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Unlocking the M13 (F1) Virion

Investigation into the role of pIII C domain of F specific Filamentous Bacteriophage in Infection

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in

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Abstract

Filamentous phage adsorb to the host cells by binding of the N2 domain of pIII to the tip of the F pilus. Binding of N1 domain of pIII to the secondary receptor (Tol A), triggers the opening of the virion by a poorly understood mechanism. Filamentous phage assembly is a secretion-like process. The assembly is terminated and virion released from the membranes by C domain of pIII. Because the infection is a reversal of assembly, it can be hypothesized that the C domain of pIII plays an active role in the infection.

To test this hypothesis, we have set up a system in which virions carried a mixture of two types of mutant pIII molecules: i) functional N1N2 domains fused to a short C domain that can be incorporated but cannot terminate assembly and release the phage from the membrane; ii) C domain only, which can terminate phage assembly, but lacks the receptor-binding domains N1N2.

The infectivity of the particles was as low as 0.21% that of the positive control setup in which virions carried a mixture of wild-type pIII and C domain. Therefore, a functional C domain covalently linked to the receptor domain N1N2 is required for infection. These findings suggest that simple binding of N1 domain of pIII to the periplasmic receptor TolA is not sufficient for infection. Rather, this interaction may, via functional C domain of pIII, trigger a conformational change required for the downstream events which result in the virion uncoating and DNA entry.

To add further weight to this model, a "microphage" producing system was designed to produce short phage particles suitable for Cryo-EM structural analysis.

Acknowledgements

Firstly I would like to thank my supervisor Dr Jasna Rakonjac for helping me through this thesis. I would also like to thank Marjorie Russel and Peter Model from Rockefeller University for supplying materials and advice. I would also like to thank all the people in the Helipad lab who have helped me through my research. I would also like to thank my friends and family for support thought out the last two years. This research was supported by the Marsden Fund Grant 02-MAU-210 from the Royal Society of New Zealand.

I think the following quote describes the path taken in this project well.

I may not have gone where I intended to go, but I think I have ended up where I intended to be.

Douglas Adams

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List of Abbreviations

AP - Alkaline phosphatase
Amp - Ampicillin
BCIP - 5-bromo-4-chloro-3-indoxyl phosphate
Cryo-EM - Cryogenic electron microscopy
Cm - Chloramphenicol
DNA - Deoxyribose nucleic acid
dsDNA - Double stranded DNA
DXMS - Deuterium exchange mass spectroscopy
E.coli - *Escherichia coli*
F - F conjugative plasmid
F+ - *E. coli* carrying F plasmid, also termed "male"
F- - *E. coli* not carrying F plasmid, also termed "female"
FAB - Fortified arabinose broth
FCS - Fluorescence correlation spectroscopy
Ff - F+ specific filamentous bacteriophage of *E.coli*, including f1, fd and M13
FGB - Fortified glucose broth
EtBr - Ethidium bromide
HA - Haemagglutinin
HRP - Horse radish peroxidase
IF - Infective form
IG - Intergenic region
IR - Interference resistant
IPTG - Isopropyl-beta-D-thiogalactopyranoside
NMR - Nuclear magnetic resonance
NBT - Nitroblue tetrazolium
OD - Optical density
PCR - Polymerase chain reaction
RF - Replicative form
sarkosyl - n-lauroylsarcosine, sodium salt
ssDNA - single stranded DNA

SIP	- selective infective phage
TB	- Tryptone broth
Tet	- Tetracycline
TEM	- Transmission electron microscopy
WT	- Wild type

Chapter 1

Literature Review

1.1 Introduction to Filamentous Bacteriophage

Filamentous phage (Inovirus) are long, thin bacteriophage that can be found infecting a wide range of different bacterial taxa including *Escherichia*, *Salmonella*, *Pseudomonas* and *Vibrio* (Model & Russel, 1988). Almost all species that are infected by filamentous phage are Gram-negative organisms.

All filamentous bacteriophage have certain features that distinguish this group. They are usually between 1-2μm in length and 6-7nm in diameter and all contain a single stranded DNA genome (Model & Russel, 1988; Webster, 1996). In filamentous phage the virion length is determined by the genome size. Thus larger the genome, longer the phage becomes. Another common feature is that filamentous bacteriophage are released from their host cells without causing lysis by a process alikened to the secretion of the virulence factors or the assembly of surface filaments.

The phage coat is composed of five proteins. There are two sets of two different minor coat proteins which are located at either end of the bacteriophage (Gray, Brown & Marvin, 1979). The dominant protein in the structure is the major coat protein. It forms the tube-like structure (shingle-like helix) that encloses the ssDNA genome (Glucksman, Bhattacharjee & Makowski, 1992).

The best studied bacteriophage are the ones that infect male (F+) strains of *Escherichia coli* (Ff) (Loeb, 1960). Members of the Ff group include filamentous phage f1, fd and M13, which are all almost identical; their DNA sequence only differing by as little as 2% (Webster, 1996). Filamentous bacteriophage found in this group infect *E.coli* by recognition and attachment to the tip of the F conjugative pilus (F). As with all other filamentous phage, infection with Ff bacteriophage does not lyse cells. However it does extend generation time of the bacterial host by about 50%. This

causes the formation of cloudy plaques and a decreases the size of colonies of infected cells.

Because they produce single stranded DNA, Ff filamentous bacteriophage have been used previously as vectors for DNA sequencing. More recently, Ff have been used for phage display technology (Smith, 1985). In parallel, Ff have been used as a model system for basic discoveries in molecular biology (rolling circle replication (Allison et al., 1977), *in vitro* translation (Konings, Hulsebos & Van den Hondel, 1975)) and cell biology (integration of proteins into the membrane (Chang, Model & Blobel, 1979), secretion from Gram-negative bacteria (Linderoth, Simon & Russel, 1997)).

1.2 The Filamentous Phage Genome

The Ff genome has been completely sequenced (M13, fl and fd) (Beck & Zink, 1981; Hill & Petersen, 1982; van Wezenbeek, Hulsebos & Schoenmakers, 1980). The genome consists of 11 genes and a short intergenic sequence, which contains the positive and negative origin of replication and the packaging or morphogenic signal (Figure 1).

The genes can be separated into three groups according to their function (Figure 1). The first group are genes that code for the proteins involved in the replication of the genome, pII, pX and pV. The second group are genes that code for the proteins which form the structure of the secreted virion, pVIII, pIII, pVI, pVII and pIX. The final group are the genes which encode the proteins that make up cytoplasmic and outer membrane complex which mediates the virus is assembly and secretion. This group consist of two proteins which encode for an inner membrane assembly and transport complex (pI and pXI) and an outer membrane pore (pIV). The genes gX and gXI have their starting AUG codon within other genes. The gX coding sequence is in frame and within gII and gXI coding sequence is in frame and within gI (Fulford & Model, 1984; Rapoza & Webster, 1995).

The level of expression of phage genes is tightly controlled and varies according to gene. Large amounts of the major coat protein pVIII and single stranded binding

protein pV are made, compared with pI, pVI, pVII, pIX which are only made in small amounts. Transcription is initiated from multiple promoters, but only terminated at few sites, resulting in numerous overlapping transcripts. Only the negative (-) strand serves as a template for transcription. Early studies using mutants determined that many mutations that lead to changes in gene expression are lethal, especially when changes involve the genes required for the formation of the assembly/export complex (pI/pXI and pIV) (Pratt, Tzagoloff & Beaudoin, 1969; Pratt, Tzagoloff & Erdahl, 1966). Therefore gene expression of the bacteriophage genome without phage assembly and export is lethal. Also the over expression of certain genes (especially pVIII) is lethal to the host cell (Pratt et al., 1969). This means that any attempts to modify phage gene expression have to be accompanied by the correct complementary modifications to prevent host death.

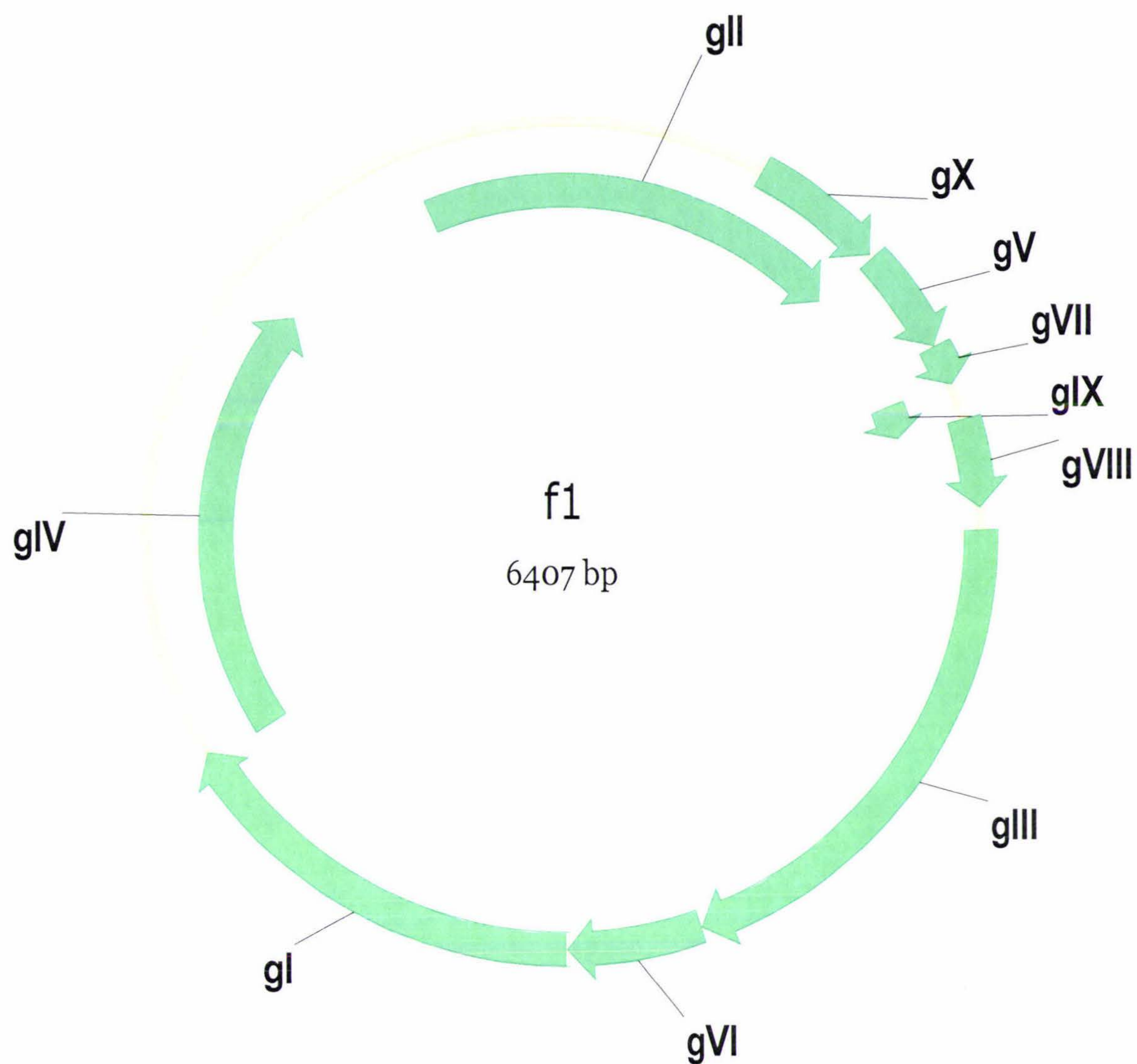
1.3 The Ff Phage Structure

Ff phage virions all have the same structure (Figure 2). The phage coat consists of five proteins (pVIII, pIII, pVI, pVII and pIX). pVIII is the major coat protein; it forms the majority of the phage coat. The other proteins are separated into two different groups; pIII and pVI are at distal end of the phage and pVII and pIX at the proximal end.

1.3.1 Structure and role of pVIII, the major coat protein

About 3000 copies of pVIII forms the tube of the Ff filament. Its structure and structural arrangement within the phage has been solved using x ray fibre diffraction and x ray crystallography (Figure 3) (Marvin, 1998; Marvin et al., 1994; Overman & Thomas, 1995; Williams et al., 1995). pVIII structure in the membrane was solved by NMR to a high resolution (Opella et al., 1980).

pVIII is a small protein of only 50 amino acids. The protein structure consists of two α- Helices (N-terminal helix and a C-terminal helix) separated by a short flexible hinge. pVIII also has an N terminal signal sequence for targeting to the SecYEG/YidC



Gene	Protein Function	No of Amino acids	Protein MW
II	DNA replication	410	46137
X	DNA replication	111	12672
V	ssDNA binding protein	87	9682
VIII	Major coat protein	50	5235
III	Minor coat protein	406	42522
VI	Minor coat protein	112	12342
VII	Minor coat protein	33	3599
IX	Minor coat protein	32	3650
I	Assembly cytoplasmic membrane	348	39502
XI	Assembly cytoplasmic membrane	108	12424
IV	Assembly outer membrane	405	43476

Figure 1: An f1 genome map showing the position of genes and a table of the gene products. Molecular weights are of mature protein products, and do not include amino-terminal signal sequence. The diagram and table was adapted from (Webster, 1996). NOTE: In Ff phage when genes are referred to a small g is added before the gene number, when a protein is referred to a small p is added in front. In this dissertation genes and proteins will be referred to using roman numerals; however some papers refer to the gene and protein in Ff phage using the letters A-K, or Arabic numerals.

Proximal End

6nm

pVII,
pIX

pVIII

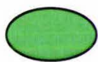
860nm

pVI,
pIII

N1N2
pIII

pVIII

pIII Transmembrane domain



N1 domain pIII

N2 domain of pIII

C1 doamin of pIII

C2 domain of pIII

Figure 2: Anatomy of Ff filamentous phage virion. Minor coat proteins pVI and pIX form capping structure at the proximal end of the virion. Major coat protein pVIII forms main cylinder of phage virion. Minor coat proteins pIII and pVI form capping structure at the distal end of the virion. Phage filament is 860nm long and 6nm wide. The electron micrograph is from (Gray et al., 1979).

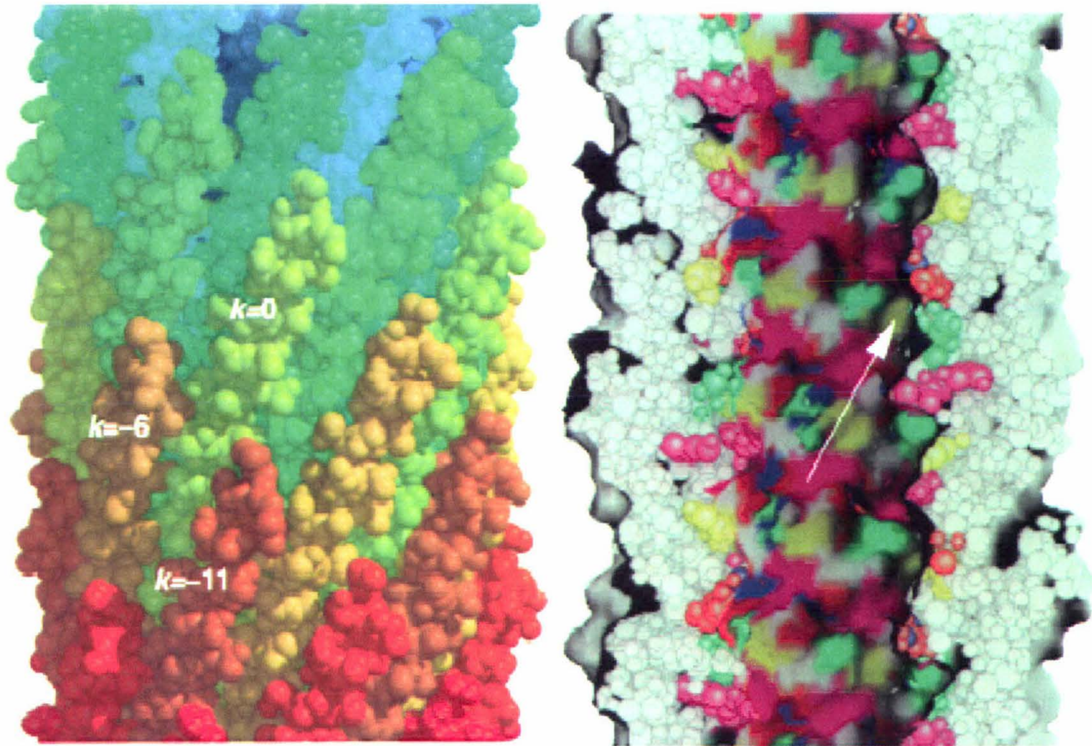


Figure 3: pVIII forms a shingle-like helix array around ssDNA genome. On the left is space-filling model of major coat protein pVIII within phage virion structure. On the right is a model of longitudinal section showing interior channel formed by pVIII around the collapsed ssDNA circular genome. The diagram is orientated with the top towards the proximal (pVII/pIX) end of the phage. pVIII is orientated with is N terminus towards the proximal end of the phage. Figure from (Welsh et al., 1998). K= 0 indicates a subunit, K =-6 and K= -11 indicate nearest neighbours to K=0 in the virion helix. The arrow shows the direction of the left handed helix.

translocation machinery in the inner membrane (Samuelson et al., 2000). The signal sequence is cleaved by signal peptidase and the mature pVIII becomes anchored into the inner membrane via the C-terminal helix. The ampipathic N-terminal helix lays on the surface of the membrane at a 90° angle relative to the C-terminal helix. During phage assembly the C-terminal helix is removed from the membrane and is packaged into the interior of the phage structure. The N-terminal helix is on the exterior of the structure. The hydrophobic side of the ampipathic helix covers the C-terminal helix of the pVIII included above it and the hydrophilic side of the helix faces the exterior of the phage. This forms a shingle-like array/helix around the ssDNA core. Each subunit of pVIII is tilted 20° relative to the main axis of the phage and there are 5 subunits of pVIII per turn of the shingle-like helix (Figure 3). The ssDNA is collapsed into a helix with phosphate groups in the centre and the bases at the periphery. Both phosphates and bases form contacts with pVIII (Day, 1969; Day et al., 1988a; Day et al., 1988b; Day et al., 1983; Day, Wiseman & Marzec, 1979; Day & Wisemann, 1978).

1.3.2 The structure and role of pVII and pIX in capping Ff phage

pVII and pIX are the proteins that are incorporated into the virion at the initiation of assembly and are the first to be extruded from the cell. They form the capping structure at the proximal end of the phage (Figure 2). Both are small proteins of only 32 (pVII) and 33 (pIX) amino acids. The structure of these two proteins has not been determined and their arrangement in the virion has not been solved. Studies using antibodies have shown that pIX will bind to antibodies when in the phage structure, but pVII will not (Endemann & Model, 1995). This suggests that pVII is buried in the structure and is inaccessible to antibodies, while pIX is exposed to the surface of the structure.

1.3.3 The structure and role of pIII and pVI

pIII and pVI form the distal end of the phage structure (Figure 2, 4A). They are required for phage stability and termination of assembly. pVI is a relatively small protein (112aa) and before inclusion into the phage is a membrane protein. During phage assembly it is removed from the membrane and included into the phage structure in a very tight complex with pIII. The complex is so tight that even after

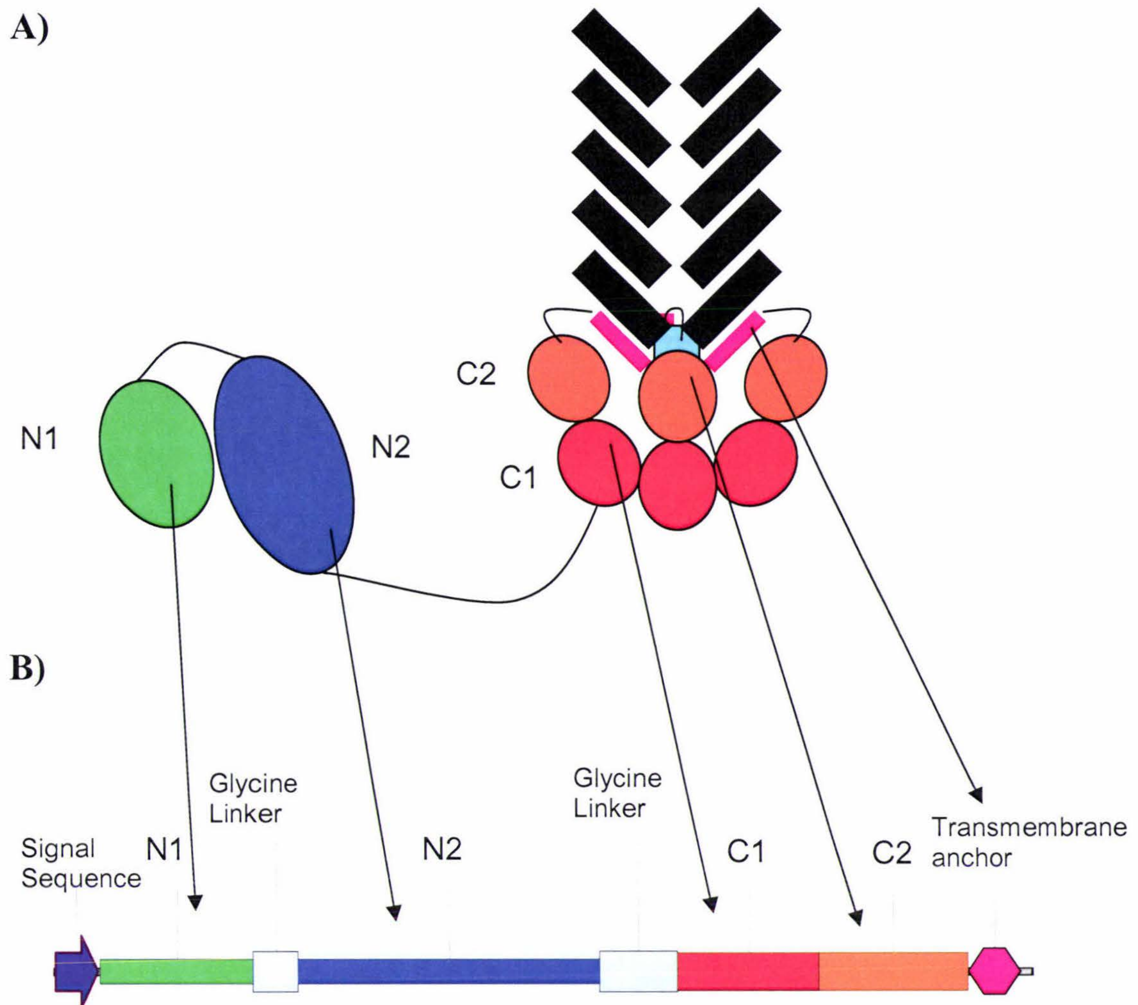


Figure 4: A) Schematic representation of the distal end of Ff filamentous phage, showing domain organisation of pIII. pVIII (Black rectangle) and pVI (Light blue rectangle) are also shown. B) pIII domain arrangement. The signal sequence is cleaved during membrane insertion. Arrows show structural domains (N1/N2) or functional sub regions (C1/C2) of pIII. Note that the C1/C2 sub domain arrangement and membrane anchor location in the virion are not known.

phage disruption using surfactants pVI and pIII remain in a complex (Gailus & Rasched, 1994).

pIII is a large protein of 406 amino acids (424aa including signal sequence). The structure of pIII includes two N terminal domains (N1 and N2) separated by a glycine linker and a C-terminal domain (Cd) which is separated from the N-terminal domain by another glycine linker (Figure 4B). The C domain can be separated into two distinct regions in relation to function, the C1 and the C2. The C1 region is required for phage stability, while the C2 region is involved in phage termination and release (Rakonjac, Feng & Model, 1999; Rakonjac & Model, 1998). At the C-terminal end of the C domain there is a hydrophobic membrane anchor (15aa) which has been well characterised (Davis, 1985; Davis, Boeke & Model, 1985). After transcription, the pIII protein is targeted to the inner membrane by the Sec pathway and is anchored into the cytoplasmic membrane by its C-terminal anchor domain. The N-terminal domains and most of the C-terminal domain are in the periplasm; only 5 residues at the C-terminus are in the cytoplasm.

The structure of N1 and N2 has been determined using x ray crystallography and NMR (Holliger, Riechmann & Williams, 1999; Lubkowski et al., 1998), as these domains fold independently of the C domain. The X-ray structures have also been solved of the N1 in complex with the phage co-receptor TolA (Lubkowski et al., 1999; Reichmann & Holliger, 1997). This showed that the N1/TolA binding site is covered by the N2 domain prior to F pilus binding. It is also known that the N2 domain contains the site for binding the F pilus during infection (Caro & Schnos, 1966; Stengele et al., 1990). This spatial site for F binding was located by mutagenesis studies (Deng & Perham, 2002). However the phage binding site on the F pilus tip is unknown (Manchak, Anthony & Frost, 2002). The kinetics and mechanism of the N terminal domain folding has been studied in great detail (Martin & Schmid, 2003a; Martin & Schmid, 2003b).

The structure of the C-domain has not been determined as yet. pIII has been co-purified with pVI (Gailus & Rasched, 1994) from the virion. However the complex has not been purified at the level required for crystallisation. A study of the C terminal domain using scanning mutagenesis has been carried out (Weiss et al., 2003). This

study determined that residues of the membrane anchor are required for the C domain to be incorporated into a phage.

It has been found that the C domain of pIII is essential for the release of the phage from the cell (Rakonjac et al., 1999; Rakonjac & Model, 1998). If cells are infected with ΔgIII mutant f1 or if they express a short C-terminal fragment (<93 aa) of pIII, then 99% of the phage are retained on the surface forming long cell-associated filaments. the small amount of phage virions which are produced in the infection with a Δ*gIII* mutant phage represent broken-off cell-associated filaments.

1.4 The Ff Phage Lifecycle

1.4.1 Infection of host cell

Ff filamentous bacteriophage begins the process of infecting a host *E.coli* by binding to the tip of the F pilus (Caro & Schnos, 1966) (Figure 5). This is the primary receptor for Ff phage, however it is not the only protein required by the phage to infect the bacteria. Ff phage also require interactions to occur with the cytoplasmic membrane complex of TolQ, R and A. If either the F pilus is not present due host cell lacking the F conjugative plasmid (termed F⁻ or "female" *E.coli*) or if the cytoplasmic membrane proteins Tol Q, R or A are mutated or missing then Ff phage can not infect the *E.coli* (Bradley & Whelan, 1989; Smilowitz, 1974).

The N2 terminal domain of pIII binds to the tip of the F pilus (Figure 5). Binding causes a structural rearrangement between the N domains releasing the N1 domain and exposing the TolA binding site which was previously covered by the N2 domain (Lubkowski et al., 1999; Reichmann & Holliger, 1997). The N2 domain binding to the F pilus tip also cause the F pilus to retract, bringing the phage into closer contact with the cell, allowing the interaction between N1 and TolA. The N1 domain then hinds to TolA (Lubkowski et al., 1999) after which virion coat becomes integrated into the host cell membrane and ssDNA enters the cytoplasm (Click & Webster, 1998).

There is evidence showing that the N1 and N2 domains must be covalently linked to the C-domain incorporated into the Ff virion at a specific distance for membrane insertion to occur. Paper by (Krebber et al., 1997; Spada, Krebber & Pluckthun, 1997) showed that if a large protein domain (β- lactamase) was placed between the N2 domain and the C domain then infectivity was decreased hundred fold compared with the wild type.

The data from the same publication showed that a non-covalently bound N1N2 complex to the C domain results in four orders of magnitude lower infectivity then that of the wild type phage. The authors replaced the N1N2 domains of pIII with a ligand- binding protein-C domain fusion and then expressed the ligand independently covalently as a fusion to N1N2 in an attempt to develop a protein-interaction screen named selectively infective phage (SIP). The idea is that when the ligand-binding protein binds its ligand then the pIII function should be restored and phage should become infective. However when this was tried infectivity was low (10^{-4} that of the wild type phage).

A paper by (Chatellier et al., 1999) showed that the C domain forms significant interaction with the N1N2 complex in the absence of fused interacting proteins. However the requirement of this interaction for phage infection, in the context of wild-type phage, remains unknown. When N1N2 are expressed separately to the C domain, these interactions did not increase the infectivity above the values detected by (Krebber et al., 1997; Spada et al., 1997).

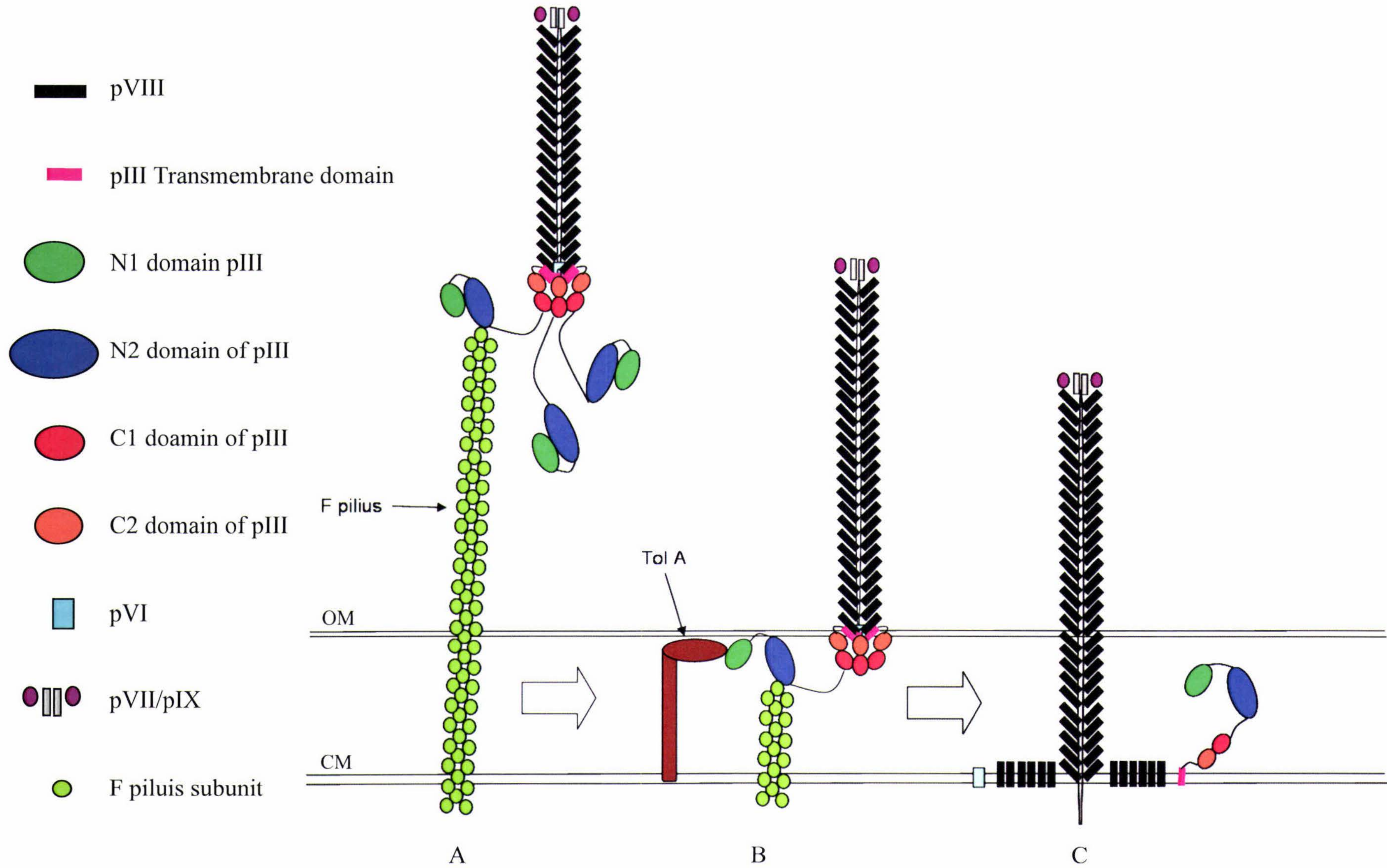

pVIII
pIII Transmembrane domain
N1 domain pIII
N2 domain of pIII
C1 doamin of pIII
C2 domain of pIII
pVI
pVII/pIX
F piluis subunit
F pilius
Tol A
OM
CM
A
B
C

Figure 5: Model of phage infection. A) Phage binds to tip of F pilus by N2 domain of pIII. F pilus retracts and N1 domain is released. B) N1 domain binds TolA. Phage structure "opens" allowing membrane insertion of phage. C) Coat proteins integrate into the inner (cytoplasmic) membrane, ssDNA genome transverses membrane and enter host cell.

1.4.2 Resistance to Filamentous phage infection caused by pIII

Cells can become resistant to Ff filamentous phage infection in many ways. One of these ways is the *in trans* expression of pIII. This is particularly a problem when using ΔpIII phage as to get phage reproduction pIII has to be expressed from a plasmid in the host cells. Resistance by the *in trans* expression of pIII occurs because the soluble N1N2 domains of pIII are believed to bind to the TolA rendering it unavailable for infection. To produce ΔgIII phage an *in trans* pIII expression system needs to be tightly controlled so that there is no pIII expression before phage infection, and a high level of induction upon phage infection.

1.4.3 Replication of phage within the Host cell

Once the Ff phage ssDNA genome enters the cytoplasm of the host cell a negative strand is synthesised by the host DNA replication machinery (Figure 6). The dsDNA form of Ff phage genome is referred to as the replicative form (RF). The replicative form of the genome is a template for replication of the phage positive strand (+) and for transcription of phage genes. During early infection newly synthesized (+) strands are recycled to create more dsDNA replicative forms. Later in the infection, (+) strands are coated with the ssDNA-binding protein pV within the cytoplasm and this complex serves as a packaging substrate for the phage assembly.

Newly synthesised phage proteins are targeted to the predetermined locations. pII, pV and pX remain in the cytoplasm and pI, pIII, pIV, pVI, pVII, pVIII, pIX, and pXI are targeted to membranes.

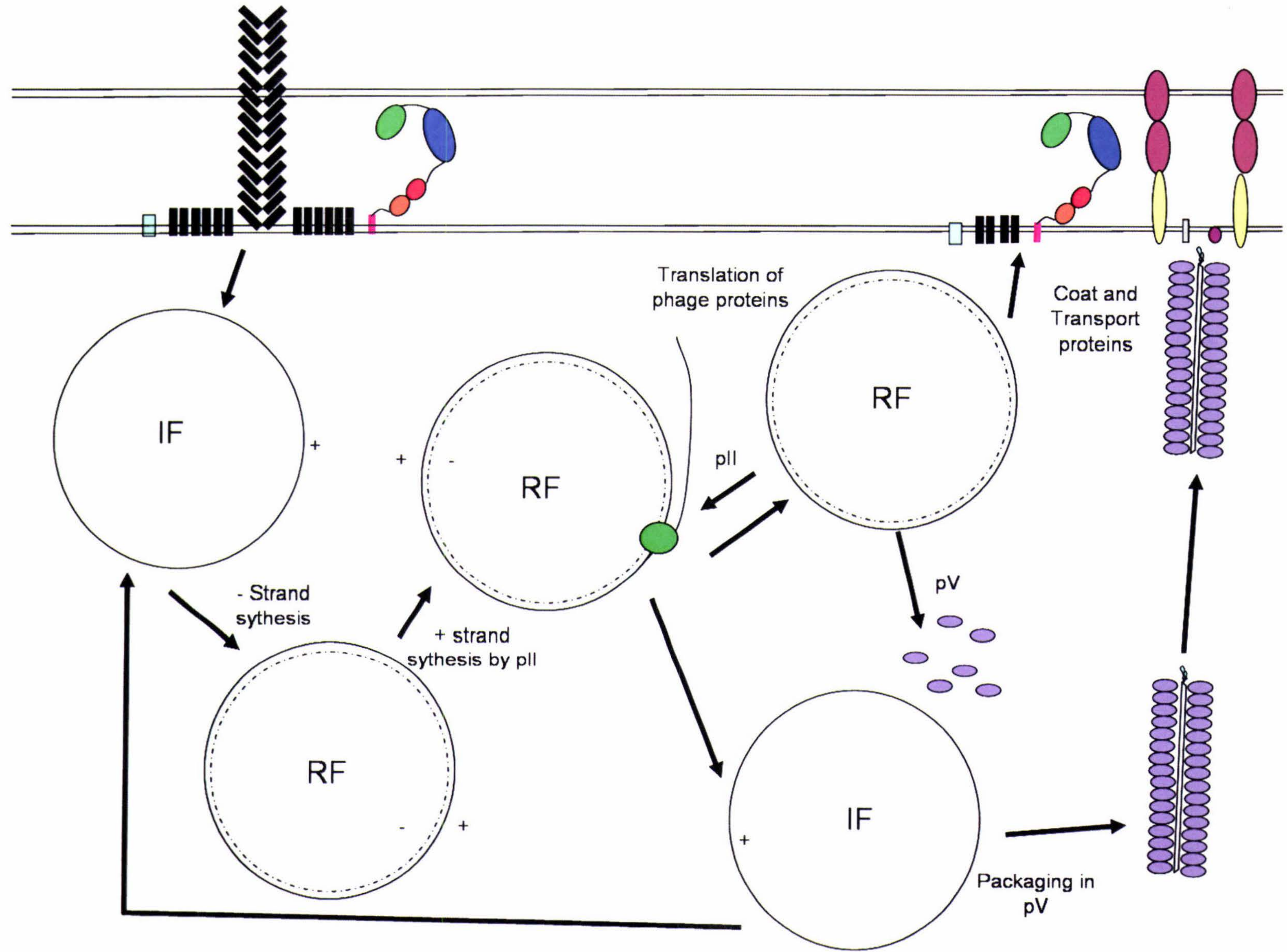

Translation of
phage proteins
Coat and
Transport
proteins
IF
+
+
-
RF
RF
pII
pV
- Strand
sythesis
+ strand
sythesis by pII
RF
-
+
IF
+
Packaging in
pV

Figure 6: Overview of replication of phage within the host cells. The ssDNA enters into cytoplasm of cell (infective form). Negative strand synthesis at the negative strand origin by host cell replication machinery results in the double stranded (replicative) form (RF). Positive strand synthesis is initiated by pII at the positive origin of replication via a rolling circle replication mechanism. During the initial period of viral infection, positive strands are used as templates for synthesis of the negative strand, to attain a set number of copies of the double stranded (replicative) form. The RF serves as a template for transcription of phage genes. Proteins pII, pV and pX stay in cytoplasm and regulate genome reproduction and packaging. pI, pIV and pXI form transport complex spanning the inner membrane and outer membrane. pVII, pIX, pVIII, pVI, pIII are inserted into the inner membrane in preparation for phage assembly. Later in the infection, positive strands are coated in pV and brought to the cell membrane assembly/transport complex for assembly and export, and virion proteins are translocated from the inner membrane into the secreted virion.

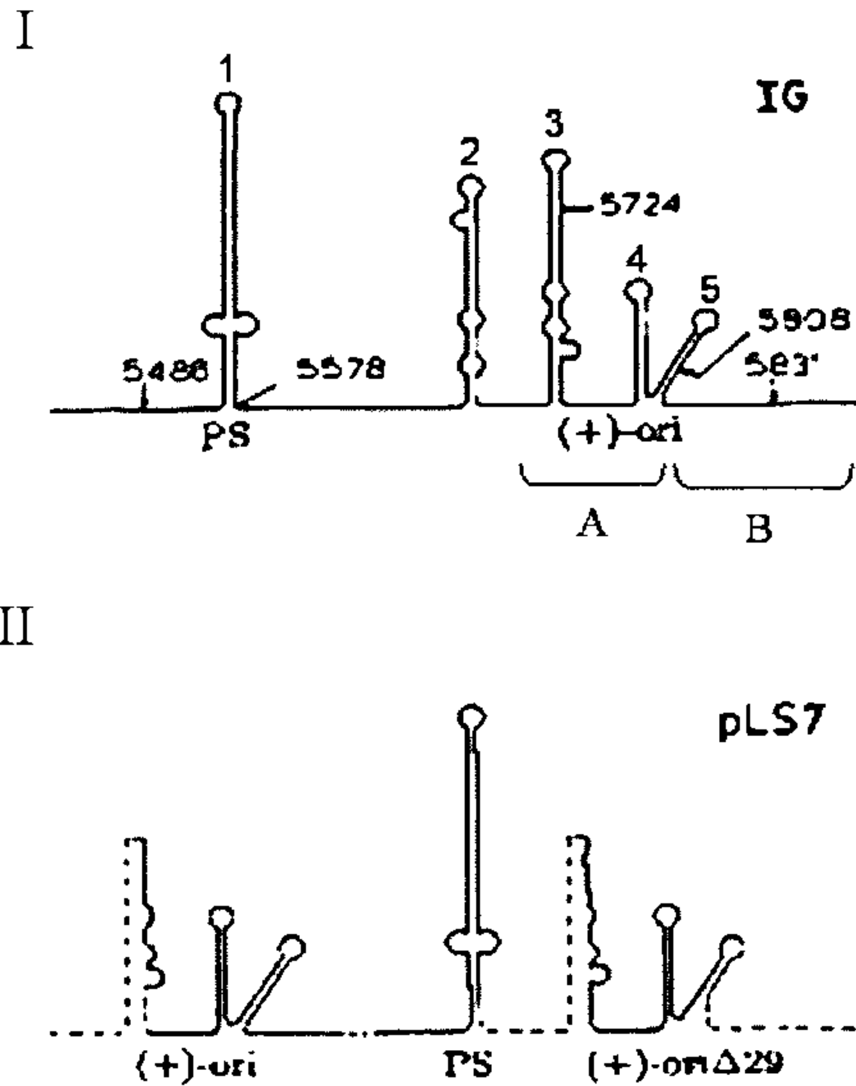


Figure 7: Modified from Spectrie et all (1992) I) ssDNA secondary structure of WT-type phage origin of replication, 1: Packaging signal; 2: negative (-) strand origin of replication; 3, 4, and 5 positive (+) strand origin of replication. A: Region essential for initiation and termination of positive strand synthesis. B: Region essential for initiation of positive strand synthesis. II) pLS7; Secondary structure of the fl origin of microphage producing plasmid (Specthrie et al., 1992)

1.4.4 The Intergenic Region and Replication

The intergenic region (IG) contains the positive and negative origins of replication and the packaging signal or morphogenic signal (Figure 7 I). It is the site at which the replication of the phage DNA is initiated. The synthesis of the second (negative) strand starts from the negative (-) strand origin of replication (Figure 7 I, hairpins labelled 2). The conversion of the single stranded (+) DNA into a double stranded DNA does not require any phage genes. The negative origin serves as a starting site where the RNA polymerase synthesises a primer, which is then used by DNA polymerase III to synthesise the (-) DNA strand. The negative strand origin is not absolutely required for phage replication. However phage replicates poorly if the negative origin is deleted.

The positive strand origin is absolutely required for phage replication and packaging (Figure 7 I, hairpins labelled 3, 4, and 5). Only the positive strand is packaged into new phage particles. Phage proteins pII and pX are required for the (+) strand synthesis. Protein pV has two functions in the Ff phage lifecycle. One is to coat ssDNA phage genomes in the cytoplasm for packaging and export, and the other is to negatively regulate the translation of pII (Fulford & Model, 1988; Michel & Zinder, 1989).

The positive origin has two important regions designated A and B (Figure 7 I). The A region (5769-5819) is the core region for positive origin synthesis. The A region is required for initiation and termination of positive strand synthesis. It also contains the pII nicking site (5780). Mutations in the A region of the positive origin reduce positive strand synthesis by 10 000 fold. The B region extends 100bp downstream of the A region. The B region is also required for initiation of positive strand synthesis, but is not required for termination of positive strand synthesis. Mutations in the B region reduce positive strand synthesis 100 fold (Dotto, Horiuchi & Zinder, 1984; Dotto, Horiuchi & Zinder, 1982; Dotto & Zinder, 1984; Zinder & Horiuchi, 1985).

The (+) strand replication is initiated by pII introducing a nick in the positive origin at position 5780. This allows DNA polymerase to bind and rolling circle replication to

take place. When one rolling circle cycle of genome is complete, pII cuts and ligates the newly displaced (+) strand to itself. This new strand can then be either coated in pV for phage export or used as a template for (-) strand replication to create another dsDNA Ff genome.

The packaging or morphogenic signal is a hairpin loop (Figure 7(I), 1). It acts as a tag for the DNA to be packaged into the phage and exported. Only the packaging signal on the positive strand is recognised and packaged (Zinder & Horiuchi, 1985). It has been noted however that filamentous phage can still be packaged without a packaging signal, the efficiency is reduced however to less than 0.1% of the normal rate (Russel & Model, 1989; Specthrie et al., 1992).

1.4.5 Microphage and pLS7

A microphage is a phage particle which is significantly shorter than the wild-type phage (Usually 45nm vs. 860nm). In Ff filamentous bacteriophage very short phage particles can be naturally produced, often after about 40 passages of the phage growth in the absence of clonal (plaque) purification. The miniphage production is due to duplication of the positive origin of replication (La Farina et al., 1987). When the positive origin is duplicated, the first pII nick allows the start of replication; when the next positive origin is reached, pII makes another cut and then ligates the two ends. This creates a small piece of single stranded DNA from ori1 to ori2 which is packaged into the phage particles. This however is inefficient and microphage only represent only a small fraction (0.1%) of the mass of the total phage produced (La Farina et al., 1987).

An engineered system of producing microphage uses the pBR322-derived plasmid pLS7 (Specthrie et al., 1992). On the plasmid pLS7 two positive origins separated by a packaging signal have been inserted, 200nt apart (Figure 7(II)). The first positive origin is wild-type; however the second positive origin has been mutated so that it can only serve as a termination signal, but not as an origin of replication (Specthrie et al., 1992). When pII binds to the wild-type + origin it cuts the strand and replication

starts. DNA replication proceeds through the packaging signal and reaches the next mutated positive origin (terminator) where pII nicks the strand and religates it to the initial nick site; this creates a 200bp piece of circular ssDNA which is packaged into the phage virion. This system however requires a helper phage to work, so full length helper phage are also produced and microphage constitute only a fraction (1%) of the total phage mass produced (Specthrie et al., 1992). Microphage are useful for structural analysis of phage termini.

1.4.6 Interference Resistance and Phage Replication

Because filamentous phage produce ssDNA they were used extensively for DNA sequencing. For sequencing, special vectors were used that carried the phage intergenic sequence and a plasmid origin of replication (phagemid vectors). To produce ssDNA for sequencing, the DNA of interest is inserted into the phagemid vector and transformed into a host cell. The host cell is then infected with a helper phage. This induces virion production of both the helper phage and the phagemid. Isolated virions could then be disassembled, ssDNA purified and sequenced. If a WT f1 ori is carried on the phagemid, the titre of both phagemid and phage produced is low due to a phenomenon called interference.

Interference in phage replication is a phenomenon in which one phage origin (phagemid, microphage origin) of replication affects another phage origin of replication (Helper phage). In filamentous phage interference is seen as a hundred fold reduction in phage titre. It is due to the secondary phage origin (phagemid or microphage) binding pII during the early replication and decreasing its availability to the helper phage (Enea & Zinder, 1982). Early replication is the point were the phage genome levels in the cell are set. If the number of phage genomes in the cell is reduced, this reduces the rate of transcription of phage proteins and lowers the phage titre. Therefore, for the efficient phagemid or microphage production the interference-resistant helper phage have to be used.

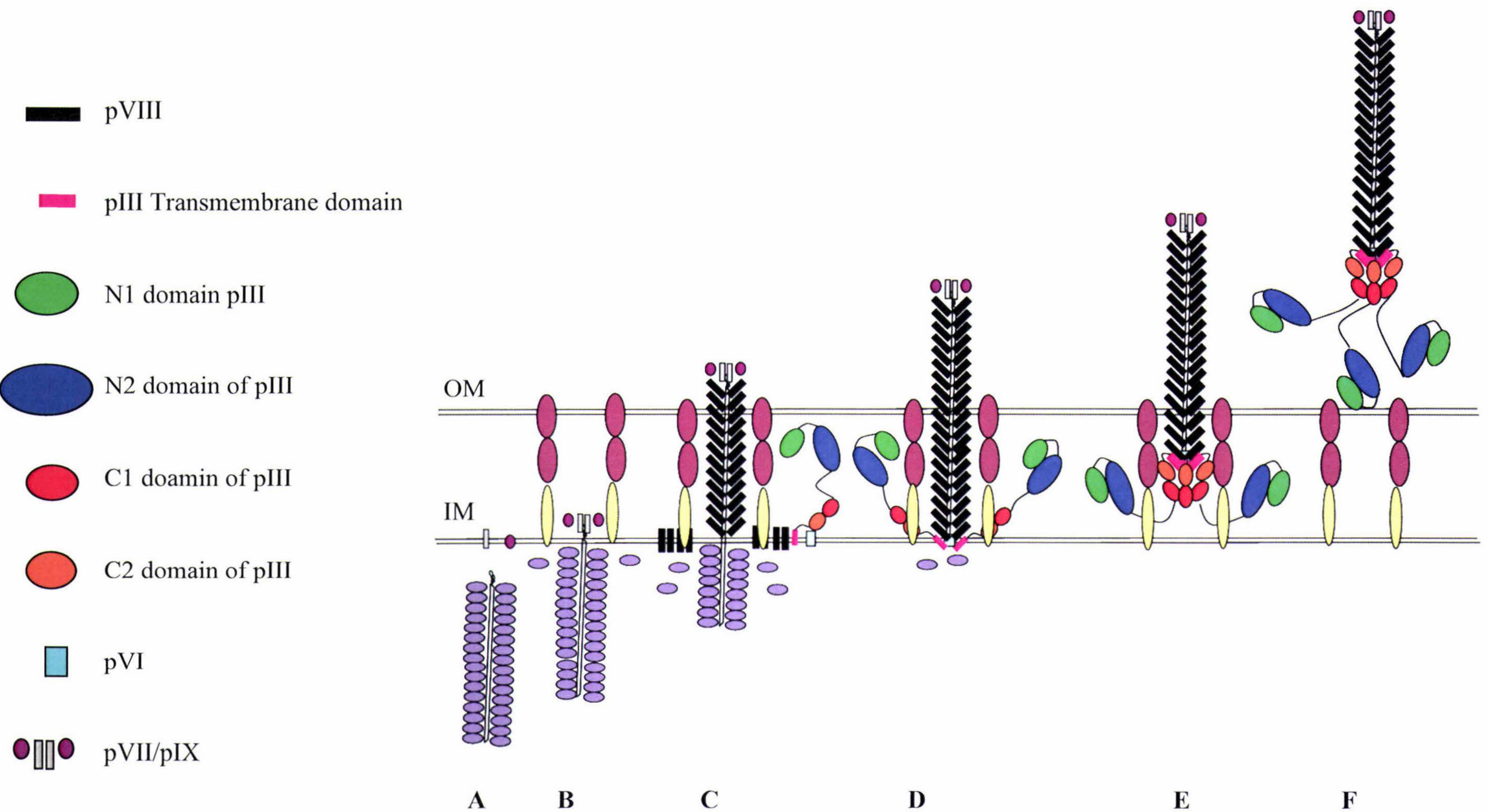

pVIII
pIII Transmembrane domain
N1 domain pIII
N2 domain of pIII
C1 doamin of pIII
C2 domain of pIII
pVI
pVII/pIX
pV
OM
IM
A
B
C
D
E
F

Figure 8: Phage assembly, termination and release from the host cell.
A) The positive strand is introduced into the export machinery starting from the packaging signal first (proximal end). B) The packaging signal forms interactions with the export machinery proteins and the virion proteins pVII, pIX and pVIII. The order in which this occurs however is unknown. C) After the initiation of assembly the phage is extruded through the cytoplasmic membrane as more pVIII monomers are added. As this happens, pV is removed from the (+) strand and recycled to the cytoplasm. The process of export requires ATP hydrolysis and thioredoxin (Feng, Russel & Model, 1997; Russel & Model, 1985; Russel & Model, 1986). D) Once the genome been fully covered by pVIII, the minor coat proteins pIII and pVI are added to the structure. E) It has been proposed that pIII undergoes a structural change in the C domain capping the phage structure and releasing it from the cell membrane (Rakonjac et al., 1999). F) The virion can now be fully released from the cell and into the growth medium.

The interference-resistant phage all carry mutations conferring interference resistance. IR1 is notation for a pair of such mutations, one within the pII regulatory sequence and the other within pII as defined by (Enea & Zinder, 1982)

1.4.7 The Ff Phage Export Apparatus and Phage Assembly/Secretion

Ff assembly and export from the cell resembles secretion of virulence factors or assembly of the bacterial surface structures in Gram-negative bacteria (Figure 8). Encoded by the phage genome are three genes that form the phage assembly and exporting machinery. pI and pXI form the assembly complex in the cytoplasmic membrane. The structure of this complex is unknown. As the phage are assembled at the cytoplasmic membrane they are extruded through a large gated pore in the outer membrane. This pore is consists of 14 pIV monomers (Linderoth et al., 1997; Opalka et al., 2003). pIV is a β-barrel protein. Its quaternary structure is a large ring with an internal gated pore (8nm).

ssDNA genomes coated by pV dimers are brought to the phage export complex (Figure 8A). The packaging signal (proximal end) is placed into the phage assembly complex in the membrane were it forms and interaction with pVII, pXI, and pVIII (Figure 8B). Then as the ssDNA genome passes through the inner membrane complex, pV is removed and replaced by pVIII, forming a shingle-like helical tube around the ssDNA genome (Figure 8C). When the phage DNA is fully packaged, pVI and pIII are added to the phage (Figure 8D) causing the termination of elongation and the release of the phage (Figure 8E, F). The mechanism by which the pIII/pVI complex achieves this is not understood as yet.

Currently the structure of the C-terminal region of pIII is unknown. It is unknown how the structure changes as it is included into the elongating phage and how it facilitates assembly termination and release. Genetic evidences suggest that the C-terminal region of pIII is involved in the termination of elongation and release of the phage (Rakonjac et al., 1999; Rakonjac & Model, 1998; Zacher et al., 1980). In Rakonjac J (1999) it was shown that if the C domain was truncated from 153 to only the final 93 residues, then termination of the phage could still occur. If the truncation was to the final 83 residues of the C domain, phage termination did not occur. In this

case the phage was not released from the cell, and elongation continued as cell associated filaments.

1.5 Aims of the Project

Because the infection is a reversal of the assembly process, it can be hypothesized that the mechanism of f1 entry involves a conformational change of the C domain of pIII which is the reverse of the one occurring during phage assembly. However this hypothesis can not be tested directly due to the lack of knowledge of pIII C domain structure. But if the stated hypothesis is true then a functional C domain of pIII should be required for infection. This re-formulated hypothesis is testable by structure-function analysis approach and is this project's primary objective.

To test this hypothesis, a system was be set up in which virions carried a mixture of two types of mutant pIII molecules: i) functional N1N2 domains fused to a short C domain that can be incorporated but cannot terminate assembly and release the phage from the membrane; ii) C domain only, which can terminate phage assembly, but lacks the receptor-binding domains N1N2 and therefore can not mediate infection by itself. If the hypothesis is correct then this system should produce normally terminated virion particles which are non infectious. Therefore, the first aim is to test infectivity of the particles, as produced particles should be non infectious. The second aim is to examine whether the existing system produces normally terminated particles that carry designed pIII mutants.

The secondary objective of the project is to improve the microphage producing system for future structural work on pIII. This is to be carried out starting from the microphage-producing plasmid pLS7 (Specthrie et al., 1992). The length/diameter ratio (860nm/6nm) of Ff filamentous phage is too high for current imaging techniques to image only the pIII/pVI tip of the phage. The length to diameter ratio of filamentous phage can be reduced by making microphage (45nm/6nm). In a microphage the pIII/pVI tip comprises of a much larger portion of the phage length than in a full length phage. This makes microphage a much more suitable object for structural studies than the wild type Ff filamentous phage.