Unlocking the M13 (F1) Virion

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

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Nicholas James Bennett
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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.

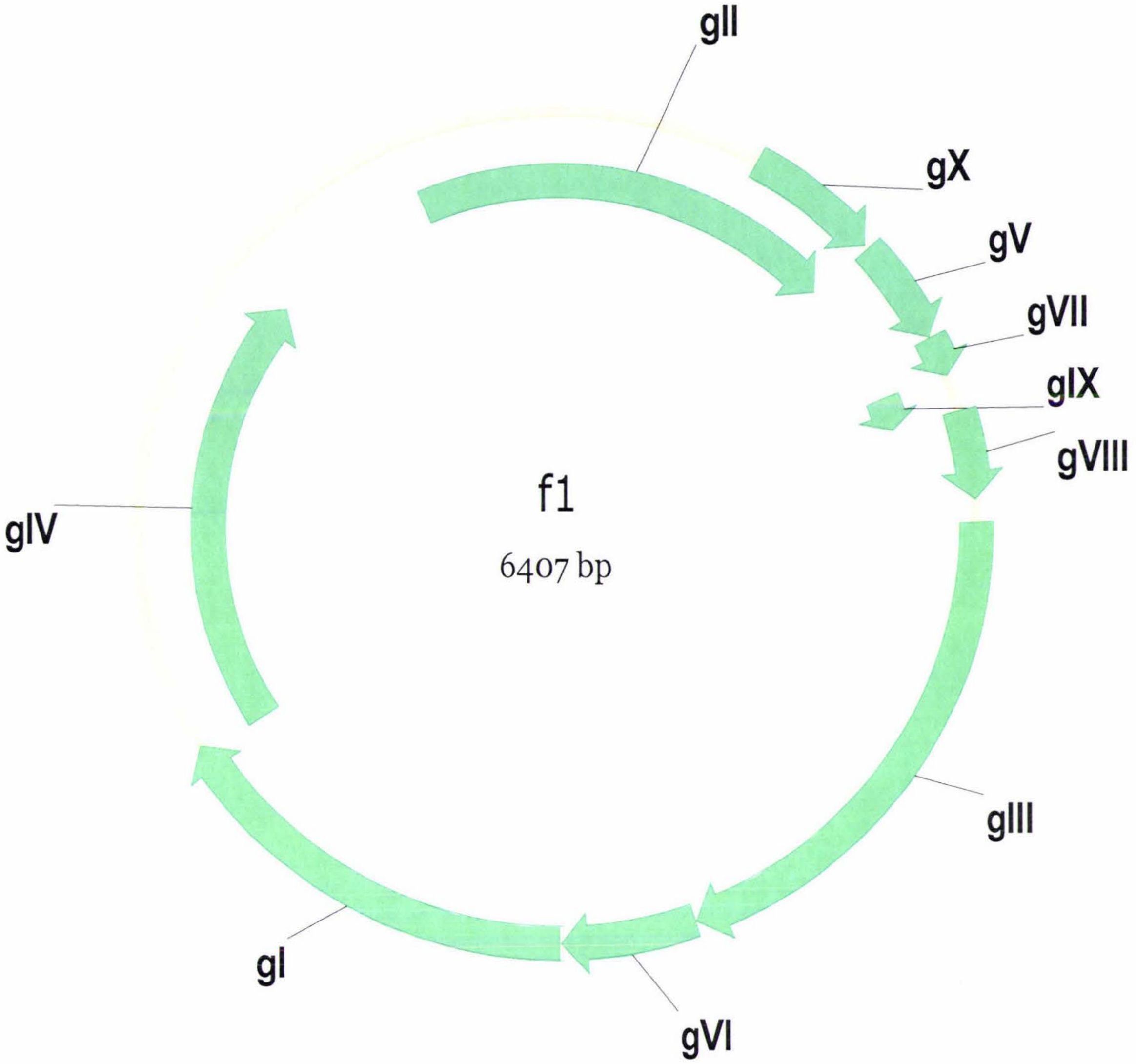
1.3 The Ff Phage Structure

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

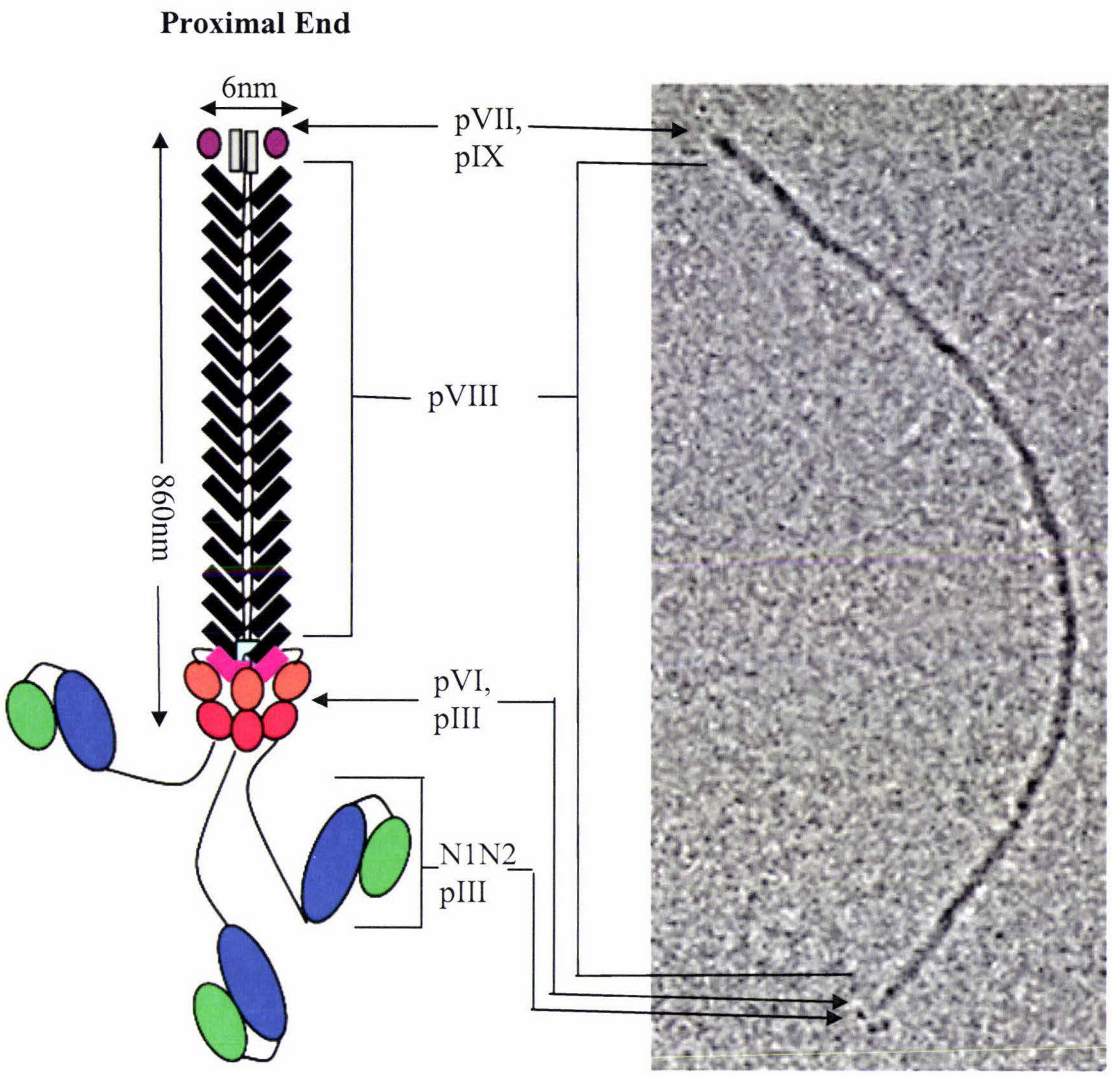
About 3000 copies of pVIII forms the tube of the Ff filament. Its structure and structural arrangement within the phage has been solved using x ray fibre diffraction and x ray crystallography (Figure 3) (Marvin, 1998; Marvin et al., 1994; Overman & Thomas, 1995; Williams et al., 1995). pVIII structure 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.



▮ 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).

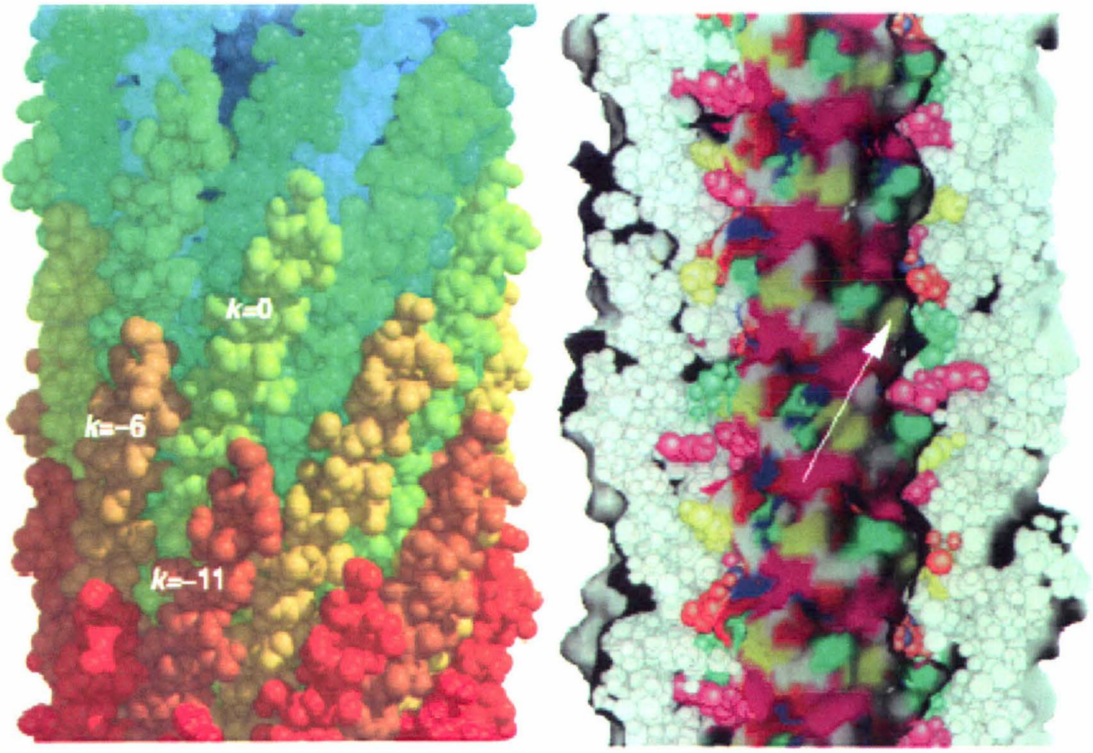


Figure 3: pVIII forms a shingle-like helix array around ssDNA genome. On the left is space-filling model of major coat protein pVIII within phage virion structure. On the right is a model of longitudinal section showing interior channel formed by pVIII around the collapsed ssDNA circular genome. The 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

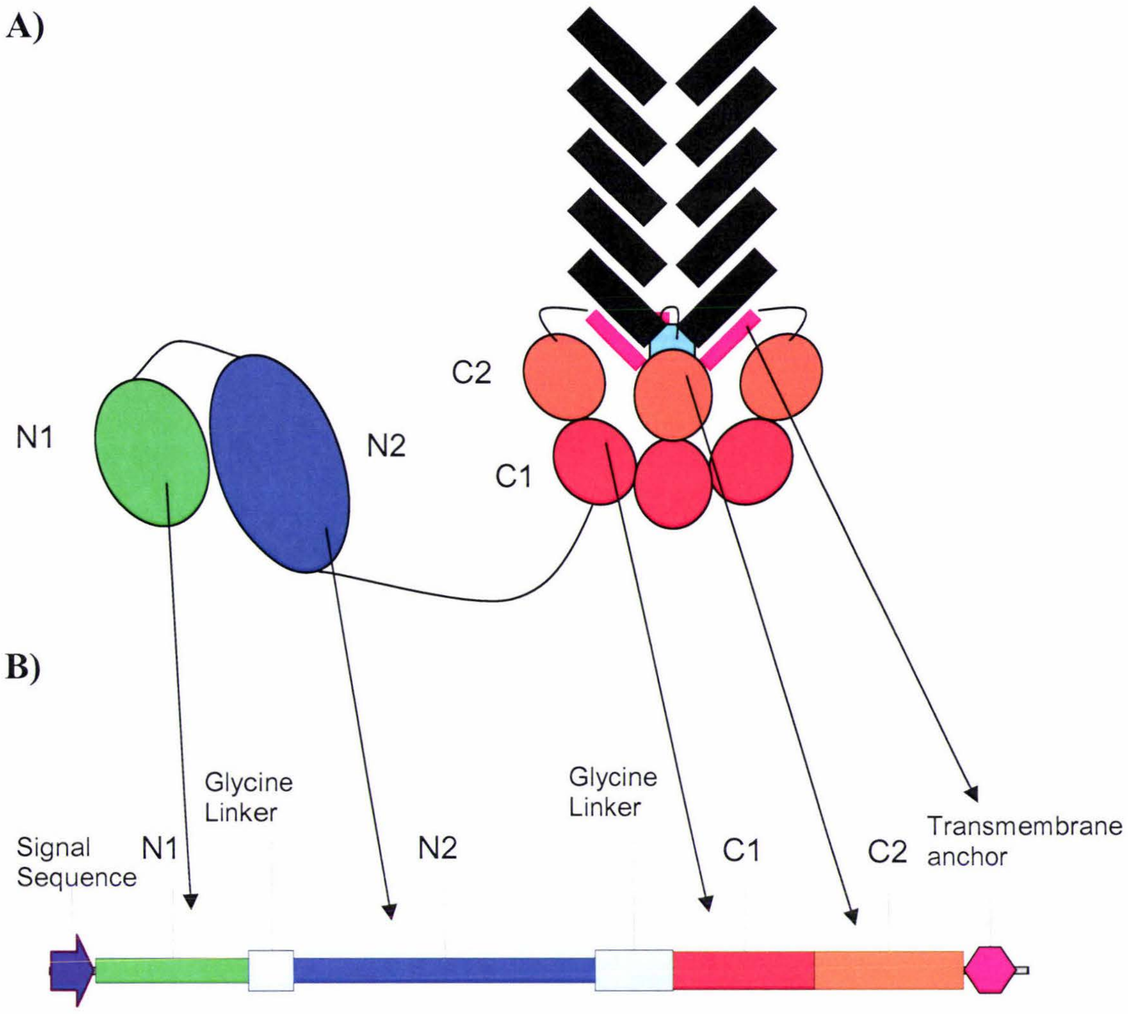


Figure 4: A) Schematic representation of the distal end of Ff filamentous phage, showing domain organisation of pIII. pVIII (Black rectangle) and pVI (Light blue rectangle) are also shown. B) pIII domain arrangement. The signal sequence is cleaved during membrane insertion. Arrows show structural domains (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 fl 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).

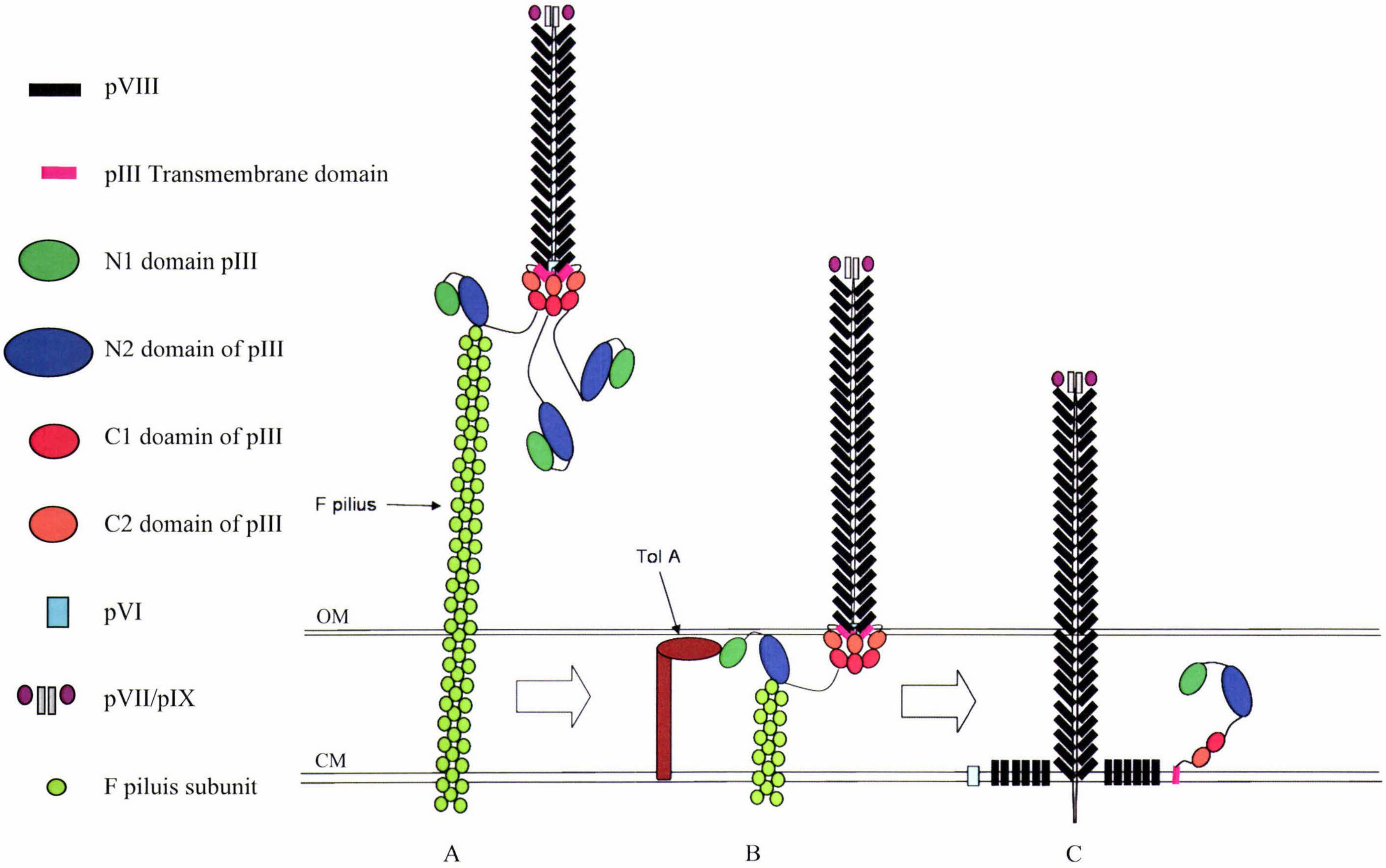

pVIII
pIII Transmembrane domain
N1 domain pIII
N2 domain of pIII
C1 doamin of pIII
C2 domain of pIII
pVI
pVII/pIX
F piluis subunit
F pilius
Tol A
OM
CM
A
B
C

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.

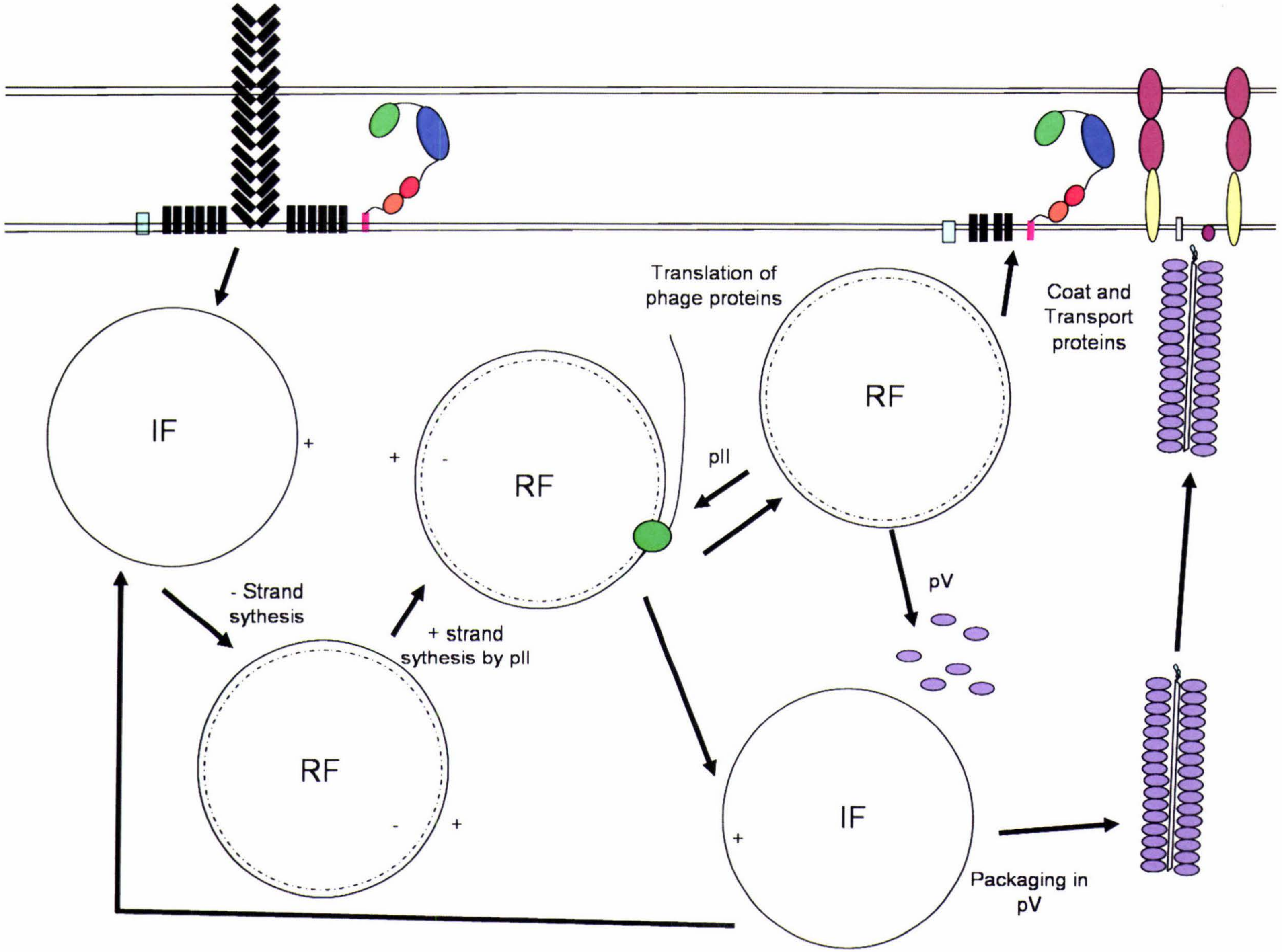

Translation of phage proteins
Coat and Transport proteins
IF
+
+
-
RF
pII
RF
- Strand sythesis
+ strand sythesis by pII
pV
RF
-
+
+
IF
Packaging in pV

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.

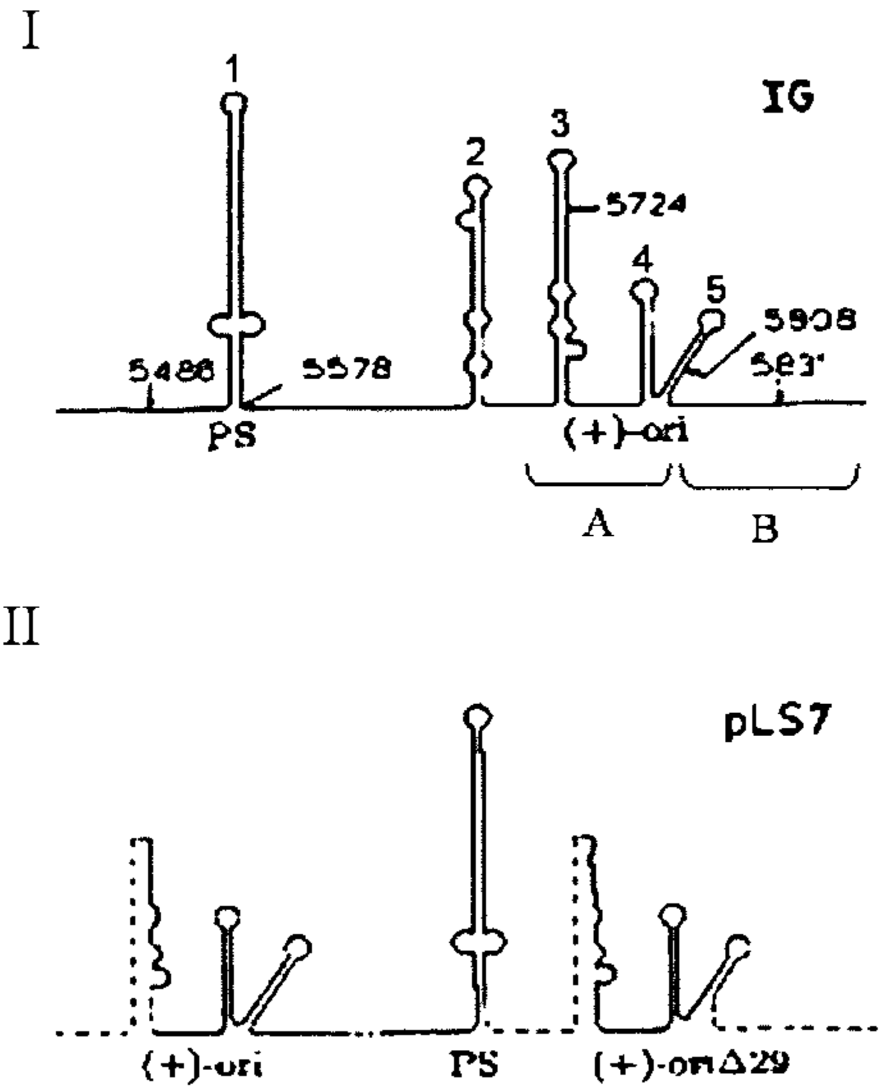


Figure 7: Modified from Spectrie et all (1992) I) ssDNA secondary structure of WT-type phage origin of replication, 1: Packaging signal; 2: negative (-) strand origin of replication; 3, 4, and 5 positive (+) strand origin of replication. A: Region essential for initiation and termination of positive strand synthesis. B: Region essential for initiation of positive strand synthesis. II) pLS7; Secondary structure of the f1 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 fl 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.

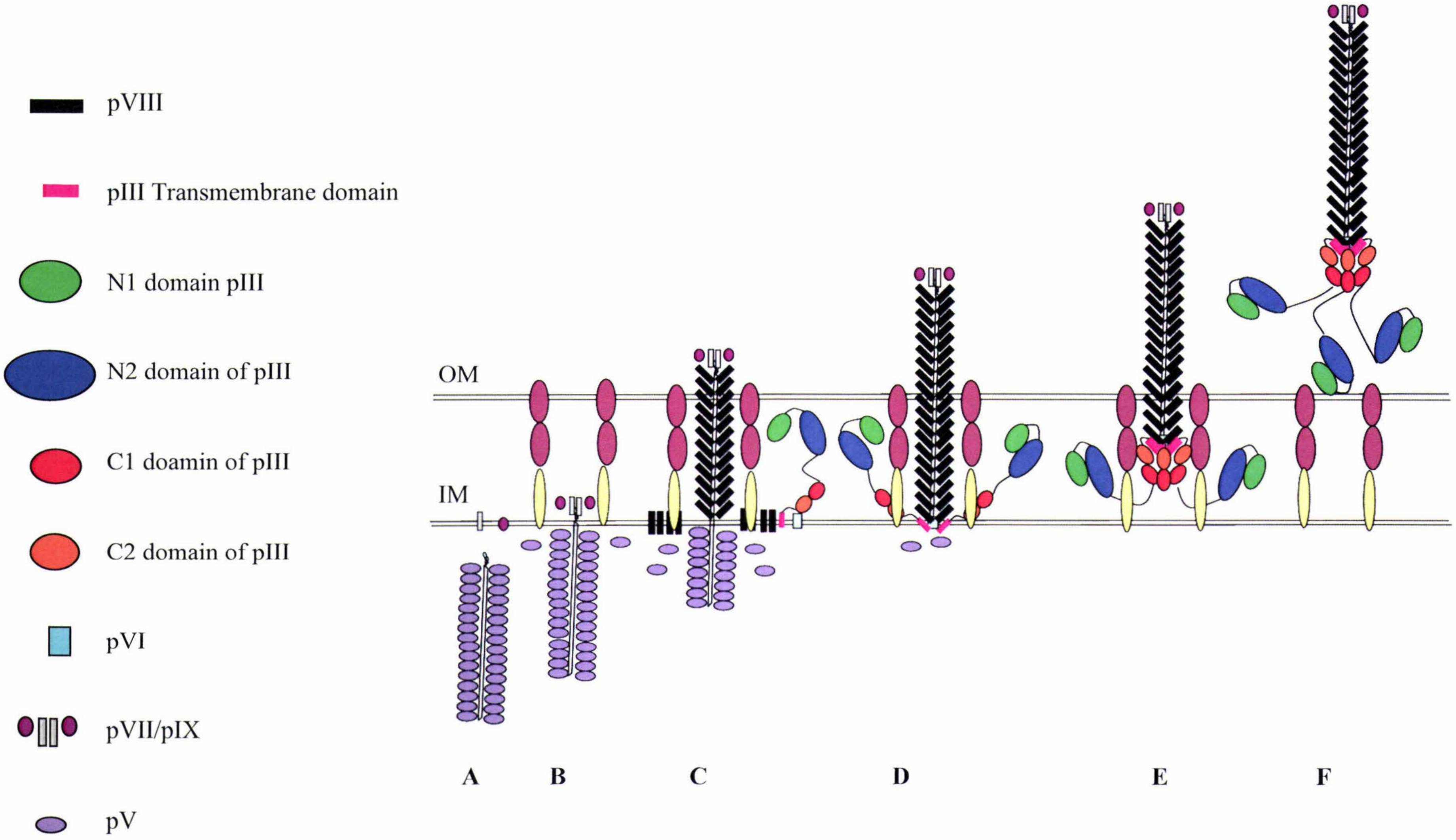

pVIII
pIII Transmembrane domain
N1 domain pIII
N2 domain of pIII
C1 doamin of pIII
C2 domain of pIII
pVI
pVII/pIX
pV
OM
IM
A
B
C
D
E
F

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 fl 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.

Chapter 2

Materials and methods

2.1 Bacterial Strains, Culture Condition and Chemicals

Bacterial strains are listed in Table 1. Phage strains are listed in Table 2. All plasmids used in this thesis are listed in Table 3. All primers used in this thesis were from Invitrogen and are listed in Table 4. *E.coli* was cultivated in TB (10g Bacto tryptone, 1g yeast extract, 4g NaCl, 1g glucose), 2×YT (Gibco), FB (25g of Bacto tryptone, 7.5g yeast extract, 6.0g NaCl, 1g of glucose, 0.05M Tris HCL, pH 7.6 per litre), FGB (25g of Bacto tryptone, 7.5g yeast extract, 6.0g NaCl, 2g of glucose, 0.05M Tris HCL, pH 7.6 per litre) or FAB (25g of Bacto tryptone, 7.5g yeast extract, 6.0g NaCl, 2g arabinose, 0.05M Tris HCL, pH 7.6 per litre). Antibiotics used were ampicillin (Amp), 100μg/ml, tetracycline (Tet), 15μg/ml and chloramphenicol (Cm), 25μg/ml. *E.coli* cultures were incubated at 37°C. Liquid cultures were aerated by shaking (200rpm) unless otherwise indicated. All analytical grade chemicals were sourced from either Sigma or BDH.

2.2 Molecular Biology Tools

All of the restriction enzymes were sourced from either New England Biosciences (NEB) or Roche. Plasmid DNA purification was carried out using: Roche miniprep kit, Invitrogen 5 minute Fast prep kit, or Qiagen Midi Prep kit. Gel slice purification was carried out using the protocol outlines by (Heery, Gannon & Powell, 1990) and concentrated using ethanol precipitation. *PWO* polymerase (Roche) was used to amplify all fragments aimed for cloning. This proof-reading polymerase has 10× increased fidelity in comparison to Taq polymerase. All molecular biology techniques were carried out according to protocols found in Molecular Cloning (Sambrook, Fritsch & Maniatis, 1989) or manufacturer's instructions.

Table 1

Bacterial Strains

Strain	Genotype
Top10	F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*Δ139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G
K833	F+ *sup*F
K1822	F+, *hsd*R, *mcr*B, araD139, Δ*ara*ABC-leu)7697, *lac*I74, *gal*V, *gal*K, *str*A, *thi*
K561	*Hfr*C, λ+, *rel*A1, *spo*T1 T2R, *Omp*F627, *fad*L701, *sup*D, *lac*Iq
TG1	Δ(lac-pro), *sup*E44, *thi*, *hsd*R 5, F′ *tra*D36, *pro*A+B+, *lac*Iq,*lac*ZDM14
K91	F+, HFR, λ+, *rel*A1, *spo*T1 T2R, *Omp*F627, *fad*L701
K1762	K561 (pJARA122 and pJARA131)
K1271	K91(pLS7)
K1926	TG1 (pJARA200)
K1923	K1822 (pJARA24, pBAD hisB)
K1924	K1822 (pNJB4, pJARA24)
K1925	K1822 (pNJB5, pJARA24)

Table 2

Phage Strains

Strain	Genotype
f1	Wild Type
f1d3	f1 ΔgIII
R21	*gII* ^{am}20
R408	Δ*PS*, IX, T30A, *IR*1, *gtrx*A2
R408d3	Δ*PS*, IX, T30A, *IR*1, *gtrx*A2 ΔgIII
R474	*ΔPS* VII 127V
VCSM13	Stratagene (Helper phage M13/R:: pA15ori, Km^R)
VCSM13d3	VCSM13 Δ*gIII*

Table 3

Plasmids

Name	Description	Reference
pJARA24	pGZ119EH, *tac*-pelB-C pIII	(Rakonjac et al., 1999)
pJARA112	pBR322, *psp*-gIII	(Rakonjac, Jovanovic & Model, 1997)
pJARA131	pGZ119EH, *psp*FABCD operon	(Rakonjac et al., 1997)
pJARA200	pND372, *lac*-gIII	(Rakonjac & Model, 1998)
pLS7	pBR322, fl IG (ori+ Δ102, PS, ori+Δ)	(Specthrie et al., 1992)
pNJB2	pACYC184, *tac*-gII	This Study
pNJB 4	pBAD his B, $araP_{BAD}$-NdC83 gIII	This Study
pNJB5	pBAD his B, $araP_{BAD}$-pIII	This Study
pNJB6	pCR ZeroBlunt TOPO, fl IG (ori+ Δ102, PS, ori+Δ)	This Study
pNJB7	pCR ZeroBlunt TOPO, fl IG (repaired microphage origin; ori+, PS, ori+Δ)	This Study
pGZ119EH	*Col*D, Cm^R, *lac*I^q, *ptac*	(Lessl et al., 1992)

Table 4

Primers Used in Study

Name	Sequence	Restriction Sites/Fusion Partner	Target
NB2	CCA CGA TGC GTC CGG CGT AGA	Sequencing	pLS7
NB9	CAT GGC ATG GCT AAA AAA TTA TTA TTC GCA ATT CCT TTA G	*Nco*I	gIII
NB10	CGT CAC CGA CTT GAG CCA TTT GGG ATT CAT AAT CAA AATCAC CGG AAC CA	NB11	gIII
NB11	TGG TTC CGG TGA TTT TGA TTA TGA ATC CCA AAT GGC TCA AGT CGG T	NB10	gIII
NB12	TCG GAA TTC TAT TAA GAC TCC TTA TTA CGC AGT AT	*Eco*RI	gIII
NB14	TCC CCG CGG CCG ACA CCA TCG AAT GGT GCA A	*Sac*II	*tac*
NB15	AAA ACT AGC ATG TCA ATC ATA TGC TAT GGT CCT TGT TGG TGA AG	NB16	*tac*-pII
NB16	ACC AAC AAG GAC CAT AGC ATA TGA TTG ACA TGC TAG TTT TAC GAT	NB15	gII-*tac*
NB17	TCC CTC GGG TTA TGC GAT TTT AAG AAC TGG CTC A	*Ava*I	gII
NB26	AGA CGT TTT CCA GTT TGG AAC AAG		pLS7
NB27	GTC CAA GCT TGC CCG AGA TAG	*Hind*III	pLS7
NB28	CCT ATA AAA ATA GGC GTA TCA CGA G		pLS7
M13 Forward	GTA AAA CGA CGG CCA G	Sequencing	pCR zeroBluntII
M13 Reverse	CAG GAA ACA GCT ATG AC	Sequencing	pCR zeroBluntII
T7	ATT AAC CCT CAC TAA AGG GA	Sequencing	pCR zeroBluntII
T3	TAA TAC GAC TCA CTA TAG GG	Sequencing	pCR zeroBluntII

2.3 Construction of Plasmids

Plasmids coding for WT and mutant pIII that carried the N1 and N2 domains were derivatives of pBAD hisB (Invitrogen). Plasmid pNJB3 coding for WT pIII (Figure 11B) was constructed in two steps: first, the gIII fragment was amplified by PCR with f1 phage RF DNA as a template, using oligonucleotides NB9 and NB12. The primer NB9 contains an NcoI site and primer NB12 contains an *Eco*RI site. The PCR-amplified pIII was then cloned into *Nco*I/*Eco*RI-digested vector pBAD his B (Invitrogen). This placed the gIII WT under the control of the arabinose promoter ($araP_{BAD}$).

Plasmid pNJB4, coding for mutant pIII with N1N2 domains fused to the non-functional C-terminal fragment (83 residues), named pIII NdC83 through this thesis (Figure 11A), was constructed by overlap extension PCR (Horton et al., 1989). First round PCR primer pairs were: NB9/NB10 and NB11/NB12 and the template for both reactions was f1 phage RF DNA. The second round of PCR was carried out using the primer pair NB9 and NB12 and the templates were the two PCR fragments from the first round of PCR. The product of the second round of PCR was then cloned into pCR® -Blunt II -TOPO® (Invitrogen) and the inserts of three independent transformants were sequenced. The gIII *Nco*I/*Eco*RI fragment of an error-free clone was excised from the pCR® -Blunt II -TOPO® backbone and placed under the control of the $araP_{BAD}$ promoter by cloning into *Nco*I-*Eco*RI-digested vector pBAD his B (Invitrogen).

Plasmid pNJB2 is a pACYC184 derivative containing a *tac*-gII fusion. The *tac*-gII fusion was created by overlap extension PCR (Horton et al., 1989). In the first round of overlap extension PCR the primers NB14/ NB15 and template pMAL-2X (NEB) were used to amplify *lac*I^q and the *tac* promoter. Primers NB16/NB17 and template DNA from f1 phage particles (infectious form) were used to amplify gII. The second round of PCR was carried out using primers NB14 and NB17 and fragments obtained in the first round of PCR. The band was gel- purified and digested with *Sac*II and *Ava*II. The fragment was then ligated into SacII/*Ava*II-digested pACYC184 (NEB).

The microphage-producing plasmid pNJB7 is a pCR® -Blunt II -TOPO® (Invitrogen) derivative containing a microphage producing fragment from pLS7 (Specthrie et al., 1992). The microphage producing fragment was amplified by PCR using primers NB26/NB28 and plasmid pLS7 as a template. The amplified fragment was cloned into pCR® -Blunt II -TOPO® (Invitrogen), using TOPO® cloning kit. Fragments from three independent clones were examined by sequencing. Previous sequencing of pLS7 identified an error in the + ori (Base pair 102 of the f1 IG). The same error was identified in the three sequenced clones. This mutation was repaired by PCR mutagenesis of the TOPO clone (pNJB6), using oligonucleotide pair NB27/ vector-derived T3 primer. The fragment was then digested with *Hind*III/*Pst*I and inserted into *Hind*III/*Pst*I sites of pNJB6. *Hind*III was a unique site derived from the f1 origin insert. Inserts from three independent transformants were then sequenced to ensure correct insertion of fragment.

2.4 Phage Protocols

2.4.1 Phage Stocks

All working stocks of phage f1, f1d3 and R21 were prepared by the following method. An exponentially growing culture (around 10^8 cells/ml) of the appropriate bacterial strain in either TB or 2×YT was infected with phage at an m.o.i 20. The phage culture was then incubated for four hours. Cells were removed by centrifugation (7000rpm/15 min/SS34). The supernatant was then heated to kill all remaining bacterial cells (65°C/20 min) and centrifuged again (7000rpm/15 min/SS34). Phage stocks were titred as described (2.4.3) and stored a 4°C.

2.4.2 Growth Experiments for producing phage with mutant pIII

Exponentially growing cultures ($OD_{600} = 0.2$) of strains K1923, K1924, and K1925 grown in FGB medium were infected with f1d3 phage at a multiplicity of infection of

100 phage per cell, for 1 hour (without shaking; Figure 9). Infected cells were separated from unabsorbed phage by two rounds of centrifugation (5000rpm/10min/30°C), resuspended in FAB medium supplemented with Amp (100μg/ml), Cm (25μg/ml) and IPTG (0.1mM) and incubated for 4 hours. Following the incubation period, the phage culture was centrifuged (7000rpm/20min/4°C). Supernatant containing the released phage was collected for further analysis.

Bacterial pellets were resuspended in TE (10mM Tris, 2mM EDTA, pH 7.6) at 50 OD_{600} units per millilitre and frozen at -80°C and later used for analysis of proteins by SDS-PAGE and Western blotting (not shown).

2.4.3 Concentration of Virions by PEG precipitation

Culture supernatants from infected cells obtained as described above were first treated with DNase I (1μg/ml) and RNase A (1μg/ml) for 30 minutes at room temperature. Phage were then concentrated by an overnight precipitation in PEG8000 (2.5% w/v) and NaCl (0.5 M) at 4°C. Precipitate was collected by centrifugation (10000rpm/45min/4°C). The pellet was resuspended in 400μl of TE (10mM Tris, 2mM EDTA, pH7.6) buffer.

2.4.4 Phage enumeration by plate titering

Phage were titred on either TG1 (f1), K1762 (f1d3) or K833 (R21). Phage were first titred by a quick method. 10μl drops of phage dilutions were placed on the surface of a lawn of the appropriate *E.coli* strain on 2×YT agar plates. Plaques in the area of absorbed drops were counted to determine titre. Once a rough titre was determined using this quick method, the titre was determined more accurately by standard phage plating method. At least three hundred plaques from the total of three plates were counted to determine the titre.

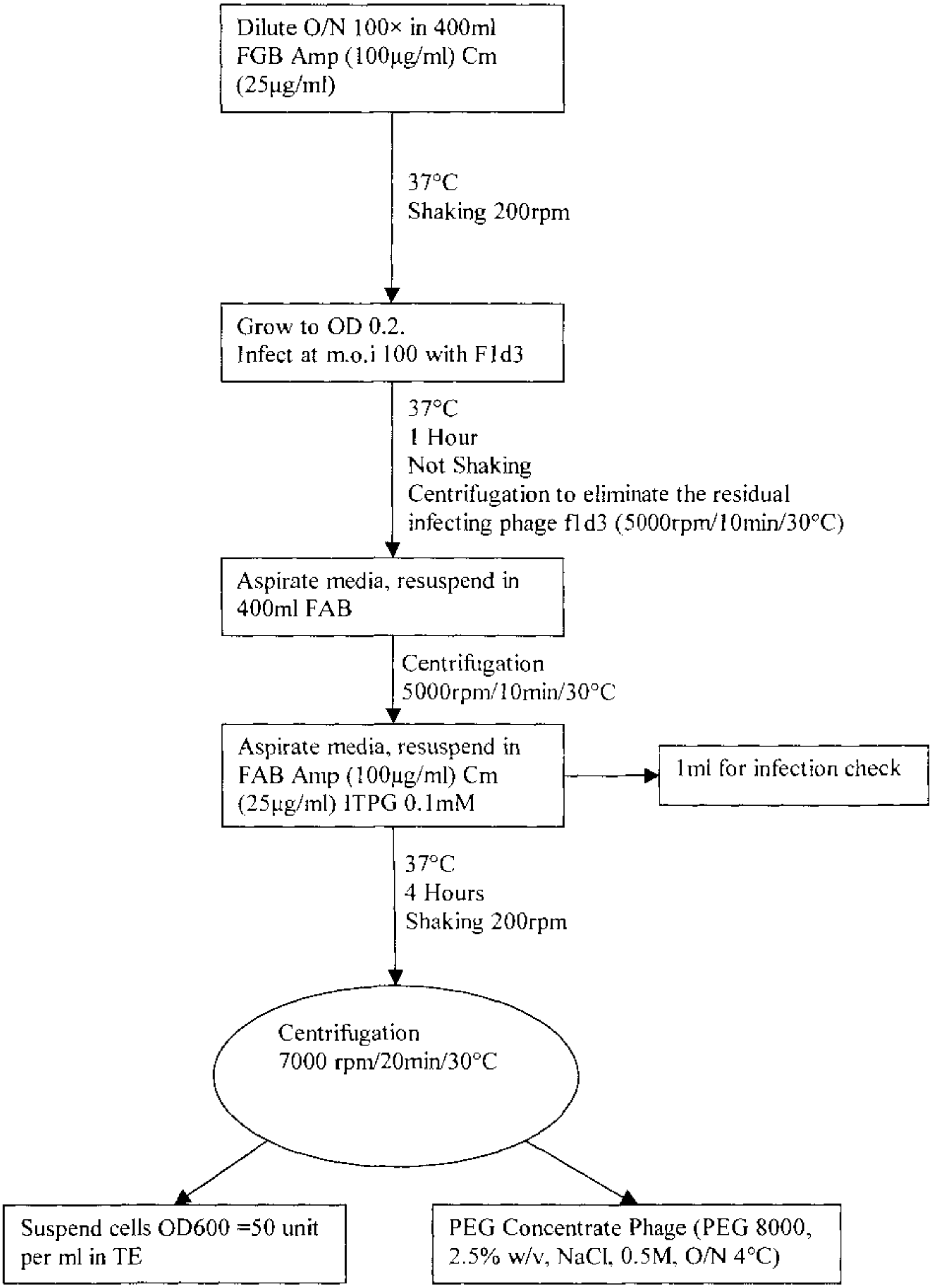


Figure 9: Flow diagram of the production of the virions carrying combination of pIII constructs.

2.5 Agarose gel electrophoresis of the phage and quantification

A genome equivalent is a measure of particle mass, and is defined as a particle (or its portion) containing one encapsulated genome (Figure 10). Thus, a particle containing ten genomes represents ten genome equivalents, as do ten particles containing one genome each (Rakonjac & Model, 1998). In all experiments, the number of genome equivalents was determined from agarose electrophoresis of phage ssDNA, released from the SDS disassembled virions (Nelson, Friedman & Smith, 1981). Prior to electrophoresis, virions were disassembled by incubation in SDS-containing buffer (1% SDS, 1×TAE buffer at 70°C for 20 min). After electrophoresis, phage ssDNA was stained with the EtBr and quantified densitometrically. Since the amount of ssDNA in a band is not linearly proportional to the intensity of the fluorescence, every gel contained a set of twofold dilutions of a standard, typically 1280 to 40 ng per lane, used for calibration. Purified phage ssDNA from f1d3, the concentration of which was determined spectrophotometrically, was used as a standard. The gel was photographed with a CCD camera (BIORAD), and quantitative analysis preformed using software packages Image Gauge (Fuji Film), and Excel (Microsoft). A second order polynomial function was used to fit the standard curve over the range. Conversion of the calculated amount of ssDNA (ng) in the sample into the amount genome equivalents was carried out based on the molecular mass of ssDNA genome, which was again calculated from the base composition and length. For f1d3, 1ng of ssDNA is equal to 3.82×10^8 genome equivalents (See Appendix 1).

Native virion agarose gel electrophoresis was used to separate virions of various lengths and to detect free phage DNA when the stability of phage was analysed (Nelson et al., 1981). Samples were loaded onto low density agarose gels (0.6%) in DNA loading buffer (1× TAE, 5% Glycerol, 0.25% BPB) at 7.6×10^{10} genome equivalents per lane. When stability of the phage was analysed, the detergent sarkosyl (0.01%) was added to the loading buffer and the sample was incubated at room temperature for ten minutes prior to loading.

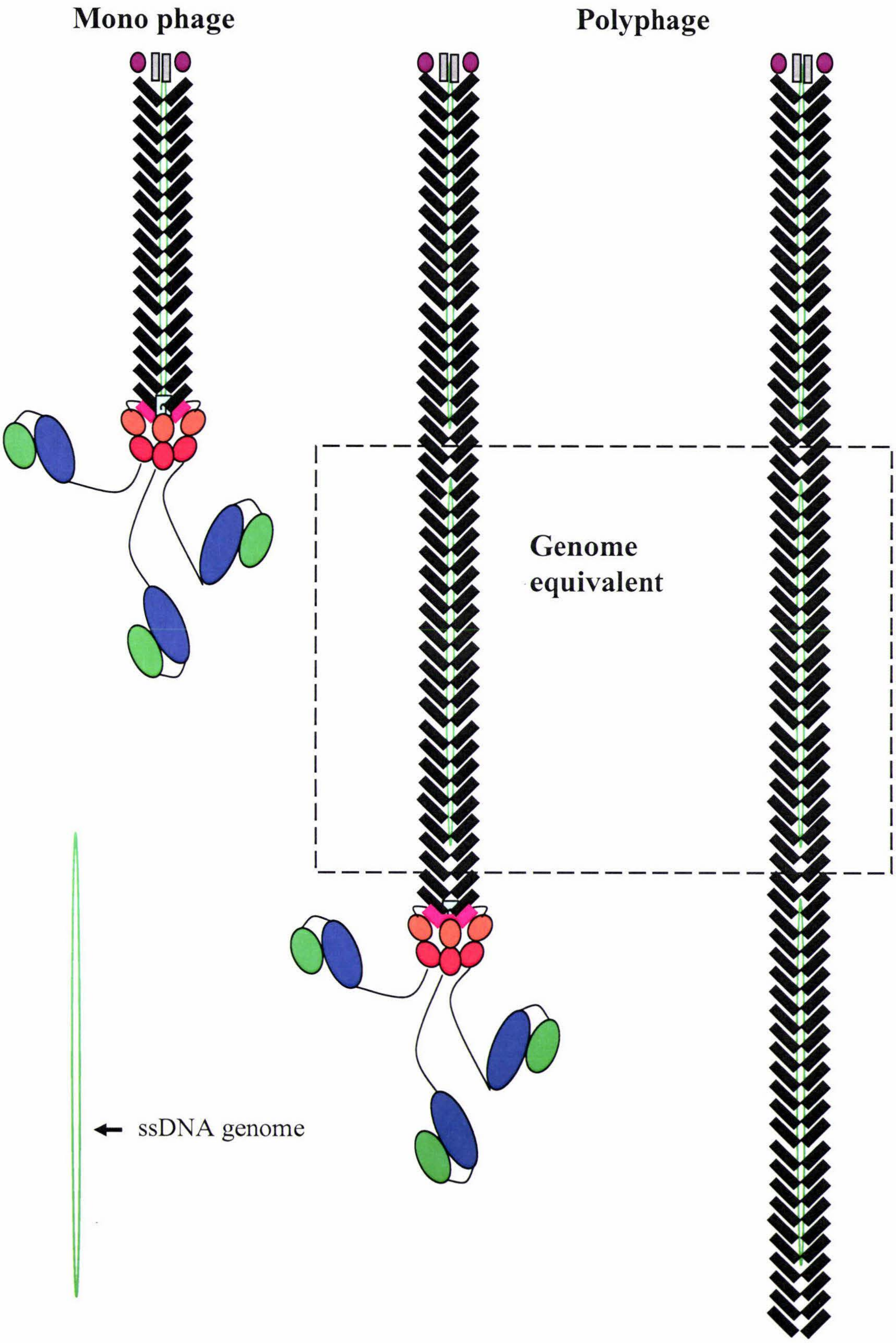

Mono phage
Polyphage
Genome equivalent
ssDNA genome

Figure 10: A diagram showing a genome equivalent. A monophage is a virion which contains only one ssDNA genome. A polyphage is a virion particle which carries multiple copies of ssDNA genome. A genome equivalent is a portion of a phage or phage like filament that contains one ssDNA genome (Rakonjac & Model, 1998).

Electrophoresis was preformed at 3 V/cm for sixteen hours. After electrophoresis, free phage ssDNA was first detected by staining the gel in EtBr. To detect the position of the particles in the gel, virions were disassembled by soaking the gel in alkaline buffer, neutralised in 0.45M Tris pH7.1, and then the phage DNA was stained in EtBr.

2.6 Protein Electrophoresis and Western Blots

Proteins from the phage samples were separated by SDS-PAGE, using either glycine (Laemmli, 1970) of tricine (Schägger & von Jagow, 1987) gel systems, transferred to nitrocellulose filters, and then detected using appropriate antibodies. Protocols varied slightly, depending on the antibody used. With antibody R164 (anti-pIII; Rakonjac *et al.*, 1997), an anti-rabbit antiserum conjugated to HRP was used and detected with the ECL Plus detection system (Amersham Corp., Arlington Heights, IL). With antibody 19-38 (anti pVI; Endemann & Model, 1995), an anti-rabbit antiserum conjugated to AP was used and detected using substrates NBT and BCIP in alkaline buffer (Blake et al., 1984). Antibody R164 was raised against the C-terminal decapeptide of pIII (FANILRNKES). Therefore it is able to detect all C-terminal fragments of pIII (Rakonjac et al., 1997). Antibody 19-38 was raised against the C-terminal peptide of pVI, has been described (Endemann & Model, 1995). The basic buffer was TBS (30mM Tris, 150 mM NaCl, pH8.0) with 0.05% Tween 20. Blocking and antibody binding buffers also contained 5% non-fat dry milk.

Results

There are two main aims presented in this thesis. The first one is to examine the involvement of the C domain in the infection process of Ff filamentous phage (Chapter 3) and the second is to design a microphage-producing system for the examination of the structure terminus of phage that carries pIII (Chapter 4).

Chapter 3

The Involvement of the pIII C domain in Ff Filamentous Phage Infection

3.1 Experimental System for Production of Mutant (NdC83/Cd) Phage

To test weather a termination-competent C domain is required for infection, a system for producing mutant phage was constructed (Fig.11). Phage particles were generated by infection with an f1 phage mutant with a complete deletion of gIII (f1d3).

This phage system involved the construction of a mutant pIII carrying a truncated C domain which was not able to terminate phage assembly by itself, fused to functional N1N2 domains which were required for infection. The C domain is truncated so that only the final 83 residues are remaining. This C-terminal fragment is sufficient for incorporation of pIII into the phage virion, but can not mediate the termination of assembly and release of the phage virion from the membranes (Rakonjac et al., 1999). To construct this mutant (named NdC83 throughout this thesis) the sequence coding for N1N2 domains was fused to the sequence coding for truncated C domain by overlap-extension PCR (Horton et al., 1989) (Figure 11A). The mutant gIII was then placed under the control

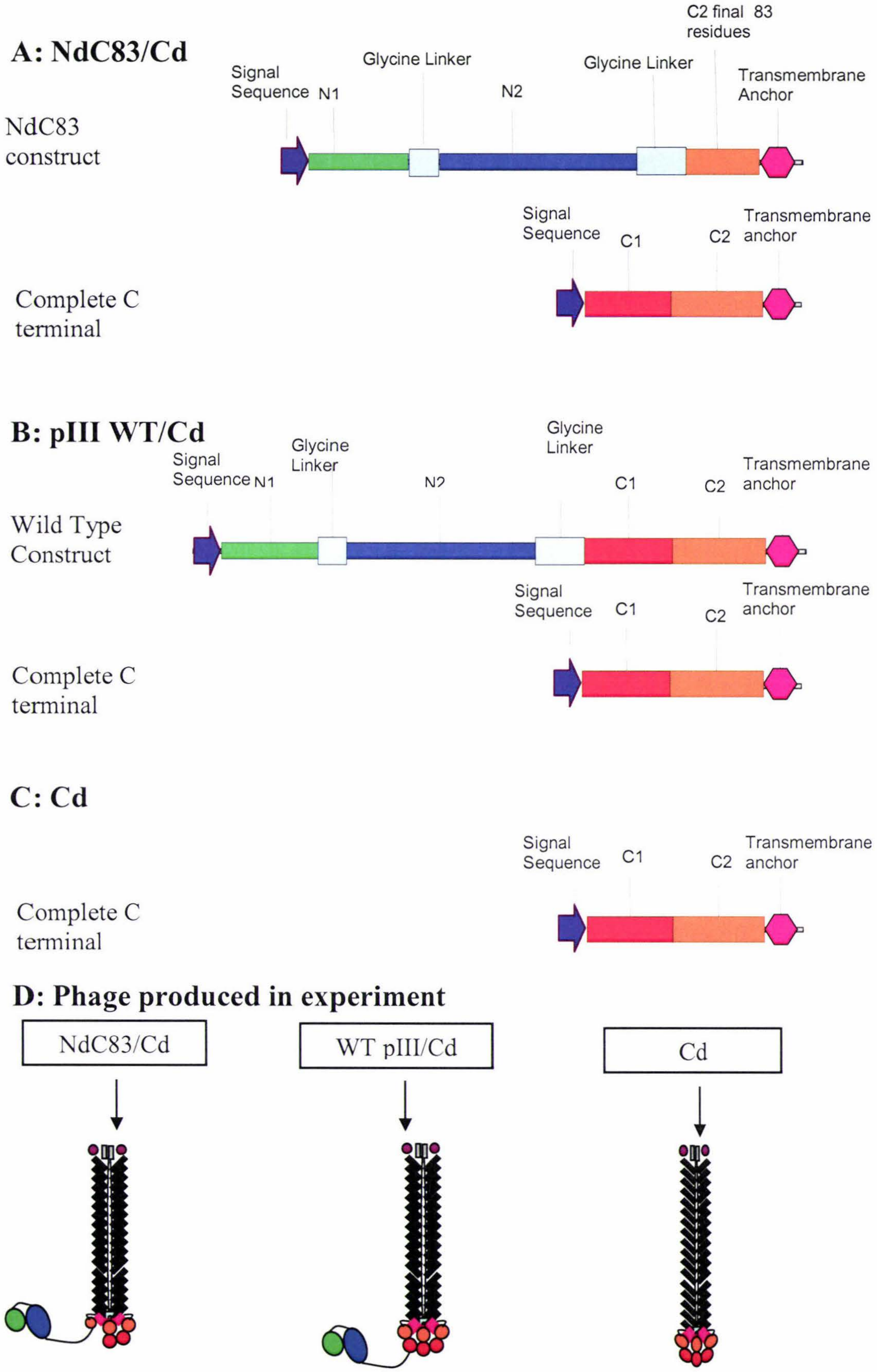


Figure 11: A, B and C: Constructs used in the NdC83 truncation mutant experiment. D) Schematics representation of types of phage particles produced by experimental system.

of the arabinose promoter (*araP*$_{BAD}$) on pBAD his B. The resulting plasmid was named pNJB4. The *araP*$_{BAD}$ promoter, known to be very tightly regulated (Ogden et al., 1980), was used because N1N2 domains of pIII, even if expressed at a low amount, render host cells resistant to phage infection (Boeke, Model & Zinder, 1982).

NdC83 construct is not sufficient to allow phage assembly termination. To achieve phage assembly termination, a second type of mutant pIII was expressed in the same host cell on a compatible plasmid (pGZ119EH; *colD ori*; (Lessl et al., 1992) under the control of a *tac* promoter. This mutant pIII is devoid of the N1N2 domains required for the receptor binding and infection, but carries complete pIII C domain (referred to as Cd throughout this thesis) (Figure 11A (Rakonjac et al., 1999). The phage particles carrying NdC83 and Cd (labelled NdC83/Cd though this thesis) were generated by infection with gene III deleted phage, f1d3. With the NdC83 construct and the complete C domain, phage assembly should terminate efficiently, however if our hypothesis is correct, the produced virions should be non infectious. The schematic representation of the virion particles is shown in Figure 11D.

The positive control construct for this system was wild-type pIII under the arabinose promoter (*araP*$_{BAD}$). Particles were generated in combination with the same complete C domain (Cd) from the same plasmid that was used to create the NdC83/Cd particles (Figure 11B). Particles created with wild-type pIII (labelled WT pIII/Cd throughout this thesis) should be infectious and provide a reference for comparison to NdC83/Cd-carrying particles. The negative control for this system is complete C domain (labelled Cd through this thesis) expressed from the same construct (Figure 11C) used to generate NdC83/Cd and WT pIII/Cd particles. This system terminates assembly efficiently, however the produced phage virions are non infectious due to the lack of the N1N2 domains (Figure 11D).

The host strain K1822 lacks the arabinose operon; allowing the tight control of the *araP*$_{BAD}$ promoter of the pBAD-derived constructs. The vector pBADhisB from which NdC83 and WT pIII are expressed also carries the *araC* gene of the arabinose operon required for regulation of *araP*$_{BAD}$ promoter. In addition, K1822 is F+, therefore it can be infected by f1 phage.

3.2 Production of mutant phage

The outline of the protocol for production of mutant phage is presented in Figure 9. For growing the mutant phage, many factors needed to be taken into consideration to ensure a high phage titre.

As described in the introduction, NdC83 and WT pIII expression renders cells resistant to phage infection. Therefore, these two proteins were expressed from the tightly-regulated $araP_{BAD}$ promoter. In order to minimise the $araP_{BAD}$ expression before induction, a minimal medium, supplemented with glucose is recommended (Guzman et al., 1995). However in a trial experiment in which minimal medium was used, the infection rates were very low (<1% of the cells in the culture). Therefore, a different medium, a modified broth, (FGB, Fortified glucose broth), based on fortified broth (FB) was used. FGB contains 0.2% glucose which mediates the catabolic repression of arabinose promoter ($araP_{BAD}$). With this system phage infection levels in all strains were improved (40-80%). The FB broth base, which is buffered and richer than typical f1 broth 2xYT, was chosen is because it extends the exponential growth phase of the culture, allowing growth to a higher cell density in comparison to that in the 2xYT medium. Because there are more cells in the culture at the end of the experiment, there is a marked increase in phage production.

After phage infection, the production of pIII mutants needed to be induced. Moreover, the residual f1d3 phage from the stock had to be removed because they interfere with the infectivity assays. To achieve this, cells were pelleted by centrifugation and resuspended in FAB. In this broth the sugar glucose was replaced with arabinose (0.2%). Removal of glucose releases the catabolic repression of $araP_{BAD}$ and addition of arabinose causes full induction of the arabinose promoter and the expression of pIII. Also to induce the *tac* promoter controlling the Cd construct, IPTG (0.1mM) was added.

A secondary consideration was the amount of f1d3 phage required to achieve a high level of infection. Early trials using m.o.i of 20-50 showed a very low infection efficiency (<1%). To achieve a high rate of infection a m.o.i of 100 was required. Therefore the culture contained 100 infectious phage particles per cell. In an exponentially growing culture with an OD_{600} there are 1×10^{8} cells per ml; therefore 1×10^{10} virions per ml of culture were needed. The experiment used 400ml culture, so a total of 4×10^{12} virion particles per culture were used.

The excess of f1d3 phage from the stock are infectious and would therefore interfere with infectivity assays. Therefore, they had to be removed from the sample by washing. Preliminary experiment with just one wash step showed that relatively high residual f1d3 was retained (not shown). To further decrease the number of these phage, the second wash immediately after the first one was introduced, decreasing the number of infectious particles in the negative control by two orders of magnitude relative to the single-wash experiment (see below, 3.3.).

The third consideration was at what point to infect the cells. The f1 phage infection and production are efficient only during exponential phase of growth. Once cells are in stationary phase, infection is not possible and the phage virion production is minimal. Preliminary experiments showed that an $OD_{600} = 0.1$-0.2 was optimal for infection and phage production (not shown). This is near the start of exponential growth phase, ensuring the maximum possible time for phage production could be utilised. After infection and two wash steps to eliminate glucose and the f1d3 from the stock, cells were incubated for four hours. During that time the culture growth reaches a plateau.

At the end of incubation, produced phage particles were separated from the cells by centrifugation. To achieve sufficiently high concentration for further analysis phage were concentrated 1000 times using PEG precipitation.

3.3 Analysis of Infectivity of Mutant Phage Particles

To observe the effect of the mutant pIII (NdC83) on phage infection, the infectivity of each phage sample had to be determined. Infectivity is defined as the number of the infectious virion particles per total amount of the phage. Therefore, it is calculated as a ratio of the infective phage titre to the number of phage particles in a sample expressed as genome equivalents (defined in Materials and Methods, 2.5; Fig 13). For comparing of the mutant phage sample to the positive control, the relative infectivity of the NdC83/Cd (test sample), a ratio of this sample's infectivity to a reference sample's infectivity, was calculated. In this experiment the reference sample is the positive control, WT pIII/Cd. To calculate the infectivity and relative infectivity, two values need to be known: Firstly the infective titre of the samples (the ability to penetrate a host cell and replicate) and secondly, the virion concentration in the sample needs to be known. The former was measured by titering of the samples and latter by densitometry of the phage.

3.3.1 Analysis of Phage Infective Titre

To determine the infective titre of the produced phage, plate titering was carried out on strain K1762. This is an *E.coli* strain specially constructed for titering ΔgIII fl phage, including the phage used in this experiment, fld3 (Rakonjac et al., 1997). This strain contains two plasmids: pJARA112, and pJARA131. pJARA112 carries WT gIII under the control of the *psp* (Phage Shock Protein) promoter (Brissette et al., 1990; Brissette et al., 1991). This promoter is uninduced before phage infection and induced only after a cell is infected with fl phage. Because pIII renders cells resistant to fl infection, this infection-dependent mode of promoter regulation for expressing pIII allows multiple rounds of phage infection required for plaque formation. pJARA131 carries a copy of the *psp* operon. This further reduces the background expression rate of *psp*-gIII fusion, most likely due to the increased amount of the negative regulators of the *psp* promoter expression coded by the operon (Model, Jovanovic & Dworkin, 1997).

Phage were first titered using a fast method (Materials and Methods) to obtain the rough titre estimation. Based on this preliminary titre, dilutions of the sample required for high accuracy titering (100-300 plaques per plate) were estimated. Phage plating was carried out in triplicate for each sample, to further increase the accuracy of the titres.

Table 5

Raw Data For Phage Infective Titre

	Count on plate	Dilution	Volume plated	Infective titre (per ml)	Average infective titre (per ml)	Standard Deviation	Error %
Cd	43	1.00×10^{06}	0.02	2.15×10^{9}			
	58	1.00×10^{06}	0.02	2.90×10^{9}	2.68×10^{9}	4.65×10^{8}	0.17
	30	1.00×10^{06}	0.01	3.00×10^{9}			
NdC83/Cd	77	1.00×10^{06}	0.01	7.70×10^{9}			
	55	1.00×10^{06}	0.01	5.50×10^{9}	7.03×10^{9}	1.33×10^{9}	0.19
	79	1.00×10^{06}	0.01	7.90×10^{9}			
WT pIII/Cd	51	1.00×10^{06}	0.05	1.02×10^{13}			
	67	1.00×10^{06}	0.1	6.70×10^{12}	8.45×10^{12}	2.47×10^{12}	0.29

Phage titering showed that the infective titre of the NdC83/Cd and the negative control was 0.08% and 0.03% respectively, that of the positive control sample WT pIII/Cd (Table 5, Figure 14).

3.3.2 Analysis of Concentration of Phage Genome Equivalents

Since a low infective titre could be caused by multiple factors, including poor efficiency of phage particle production, it was required to determine the concentration of phage virions in the samples. This determined whether the phage virions were present in the sample, but were non infectious, or just not present. The concentration of phage particles in the samples was analysed agarose gel electrophoresis of SDS-disassembled vision particles (Nelson et al., 1981)(Materials and Methods), followed by densitometry of gel bands. Phage virion concentration is expressed as the number of genome equivalents, defined in the material and methods section (2.5 and Figure 10).

Typically two gels were used. The first gel was used to estimate the concentration of phage so that dilutions could be made to ensure optimal loading concentrations for densitometry and to prevent gel band spreading due to overloading. The second gel with adjusted loading concentrations was used for quantification (Figure 12).

Gel band density was analysed and collated (Table 6). Using the standard curve (Figure 13) derived from densitometric measurements of the f1d3 ssDNA standard dilutions of known concentrations (Table 6), the amount of phage ssDNA in the samples was calculated (Table 7). The amount of ssDNA was converted to the number of genome equivalents (1ng ssDNA for f1d3 = 3.82×10^8 genome equivalents; see Appendix 1). Calculation showed that all phage samples contained highly concentrated phage ssDNA ($\sim 1 \times 10^{14}$ genome equivalents per ml of concentrated sample). Therefore, phage assembly is effieient, and ssDNA derived from the released virions was present at a high concentration within all samples (Table 7, Figure 14).

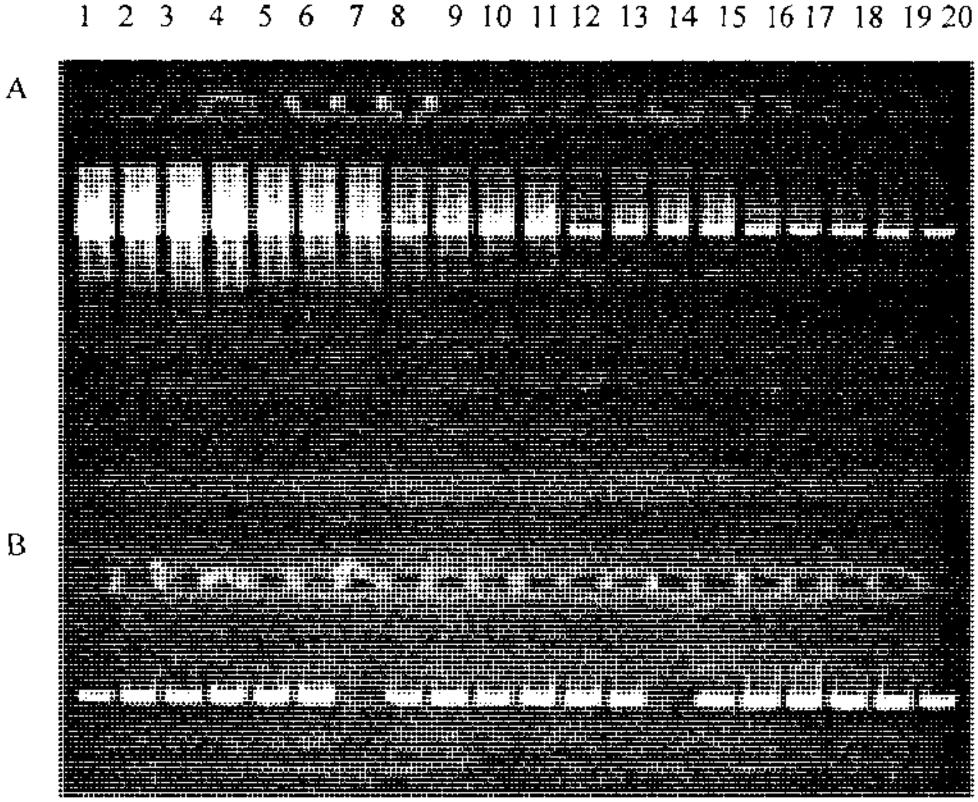


Figure 12: Quantification of phage ssDNA A) Dilutions of calibration standards of purified f1d3 ssDNA. Lanes 1-4, 1280ng per lane; Lanes 5-8, 640ng per lane; Lanes 9-12, 320ng per lane; Lanes 13-16, 160ng per lane; Lanes 17-20, 80ng per lane. B) SDS-disassociated phage samples. Lanes 1-6, Cd Phage (4μl of 10× dilution per lane); Lanes 8-13, NdC83/Cd phage (1μl per lane); Lanes 15-20, WT pIII/Cd phage (4μl of 10× dilution per lane).

Table 6

Densitometry data of the standard dilutions

Standard	Density	Background	Corrected	ssDNA loaded(ng) (*)	ssDNA calculated
640	60002	31563	28439	640	680
	58926	31563	27363	640	630
	59262	31563	27699	640	640
320	52139	31563	20576	320	360
	51131	31563	19568	320	320
	50957	31563	19394	320	320
160	44434	31563	12871	160	160
	43968	31563	12405	160	150
	45238	31563	13675	160	180
80	37458	31563	5895	80	85
	35938	31563	4375	80	82
	35665	31563	4102	80	82

(*) Values obtained using the standard curve equation (Fig 13)

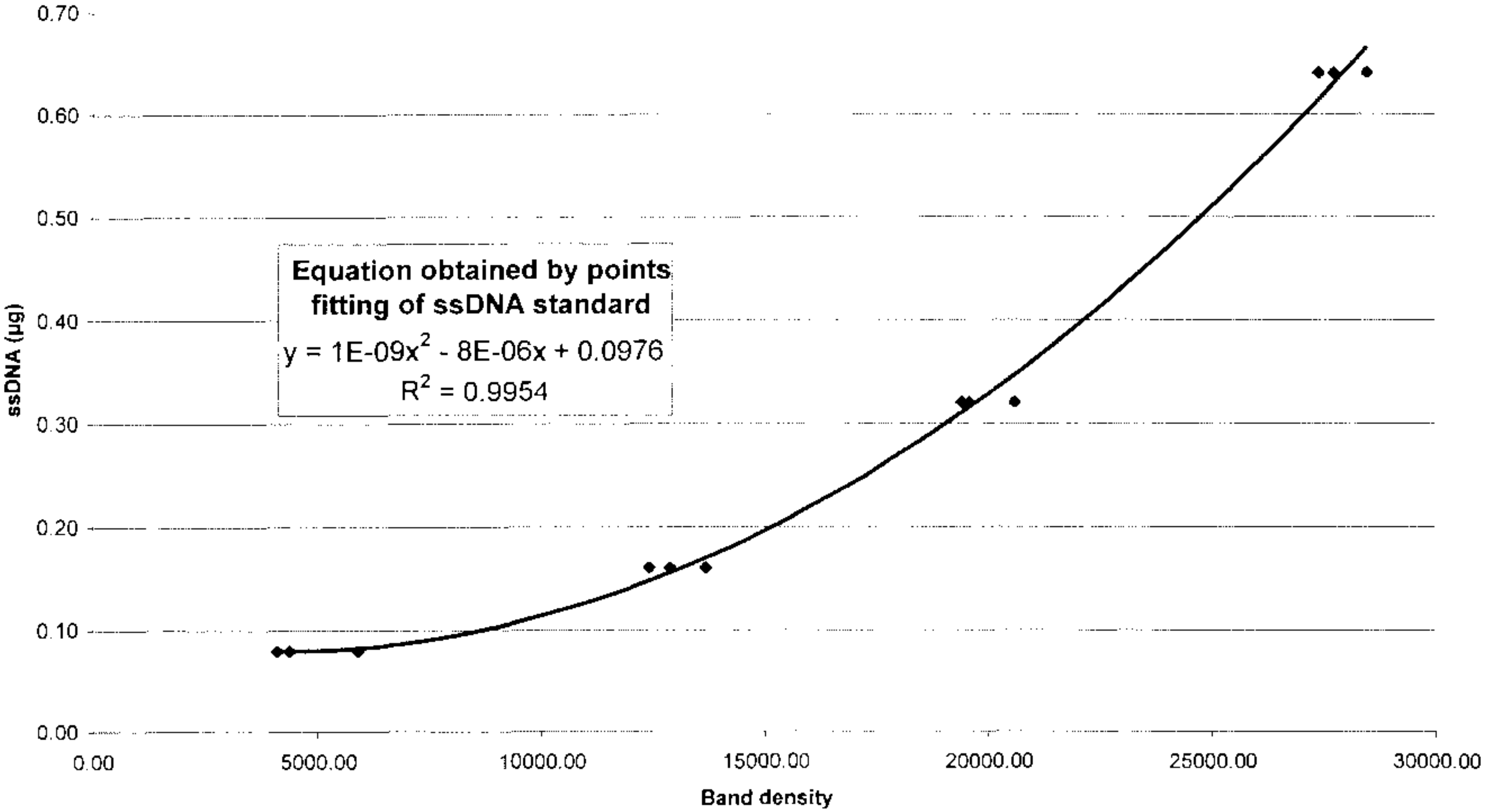


Figure 13: Standard curve of gel band density to ssDNA standard.

Table 7

Densitometry and virion quantification

Sample	Density	Background	Corrected	ssDNA calculated (ng)	Genome Equivalents per well	Sample loaded (μl)(*)	Genome Equivalents/ml (**)	Average Genome Equivalents/ml (**)	Standard Deviation	Percentage Error
NdC83/Cd	45751	31563	14188	190	7.1×10^{10}	1	7.1×10^{13}			
	49572	31563	18009	280	1.1×10^{11}	1	1.1×10^{14}			
	47723	31563	16160	230	8.8×10^{10}	1	8.8×10^{13}			
	50928	31563	19365	320	1.2×10^{11}	1	1.2×10^{14}			
	50416	31563	18853	300	1.2×10^{11}	1	1.2×10^{14}			
	49221	31563	17658	270	1.0×10^{11}	1	1.0×10^{14}	1.0×10^{14}	1.9×10^{13}	18.53%
Cd	35607	31563	4044	82	3.1×10^{10}	0.4	7.8×10^{13}			
	45077	31563	13514	170	6.6×10^{10}	0.4	1.6×10^{14}			
	45369	31563	13806	180	6.8×10^{10}	0.4	1.7×10^{14}			
	50250	31563	18687	300	1.1×10^{11}	0.4	2.8×10^{14}			
	48453	31563	16890	250	9.5×10^{10}	0.4	2.4×10^{14}			
	51458	31563	19895	330	1.3×10^{11}	0.4	3.2×10^{14}	2.1×10^{14}	8.9×10^{13}	42.46%
WT pIII/Cd	45118	31563	13555	170	6.6×10^{10}	0.4	1.7×10^{14}			
	52029	31563	20466	350	1.3×10^{11}	0.4	3.4×10^{14}			
	52513	31563	20950	370	1.4×10^{11}	0.4	3.5×10^{14}			
	50205	31563	18642	300	1.1×10^{11}	0.4	2.8×10^{14}			
	49735	31563	18172	280	1.1×10^{11}	0.4	2.7×10^{14}			
	43481	31563	11918	140	5.5×10^{10}	0.4	1.4×10^{14}	2.6×10^{14}	8.8×10^{13}	34.28%

(*) Dilutions were adjusted so that total loaded volumes were all 20μl/lane

(**) per ml of PEG-concentrated sample

3.3.3 Infectivity of particles containing mutant pIII is extremely low

Using the data above the infectivity of each sample was calculated (Table 8). Infectivity is expressed as infectious particles per genome equivalent (ip/ge) Calculation showed that the infectivity of the NdC83 mutant was very low (0.0070% ip/ge). This meant that only 0.0070% of total phage genomes in the sample were able to infect a host cells. This value was within the same order of magnitude to that of the negative control (0.0013%). Since the negative control is non-infectious due to the absence of the N terminal domains. infective virions in this sample represent residual phage f1d3 stock that were not removed in the washing steps. The infectivity of the positive control is much higher (3.28%) than that of the NdC83/Cd particles or the negative control Cd particles.

To compare the samples, the relative infectivity to the positive control was calculated. When the relative infectivity of mutant phage sample to the positive control was calculated it was (Table 8, Figure 15) 0.21%. This is over a 450 fold reduction in infectivity. This value is within the same order of magnitude as that of the negative control (0.04%). Therefore, compared to WT pIII sample, the mutant phage are of three orders of magnitude lower infectivity. Alternatively, residual infectivity in the mutant phage may be due to the background helper phage that was not removed during the washing steps in phage production.

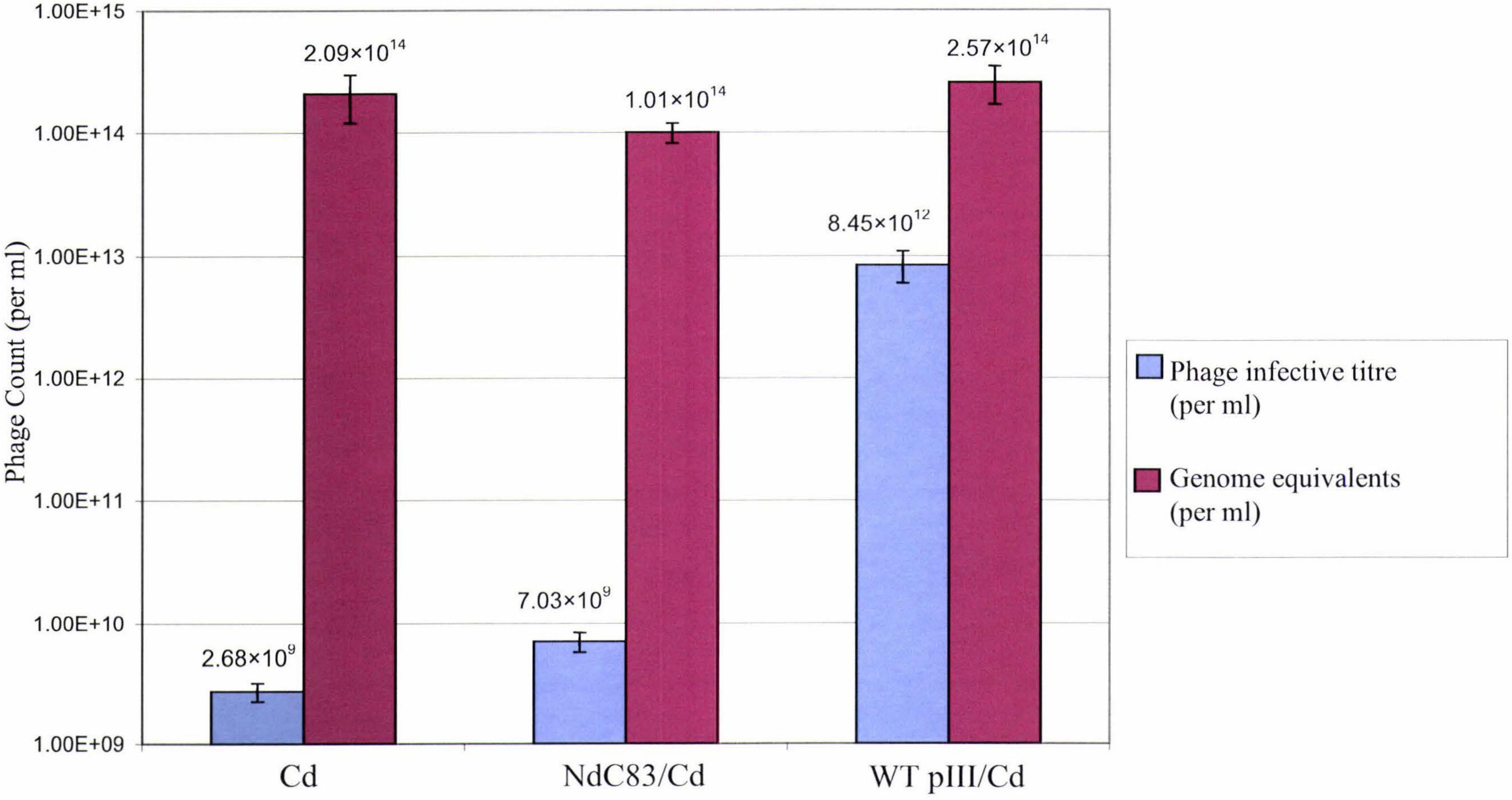


Figure 14: Comparison of infective titre to the amount of virions (genome equivalents) in concentrated phage samples

Table 8

Infectivity (titre vs. the amount of phage)

	Phage infective titre (per ml)	Standard Deviation	Monophage Genome Equivalents (per ml)	Standard Deviation	Infectivity	Relative Infectivity
Cd	2.7×10^{9}	4.6×10^{8}	2.08×10^{14}	8.9×10^{13}	0.0013%	0.04%
NdC83/Cd	7×10^{9}	1.3×10^{9}	1.00×10^{14}	1.9×10^{13}	0.0070%	0.21%
WT pIII/Cd	8.5×10^{12}	2.5×10^{12}	2.57×10^{14}	8.8×10^{13}	3.28%	100.00%

3.4 Analysis of Phage Assembly Termination and Phage Stability

Previous experiment showed that phage ssDNA was present in high amount in the (NdC83/Cd) sample. However, it was possible that the low infectivity of NdC83/Cd particles was due to assembly of defective (structurally unstable) virions, which are usually significantly longer than properly terminated virions, and sensitive to detergent sarkosyl. Therefore, the size and stability of NdC83/Cd particles was examined.

Native virion electrophoresis determines the size of the virions. It also detects free phage ssDNA released by unstable virions either spontaneously or after the addition of ionic detergent sarkosyl.

If the phage particles are not terminating through action of pIII, but rather are result of release of cell-associated unterminated particles by mechanical shearing, they are located as a smear under the well. Phage virions whose release from the cell is mediated by pIII, typically run as a ladder of bands that are carrying from 1 to 20 phage genomes. Phage carrying more than one genome are sometimes referred to as "polyphage" (Figure 10). In a wild-type f1 culture >90% of virions produced are monophage. In ΔgIII phage complemented with pIII expressed from a plasmid the amount of polyphage is elevated. All gels were run with the same amount of virions per well. All samples have very similar amounts of monophage (Figure 16A); therefore termination is carried out equally well in all samples.

The phage termination complex's stability was also measured by native phage gel electrophoresis. To measure the complex's stability, phage are mixed with the detergent sarkosyl (0.01%) prior to loading. Any phage in which the termination complex is not formed correctly will be disrupted by this treatment. When unstable disrupted phage are run out on the gel the released ssDNA will run at the same level as purified free phage ssDNA. If all the phage virion bands are eliminated by sarkosyl treatment, then the phage termination complex is not stable. However it is usual to see a weak ssDNA band in samples of complemented f1d3, due to a fraction of broken polyphage or cell associated filaments which carry multiple copies of the ssDNA

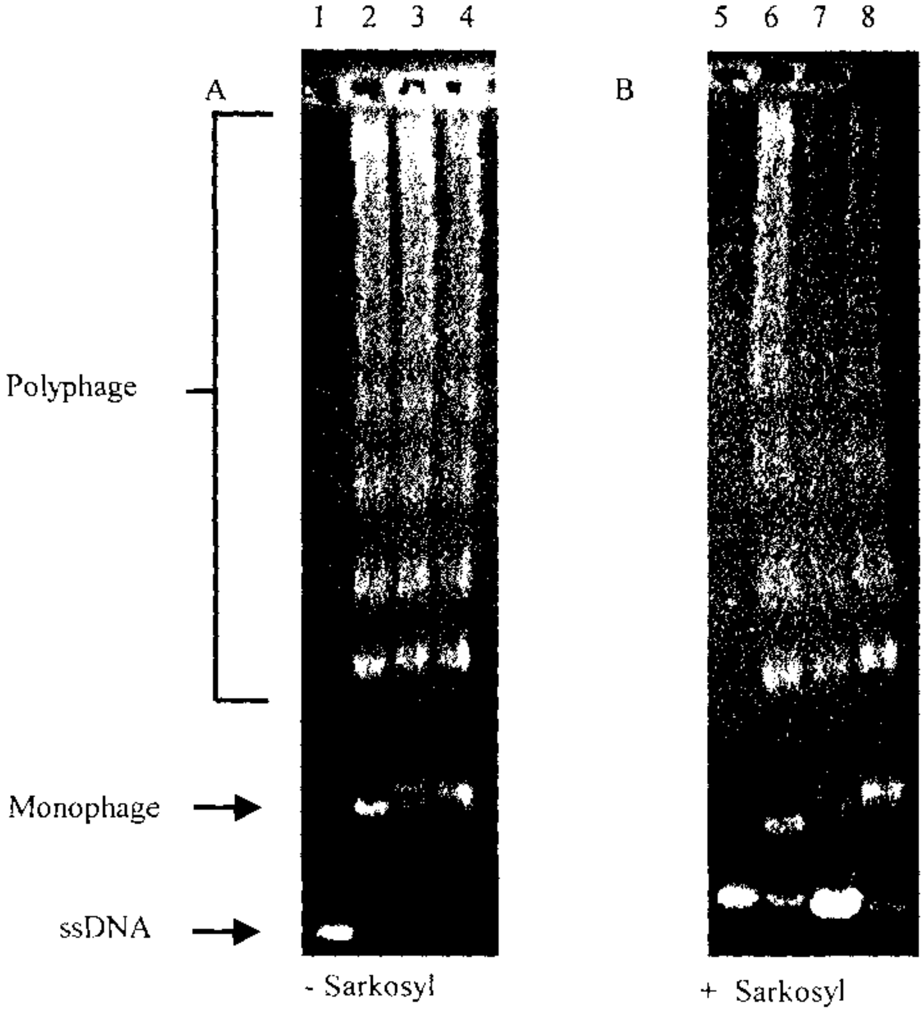


Figure 16: A) Native phage gel showing phage termination efficiency. Lane 1, purified ssDNA (80ng); Lane 2, Negative Control Phage; Lane 3, NdC83 phage; Lane 4, WT pIII phage. B) Native phage gel with Sarkosyl dissociation prior to loading showing phage stability. Lane 1, ssDNA (80ng); Lane 2, Negative Control Phage; Lane 3, NdC83 phage; Lane 4, WT pIII phage. All lanes were loaded with 7.6×1010 genome equivalents

genome. Majority of the N1N2C83/Cd particles were stable in sarkosyl. However, this sample contained slightly larger portion of the unstable particles than did negative control (Cd) or the positive control (WT pIII/Cd ; Figure 16B). This may be due to the increased amount of particles in the lane containing the NdC83/Cd sample, slightly decreased stability of the particles that carry majority of destabilizing NdC83 molecules, or a larger portion of broken off cell-associated filaments in this sample relative to the other two. This difference however, can not account for the three order of magnitude decrease in infectivity relative to the WT pIII control. Therefore, decreased stability is not the reason for low infectivity of NdC83/Cd virions.

3.5 NdC83 is incorporated into the Virions

A reason for low infectivity of the NdC83/Cd particles could have been degradation of NdC83 fusion in the host cells and/or poor incorporation into the virion.

To demonstrate that NdC83 fusion is incorporated into the virions, its presence was examined by western blotting of virion samples using an anti-pIII antibody (R164) (Rakonjac et al., 1997). This antibody is raised against the 10 terminal residues of pIII, therefore it recognises pIII mutants used in the experiment. The western blot showed that the NdC83 fusion was incorporated into the NdC83/Cd virions with the same efficiency as was the WT pIII expressed from the same vector in the positive control particles (WT pIII/Cd, Figure 17A).

3.6 Infection complex is forming correctly

The low infectivity could be explained if the termination complex was not forming correctly due to the poor efficiency of incorporation of pVI, which interacts with pIII and forms the termination complex. To examine this possibility, presence of pVI was also examined. Western blotting showed that pVI was incorporated efficiently into the virion carrying NdC83, showing that the phage were forming the pIII-pVI complex correctly (Figure 17B). In summary, the NdC83/Cd particles were either non-infectious or of a very low infectivity due to inability of the NdC83 to mediate the infection.

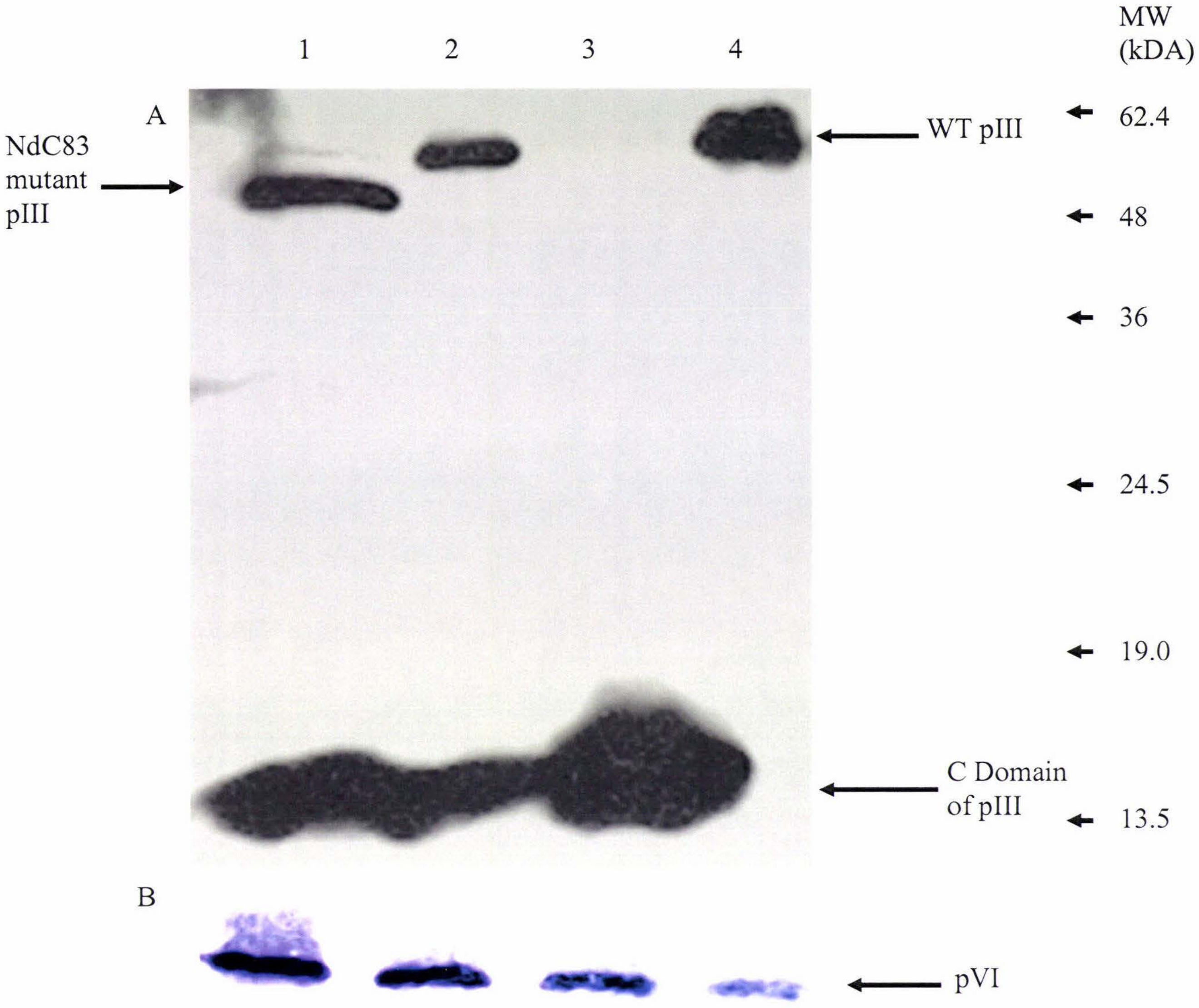


Figure 17: A) Western blots showing presence of mutant pIII in produced phage. Lane 1, NdC83 phage (3.2×10^{10} genome equivalents); Lane 2, WT pIII phage (3.2×10^{10} genome equivalents); Lane 3, Negative control phage (4.6×10^{11} genome equivalents); Lane 4, F1 (2.5×10^{10} phage). B) Western blot showing presence of pVI in produced phage. Lane 1, NdC83 phage (2.0×10^{10} genome equivalents); Lane 2, WT pIII phage (2.0×10^{10} genome equivalents); Lane 3, Negative control phage (2.0×10^{10} genome equivalents); Lane 4, F1 (1.17×10^{9} phage).

Chapter 4

Construction of a Microphage producing-system for structural studies.

Mutant analysis in this thesis and previous work (Rakonjac et al., 1999; Rakonjac & Model, 1998), suggest that the C domain is essential for infection and assembly, respectively. Structural analysis of the protein and domain organization of the pIII/pVI complex is required to begin deciphering the exact structural role of Cd in the fl infection and assembly. However nothing is known about the structure of the C domain of pIII and the complex it forms with pVI at the end of the virion. The reason for that is a large length to width ratio of the virions (860nm x 6 nm), which prevents the structural analysis of the termini, and tight association of Cd with the rest of the virion. Therefore, preparation of very short phage particles is necessary for structural analysis of the pIII C domain and the termination complex.

4.1 Analysis of existing microphage producing plasmid pLS7

It was originally planned to use an existing microphage producing plasmid, pLS7, to create microphage. pLS7 is a derivative of pBR322, carrying a microphage producing region. It was constructed in 1992 by Specthrie et al. However, the analysis of the plasmid which was acquired by our lab showed that pLS7 was twice that of the reported size of 4kb. Upon sequencing it was also noticed that recombination had occurred within the plasmid. Most importantly, one base was missing (bp 102) from the positive origin of replication required for the microphage production (Figure 18).

Using various helper phage (R474, R408, and VCSM13) in combination with pLS7 microphage production and isolation was attempted. Produced phage stocks were analysed by SDS disassembly agarose gels and Southern blots, using pLS7 as a probe (not shown).

```
                                   1                                                  50
Microphage Sequence Predicted      CCATCGCCCTGATAGACGGTTTTTCGCCCTTTGACGTTGGAGTCCACGTT
pLS7.NB2 sequencing                CCATCGCCCTGATAGACGGTTTTTCGCCCTTTGACGTTGGAGTCCACGTT

                                   51                                                100
Microphage Sequence Predicted      CTTTAATAGTGGACTCTTGTTCCAAACTGGAACAACACTCAACCCTATCT
pLS7.NB2 sequencing                CTTTAATAGTGGACTCTTGTTCCAAACTGGAACAACACTCAACCCTATCT

                                   101                                               150
Microphage Sequence Predicted      CGGGCAAGCTTGGACGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGTGT
pLS7.NB2 sequencing                C-GGCAAGCTTGGACGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGTGT
                                    ↑

                                   151                                               200
Microphage Sequence Predicted      GGTGGTTACGCGCAGCGTGACCGCTACACTTGCCAGCGCCCTAGCGCCCG
pLS7.NB2 sequencing                GGTGGTTACGCGCAGCGTGACCGCTACACTTGCCAGCGCCCTAGCGCCCG

                                   201                                               250
Microphage Sequence Predicted      CTCCCGGGATCGGAATTCCGGCCATCGCCCTGATAGACGGTTTTTCGCCC
pLS7.NB2 sequencing                CTCCCGGGATCGGAATTCCGGCCATCGCCCTGATAGACGGTTTTTCGCCC

                                   251                                               300
Microphage Sequence Predicted      TTTGACGTTGGAGTCCACGTTCTTTAATAGTGGACTCTTGTTCCAAACTG
pLS7.NB2 sequencing                TTTGACGTTGGAGTCCACGTTCTTTAATAGTGGACTCTTGTTCCAAACTG

                                   301
Microphage Sequence Predicted      GAAAACGTCT
pLS7.NB2 sequencing                GAAAACGTCT
```

Figure 18: Sequence Data Showing Mutant Residue in pLS7. Arrow points to missing residue that was corrected by PCR mutagenesis.

No microphage DNA or particles could be detected in any sample (not shown). The lack of the microphage production was most likely due to the mutation in the positive f1 origin of the microphage-producing plasmid pLS7. Therefore, the microphage-producing fragment had to be reverted to wild type. The microphage-producing region from pLS7 was first cloned into pCR®-BluntII-TOPO® (Invitrogen) and repaired to the original sequence by PCR mediated mutagenesis. Sequencing has shown that the microphage sequence has been corrected. The plasmid is now ready for testing of the microphage production.

4.2 Construction and Testing of the *tac*-gII fusion plasmid pNJB2

The f1 origins on plasmids interfere with helper phage replication, decreasing overall phage and microphage production. The interference is due to plasmid-derived f1 ori titering out replication protein pII. To add more pII to the system we constructed a plasmid in which pII was expressed under the control of the *tac* promoter. The *tac*-gII promoter fusion plasmid was constructed using overlap extension PCR (as described in material and methods) (Horton et al., 1989)

To test whether the pNJB2 plasmid was expressing the *tac*-gII fusion was tested by assaying its ability to complement a gII amber mutant phage, R21. The gII amber mutation is a single point mutation which changes a codon 20 of gII into a stop codon and produces a non-functional truncated pII protein. Because this mutation is a point mutation there is a high level of reversion (as determined by titering on a non-suppressor strain K561, Table 9). The R21 stock was prepared on a *supF* strain (K833) which allows reading of the stop codon as amino-acid tyrosine and restoring pII production. To test functionality of pNJB2, plasmid DNA of four independent clones was purified and transformed into a non suppressor strain (K561), which is suitable for testing complementation of gII amber mutation by *tac*-gII fusion from the plasmid. This showed that the *tac*-gII fusion plasmid was able to complement gII amber mutation (Table 9). To determine the level of pII induction needed for complementation the cells were also grown at differing concentrations of ITPG (Table 9).

Results showed that pNJB2.3 and 2.4 were able to complement R21 amber mutation and the *tac*-gII constructs needed no induction. This is consistent with the fact that the *tac* promoter has relatively high activity in the absence of induction.

In conclusion, a microphage-producing system consisting of a microphage-producing plasmid and a pII-producing plasmid has been successfully constructed.

Table 9
Complementation of pII Amber mutant by pNJB2 isolates

Strain	ITPG conc (mM)	Plaque Count	Dilution	Volume Plated (ml)	Titre (phage/ml)
K833 (Suppressor Strain)	0	80	1×10^{-7}	0.01	8.0×10^{10}
K561 (Non Permissive Strain)	0	37	1×10^{-3}	0.01	3.7×10^{6}
K561/pNJB2.1	0	79	1×10^{-7}	0.01	7.9×10^{10}
	0.01	89	1×10^{-5}	0.01	8.9×10^{8}
	0.1	2	1×10^{-7}	0.01	2.0×10^{9}
K561/pNJB2.2	0	62	1×10^{-5}	0.01	6.2×10^{8}
	0.01	1	1×10^{-7}	0.01	1.0×10^{9}
	0.1	58	1×10^{-5}	0.01	5.8×10^{8}
K561/pNJB2.3	0	19	1×10^{-9}	0.01	1.9×10^{12}
	0.01	59	1×10^{-9}	0.01	5.9×10^{12}
	0.1	64	1×10^{-9}	0.01	6.4×10^{12}
K561/pNJB2.4	0	38	1×10^{-9}	0.01	3.8×10^{12}
	0.01	55	1×10^{-9}	0.01	5.5×10^{12}
	0.1	52	1×10^{-9}	0.01	5.2×10^{12}

Chapter 5

Discussion

5.1 The role of the pIII C domain in infection

Although binding of the phage to cellular receptors has been dissected in detail (Click & Webster, 1997; Deng & Perham, 2002; Lubkowski et al., 1999; Marvin & Hoffmann-Berling, 1963; Reichmann & Holliger, 1997) the mechanism of "opening" of the Ff virion that results in DNA entry into the host cytoplasm is not understood.

To begin understanding the mechanism of the f1 virion opening, this thesis tested the hypothesis that the C domain of pIII is required for phage infection. A mutant pIII (NdC83) that is unable to terminate phage assembly, but still carried the receptor-binding N terminal domains of pIII, was constructed and used to examine the requirement of a functional C domain covalently linked to the receptor-binding domains for phage infection. Because the virions have to be terminated in order to be released from the cells, the functional C domain was supplied *in trans*.

The produced phage terminated correctly and carried the NdC83 fragment. But as predicted by the starting hypothesis, were not infectious. Therefore the same protein domain that terminates (closes) and releases the virion from the membrane during assembly, also mediates its opening and insertion into the host membrane during infection.

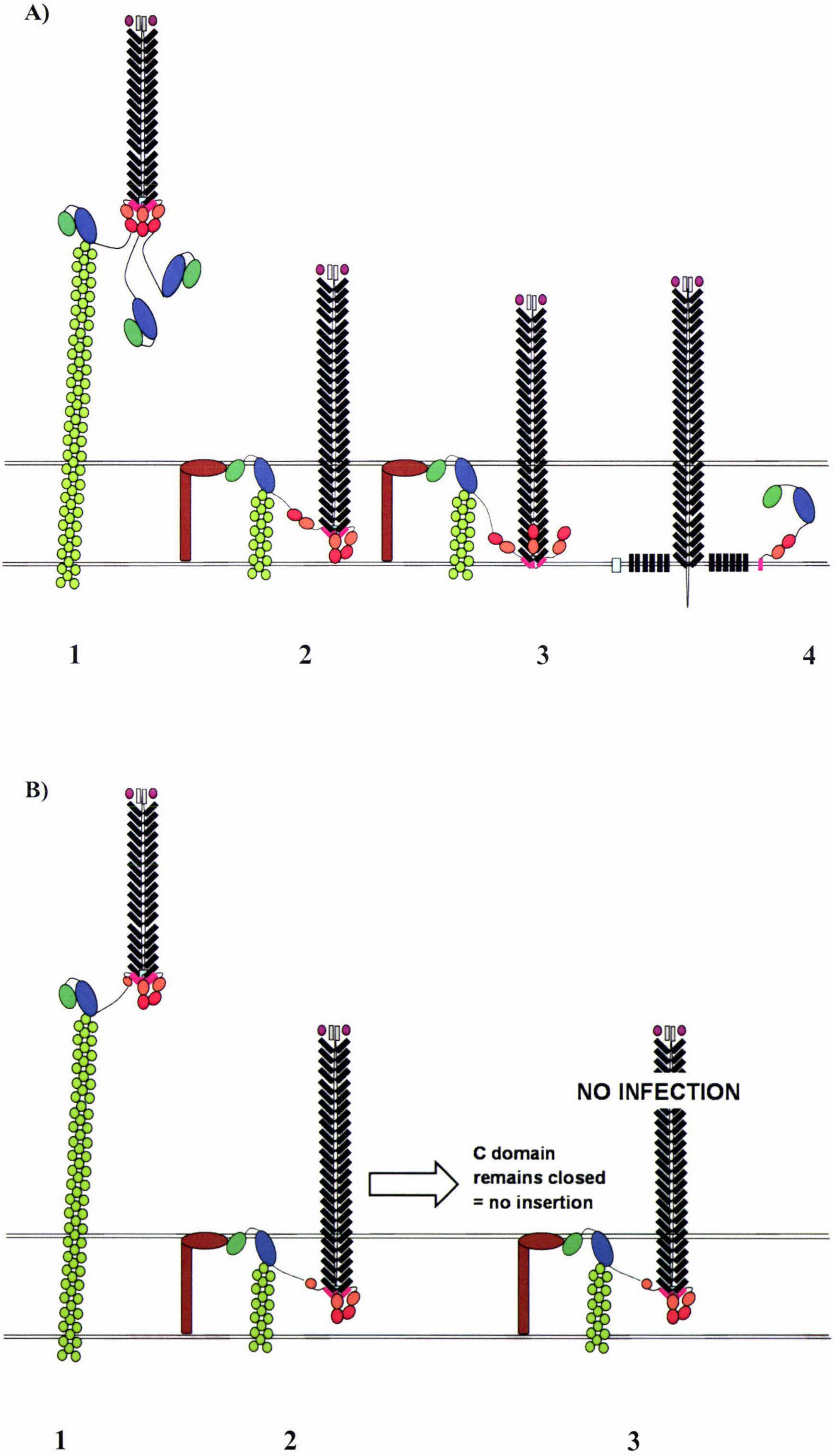

A)
1
2
3
4
B)
NO INFECTION
C domain
remains closed
= no insertion
1
2
3

Figure 19: A) A revised model for infection of *E.coli* by Ff filamentous phage. 1: Phage binds to F pilus tip. 2: Pilus retracts, N1 domain is released from N2 allowing binding of TolA. 3: Conformational change in C domains causing "unlocking" of the virion and insertion into membrane. 4: Integration of virion proteins into the inner membrane, transport of phage ssDNA genome into cytoplasm. B) Model for NdC83 phage. 1: Phage binds to F pilus tip. 2: Pilus retracts, N1 domain released from N2 allowing binding of TolA. 3: No conformational change in C domain upon TolA binding. No membrane integration of virion proteins, no infection.

The low infectivity of the NdC83/Cd virions, in which the secondary receptor-binding domain N1 is fused to a non-functional C-terminal fragment, suggests that the secondary receptor TolA is not a simple tether for the infecting phage, but rather has a role in triggering conformational change that results in DNA entry and coat protein integration into the membrane. Based on this observation, a model of the fl phage entry could be proposed: binding of the N1 domain to the secondary receptor TolA generates a signal that is transmitted to the C domain of the same molecule, resulting in its conformational change, which is communicated to the other pIII subunits in the complex (Figure 19A). The conformational change of the C domains of pIII subunits exposes the hydrophobic membrane anchors of pIII, resulting in anchoring the virion in the inner membrane, integration of the coat protein into the membrane and release of the DNA into the host cytoplasm. If the C domain is not functional in assembly, it is also incapable of undergoing the conformational change required for opening of the virion (Figure 19B). The failure of the virion to open could also be due to failure of mutated Cd to "communicate" the contact of the receptor to other (intact) Cd subunits in the virion.

Alternative model for lack of infectivity Ff mutant NdC83/Cd particles would be that shortening of the pIII in NdC83 by 70 residues may have prevented TolA bound virions reaching a particular point in the inner membrane required for membrane anchoring. However, data from (Krebber et al., 1997; Spada et al., 1997), where the infectivity is decreased by 100× due to insertion of β lactamase between N2 and Cd, extending the distance between the two domains, argues against this alternative hypothesis. Moreover, low infectivity (10^{-4}) observed in particles in which N1N2 are non-covalently associated to the Cd strongly argues in favour of a conformational change.

All together, findings in this thesis define the domain requirement for phage entry and suggest a model of infection analogous to anchoring of eukaryotic viruses into the host membrane by exposure of fusogenic peptide after interaction with the secondary receptor.

This model proposes conformational changes in pIII similar to that of the enveloped viruses of animals (e.g. HIV) in which binding to the secondary receptor results in exposure of hydrophobic fusogenic peptides which insert into the host cell membrane (Furuta, Wild & Weng, 1998; Sattentau & Moore, 1991). This is reasonable because logistically f1 infection in which the coat protein becomes integrated into the host membrane is similar to that of the enveloped viruses.

One of the best studied proteins that use fusogenic peptide to aid viral infection is haemagglutinin (HA) from the influenza virus (Myxovirus). The haemagglutinin protein forms a spike like trimers on the surface of the influenza virus. When the tip of the HA spike binds to it cellular receptor, which in this case is sialic acid and the local pH is lowered to pH 5, the HA spike undergoes a structural rearrangement. This in turn causes the exposure of a trimer of highly hydrophobic fusogenic peptide which insert into the host cell membrane. (Weis et al., 1988; Wiley & Skehel, 1987).

5.2 Further Functional Analysis of the C Domain Role in Phage Infection

To further validate the role of the C domain in phage infection a series of further mutants should be made for structure function analysis studies. Due to time constraint, it was not examined whether the region of the C domain required for phage infection is the same as for phage termination/release. It is a possible that a larger portion of the C domain is required during the phage infection than during the phage termination/release. For example, the rest of the C2 and maybe part of the C1 subdomain could be involved in infection. The region of the C domain that is required for infection could be determined by producing a series of nested deletions of C domain fused to complete N1N2 domains, so that the functionality in the infection can be assayed using the same experimental setup as described in this thesis. It is also possible in a system with assembly-competent mutants to dispense with the Cd construct. C domain truncations containing the final 93 residues of the C domain can terminate assembly effectively.

Once the region of the C domain that is required for infection has been defined it would be possible to use either shot-gun or targeted PCR mutagenesis to determine which amino acid residues are important during the infection process. This experiment was previously used to determine the residues required for pIII inclusion into phage virions (Weiss et al., 2003). Further, isolation of intragenic suppressors of this mutation would allow identification of the interacting residues within the C domain.

5.3 Plans for Structural Analysis of pIII/pVI Complex

The best way to determine how the C domain of pIII mediates assembly termination and infection is to provide some form of structural information. Generally the best methods for determining protein structure are of the high (Å) resolution, eg X-ray crystallography or Nuclear Magnetic Resonance (NMR). However these two methods require purified protein (or protein complexes). The pIII/pVI complex only folds correctly within the phage structure, and currently purification of the pIII/pVI complex from phage filaments at a scale large enough for crystallography has not been achieved. The pIII/pVI complex is too large for NMR, therefore this method can not be used to determine its structure.

The major problem in determining pIII/pVI complex structure within the phage is the high length to width ratio (860nm×6nm) of the filament. This creates a high amount of background noise (Bahns, Liu & Chen, 2004) through fibre torsion and movement. Also in wild type filamentous phage pIII/pVI are only a small fraction of the total protein mass of a virion (pIII = 1.20% and pVI = 0.34%, Appendix 1). To reduce the length to width ratio of filamentous phage it is proposed to make a microphage producing system based on plasmid pLS7 (Specthrie et al., 1992). These microphage can then be used for multiple molecular techniques, including Cryo-Electron Microscopy (Conway & Steven, 1999; Yeager, Unger & Mitra, 1999), and Hydrogen-Deuterium Exchange Mass Spectroscopy (DXMS) (Englander et al., 2003).

5.4 Effect of Mutation in Microphage Producing Region

The microphage analysis was attempted; it was not achieved due to a mutation in microphage producing plasmid pLS7. There was a single base deletion in position 102 of the first positive f1 origin. The missing base is near the 3' end of the B region of the (+) origin required for initiation of (+) strand synthesis. Therefore, it is possible that the deletion caused either a complete lost of function, or reduced the microphage production to a level which was undetectable. The microphage-producing fragment has been reconstructed using PCR mutagenesis to generate a new plasmid pNJB7.

5.5 Proposed Microphage Production and Purification System

The system for the production of microphage for structural analysis has been designed. It combines both the microphage plasmid pNJB7 and the pII producing plasmid pNJB2 in the same host cell, using an appropriate helper phage to induce phage production. This system should have two advantages over pLS7 system described by (Specthrie et al., 1992).

First, *in trans* expression of pII should increase the level of rolling circle replication by the microphage positive origin. This in turn would create more packaging substrates and therefore increase the microphage production.

Second, microphage-producing region of pLS7 was transferred into pCR®-Blunt-TOPO® which is a high copy number plasmid (pUC origin, 200 copies per cell). pLS7 is a derivative of pBR322 and is a low copy number plasmid (20 copies per cell). An increase in plasmid copy number increases the amount of microphage origins available for microphage production and therefore should increase the amount of microphage produced.

To examine microphage production, concentrated phage samples will be analysed by agarose gel electrophoresis of SDS-disassociated particles and Southern blotting. Also, episomal DNA will be purified from the microphage-producing cells to examine whether microphage genomes are present in the cells. Phage preparations will also be analysed under negative stain TEM to confirm virion length.

If produced, microphage will then be separated from helper phage by either size exclusion chromatography (Zakharova et al., 2005) or differential PEG precipitation (Sambrook et al., 1989; Specthrie et al., 1992). This will be followed by Caesium Chloride gradient centrifugation to separate phage from the secreted proteins and other contaminants. Purified microphage can be then subjected to Cryo-EM analysis (Conway & Steven, 1999), and other biochemical analyses.

Alternatively, the pIII/pVI complex could be isolated and purified from virions for X-ray crystallography. Gailus & Rasched (1994) showed that pIII/pVI form a structural entity which can be purified from filamentous phage. The authors purified the pIII/pVI complex from filamentous phage by disrupting the phage with chloroform and sodium deoxycholate. They then purified the complex using gel filtration. The purified complex could still bind pIII and pVI antibodies. However it is unknown if the purified complex is still folded in its native form.

The major problem in using this system would be producing pIII on a scale large enough for crystallisation trials, since the complex only represents 1.5% of the total phage mass in wild type Ff phage.

5.6 Discussion of Accuracy of Data and Characteristics of the Experimental System

Three issues relevant to interpretation of the data have been identified; A) Accuracy of measurements; B) Folding of the N1N2 domains in the NdC83/Cd particles; C) The relatively low infectivity of the positive control particle, WTpIII/Cd. These issues are discussed in the following three sections, 5.6.1, 5.6.2, and 5.6.3

5.6.1 Accuracy of Methods and Statistics

Some of the methods used in the evaluation of phage infectivity are not perfect and have a known level of error. Phage plate titering is an accurate technique in evaluating phage infective titre and phage concentration. However, in this case there it is one possible source of error. To titre f1d3 a special strain called K1762 is used, which contains two plasmids. Occasionally when using this strain the phage failed to form plaques efficiently. This is believed to be because of recombination of the plasmids, causing either the cell to become non-permissive to f1d3 (due to the loss of pIII or to increase of pIII expression in non-infected cells), dramatically reducing the apparent phage titre of a sample. To avoid this effect all titering on K1762 was carried out with an extra plate containing f1d3 and f1 of known titres. f1 was also titered on TG1 at the same time. If the titre of f1 on both plates matched, and f1d3 matched the known titre then it was concluded that titre on strain K1762 was accurate. To avoid titering failure on K1762, only freshly streaked plates and overnight cultures were used for titering.

Another source of error was quantification of the virion amount. Firstly, the method used measures the concentration of phage genome equivalents and not the total phage particle concentration. Because of the presence of polyphage the concentration of genome equivalents is higher than the phage particle concentration. This was alleviated by using as a positive control the phage with the same particle length distribution as that of the sample. Currently this is the best way of measuring the virion concentration of a filamentous phage sample.

A further source of error in virion quantification is the densitometric analysis, which usually has a high error rate due to uneven band measuring. To minimise the error, firstly all measurements of bands were taken using the same area, and secondly the background density was removed from each band by measuring the equal area below it. This should limit the error caused by uneven background fluorescence and band spreading. The other source of error in densitometric analysis is in measurement of saturated pixels. Even if a pixel's brightness exceeds the saturation point of a CCD camera, it is nevertheless measured at the maximum brightness value. Therefore, at high loadings a certain amount of pixels is fully saturated and is not recorded accurately. To avoid this effect, gels were loaded with enough phage to visualise bands after staining with ethidium bromide, but not enough to reach pixel saturation. Also gel pictures were taken at the maximum possible resolution, to ensure the highest amount of pixels possible for analysis, and all values were average over multiple samples. However, the calculations showed that the error was still in the range of ±20-40%. Nevertheless this inaccuracy does not interfere with interpretation of the data, because the difference in infectivity was about three orders of magnitude (Figure 14, Table 8).

5.6.2 Evidence for the Correct Folding of the N Domain of pIII in the Mutant Particles.

The three orders of magnitude reduction in the infectivity in the mutant sample relative to the positive control could also be explained by the incorrect folding of the N terminal domains of pIII. If the N terminal domains were misfolded then they may be unable to bind to the receptors (F pilus tip or TolA). This would render the resulting phage non infectious. However, in these experiments it is assumed that the N1N2 terminal domains should be folded correctly based on ample evidence from the literature of correct folding independently of Cd. There are multiple NMR and X ray crystal structures of the N terminal domains (Holliger et al., 1999; Lubkowski et al., 1998; Lubkowski et al., 1999) which were produced in the absence of Cd. The best example is the paper by Holliger et al (1999) in which the N1N2 domains, used for structure determination, folded properly when expressed without C domains and targeted to the periplasm. In favour of robustness of the N1N2 folding, Lubkowski et al (1998) and (1999) used N1N2 domains which had been purified from cytoplasmic inclusion bodies. The N1N2 domains from the inclusion bodies were refolded by redox shuffle before crystallization. The structure matched that of the spontaneously folded periplasmic N1N2. The refolded N1N2 domains were also shown to bind the primary and secondary receptors, therefore they retained their biological activities (Reichmann & Holliger, 1997). In summary, all these findings show that the N domains can fold completely independently of the C domain.

Recently more evidence that the N domains fold independently of the C domain can be found in a series of papers (Martin & Schmid, 2003a; Martin & Schmid, 2003b). These papers used a variety of techniques and N domain mutants to analyse the folding kinetics of the N domain and demonstrate that the N domain can be used as a model system for folding kinetics and domain docking of two-domain proteins.

Correctly folded and functional N1N2 domains result in inhibition of infection of the cells that express them (Boeke et al., 1982). The fact that NdC83 construct rendered the cells difficult to infect (<1%) unless the expression was repressed by increasing the glucose concentration and even then only 50% of cells were infected (as opposed to 100% in parallel culture without NdC83 construct), confirms that N1N2 domains of NdC83 are functional.

5.6.3 Comparison of Infectivity of WTpIII/Cd to F1d3

The positive control (WTpIII/Cd) which carried a mixture of full length pIII and C domain was calculated to have a very low infectivity (3.28%). This, however was a similar value to the f1d3 virions that carry exclusively full length pIII (7%). Therefore the low infectivity is a characteristic of complemented f1d3 particles. The low infectivity can be attributed to the increase of the fraction of polyphage in f1d3 sample. The extended length of the particles is most likely due to the spatial separation of pIII production relative to phage assembly. If infective, a polyphage virion will only add one infection unit per particle, but can consist of more than one genome equivalent per particle. Therefore the infectivity defined as a ratio of infectious titre to the number of genome equivalents is expected to be significantly lower than that of a short wild type virion.

The small difference in infectivity between f1d3 and WT pIII/Cd (7% *vs.* 3.28%) could be due to reduced number of copies of the N1N2 domains in the WTpIII/Cd particles, because phage produced using this system carry a mixture of pIII molecules. Out of the possible five copies of pIII, at least 2 or 3 would be Cd, which lacks N1N2 domains required for infection. As a consequence, virions in the WT pIII/Cd sample are not uniform in the terms of the ratio of WT pIII to Cd. This means that some virions will have a majority (or all) of WT pIII, while some may have a majority (or all) Cd. Therefore, certain amount of virions will have no WT pIII at all, and will be non-infectious. In contrast, each virion of f1d3 phage complemented with wild-type pIII carries a full complement of five wild-type pIII molecules therefore it carries five copies of the N1N2 domains, therefore there should be no non-infectious virions in the population.

Chapter 6

Conclusions

An assembly-incompetent C-terminal domain covalently linked to the N domain is not sufficient for fl infection. This shows that the C domain of pIII plays a key role in phage infection.

From this experiment a model for the mechanism of fl infection can be proposed: Binding of the N1 domain to TolA triggers a conformational change or structural rearrangement to the functional C terminal domain, causing the "opening" of the phage structure to allow the release of phage DNA into the cytoplasm and insertion of the virion proteins into the inner membrane.

In order to confirm or reject the proposed model, further truncation mutants will need to be constructed to determine the minimum requirement for the C domain for infection. Also for additional support of the model the structure of the C terminal domain in the virion and in the host cell membrane will have to be determined.

As a first step toward structural analysis of the C domain of pIII, cryo-EM analysis of very short phage particles "microphage" is going to be carried out. A microphage producing system was constructed that consists of two components: i) the microphage origin inserted into a high copy number vector and ii) pII expressed *in trans* from a compatible plasmid. However, due to time constraints this system still awaits testing.

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Appendix 1

General Statistic of Ff Filamentous Phage

base	mol w	# in f1wt	total mw f1 wt	total mw f1d3	# in VCSM13d3	total mw VCSM13 d3
A	313.2	1574	492976.8	400896	1888	591321.6
T	304.2	2210	672282	551210.4	2399	729775.8
G	329.2	1325	436190	333479.6	1508	496433.6
C	289.2	1298	375381.6	286430	1597	461852.4

	f1	f1d3	VCSM13 d3
Gram-mole (g)	1976830	1572016	2279383.4
Molecules (Avogadro's number)	6.02×10^{23}	6.02×10^{23}	6.02×10^{23}
Molecules/gram DNA	3.05×10^{17}	3.83×10^{17}	2.64×10^{17}
Molecules/mg DNA	3.05×10^{11}	3.83×10^{11}	2.64×10^{11}
Genomes/ug DNA	3.05×10^{11}	3.83×10^{11}	2.64×10^{11}

Other physical measures (based on Day L.A. (1969))

Extinction coefficient (Day)
ε =3.84 ml /(mg*cm) at 269 nm (1mg/ml f1 in 1 cm cuvette has OD=3.84)

Virion composition
12% DNA
88% protein

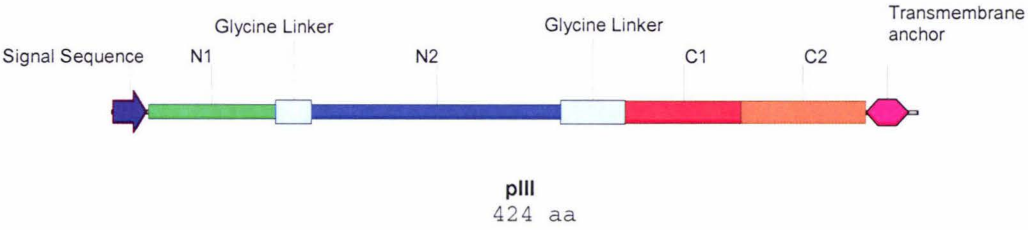
DNA	proteins	Total mass (DNA+ proteins)	pVI	pIII
1 µg	7.33 µg	8.33 µg	0.025 µg	0.088 µg

Mass ratio of pIII to pVIII in a monomeric virion is 0.012
Mass ratio of pVI to pVIII in a monomeric virion is 0.0034

Appendix 2

Protein Sequences and Domain Arrangements

Wild Type pIII sequence and structure



```
  1 VKKLLFAIPL VVPFYSHSAE TVESCLAKPH TENSFTNVWK
 41 DDKTLDRYAN YEGCLWNATG VVVCTGDETQ CYGTWVPIGL
 81 AIPENEGGGS EGGGSEGGGS EGGGTKPPEY GDTPIPGYTY
121 INPLDGTYPP GTEQNPANPN PSLEESQPLN TFMFQNNRFR
161 NRQGALTVYT GTVTQGTDPV KTYYQYTPVS SKAMYDAYWN
201 GKFRDCAFHS GFNEDPFVCE YQGQSSDLPQ PPVNAGGGSG
241 GGSGGGSEGG GSEGGGSEGG GSEGGGSGGG SGSGDFDYEK
281 MANANKGAMT ENADENALQS DAKGKLDSVA TDYGAAIDGF
321 IGDVSGLANG NGATGDFAGS NSQMAQVGDG DNSPLMNNFR
361 QYLPSLPQSV ECRPFVFGAG KPYEFSIDCD KINLFRGVFA
401 FLLYVATFMY VFSTFANILR NKES
```

NdC83 Sequence and Structure

```
  1 MAKKLLFAIP LVVPFYSHSA ETVESCLAKP HTENSFTNVW
 41 KDDKTLDRYA NYEGCLWNAT GVVVCTGDET QCYGTWVPIG
 81 LAIPENEGGG SEGGGSEGGG SEGGGTKPPE YGDTPIPGYT
121 YINPLDGTYP PGTEQNPANP NPSLEESQPL NTFMFQNNRF
161 RNRQGALTVY TGTVTQGTDP VKTYYQYTPV SSKAMYDAYW
201 NGKFRDCAFH SGFNEDPFVC EYQGQSSDLP QPPVNAGGGS
241 GGGSGGGSEG GGSEGGGSEG GGSEGGGSGG GSGSGDFDYE
281 SQMAQVGDGD NSPLMNNFRQ YLPSLPQSVE CRPFVFGAGK
321 PYEFSIDCDK INLFRGVFAF LLYVATFMYV FSTFANILRN
361 KES*
```

Pairwise Alignment showing deletion in NdC83

```
                1                                      40
NdC83      (1)  MAKKLLFAIPLVVPFYSHSAETVESCLAKPHTENSFTNVW
 pIII      (1)  -VKKLLFAIPLVVPFYSHSAETVESCLAKPHTENSFTNVW

                41                                     80
NdC83     (41)  KDDKTLDRYANYEGCLWNATGVVVCTGDETQCYGTWVPIG
 pIII     (40)  KDDKTLDRYANYEGCLWNATGVVVCTGDETQCYGTWVPIG

                81                                    120
NdC83     (81)  LAIPENEGGGSEGGGSEGGGSEGGGTKPPEYGDTPIPGYT
 pIII     (80)  LAIPENEGGGSEGGGSEGGGSEGGGTKPPEYGDTPIPGYT

                121                                   160
NdC83    (121)  YINPLDGTYPPGTEQNPANPNPSLEESQPLNTFMFQNNRF
 pIII    (120)  YINPLDGTYPPGTEQNPANPNPSLEESQPLNTFMFQNNRF

                161                                   200
NdC83    (161)  RNRQGALTVYTGTVTQGTDPVKTYYQYTPVSSKAMYDAYW
 pIII    (160)  RNRQGALTVYTGTVTQGTDPVKTYYQYTPVSSKAMYDAYW

                201                                   240
NdC83    (201)  NGKFRDCAFHSGFNEDPFVCEYQGQSSDLPQPPVNAGGGS
 pIII    (200)  NGKFRDCAFHSGFNEDPFVCEYQGQSSDLPQPPVNAGGGS
```

```
         241                                     280
NdC83  (241) GGGSGGGSEGGGSEGGGSEGGGSEGGGSGGGSGSGDFDYE
 pIII  (240) GGGSGGGSEGGGSEGGGSEGGGSEGGGSGGGSGSGDFDYE

             281                                  320
NdC83  (281) ----------------------------------------
 pIII  (280) KMANANKGAMTENADENALQSDAKGKLDSVATDYGAAIDG

             321                                  360
NdC83  (281) ----------------------SQMAQVGDGDNSPLMNNF
 pIII  (320) FIGDVSGLANGNGATGDFAGSNSQMAQVGDGDNSPLMNNF

             361                                  400
NdC83  (299) RQYLPSLPQSVECRPFVFGAGKPYEFSIDCDKINLFRGVF
 pIII  (360) RQYLPSLPQSVECRPFVFGAGKPYEFSIDCDKINLFRGVF

             401                   426
NdC83  (339) AFLLYVATFMYVFSTFANILRNKES
 pIII  (400) AFLLYVATFMYVFSTFANILRNKES
```