proteins, but is entirely dispensable for IncQ transfer.

To test for specific contacts between the T4CP and DNA or protein substrates, I propose using co-immunoprecipitation (Co-IP) and cross-linking studies as well as a two-hybrid system designed for testing interactions within specific protein domains. The goal of these Co-IP studies is to isolate complexes of T4CP domains with the substrate. Chemical cross-linking would be used to stabilize any transient or otherwise weak interfaces. Additional Co-IP with mutant T4CPs deleted of specific domains or motifs, or containing amino acid substitutions, would further confirm the results by demonstrating a lack of interaction.

A potential issue with this proposed line of work is that VirD4-like coupling proteins form homohexamers in a working T4SS [11, 13]. It is likely that this structure is required for interaction, and any deletion or other alteration that disrupts hexamer formation could give false data. Additionally, the use of T4CP fragments in Co-IP would not exhibit this hexamer formation and may not interact with substrates. Therefore, another possible study would use bioinformatic analysis to identify potential interacting interfaces on the coupling protein and introduce a series of cysteine mutations to complete cross-linking studies with the substrate. This has the added benefit of ascertaining the specific residues required for interaction and translocation of substrates.

How is the CTE contributing to substrate secretion?

The data in this thesis, as well as other studies, have identified the C-terminal extension on some coupling proteins as playing a functional role in substrate secretion [72, 74]. Additionally, we know that the CTE is not necessarily required, as several T4CPs function adequately without the presence of this domain (e.g. TraJ_{PKM101} and TrwB_F) [74]. The studies presented here indicate that, when present, the CTE can act to either enhance
or inhibit transfer of both DNA and protein substrates. Furthermore, this interaction between the substrate and the CTE of the T4CP seems to be very substrate specific.

In order to facilitate the study of the CTE and its contribution to substrate secretion, I propose making a series of GST fusions with CTEs from several different coupling proteins. I would then use these to perform a GST pull-down with cell lysates to isolate any interacting substrates, followed by analysis with SDS-PAGE and eventually mass spectrometry. This kind of study would also help to identify if there is a particular motif that the CTE requires for interaction, which would be useful for *in silico* identification of potential substrates.

This project arose due to interest from those in the Rickettsial field in finding a surrogate system to confirm that candidate effectors are being translocated through a T4SS. To that end, I was able to demonstrate that the native pKM101 T4SS is adequate for translocation of a diverse set of substrates from a variety of bacterial species. Additionally, the chimeric T4CPs generated provide an additional tool for demonstrating translocation as well as providing further insight into specific T4CP/substrate interactions. The findings presented in this thesis should generate great interest from the Rickettsial field and lead to much fruitful collaboration between the Christie lab and Rickettsial labs.
References


Trista Marie Berry was born in Nampa, Idaho on June 14, 1984, the daughter of Kolona Faye Morin and Lee Allen Morin. After completing her work at Grant Union High School, John Day, Oregon in 2002, she entered Pacific University in Forest Grove, Oregon. She received the degree of Bachelor of Science with a major in Cellular and Molecular Biology from Pacific in May 2006. For the next four years, she worked as a research assistant and study coordinator in the Department of Dermatology at Oregon Health & Science University, completing clinical trials for the treatment of eczema and psoriasis. In August of 2010 she entered the Graduate School of Biomedical Sciences at the University of Texas Health Science Center at Houston. Here she joined the Microbiology and Molecular Genetics department and the laboratory of Dr. Peter J. Christie where she studied bacterial secretion systems.

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