Department of Immunotechnology
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THE BIOLOGY OF FILAMENTOUS PHAGE INFECTION

IMPLICATIONS FOR DISPLAY TECHNOLOGY

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ORIGINAL PAPERS

I Nilsson N, Karlsson F, Rakonjac J and Borrebaeck CAK (2002) Selective infection of *E. coli* as a function of a specific molecular interaction J Mol Recognit 15, 27-32.

II Karlsson F, Nilsson N, Borrebaeck CAK and Malmborg A-C (2003) The mechanism of bacterial infection by filamentous phages involves molecular interactions between TolA and phage protein 3 domains. J Bacteriol 185, 2628-2634.

III Karlsson F, Malmborg-Hager A-C and Borrebaeck CAK (2004) Identification of mutations that segregate membrane integrity and phage receptor function of the *Escherichia coli* TolA molecule. Submitted for publication.

IV Karlsson F, Malmborg-Hager A-C, Albrekt A-S and Borrebaeck CAK (2004) Genome-wide comparison of phage M13 infected vs. uninfected *Escherichia coli*. Submitted for publication.

ABBREVIATIONS

aa	amino acid
BIA	(real-time) biospecific interaction analysis
CT	C-terminal domain of phage minor coat protein pIII
cDNA	complementary DNA
DNA	deoxyribonucleic acid
dsDNA	double stranded DNA
ELISA	enzyme-linked immunosorbent assay
Ff	F-specific filamentous
IG	intergenic
mRNA	messenger RNA
N1	the first N-terminal domain of phage minor coat protein pIII
N2	the second N-terminal domain of phage minor coat protein pIII
N1N2	both N-terminal domains of pIII, including flexible linker
ORF	open reading frame
PS	packaging signal
PCR	polymerase chain reaction
RF	replicative form
RNA	ribonucleic acid
SAP	selection and amplification of phage
scFv	single-chain antibody fragment variable
SIP	selectively infective phage
SPR	surface plasmon resonance
ssDNA	single stranded DNA

1 INTRODUCTION

There is a constant demand for molecules suitable for diagnosis and/or treatment of illness. In order to devise the molecules that will carry out these tasks, an array of biotechnological tools are available. One such tool is based on the use of bacteria and a special kind of bacterial virus. If we learn more about the relationship between this virus and the bacteria, it will be feasible to improve the design of the tool. Hopefully, this will ensure a more rapid process of finding the desired molecules.

Viruses that can infect bacteria are called bactoriophage and are harmless to humans. Like all viruses, bacteriophage (phage) are metabolically inert in their extra-cellular form and reproduce only by infecting and exploiting the metabolism of the host bacteria. Upon infection, the viral genomic material is introduced into the host cell, where it directs the production of progeny phage. Most often, progeny phage are assembled in the cytoplasm of infected cells in large numbers, which cause the host cell to burst, killing it and then infecting more bacteria (Calendar, 1988). There are innumerable types of phage, each capable of eradicating its host bacterial species. However, some special phage are assembled at the cell surface and co-ordinately exported by a secretory mechanism, a process that leaves the host cell fully viable. The secreted bacteriophage belong to the family of filamentous phage (called so due to their morphology) that are specific for the Gram-negative bacteria *Escherichia coli* (*E. coli*) carrying F-pili (Hoffmann-Berling *et al*., 1963; Loeb, 1960; Marvin and Hoffmann-Berling, 1963; Zinder *et al*., 1963).

Some 25 years after filamentous phage were first isolated, it was reported that a genetic modification, the insertion of a foreign DNA fragment into the phage genome, yielded a phage particle displaying the foreign polypeptide sequence as a fusion to the coat protein on its surface (Smith, 1985). Furthermore, the displayed peptide was accessible to specific recognition by an antibody towards the peptide. This resulted in an enrichment of the peptide-displaying phage from a mixture of wild-type (non-binding) phage and display-phage particles. In addition, the methodology ensured the direct physical link between phenotype (polypeptide) and genotype (DNA), meaning that the displayed molecule had a tag, the DNA sequence encoding that

molecule, which could be addressed through DNA sequencing. Therefore, the report by George Smith in 1985 is considered the first description of phage display technology.

It was soon realised that it would be possible to build up large pools of genetic variants, i.e. DNA libraries, by combining several new technologies. Consequently, recombinant DNA technology, which includes the precise cutting (by restriction enzymes) and rejoining (by ligases) of DNA pieces (Linn and Arber, 1968), oligonucleotide-directed mutagenesis (Hutchison *et al*., 1978) and the DNA amplification technique known as the polymerase chain reaction (PCR) (Saiki *et al*., 1985), were used to create DNA libraries. Only a few years after the publication by George Smith, the first phage-displayed random polypeptide libraries were assembled (Cwirla *et al*., 1990; Devlin *et al*., 1990; Scott and Smith, 1990). These reports were followed by others, which demonstrated that properly folded and functional proteins could also be displayed on the surface of filamentous phage (Bass *et al*., 1990; McCafferty *et al*., 1990).

Many different types of molecules can be displayed on the surface of phage. Most common is display of some sort of protein with an intrinsic capacity of recognising and binding target molecules with a high degree of specificity, such as antibody fragments. The possibility to display antibody fragments on the surface of phage is of particular interest, since these molecules have great potential as reagents in diverse settings like diagnostics, biological chemistry and as agents suitable for therapeutic applications (Borrebaeck, 2000; Borrebaeck and Carlsson, 2001; Bradbury *et al*., 2004; Hudson, 1998; Hudson and Souriau, 2001). Notably, up to 30 % of human antibodies in clinical development have been isolated using phage display technology (Kretzschmar and von Ruden, 2002). Because of these potentials, antibody fragments are the most common scaffolds diversified and used for selection processes in the field of phage display technology.

Proteins that bind to their target with a high binding strength (affinity) and a high degree of specificity are much desired for diagnostic and biochemical purposes. Therefore, attention has been drawn to the creation of protocols that increase the chance of obtaining high affinity reagents, using various display methods. One approach is to move to cell-free systems (Hanes

and Plückthun, 1997; Nord *et al*., 2003), allowing for larger libraries, which theoretically increases the likelihood of finding high affinity binding proteins. Another is to modify the selection procedure of the phage display technology, by studying the requirements for the infection process of filamentous phage, in hope of revealing key mechanistic events suitable to manipulation.

In this thesis, I will summarise and interpret data from independent investigations of the infection process of filamentous phage, as studied by differing approaches. In addition, I will present the results from my own studies in this area and how they may affect the development of phage display technology. My experimental work has resulted in four original papers (reports) that deal with aspects of both the phage infection mechanism and phage display technology. The first report describes an evaluation of the parameters important for a special application of phage display, called selective infection (PAPER I), the second and third present the results from studies of phage and host protein interactions during phage infection (PAPER II and III) and the last is a description of the effects phage infection had on the transcription of host genes (PAPER IV).

In experiments reported in PAPER I, a correlation between the affinity of the interacting pairs and infection efficiency of selective infection was detected, and a phage format that allows multiple display of antibody fragments on each phage was found to be superior to one that does not allow such multiple display. PAPER II describes the molecular interactions between phage coat protein pIII and the bacterial co-receptor protein of phage infection, TolA, as assayed by real-time bio-specific interaction analysis. The binding affinities between these proteins and their different domains were characterised and novel interactions were detected allowing us to make a refined hypothetical model of the infection mechanism of filamentous phage. In PAPER III, the bacterial co-receptor, TolA, which is also required for outer membrane integrity of *E. coli*, was studied. It was found that the membrane integrity and phage receptor functions of TolA could be segregated. PAPER IV describes the changes in gene expression of phage infected *E. coli*, as monitored by global transcription analysis and it was demonstrated that several host genes were co-ordinately affected. I will also discuss how the results of these studies have provided insights into several different aspects of

the phage infection process, as well as the possible implications of the results for phage display technology. But first, the biology of filamentous phage and the phage display technology will be introduced.

2 BIOLOGY OF THE Ff PHAGE

Filamentous bacteriophage appear as thin (6-10 nm) and long (900 nm), filamentous particles (Figure 1) containing circular single stranded (ss) DNA (Hoffmann-Berling *et al*., 1963; Marvin and Hoffmann-Berling, 1963; Newman *et al*., 1977). The actual length of the particle is determined by the length of the DNA it has encapsulated (Marvin, 1990). The filamentous phage belongs to a group of related viruses which only infect gram-negative bacteria via specific adsorption to the tip of bacterial structures called F-pili (Loeb, 1960). The F-pili (also called sex-pili) are normally involved in the transmission of the F plasmid DNA, or chromosomal DNA containing the integrated plasmid DNA, from one bacterium to another (Frost *et al*., 1994; Gaffney *et al*., 1983; Hayes, 1952). Most information about this type of filamentous phage derives from the very similar members of the same family of F-specific filamentous (Ff) phage, classified as Inoviridae, of the genus Inovirus, i.e. phage M13, f1 and fd, which infect *Escherichia coli* (*E. coli*) (Marvin and Hohn, 1969). The genomes of these three bacteriophage have been completely sequenced and are 98 % homologous (Beck *et al*., 1978; Beck and Zink, 1981; Hill and Petersen, 1982; van Wezenbeek *et al*., 1980). Because of their similarity and their dependence on the F-pilus for infection, the term Ff phage will be used throughout when referring to M13, fd and f1 biology, unless otherwise stated.

Unlike other bacterial viruses, the Ff phage does not kill its hosts, but establish a relationship in which new virions are continually released (Hoffmann-Berling and Mazé, 1964). Because of this non-lytic mode of release it is possible to grow high-titre cultures of the virus. Also, the growth curve of Ff phage shows a rapid increase of virus after a short latent period (Brown and Dowell, 1968; Hoffmann-Berling *et al*., 1963; Marvin and Hohn, 1969). The first progeny phage particles are released about 15 minutes after infection (Hofschneider and Preuss, 1963), and the rate of production is exponential for the first 60 minutes, after which it becomes linear as the bacteria enter stationary phase (Brown and Dowell, 1968; Hoffmann-Berling *et al*., 1963; Marvin and Hohn, 1969). This rate of production implies that roughly 1000 phage per bacterium are produced within the first hour after

infection. Although infected cells can continue to grow and divide indefinitely, the process causes the infected cells to continue growth at a rate significantly lower than uninfected cells (Brown and Dowell, 1968; Hoffmann-Berling *et al*., 1963; Salivar *et al*., 1964), which is why plated cultures of Ff phage-infected cells yield turbid plaques. The Ff phage are relatively simple molecular complexes and they have therefore been extensively characterised. At the Department of Immunotechnology, a derivative of Ff phage M13 is the most frequently used phage. Bacteriophage M13 was first isolated from wastewater in Munich 1963 (Hofschneider, 1963). In the following sections, the life cycle, genetics and structural organisation of the Ff phage is summarised, with a focal point on factors of importance for phage display technology.

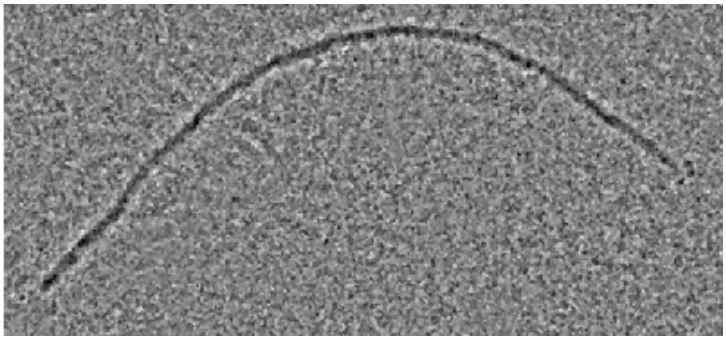

FIGURE 1. An electron micrograph of a filamentous phage. The globular domains of the adsorption complex of the virion (the pIII) are visible at the right end of the viral particle. The picture was taken by Dr. C. Brown and kind permission to reprint it was given by Professor D. A. Marvin, University of Cambridge, UK.

2.1 PHAGE COAT PROTEINS

The most abundant coat protein of the virus is encoded by gene VIII. The roughly 2700 copies of pVIII make it the major coat protein (Newman *et al*., 1977; Pratt *et al*., 1969). The minor coat proteins are located at the ends of the phage particle, and are present at only a few copies each, as can be seen in Figure 2. The distal end, which is assembled first, contains the pVII and pIX, and the proximal end, which enters the host first, contains pVI and pIII (Lopez and Webster, 1983). Estimations based on symmetry have proposed that there

are five copies of each of the minor coat proteins, although the distal end seems to have three copies each of pVII and pIX (Makowski, 1992; Simons *et al*., 1981). These two proteins (33 and 32 residues) are required for phage to be assembled and released, and in the absence of either no infectious particles are formed (Lopez and Webster, 1983; Pratt *et al*., 1969). The two remaining minor coat proteins pVI and pIII, are necessary for particle stability and infection (Gailus *et al*., 1994; Grant *et al*., 1981a; Grant *et al*., 1981b; Gray *et al*., 1978; Lopez and Webster, 1983; Rakonjac and Model, 1998).

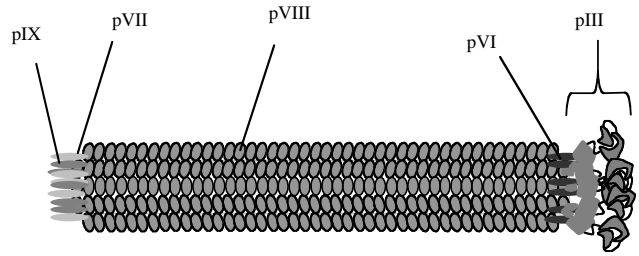

FIGURE 2. The localisation of the Ff phage coat proteins. At the left end, the minor coat proteins pVII and pIX are located, while the major coat protein, pVIII, is spread along the entire length of the particle. At the right end, which penetrates the bacteria upon infection, the minor coat proteins pVI and pIII are located.

Not much is known about the appearance of pVI on the surface of the phage particle, but at least a portion of the protein seem to be surface exposed, and pVI and pIII are known to assemble in the membrane prior to incorporation into phage particles (Gailus and Rasched, 1994; Rakonjac *et al*., 1999). In contrast, the most commonly used coat protein for phage display, pIII (Kay and Hoess, 1996; Russel *et al*., 2004; Smith and Petrenko, 1997), has received a lot more attention, primarily because of its role in phage infection. Phage protein pIII is synthesised with an 18-residue amino-terminal signal sequence and requires a bacterial secretion and protein export system (Sec) for insertion into the membrane (Rapoza and Webster, 1993). After removal of the signal sequence, mature pIII, as shown in Figure 3, is obtained. This molecule is composed of 406 residues and form three distinct, folded domains, which are separated by glycine-rich, flexible, regions. The 149-residue carboxy-terminal

(CT) domain (residues 256-406) anchors pIII to the phage particle and its membrane anchor region (residues 378-406) is probably buried within the phage particle (Marvin, 1998; Rakonjac *et al*., 1999), while the two remaining amino-terminal domains are surface exposed. The amino (N) terminal domains 1 and 2 (N1 and N2) interact intramolecularly and form a N1N2 complex (residues 1-217), as shown by X-ray crystallography and NMR spectroscopy (Holliger *et al*., 1999; Lubkowski *et al*., 1998). The N1 and N2 domains of pIII play important roles during the infection process of Ff phage, by mediating infection (Armstrong *et al*., 1981; Boeke *et al*., 1982; Crissman and Smith, 1984; Jakes *et al*., 1988; Rasched and Oberer, 1986; Riechmann and Holliger, 1997; Stengele *et al*., 1990).

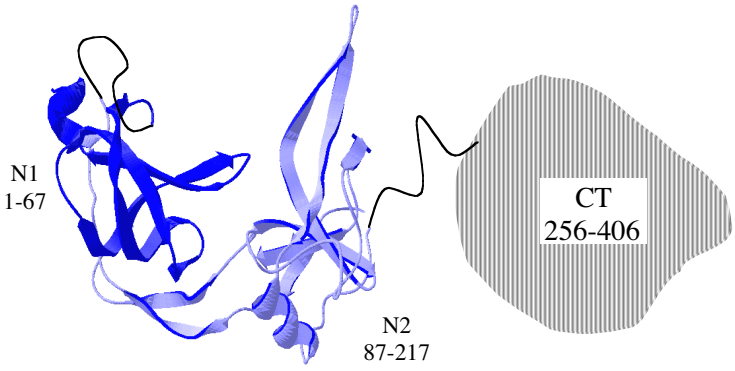

FIGURE 3. The domain structure of the adsorption protein pIII. The amino acids creating each domain are indicated by their number in the sequence. The N1 (blue) and N2 (light blue) domains are involved in inter-domain contacts, as determined by X-ray crystallography (Lubkowski *et al*., 1998). The N1 domain mediates binding to the bacterial co-receptor protein TolA (Riechmann and Holliger, 1997). N2 binds to F-pilus via residues located on the outer rim of N2, and not in the central cavity formed in the interface between N1 and N2 (Deng and Perham, 2002). The CT (shaded gray) is required for virion assembly and its topology is unknown. The flexible linker regions, containing tandem repeat sequences of GGGS and EGGGS

(van Wezenbeek and Schoenmakers, 1979), are indicated as black lines between the domains.

2.2 INFECTION MECHANISM

Filamentous phage infection of *E. coli* starts with the adsorption to the bacterial structure called the F-pilus, which is of long and slender conformation not unlike the one of phage. The infection process eventually leads to phage DNA translocation by a mechanism that remains uncertain. However, the events prior to this final step are well characterised and shown in Figure 4. The F-pilus extends from the cell envelope at contact sites between inner and outer membranes, called adhesion zones (Bayer, 1968a, b). The initial phage binding to the tip of the F-pilus is strong (Deng *et al*., 1999; Stengele *et al*., 1990; Tzagoloff and Pratt, 1964) and achieved by residues on the outer rim of the N2 domain of pIII (Deng and Perham, 2002). The glycine rich linker region between N1 and N2 (residues 69-86) may also be involved via an unknown mechanism (Nilsson *et al*., 2000). After phage adsorption, the natural process of pilus retraction brings the phage closer to the bacterial membrane (Jacobson, 1972; Marvin and Hohn, 1969). It has been suggested that the binding to the F-pilus leads to a conformational change in N2 that releases N1 from the N1N2 complex (Deng and Perham, 2002; Riechmann and Holliger, 1997). Thus, after pilus retraction, the N1 domain is free to interact with the bacterial co-receptor TolA as seen in Figure 4 (Riechmann and Holliger, 1997). The TolA protein is membrane bound and encoded by the *tolA* gene of *E. coli,* also located at adhesion zones (Guihard *et al*., 1994). TolA is believed to form a complex with two other membrane bound proteins, TolQ and TolR (Webster, 1991). Host cells that lack of any of the inner membrane proteins TolQ, TolR and TolA, are tolerant to phage infection (Click and Webster, 1997, 1998; Russel *et al*., 1988; Sun and Webster, 1986). From its anchor point in the cytoplasmic membrane, the TolA protein extends into the periplasm (Levengood *et al*., 1991), where it interacts with N1 during phage infection. The putative complex formed by the three Tol proteins (Q, R and A) mediates incorporation of phage coat proteins (pIII and pVIII) into the cytoplasmic membrane and the translocation of the ssDNA by an incompletely understood mechanism (Armstrong *et al*., 1983; Boeke and Model, 1982;

Russel *et al*., 1988; Smilowitz, 1974; Trenkner *et al*., 1967). Similarly, it is not known if the TolA co-receptor molecules interact with each other during the infection process. In a few of the following chapters some light will be shed on these issues.

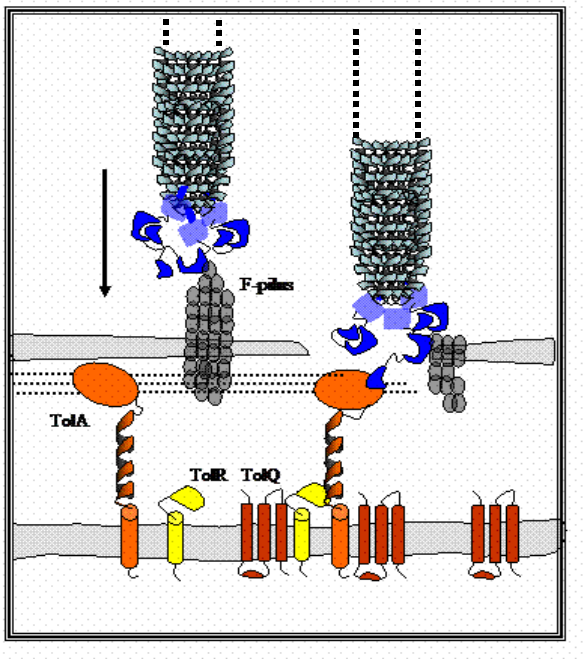

FIGURE 4. Schematic representation of the tip of an Ff phage binding to *E. coli* receptor (F-pilus) and co-receptor (TolA) during the initial steps of the infection process. The infection process is initiated by interaction of the N2 domain of pIII (blue) with an F-pilus projecting from the *E. coli* cell surface. This interaction releases N1 from the inter domain interaction with N2, allowing it to bind to the C-terminal domain of the bacterial protein TolA. The upper and lower grey horizontal bars are outer and inner membranes of *E. coli*, respectively. The dotted line is the peptidoglycan layer. For clarity, the F-pilin subunits of the F-pilus are only shown when they are part of the F-pilus.

2.3 GENES, GENE EXPRESSION AND REPLICATION

The infecting Ff phage contains a circular ssDNA molecule of 6407 (6408 for fd) nucleotides, which encodes 11 proteins from 9 open reading frames

(ORFs) (Beck *et al*., 1978; Beck and Zink, 1981; Day and Berkowitz, 1977; Guy-Caffey *et al*., 1992; Rapoza and Webster, 1995; Rasched and Oberer, 1986; van Wezenbeek *et al*., 1980). All 11 proteins are necessary for DNA replication and encapsulation (Figure 5) (Hohn *et al*., 1971; Rapoza and Webster, 1995). Five of the genes encode structural proteins (III, VI, VII, VIII and IX), three are required for phage DNA replication (II, V and X) and the remaining encode products due for assembly and secretion reactions (I, IV and XI) (Rapoza and Webster, 1995; Rasched and Oberer, 1986). One large and one small non-coding intergenic (IG) region, containing signals for initiation of DNA synthesis and termination of RNA synthesis, as well as an imperfect 32-bp hairpin containing the packaging signal (PS) for encapsulation of phage DNA (Dotto and Zinder, 1983; Schaller *et al*., 1969; Webster *et al*., 1981), complete the genome of the Ff bacteriophage.

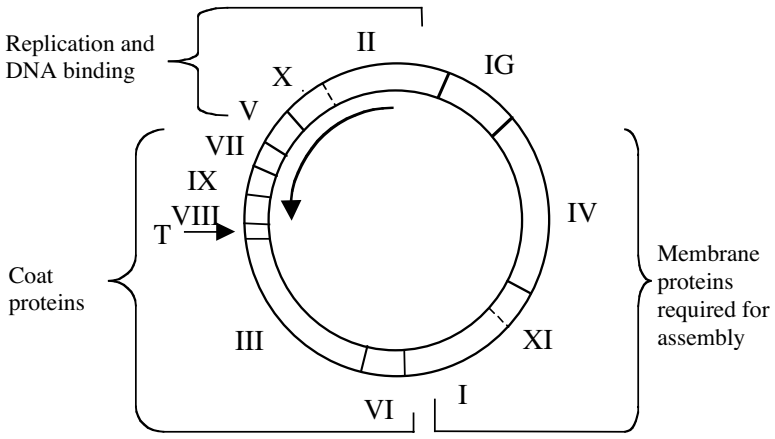

FIGURE 5. Genetic map of the Ff phage. The nucleotide sequence of the Ff genome is numbered counter clockwise from the unique *Hind*II restriction site within gene II (start of inner circle arrow). Also, the direction of transcription is indicated by the inner circle arrow. The position of the common termination site or stem loop (T) and the intergenic region (IG) (containing the packaging signal, PS) are also shown. For the two pairs of in-frame overlapping genes (II/X and I/XI), the positions are shown (broken lines) for the translation starts from which gene X and XI proteins are produced (Guy-Caffey *et al*., 1992; Yen and Webster, 1981). The gene products are grouped based on their known functions, as seen outside the brackets. The encoded

proteins have differing sizes, which is indicated by the differing sizes of the "gene boxes" in this figure.

Once the viral ssDNA has entered the host, the RNA and DNA polymerases of the host convert it into a double stranded (ds) DNA molecule (Brutlag *et al*., 1971; Geider and Kornberg, 1974; Kaguni and Kornberg, 1982; Vicuna *et al*., 1977). The dsDNA is super-coiled and called replicative form (RF), which is the template for phage gene expression leading to further replication (Lin and Pratt, 1972; Mazur and Model, 1973; Tseng *et al*., 1972). Phage pII nicks the (+) strand of the RF, at a specific site within the IG region, and host polymerase III is involved in elongation of the strand, thus enabling viral strand synthesis, and hence RF replication (Geider and Kornberg, 1974; Meyer and Geider, 1979; Model and Zinder, 1974). A host replicase, Rep, displaces the (+) strand as the new (+) strand is synthesised by a rolling circle mechanism (Gilbert and Dressler, 1968; Meyer and Geider, 1982). The newly synthesised viral strand is then re-circularised by pII (Meyer and Geider, 1979). The (–) strand is synthesised via an RNA primer generated by the host RNA polymerase, which initiates synthesis at a site within the IG region (Horiuchi *et al*., 1997).

During the period immediately after infection, newly synthesised single strands are instantaneously converted to RF, and both RF and phage proteins increase exponentially (Marvin and Hohn, 1969). When the concentration of pV begins to build up, this ssDNA binding protein starts to bind to newly generated (+) strands by the formation of DNA-binding pV-dimers (Alberts *et al*., 1972; Cavalieri *et al*., 1976). The binding of pV dimers to ssDNA prevents polymerase access and blocks the conversion to RF, by the formation of a complex morphologically similar to the assembled phage (Pratt *et al*., 1974). At this stage, the pX protein, identical to the 111 C-terminal residues of pII (van Wezenbeek *et al*., 1980), ensures stable accumulation of single strands (Fulford and Model, 1984). The pV/ssDNA complex has an orientation that allows the PS to protrude from one end of the complex, making it the substrate for phage assembly (Bauer and Smith, 1988).

Gene expression of phage proteins is carefully regulated by diverse mechanisms that ensure an appropriate amount of each protein. Although all

phage proteins are generated simultaneously, there are e.g. differences in promoter and ribosome binding strength or accessibility. Genes III, VI, I and IV are transcribed by a weak promoter (La Farina and Model, 1983) and in addition there is a weak termination signal at the end of the gene preceding gI (La Farina and Vitale, 1984; Moses and Model, 1984), which limits gI transcription, and gI also contains a large number of infrequently used codons. Other examples of strategies to differentiate the gene expression levels of Ff phage, is to have overlapping transcripts from multiple promoters and multiple RNA processing events, which affect the abundance of RNAs (Goodrich and Steege, 1999). These measures ensure the required high levels of pV and pVIII. In addition excess pV bind gII and gX mRNAs (Oliver *et al*., 2000), thus repressing their translation. The net effect is a lower synthetic rate of pII and (+) strand (Fulford and Model, 1988a, b), leading to a linear rate of phage production during the later stage of the proliferation process.

2.4 Assembly reactions

One of the hallmarks of Ff phage proliferation is its mode of secretion, where nascent phage are assembled and released through the cytoplasmic and outer membranes of infected cells, by a channel formed by phage encoded proteins (Lopez and Webster, 1985; Marciano *et al*., 1999, 2001). In total, eight phage-encoded proteins are involved in the assembly process, and all of them are integral membrane proteins (Endemann and Model, 1995; Guy-Caffey *et al*., 1992; Ohkawa and Webster, 1981; Rapoza and Webster, 1995). Apart from the five coat proteins (III, VI, VII, VIII and IX), the assembly also involves the pI protein and its restart associate, pXI, and the outer membrane protein pIV. The process of assembly starts with the formation of an assembly site (Lopez and Webster, 1985), where the cytoplasmic and outer membranes make contact. The site is formed by the pI, pXI and pIV proteins, which interact via their periplasmic domains (Russel and Model, 1989; Russel and Kazmierczak, 1993). Protein IV is a cylindrical protein that forms a large homo-multimeric channel in the outer membrane for export of the phage (Marciano *et al*., 1999, 2001). The properties of the pIV channel may be one of the factors that limit the size of polypeptides that can be displayed on pVIII (Greenwood *et al*., 1991; Malik *et al*., 1998).

The initiation of assembly also requires the presence of the minor coat proteins pVII and pIX and the ssDNA/pV substrate, in addition to the assembly site formed by pI, pXI and pIV (Feng *et al*., 1999). The PS of the ssDNA then associates to the cytoplasmic domain of pI to start the assembly and release process, possibly by the removal of pV dimers from the ssDNA by the action of pI and/or pXI (Rapoza and Webster, 1995; Russel, 1991). The particle is elongated by incorporation of membrane embedded pVIII, which then replaces the removed pV molecules on the ssDNA by an incompletely understood process. The pVIII replacement reaction pushes the phage through the pIV-channel at the assembly site and thus translocates the DNA across the membrane. If either pIII or pVI are present in low amounts, very long phage that contain multiple unit-length phage genomes are produced and remain attached to the cell surface (Rakonjac and Model, 1998; Rakonjac *et al*., 1999). During a normal infection cycle, such phage represent 5 % of the total progeny (Rakonjac and Model, 1998; Russel and Model, 1989).

The terminal release of the phage particle is achieved by the incorporation of the membrane embedded pIII-pVI complex at the end of the particle (Rakonjac and Model, 1998). The release process has been proposed to depend on a conformational change in the pIII-pVI complex that detaches the phage from the cytoplasmic membrane (Rakonjac *et al*., 1999). At least 132 of the 149 C-terminal residues of pIII are required to form a stable virus particle; however, shorter variants of pIII are able to release nascent phage particles. The N-terminal domains of pIII can be replaced or removed without disturbing the release and stability of the phage. This feature is used for display of proteins fused to pIII. To close this chapter on the Ff phage biology, a summary of some of the major events in its proliferation cycle is described in Figure 6.

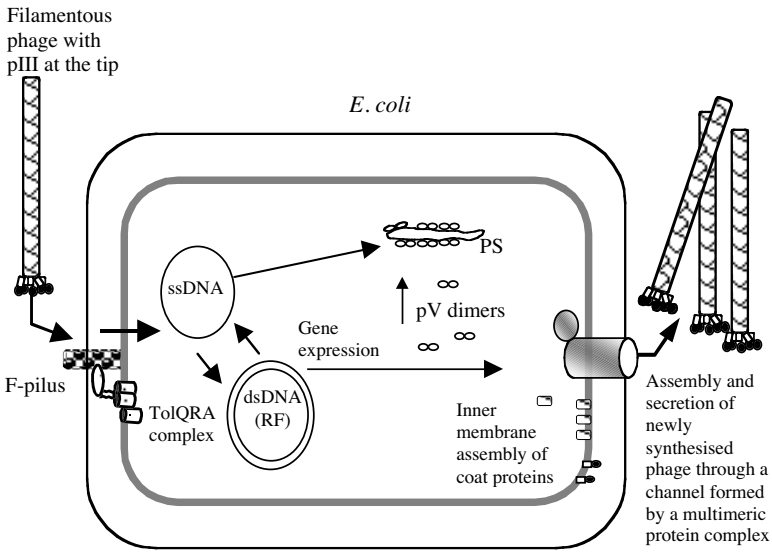

FIGURE 6. Schematic representation of the life cycle of Ff phage. Starting from the left and moving to the right the major events, leading to the release of many progeny phage, are outlined. First the phage attach to the F-pilus of bacteria via the adsorption complex (pIII), and are brought closer to the bacterial membrane, where the co-receptor molecule TolA is located in a protein complex. After DNA entry into the host, the single stranded (ss) DNA is converted into double stranded (ds), replicative form (RF) DNA, by the action of host polymerases. Gene expression is then initiated and phage proteins start to accumulate. The subsequent step of assembly is initiated by pV dimers binding to ssDNA, which prepares the ssDNA for incorporation into the phage capsid. The pV/ssDNA complex is morphologically similar to intact phage particles, and the packaging signal (PS) is exposed at one end. The assembly takes place at adhesion zones of the cell, and is carried out by a phage encoded multimeric protein complex, formed by phage proteins pI, pXI and pIV. The *E. coli* outer and inner membranes are indicated as a thin black line and a thicker grey line, respectively.

3 PHAGE DISPLAY TECHNOLOGY

Phage display technology is the process of first displaying polypeptides on the surface of Ff phage (which contain the genome of each displayed polypeptide), and then to select the fraction of displayed polypeptides that exhibit desired properties. Today, it is most often used in a scheme based on affinity selection, as depicted in Figure 7. The technology combines the use of several common molecular biology techniques and biochemical methods to establish diversity into the displayed polypeptide, as described in the Introduction. To create display, the knowledge of recombinant DNA vectors is exploited. They have the ability to accommodate segments of foreign DNA, to replicate this segment and to express the inserted segment as a protein in its *E. coli* host. Thus the host synthesises a foreign polypeptide, whose amino acid sequence is determined by the nucleotide sequence of the insert. In order for the polypeptide of interest to be displayed on the surface of a phage, the insert is spliced into the gene for one of the phage coat proteins, so that the expressed protein is a fusion of the endogenous phage coat protein and the foreign peptide. Also, the necessary diversity among the polypeptides is created so that these variant molecules can be tapped for a desired property. This results in the creation of a heterogeneous mixture of phage clones, a so called phage library. In a phage library each phage clone carries a different foreign DNA insert and therefore displays a different polypeptide on its surface. In addition, because the displayed polypeptide is solvent accessible the displayed molecule often retains the behaviour of its free counterpart. Thus, for example, phage-displayed antibody fragments maintain their affinity and binding specificity. This means that biochemical techniques applied to polypeptides in solution can be used on phage-displayed polypeptides without further modification. This is true for capture techniques based on affinity, in which an immobilised target molecule is used as bait to extract polypeptides binding to the target from the phage-displayed molecules in the phage library. The captured display-phage are then used to infect *E. coli*, which are expanded in culture, allowing the selected phage to be amplified. The amplified phage can then serve as the input phage for another round of affinity-based capture selection. Eventually, the selected polypeptides are analysed individually. This

protocol of affinity selection is the leading example of how phage display is used to obtain polypeptides with desired properties.

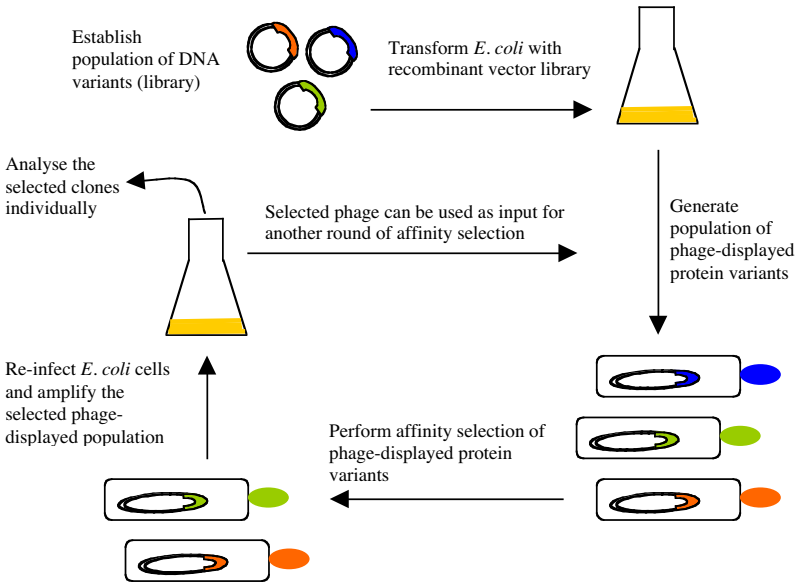

FIGURE 7. A simplified version of the phage display cycle. First a library of variant DNA sequences is created in the format of recombinant vectors. These are then introduced into *E. coli*, which after introduction of a phage genome are converted into phage particles containing the gene for the DNA-variant and displaying the encoded polypeptide on the surface of the phage, as a phage fusion protein (in this case to pIII). After affinity selection of the displayed protein variants (the phage library), those binding to the target molecule are rescued. This selected population can then either be analysed, by re-infecting *E. coli* and spreading the cells on plates to identify individual clones, or be used for another round of affinity-based selection.

3.1 CHOICE OF COAT PROTEIN AND DISPLAY FORMAT

Although all five capsid proteins have been used to display proteins or peptides (Gao *et al.*, 1999; Hufton *et al.*, 1999; McCafferty *et al.*, 1990), by far the most commonly used proteins for phage display are pVIII and pIII (Smith and Petrenko, 1997; Webster, 1996). The major coat protein pVIII builds up the tube of the phage particle and is present in thousands of copies. To achieve display on pVIII foreign sequences of interest are typically inserted at the N-

terminus, between the signal sequence and the beginning of the coding sequence for pVIII. As mentioned in a previous section (2.4), only short peptides (6-8 residues) allow efficient assembly of pVIII-display phage, due to size restrictions of the pIV-channel during assembly. However, larger proteins like antibody fragments have been displayed as fusions to pVIII (Kang *et al*., 1991; McCafferty *et al*., 1990). The limitation in the size of the display molecule is probably also related to the disruption of the interactions between pVIII molecules during assembly (Makowski, 1993).

Display on pIII is the most attractive phage display fusion, because of its tolerance to large insertions (Krebber *et al*., 1997; Smith, 1985). In pIII fusions, the foreign polypeptide must be inserted somewhere between the signal peptide sequence and the carboxy-terminal part of the coat protein that is required for incorporation into the virion. Some pIII display systems are designed so that the foreign peptide replaces the entire N-terminal domains of pIII, thus abolishing its capacity to mediate infection. To make such display phage infective, wild type pIII must be delivered from a different source, e.g. a helper phage (Barbas *et al*., 1991).

A helper phage is used together with so called phagemid genomes to generate certain types of phage display particles. The helper phage is a phage derivative, which has mutations in its origin of replication that reduce the packaging efficiency of its otherwise complete phage genome, and a phagemid genome is a vector that carries a plasmid replication origin allowing it to replicate normally in an *E. coli* host, an antibiotic resistance gene that allows selection and a Ff replication origin including the packaging signal, which is inactive until the cell becomes infected with the helper phage (Bass *et al*., 1990; Smith *et al*., 1993). Furthermore, the phagemid vectors contain the gene sequence for the gene III fusion, but lack all other structural and non-structural phage genes required for generating a complete phage. These phagemids can thus be grown as plasmids or packaged as recombinant phage particles with the aid of the helper phage, which will supply all the structural phage gene products for generating a complete phage. Because the helper phage genome is poorly packaged, nearly all the phage particles contain the phagemid genome, preserving the necessary link between the displayed protein and its gene. Another possibility, for the generation of display phage, is to use phage

vectors, in which the foreign sequence is fused to the single wild type gene for pIII, or to the gene III of an extra expression cassette, providing two sources of pIII in the same phage DNA.

The different formats of pIII display achieved by these approaches are outlined in Figure 8. First, the use of helper phage and phagemid genomes to generate display phage particles is depicted. The result of using a combination of wild type gene III from the helper phage genome and fusion gene III from the phagemid is a phage that displays a mixture of displayed molecules and wild type pIII on the phage coat (Figure 8A). The wild type pIII, delivered by the helper phage, help this display phage to maintain infectivity, because the recombinant pIII encoded by a typical phagemid vector usually lacks one and a half N-terminal domain (residues 1-198) (Armstrong *et al*., 1996; Lowman *et al*., 1991). Removal of this domain enhances the accessibility of the displayed polypeptide (Lowman *et al*., 1991) and cells expressing this domain are resistant to superinfection by helper phage (Boeke *et al*., 1982). Furthermore, the hybrid format has the advantage of permitting monovalent display (Lowman *et al*., 1991). The valency of display is of importance principally because of its impact on the ability to discriminate between binding proteins of different affinities. Multivalency confers a high apparent affinity (avidity) on weak-binding clones, while monovalency will result in selection for true affinity. Polyvalency can be achieved by using a helper phage with a deleted gene III, and a phagemid that carries a fusion to an intact, full gene III sequence as shown in Figure 8B. The use of phage vectors to generate polyvalent and monovalent display is shown in Figures 8C and 8D, respectively.

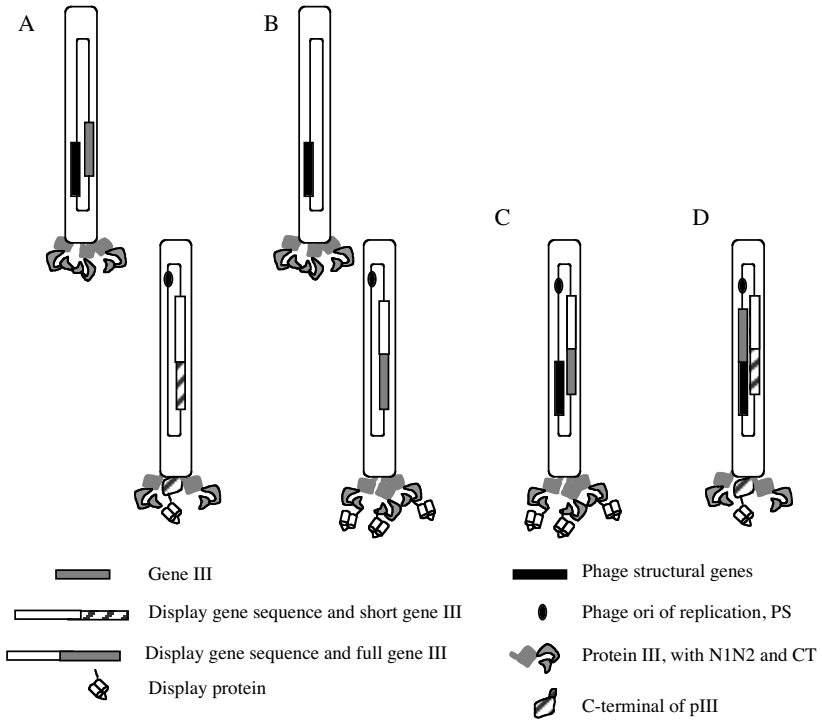

FIGURE 8. Different display formats for pIII-fusions. The helper phage (A and B) are shown in the upper end of the figure, while the display phage are shown in the lower end. A comparison of polyvalent and monovalent display, as achieved by helper phage/phagemid systems (A and B) or phage vectors (C and D), is outlined.

3.2 Libraries for Phage Display

As described in the introductory section of this chapter, phage display technology is often used with a pool of polypeptide variants, called a phage library, as starting material for the selection procedure. Prior to this, it is reasonable to confirm functional display of the polypeptide to be diversified, if it has not previously been used for display (McCafferty and Johnson, 1996; Russel *et al*., 2004). Thereafter, a large variety of techniques can be exploited to introduce diversity into the nucleotide sequence encoding the polypeptide to be displayed. The most common approach involves the powerful and versatile

oligonucleotide-directed mutagenesis, using the polymerase chain reaction (PCR). This allows either complete randomisation of residues, partial (in which the wild-type residue is retained in some proportion) or tailored randomisation, in which only a defined subset of amino acids is specified, for introduction of diversity. Other ways of obtaining diversity in your library is by way of *in vitro* recombination through DNA-shuffling (Stemmer, 1994a, b), which gives rise to new random combinations of gene segments, error prone PCR (Ling and Robinson, 1997; Zhou *et al*., 1991) or using mutator strains of *E. coli* (Schaaper, 1988), which generates random changes throughout a sequence. There are also specialised applications of phage display, in which the diversity of the library arises from a collection of genes rather than a single gene. The leading example of this is antibody V-region libraries (Clackson *et al*., 1991; Hoogenboom *et al*., 1992; McCafferty *et al*., 1990; Söderlind *et al*., 2000; Winter *et al*., 1994). Naturally, combinations of the methods described above can also be used.

Once diversity is obtained, the DNA is inserted into the phagemid for cloning by electroporation into the *E. coli* host. This allows the size of a library to be in the order of 10^9 – 10^{11} members (Söderlind *et al*., 2000; Vaughan *et al*., 1996). The host is then readily infected with a helper phage to obtain phage stocks, which finalises the creation of the phage display library. Once the phage library is obtained, the selection procedure follows.

3.3 Principles of selection and analysis of selected clones

The feasibility of obtaining large phage libraries have led to the development of a number of techniques for selecting the desired molecules from the libraries (Clackson and Wells, 1994; Hoogenboom, 1997). The most common means of doing this, is through an *in vitro* binding incubation, in which the specific phage in the library are bound to a target tethered to a solid support, and then only retained phage are eluted, after washing away excess (unspecific) phage. This process is often referred to as sorting or panning and is based primarily on affinity for selection of binding polypeptides. Similar principles guide the use of antigen coated magnetic beads (Schier *et al*., 1996), immunotubes (Marks *et al*., 1991), immobilisation onto a sensor chip normally used for kinetic analysis (Malmborg *et al*., 1996), microtitre plates

(Kretzschmar and Geiser, 1995) or targets naturally carried on a surface such as a cell membrane (Fransson *et al*., 2004; Kupsch *et al*., 1999; Marks *et al*., 1993). Another common technique for capture of the target molecule is to couple it to a chromatography column to perform affinity based chromatography selection (Clackson *et al*., 1991; McCafferty *et al*., 1990).

In general, a successful selection implies that the initial population of phage-displayed polypeptides have yielded a subpopulation typically enriched for polypeptides with improved or desired properties. This selected subpopulation can be used to re-infect fresh *E. coli* cells for preparation of a new phage stock, in which the specific phage that bound to the target are amplified, to allow further rounds of selection. During this enrichment process, the number of binding clones over non-binding clones, from each round of selection, is monitored by titration of the selected subpopulation on *E. coli* to determine the concentration of infectious particles. If the ratio between input and output phage has decreased, this is a good indication that the selection process has yielded enrichment of binding clones.

Selection parameters can often be manipulated in order to enhance the efficiency of selection, for example, the selection of antibody fragments with low binding affinity is encouraged by fewer washes, multivalent display and a high coating level of antigen, whereas the isolation of those with a high affinity for antigen is favoured by thorough washing, a low level of antigen coating and monovalent display (Barrett *et al*., 1992; Bass *et al*., 1990; Winter *et al*., 1994). The progress of affinity selection, through succeeding rounds, is ordinarily reflected in increasing affinity of individual phage clones (or of entire eluates) for the target molecule. Furthermore, the chances of finding high affinity antibody fragments from a phage library increases with the size of the library (Söderlind *et al*., 2000; Vaughan *et al*., 1996).

Analysis of the selected clones to determine their identity is carried out by simply sequencing them and by carrying out an analysis of their binding properties. The most popular and versatile technique for this initial characterisation, is the phage ELISA (Mattheakis *et al*., 1994; Ohlin *et al*., 1996). In this method, the target of interest is immobilised in wells of a 96-well plate, individual phage supernatants prepared from selected clones are added, and specific binding is detected by use of an anti-phage antibody. The

assay can also be used in a competition format to determine the specificity and relative binding affinity of selected clones.

The next level of analysis often requires the production of soluble protein. Depending on which type of phagemid is being used, this can be accomplished by expression in a non-*supE E.coli* (for phagemids that carry an amber stop codon (TAG) between the displayed sequence and gene III) or by using restriction enzymes to excise to gene III sequence from the phagemid, to allow induced expression of the remaining insert sequence (Lantto *et al*., 2002).

The display of polypeptides on Ff phage has evolved into a versatile and powerful technology, as demonstrated by the diversity of molecules displayed (Griffiths *et al*., 1994; Lucic *et al*., 1998; Nissim *et al*., 1994; Saggio *et al*., 1995; Tanaka *et al*., 1999; Verhaert *et al*., 1999). Furthermore, the display of e.g. antibody fragments have led to the identification of proteins capable of recognising a large diversity of targets, such as haptens, peptides, carbohydrates and proteins (Söderlind *et al*., 2000). In addition, the technology is cheap, requires little or inexpensive equipment, and is easily accessible for individual researchers or laboratories across the world. These factors have contributed to the wide use of the technology.

3.4 Alternative display systems

The alternatives to a traditional version of phage display technology are used essentially as solutions to specific problems or limitations (e.g. the display or the selection procedure) of the conventional use of the technology. As a first example, the problem of how to display cDNA encoded proteins on pIII was solved by using the strong interaction of the Fos-Jun leucine zipper (Crameri and Suter, 1993). The Jun sequence is displayed as an N-terminal fusion directly onto intact pIII and anchored to the phage coat whereas the Fos protein is co-secreted as a fusion protein with cDNA encoded gene products. Fos-Jun interaction and subsequent disulfide formation provides a system for C-terminal display of proteins, which has been used to identify novel genes involved in biological processes directly from cDNA libraries (Crameri *et al*., 1994; Crameri and Blaser, 1996). Alternatively, cDNA libraries may be displayed on pVI, which allows C-terminal cloning (Hufton *et al*., 1999; Jespers *et al*., 1995). A second example is the solution to problems of

displaying some proteins that are normally of intracellular location. For this, bacteriophage other than the Ff family have been used, including the lambda (Hoess, 2002), T4 (Ren *et al*., 1996) and T7 phage (Danner and Belasco, 2001). These differ from Ff phage display in that assembly takes place entirely in the cytoplasm prior to cell lysis, which means that display does not require secretion of the fusion polypeptide over the membrane before it is attached to the phage particle.

The problems of the selection procedures of phage display are often related to establishing the proper conditions in terms of target molecule concentration, incubation times and the number of rounds needed for sufficient enrichment of binding proteins. Therefore, the alternatives seek to speed up the selection procedure, and to increase the chances of finding high affinity binding proteins. One approach involves the use of display on cells rather than phage, because that will allow e.g. fluorescence activated cell-sorting (FACS) to select display cells, which may be a more rapid selection procedure than conventional panning techniques (Boder and Wittrup, 1997; Francisco *et al*., 1993). This approach has been applied on a number of different cell types (Georgiou, 2001; Wernérus *et al*., 2003; Wittrup, 2001) and has, in combination with FACS, yielded selection of high affinity scFv (Boder *et al*., 2000; Daugherty *et al*., 1998). Another approach, associated to the problems of traditional affinity selection methods, is to directly link target recognition of the display phage to infectivity of the cell, which translates into a survival advantage if infection involves the transfer of a resistance marker to the infected cell. This methodology was developed at the Department of Immunotechnology under the name selection and amplification of phage (Duenas and Borrebaeck, 1994) (later also called selective infection) (PAPER I).

4 SELECTIVE INFECTION

Selective infection is a positive selection scheme for the identification of interacting partners, based on the direct link of target recognition to infection of the cell. The principles of this method was first described in 1994 (Duenas and Borrebaeck, 1994; Gramatikoff *et al*., 1994; Krebber *et al*., 1995), and in these reports, in essence, a fusion protein consisting of the N-terminal domain of pIII and a target molecule able to interact directly with a ligand (e.g. an antibody fragment) displayed on the N-terminal extremity of the truncated pIII (CT) of the phage is used. For this, helper phage carrying a deletion in gene III are employed, which generates a display phage that lack infectivity (Figure 9). Infectivity is then restored by the interaction with the fusion protein, which recreates a functional pIII, with F-pilus- and TolA-binding capacity, on the phage. Successful infection also means that the resistance marker carried by the infecting phage is transferred to the cell, resulting in a growth advantage for infected cells on selective media. Furthermore, because the technique involves protein-protein interaction in solution, selective infection can be viewed as a variant of affinity selection that does not rely on capture on a solid support. The technology has been coined both selection and amplification of phage (SAP) (Duenas and Borrebaeck, 1994), selectively infective phage (SIP) (Krebber *et al*., 1997) and direct interaction rescue (DIRE) (Gramatikoff *et al*., 1994), but the term selective infection will be used throughout to describe this technology.

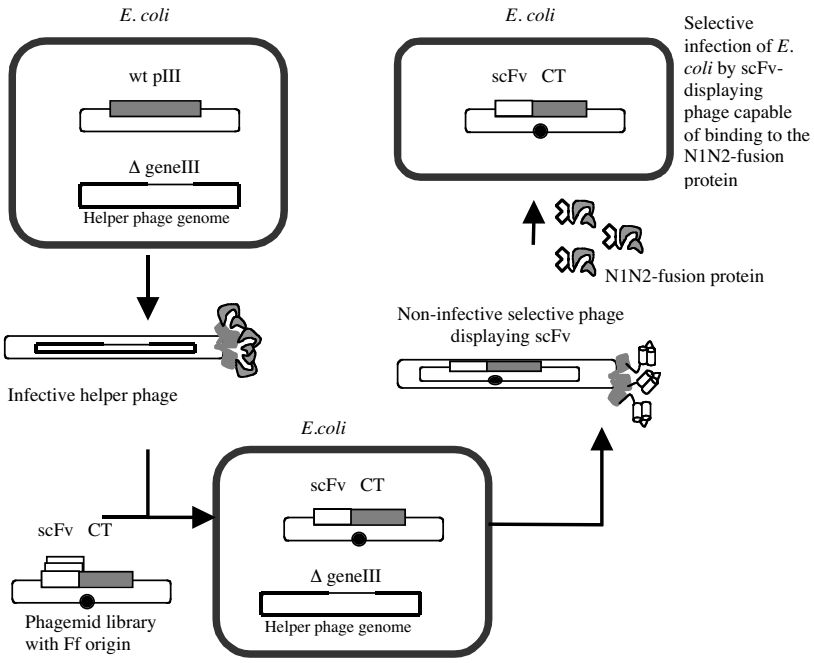

FIGURE 9. The preparation of selectively infective phage and the principle of selective infection. The helper phage genome carries a deletion in gene III, so in order to generate the infective particles needed to introduce the phage DNA into the host for subsequent generation of selectively infective phage, the helper phage is produced in a host that is doubly transformed with the deleted helper phage genome and a plasmid containing the wild type gene III, but no packaging signal. Thus, only the deleted helper phage genome should be packaged into the helper phage particles. The deleted helper phage is then used to infect e.g. an *E. coli* culture transformed with a phagemid library encoding variants of scFv. The phagemid genome carries a phage packaging signal (black dot), why the non-infective phage extruded from such cells should typically contain the phagemid genome and display a truncated pIII-polypeptide fusion, to be used in selective infection experiments. The resulting non-infective phage carries a scFv capable of binding to soluble N1N2-fusion protein, which upon cognate interaction will be able to selectively infect *E. coli*. The rescued scFv can then be identified by DNA sequencing.

4.1 Effect of fusion protein on selective infection

When selective infection of *E. coli* was first described, non-infective phage stocks displaying different antibody fragments were mixed and allowed to interact with a target molecule fused to N1 alone (Duenas and Borrebaeck, 1994). Despite that the F-pilus binding domain of pIII was missing, an enrichment of the cognate antibody-antigen pair was achieved. Later, a mechanistic dissection of the influence of a large protein insertion at various positions between the different domains of pIII, demonstrated that the presence of N2 enhanced the efficiency of selective infection, irrespective of whether N2 was on the phage or together with N1 as a soluble target fusion (Krebber *et al*., 1997). Therefore, the more recent versions of selective infection use the N1N2-fusion to restore infectivity (Figure 9).

4.2 Factors affecting the outcome of selective infection

One factor that determines the outcome of selective infection is the concentration of soluble fusion protein added to the mixture of non-infective display phage and *E. coli* cells during the selection process. It has been reported that high concentrations of fusion protein have an inhibitory effect on infectivity (Duenas and Borrebaeck, 1994; Krebber *et al*., 1997). Since then it has been realised that the N1N2-fusion concentration needs to be adjusted to achieve optimal infection conditions (Krebber *et al*., 1997). The main reason is that, at least for N1N2-fusions, the fusion protein binds to the pilus and depolymerises it at a high fusion protein concentration. Another potentially important factor for successful rescue of interacting pairs is the display format. Several copies of the displayed antibody fragment, linked to the N1N2-fusions, may be required to achieve optimal conditions for selective infection. Furthermore, the binding strength (affinity) of the protein-ligand interaction is suspected to be of importance to the outcome of selective infection experiments. This notion comes from reports demonstrating a selection of high affinity antibody fragments using the selective infection methodology, however, the results were inconclusive and could be shown to depend on e.g. advantages in folding (Duenas *et al*., 1996a; Pedrazzi *et al*., 1997; Spada *et al*., 1997).

In the experiments described in PAPER I, we used two different gene III-deleted Ff phage; the R408d3 (Rakonjac *et al*., 1997) and the VCSM13 mutant helper phage Δg3N (Gao *et al*., 1997), for the creation of selectively infective phage with different display format (Figure 10). The R408d3 helper phage/phagemid system gives rise to infective helper phage carrying pIII on its surface, encapsulating a genome lacking the entire gene III. After infection of an *E. coli* containing phagemid, the selectively infective phage produced display antibody fragments on all copies of the truncated pIII molecule (residues 256-406), encoded by the phagemid genome. The Δg3N helper phage carries a partial deletion of gene III, keeping the first 38 residues of N1 and residues 252-406 of CT. During the production of the Δg3N selectively infective phage, the genome encoded CT molecules compete for incorporation into the phage coat with the phagemid encoded CT-antibody fragment fusion protein, thus giving particles where the majority of the displaying species are monovalent (Lowman et al., 1991) (see section 3.1).

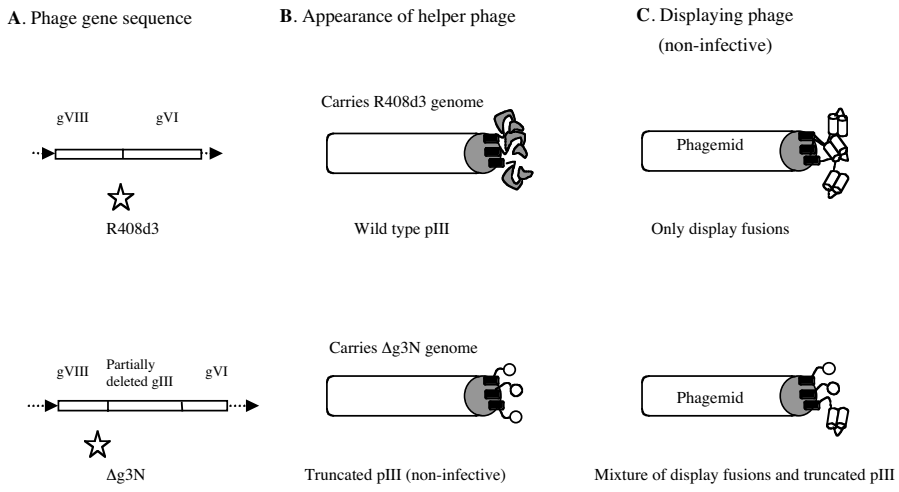

FIGURE 10. Schematic representations of the two different helper phage systems used in PAPER I. The position of the deleted gene sequence within each helper phage genome is indicated by a star (A). The R408d3 helper phage encapsulates the deleted helper phage genome and will display wild type pIII if propagated in a host carrying

pIII-complementing vectors. The Δg3N helper phage will display a truncated pIII, lacking all of N2 and keeping the first 38 residues of N1, resulting in a non-infective particle (B). The display-phage of R408d3 will only display CT-fused proteins, while the Δg3N display-phage will carry a mixture of CT-fusions and truncated pIIIs (C). The picture is adapted from Paper I with written permission from the copyright holders John Wiley & Sons Limited.

The size of the complex obtained after cognate interaction was also varied in this study, since we chose to study three different interacting pairs of molecules, of which one pair was peptide-scFv, the second a non-peptidic ring structure and scFv and the third was a protein-protein interaction. Furthermore, their respective binding affinity varied: Starting with the pair of the weakest affinity, we used a peptide (AD2) derived from glycoprotein B of human cytomegalovirus (CMV) and a scFv (AE11F) binding to this peptide with a $K_D = 6 \times 10^{-7}$ M (Lantto and Ohlin, unpublished; (Ohlin *et al*., 1993)), a fluorescein isothiocyanate (FITC) –specific scFv binding to FITC with a $K_D = 3 \times 10^{-10}$ M (Vaughan *et al*., 1996) and a papain and cystatin C interacting pair having a $K_D = 1 \times 10^{-11}$ M (Pol *et al*., 1995).

TABLE 1. The outcome of R408d3 and (Δg3N) selective infections of *E. coli* as described in PAPER I.[a]

Protein displayed on phage	Target N1N2-fusion	K_D (M)	Concentration of target N1N2-fusion at peak infection efficiency (M)	Relative increase in cfu (times background)[b]
scFv-AE11F	AD2	6×10^{-7}	1×10^{-9} (1×10^{-8})	10 (10)
scFv-FITCE2	FITC	3×10^{-10}	1×10^{-10}	250
Cystatin C	Papain	1×10^{-11}	1×10^{-10}	2100

[a] The numbers within brackets refers to the outcome of selective infection using the Δg3N display phage.

[b] The background refers to the colony forming units (cfu) obtained by mixing display phage and *E. coli*, without fusion protein.

A N1N2-fusion concentration, below the affinity constant of the interacting pair, gave the highest selective ratio after one round of selective infection for each of the different pairs tested in PAPER I (Table 1). This phenomenon was most pronounced for the peptide-scFv interaction of the weakest affinity.

Using higher concentrations of N1N2-fusion protein, the expected inhibition of selective infection was observed (PAPER I; Figure 3). Using the Δg3N display phage, selective infection was also achieved for the AE11F-AD2 binding pair, at a fusion protein concentration below the affinity constant of the interacting pair. However, the concentration required for selective infection was higher for this monovalent display system in comparison to using the same interaction pair and the R408d3 multivalent display. Therefore, monovalent display requires a (undesirably) high concentration of fusion protein, which was also demonstrated by the observation that no selective infection could be achieved with the Δg3N phage at concentrations below 10^{-8} M (PAPER I). These findings are readily explained by effects of avidity on the interaction between displayed protein and target, as the effective concentration of e.g displayed scFv is higher with a multivalent display-phage when the same concentration of mono- or multivalent phage are used. Other reports have demonstrated that multivalent display on phage improve the selection for binding proteins with low affinity (Crameri *et al*., 1994; Terskikh *et al*., 1997), and that an improved efficiency of display and antibody fragment selection can be achieved (O'Connell *et al*., 2002). Thus, in PAPER I we conclude that, for selective infection, a multivalent display format is of importance, because it allows the use of lower concentrations of fusion protein. Furthermore, we found a direct correlation between the affinity constant and infection efficiency of the interacting pairs, when using multivalent display (PAPER I).

The mechanism behind the observation that selective infection seems to select for high affinity binding pairs, is about to become clear. Previous speculations were based on the fact that N1N2-fusions could not be used at higher concentrations, otherwise it would saturate the pilus, thus only very low amounts of N1N2-fusions are needed and the system must select for high affinity pairs (Spada *et al*., 1997). Contrary to this, recent findings suggest that a strong interaction between the N-terminal domains and CT of pIII is of importance, to transfer a signal to the CT necessary for infection (Bennett and Rakonjac, 2004). From their data, it was speculated that the link between the N-terminal domains and residues 253-323 of CT needs to be of a covalent-like character to allow a conformational change in CT, which leads to the dissociation of pIII from the phage coat (J. Rakonjac, personal

communication). Consequently, these findings can be used to explain the results from the selective infection experiments presented in PAPER I, which demonstrated the highest rescue efficiency by the coupling of the interacting pair with the highest affinity.

4.3 THE FUTURE OF SELECTIVE INFECTION

Elaborate solid support protocols used in conventional phage display technology may risk the permanent trapping of high affinity binding polypeptides during the passage of phage clones over e.g. an affinity column. The advantage of using selective infection is the simple selection step that avoids the use of these solid support protocols. However, the simplified selection step of selective infection has been shown to require careful evaluation of N1N2-fusion concentrations and incubation times in order to drive the selection process in the desired direction (Duenas *et al*., 1996b; Krebber *et al*., 1997) (PAPER I). Also, the *in vitro* selective infection approach has demonstrated that there is a risk for obtaining a background of infectious particles (Pedrazzi *et al*., 1997) (PAPER I), which will have detrimental effects on library selections. Thus, there are a number of issues to resolve for the future use of the technology, before it can be a protocol of general applicability. Nevertheless, selective infection has been used to select high affinity binding proteins (Duenas *et al*., 1996a; Duenas *et al*., 1996b; Pedrazzi *et al*., 1997), and to be a powerful technique for the enrichment of proteins with the best folding and binding characteristics from a library of similar molecules (Hennecke *et al*., 1998; Spada *et al*., 1998). In addition, we have shown that the technique demonstrates a potential to select for high affinity binding proteins, when multivalent display is used (PAPER I). Thus, there can be no doubt the technology is suitable for special applications like the selection of high affinity interaction pairs, but the methodological difficulties described above and the lack of understanding for the underlying mechanism of filamentous phage infection has limited its use.

5 MOLECULAR DISSECTION OF THE Ff PHAGE INFECTION

The infection process of Ff phage depends on (exploits/parasitises) a number of bacterial proteins for the phage DNA to pass the *E. coli* membranes. First, the specificity of the Ff phage is determined by the reaction, in which N2 of pIII attaches to the tip of the F-pilus extending from the bacterial membrane (Jacobson, 1972; Marvin and Hohn, 1969). This step of the infection process also brings the phage closer to the bacterial membrane, through the rapid depolymerisation of the F-pilus. In the membrane, the products of the *tolQRA* genes are required for Ff phage infection (Sun and Webster, 1986), in addition to being necessary for membrane integrity. Also, sensitivity towards bacterial toxins specific for *E. coli* (colicins) is mediated through these proteins (Webster, 1991), which are preferentially associated with contact regions between the inner and outer membranes, so called adhesion zones (Guihard *et al.*, 1994). Thus, the entry point of the Ff phage seems to be at a site, where the distance the phage DNA would have to travel to cross both membranes, is the shortest. Once the phage has reached the bacterial outer membrane, the N1 domain binds to the TolA receptor molecule (Click and Webster, 1997; Riechmann and Holliger, 1997). In order for N1 to bind TolA efficiently, the initial interaction with the F-pilus must have taken place, since the binding site of TolA and N2 for N1 overlap (Riechmann and Holliger, 1997). The stoichiometry of the TolQRA complex required for phage DNA entry is unknown, but TolR dimerisation has been reported (Journet *et al.*, 1999), and the TolQ and R proteins have been shown to act as motor proteins, energising the TolA molecule to form a transmembrane link via binding to an outer membrane protein (Cascales *et al.*, 2001). Following pIII and TolA interaction, pIII crosses the outer membrane and inserts its CT in the inner membrane (Boeke and Model, 1982; Endemann and Model, 1995). The interactions between the pIII domains and TolA important for this process were the focus of the study in PAPER II.

5.1 pIII AND TOLA DURING INFECTION

Not only pIII but also the 421 amino acid TolA has a sophisticated domain organisation (Levengood *et al.*, 1991). The first domain of TolA (TolAI) is the

N-terminal membrane anchor domain, which spans the inner membrane once. The second domain (TolAII), consists of a segment of repetitive sequences, creating an extended helix, which is estimated to be of a length that would allow it to span the periplasm (Levengood *et al*., 1991). The C-terminal third domain of TolA (TolAIII) consists of a number of short helices and ß-strands, forming a globular fold (Lubkowski *et al*., 1999), which can bind to N1. Two short stretches of glycine residues are preceding TolA domains II and III, which might confer limited flexibility to the protein (Levengood *et al*., 1991). Each of these TolA domains and the N1, N2 and N1N2 domains of pIII were prepared as purified polypeptides as described in PAPER II. The objective was to measure the binding kinetics of the interactions between these polypeptides in real time using the surface plasmon resonance (SPR) technology (Liedberg *et al*., 1995). The principle of the analysis is that one of the interacting counterparts is immobilised on a sensor chip surface , while the other passes in a continous flow in the BIAcore™ (Jönsson *et al*., 1991).

In PAPER II, Table 1, the affinities for the interactions between N-terminal domains of pIII and bacterial TolA are presented. The affinity constant of the known interaction between N1 and TolAIII was determined to be in the micro-molar range ($K_A = 1 \times 10^6$ M^{-1}). This is the first measurement of the kinetics of the interaction between TolAIII and N1 reported, although soluble TolAIII has been shown to inhibit the interaction between N1-phage and TolAIII *in vitro*, at concentrations down to 0.7 micro-molar (Riechmann and Holliger, 1997). A previously unknown interaction between N2 and TolA was also detected. Our results showed that this interaction was dependent on TolAII, since no other TolA domain was able to bind immobilised N2. In Table 2 of PAPER II, the affinities of interactions between the different domains of TolA are presented. Intact TolA was found to bind to itself, as did each of the separate domains. Furthermore, TolAI and III, as well as TolAII and III had affinity for each other, indicating the possibility of TolA-TolA functional interaction. Thus, using real-time bio-specific interaction analysis (BIAcore™), we demonstrated that both N-terminal domains of pIII contribute to the overall affinity of these domains for TolA. Also, the extensive interactions between different domains of TolA suggested that the TolA protein have the capability of interacting with itself in the bacterial membrane. In an attempt to weld the results from the

BIAcore™ analysis with previously reported interactions between pIII and the TolQRA proteins, a refined infection mechanism model was hypothesised.

5.2 A NOVEL MODEL FOR THE Ff PHAGE INFECTION MECHANISM

In previous models of the Ff phage infection mechanism (Click and Webster, 1998; Lazzaroni *et al*., 1999; Riechmann and Holliger, 1997; Webster, 1996), the knowledge that the TolQRA proteins interact with each other is taken into account (Derouiche *et al*., 1995; Germon *et al*., 1998; Journet *et al*., 1999; Lazzaroni *et al*., 1995). In contrast, the knowledge of TolQ, TolR and TolA localisation to adhesion zones was not considered in these models. Also, most of these previous models propose that this protein complex (and potentially also pIII) is involved in the formation of a channel for phage DNA to cross the cytoplasmic membrane (Click and Webster, 1998; Lazzaroni *et al*., 1999; Riechmann and Holliger, 1997).

The new model, presented in Figure 11, tries to combine the knowledge from the above mentioned previous studies with the findings presented in PAPER II. It should be mentioned however, that the data from PAPER II was generated by measuring polypeptide interaction *in vitro*, which may not directly translate into an *in vivo* situation. Nevertheless, in the new model for the early events in Ff phage infection of *E. coli*, we depict the F-pilus retraction process at the adhesion zones of outer and inner membranes. This localisation would allow a more compact state of assembly for the TolA protein, thus keeping the two membranes closer together, in addition to allowing N2 to make contact with the TolAII at the adhesion zone. The model also suggests that the N2-TolAII interaction help orient pIII correctly into the inner membrane, and that the cooperation of more than one TolA molecule help achieve the insertion of pIII into the inner membrane. Furthermore, TolA makes several interactions with the translocation domains of pIII during infection, similar to what has been seen for colicins during their TolA mediated insertion into the cytoplasmic membrane (Journet *et al*., 2001).

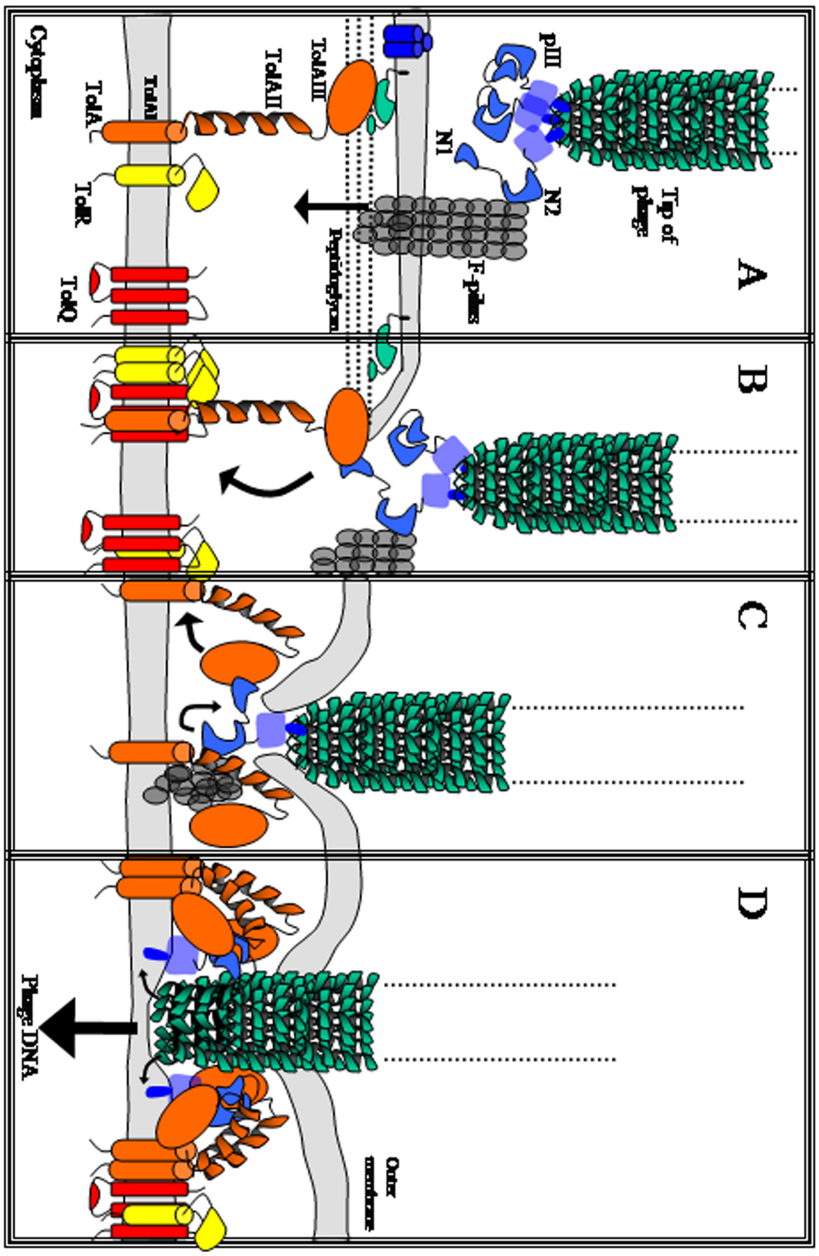

FIGURE 11. Model for the early events in the Ff phage infection of *E. coli*. First, the N2 domain of pIII interacts with the F-pilus, outside the bacterial membranes. The

outer membrane proteins OmpF (blue cylinders) and Pal lipoprotein (green, anchored to the peptidoglycan and outer membrane) are also included in the figure, as there have been reports of TolA interacting with these proteins prior to infection (Cascales *et al*., 2000; Lazdunski *et al*., 1998) (A). After F-pilus retraction, a process which might bring the two membranes closer together, the N1 domain binds to the C-terminal domain of TolA, TolAIII, (B). Both N-terminal domains of pIII are now in proximity to TolA domains. TolA can assume a more compact state of assembly, as a consequence of the two membranes being closer together, at which stage the N2 and TolAII domains have the possibility to interact (C). Finally, the pIII is inserted into the inner membrane, via cooperating TolA molecules, leading to opening of the phage coat and phage DNA entry into the bacterial cytoplasm (D). This process also involves the insertion of pVIII into the inner membrane by an unknown mechanism. The picture is adapted from Paper III, with written permission from the copyright holders.

Interestingly, TolA may drive newly synthesised membrane components across the periplasm (Derouiche *et al*., 1996; Lazzaroni *et al*., 1999; Levengood-Freyermuth *et al*., 1993; Rigal *et al*., 1997) through the C-terminal interaction with Pal, the peptidoglycan associated lipoprotein (Cascales *et al*., 2000). In this context the long, amphipathic, coiled-coil central domain, TolAII, has been proposed to interact with hydrophobic compounds and drive them through the peptidoglycan network (Cascales *et al*., 2000; Derouiche *et al*., 1999; Levengood *et al*., 1991). Therefore, the TolA protein seems to be involved in the transport of outer membrane components through the periplasm. Furthermore, this capacity to mediate transport of components from the cytoplasmic membrane to the outer membrane can be run in the reverse direction as demonstrated by the TolA-driven insertion of Ff phage pIII and group A colicins in the cytoplasmic membrane (Lazzaroni *et al*., 2002; Webster, 1991). Also, the recent findings that TolQ and TolR can function as a motor, energising TolA to drive macromolecules through the cell envelope (Cascales *et al*., 2000; Cascales *et al*., 2001; Gaspar *et al*., 2000; Germon *et al*., 2001), can be interpreted in favour of a mechanistic model in which TolA function as a bidirectional transport protein. TolA thus facilitates the entry of colicins and Ff phage, as well as the outwards translocation of membrane components from the inner membrane.

Phage assembly involves the formation of a channel (Section 2.4), which newly synthesised phage pass through. It is not known whether phage infection also requires the formation of a channel, but it has been proposed, both because of the channel formation during phage assembly and because translocating colicins form channels (Lazzaroni *et al*., 2002; Riechmann and Holliger, 1997; Webster, 1991). In parallel, the infection process of filamentous phage can also be discussed in a comparison to F-pilus assembly/retraction mechanisms. The morphological similarities of phage filaments and conjugative pili, when phage filaments are assembled without pIII, have led to the proposal that the two are likely to be evolutionarily related (Rakonjac and Model, 1998). Furthermore, in a recent study (Bidlack and Silverman, 2004), where it was found that F-pilus positive cells are more sensitive towards bile salts, the authors suggested that the sensitivity occurs when F-pilin subunit secretion transiently opens the cell envelope to the surrounding medium, allowing entry of the anionic detergents. Such transient opening may also occur during phage entry, when the pilus is retracted. This would lend further support to a "channel theory". However, the results presented by Bidlack and Silverman suggest that the pilus retraction process and the pilin subunits are more important in the formation of such a channel, than the Tol proteins and pIII, which were the previous prime suspects of channel forming proteins (Click and Webster, 1998; Lazzaroni *et al*., 1999; Riechmann and Holliger, 1997).

During phage assembly and infection, a stage can be envisaged in which the phage is attached to the cell surface, with the phage head (pIII/pVI) buried in the membrane, thus resembling cell surface-attached F-pili. However, the movement of the phage proceeds in different directions (away from the cell surface *versus* towards the cell surface) depending on whether the phage is being assembled or is infecting the cell. The driving forces of these opposing reactions can probably be found in the formation of the different membrane complexes responsible for the separate reactions (the pIV-channel for assembly and the TolQRA and pilus proteins for infection), but also in the differences in membrane concentration of phage proteins (mainly pVIII). This behaviour of the infecting and assembling Ff phage can be seen as an anology to the process of F-pilus assembly/retraction. In F-pilus assembly, a membrane

complex of assembly proteins (encoded by the F plasmid DNA), large enough to span the bacterial envelope, has been proposed to be involved in the pilus elongation process (Frost *et al*., 1994). This elongation process is achieved by the incorporation of F-pilin subunits into the growing filament. The F-pilin subunit share a similar size and fold (α-helical) with the phage pVIII molecule (Armstrong *et al*., 1980; Frost *et al*., 1994; Paranchych *et al*., 1978), and the assembly of F-pilin subunits into pilus and pVIII subunits into phage particles appear from an inner membrane pool (Laine *et al*., 1985; Moore *et al*., 1981a, b; Rapoza and Webster, 1995; Russel, 1991). These similariteies of F-pilus and phage assembly mechanisms can be translated into mechanistic ideas of relevance for the infection process of Ff phage. Perhaps the F-pilus retraction system is used for the insertion of pVIII into the inner membrane, stripping off the phage coat and releasing the naked ssDNA into the host. In such a mechanism the function of TolA would be required for the uncapping of the phage coat, by multiple interaractions with the pIII (PAPER II), thus enabling pVIII to be accessible for membrane insertion. Therefore, future studies of the proposed F-pilus assembly/retraction complex may provide more leads that will help determine the last steps in the mechanism by which Ff phage penetrate the bacterial membranes.

5.3 TOLA FUNCTION AND IMPORTANCE

In addition to its occasional role as the co-receptor for the Ff phage pIII during phage infection, the normal function of TolA is to maintain membrane integrity and probably to aid the transport of molecules through the cell envelope (Lazzaroni *et al*., 1999). To carry out these multiple functions, TolA depends on interactions with other proteins. It has been shown that mutations in the *tol-pal* gene cluster, encoding TolQ, TolR, TolA, TolB (a periplasmic protein) and Pal (a peptidoglycan associated lipoprotein), result in a pleiotropic phenotype including hypersensitivity towards detergents and bile salts, release of periplasmic content into the external milieu (Lazzaroni *et al*., 1989; Lazzaroni *et al*., 1999; Webster, 1991) and the formation of outer membrane vesicles at the cell surface (Bernadac *et al*., 1998). The TolQ, R and A and TolB and Pal form two complexes (TolQRA and TolBPal) that are connected by the interaction between Pal and TolA, which requires the proton

motive force, TolQ and R proteins (Cascales *et al*., 2000). Furthermore, the periplasmic TolAIII domain interacts with the N-terminal domain of TolB (Dubuisson *et al*., 2002; Walburger *et al*., 2002). The exact role of the Tol-Pal proteins (the Tol system) in maintaining outer membrane integrity and transport functions are still largely unknown. Nevertheless, these proteins are believed to play an important role because *tol-pal* gene clusters have been found in many other Gram-negative bacteria (Sturgis, 2001). In a wider perspective, the function of TolA, and the Tol system, is of relevance to public health, since the Tol proteins have been found to be involved in the virulence of *Salmonella typhimurium* (Bowe *et al*., 1998) and in the phage-mediated import of the cholera toxin gene into *Vibrio cholerae* (Heilpern and Waldor, 2000).

To learn more about which epitopes on the TolA protein that are functionally important for phage infection and possibly also about the different functions of TolA, we decided to introduce mutations in a part of TolA known to be involved in contacts with N1 of phage pIII during phage infection (Lubkowski *et al*., 1999). We created four limited substitution libraries of TolA, targeting six amino acid residues in the C-terminal domain of TolA (TolAIII) (Table 2). The residues at these positions (415-420) build up a β-strand of TolAIII, located in a concavity of the TolAIII molecule that accommodates N1 during phage infection (Figure 12). The substitutions introduced in all four libraries were mainly restricted to related residues, in order not to destabilise the structure and to maintain similar but modified interactions, introducing the possibility to affect the binding of TolA to phage or outer membrane proteins. TolA-negative *E. coli* were then transformed with library vectors, and the functionality of these different libraries was tested by growth on media containing detergent and sensitivity to M13 phage infection.

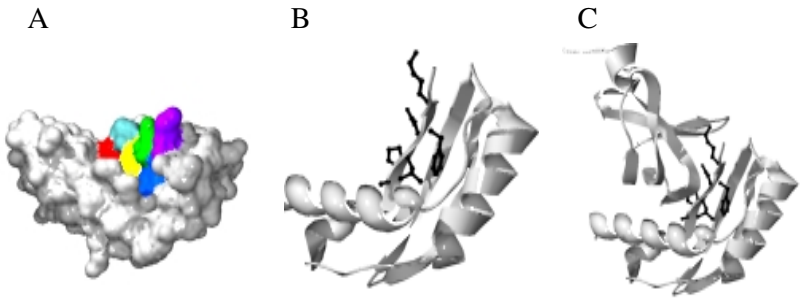

FIGURE 12. Three different views of the TolAIII three-dimensional structure, with the residues targeted by the study presented in PAPER III highlighted. In A) the TolAIII surface is shown, and residues 415-420 are highlighted by separate colourings from left to right; Alanine 415 (red), Proline 416 (turquoise), Leucine 417 (yellow), Aspartic acid 418 (light green), Phenyl alanine 419 (blue) and Lysine 420 (purple). In B) a ribbon model is shown, with the side chains of residues 415-420 exposed. (The TolAIII molecule is slightly trimmed.) In C) the binding of the Ff phage pIII N1 domain to TolA during phage infection is shown. The orientation of TolA is the same as in B).

TABLE 2. The allowed sequence variation in the four different TolA libraries created.[a]

Position	Wt amino acid	Allowed residues in TolA-lib	Allowed residues in lib-416	Allowed residues in lib-417	Allowed residues in lib-418
415	**A**	D, V, G, E	D, V, G, E	D, V, G, E	D, V, G, E
416	**P**	R, Q, L, H	**P**	R, Q, L, H	R, Q, L, H
417	**L**	R, Q, P, H	R, Q, P, H	**L**	R, Q, P, H
418	**D**	A, V, G, E	A, V, G, E	A, V, G, E	**D**
419	**F**	S, L	S, L	S, L	S, L
420	**K**	T, N, I, M, R, S, **K**	T, N, I, M, R, S, **K**	T, N, I, M, R, S, **K**	T, N, I, M, R, S, **K**

[a] The wild type TolA amino acids are shown in bold type.

5.4 SEPARATION OF TOLA FUNCTIONS

The results from PAPER III showed that few TolA mutants were tolerated when the TolA membrane integrity function was challenged (PAPER III; Table 2a).

In contrast, considerable sequence alteration was tolerated by different TolA-variants still capable of mediating phage infection (PAPER III; Table 2a), leading to the identification of clones that displayed a segregation of membrane integrity and phage receptor function (PAPER III; Table 3). We also performed a sequence alignment of TolA from closely related species, and identified a conserved sequence, involving the amino acids at positions 415, 417 and 419, that are crucial for TolA membrane integrity functionality (PAPER III; Figure 3). Furthermore, the dependence on L417 over the other targeted residues was demonstrated by the finding that TolA molecules lacking this residue were unable to sustain membrane integrity (PAPER III; Figure 2 and Table 2a).

Our results showed that the phage receptor function of TolA was more tolerant to the sequence variation introduced by the chosen mutations. Because the stability of the different TolA mutants is the same irrespective of which function was challenged, this behaviour may be attributed to either differences in the mechanisms of the two functions or to the influence of individual amino acid residues for the binding to phage or membrane proteins. The influence of individual amino acids for the preservation of phage receptor function in TolA, can be discussed from the knowledge of phage pIII N1 contact residues, and compared to contact regions for outer membrane proteins on TolA. In the first case, we know from the crystal structure of N1 and TolAIII in complex, that the introduced mutations targeted the TolA residues normally in close contact with the N1 molecule during phage infection (Lubkowski *et al*., 1999). The isolation of functional phage receptors, which were mutated at these positions, implies that either additional contacts (presumably in the α-helix formed by TolA residues 335-349) contribute enough to allow N1 binding, or that proper formation of a beta-strand overrides the effect of individual N1 contact residues in the 415-420 region of TolAIII. In comparison, the 415-420 region of TolA has not been reported to be involved in any contacts with e.g. Pal or TolB, suggesting that the introduced mutations have not affected the TolA binding to outer membrane proteins. Therefore, the most plausible explanation for the observed segregation of TolA functions is the differences in the mechanisms of phage receptor function and membrane integrity function. The phage infection process requires TolA interaction with the TolQ

and R proteins (Click and Webster, 1998), and possibly TolA-TolA interactions (PAPER II). In addition, there are only a few F-pili per bacterium (Brinton, 1965; Caro and Schnös, 1966), corresponding to the two or three adsorption sites found for Ff phage (Hsu, 1968; Tzagoloff and Pratt, 1964). Consequently, only a few functional TolA molecules will be needed to carry out the phage receptor function. Contrary to this, the TolA membrane integrity function depends on a number of additional TolA interactions (with e.g. Pal and TolB) (Cascales *et al*., 2000; Dubuisson *et al*., 2002), in which the TolA molecules need to be evenly distributed along the entire *E. coli* cytoplasmic membrane. Thus, if the introduced mutations affected the stability of the TolA molecule, leading to fewer functional TolA in the cytoplasmic membrane, it is only natural that the membrane integrity function would be most hampered. Therefore, the identification of mutants with segregated TolA functions is evidence of the mechanistic differences between the TolA membrane integrity and phage receptor functions. The technological and biological implications of the findings presented in PAPER III will be summarised in chapter 7, Concluding remarks.

6 ALTERED TRANSCRIPTION OF PHAGE INFECTED CELLS

Bacterial cells protect themselves through their cell membrane, a formidable physical barrier, but what are the cellular responses when this barrier has been penetrated by e. g. infecting Ff phage? The bacteria normally live in an environment where it is necessary to adapt rapidly to changes in e.g. temperature, pH and nutrient access (Gross, 1996; Hengge-Aronis, 1999; Slonczewski and Foster, 1996). The responses to such changes are called stress responses, and are often aimed at adapting the metabolism to the environmental changes that may be the cause of the stress. During Ff phage infection, a number of bacterial proteins are engaged and, as seen in the previous sections of this thesis (2.2 and 2.3), not only the membrane proteins involved in the infection process, but also host RNA and DNA polymerases are used by the phage. For example, the processing of phage mRNA requires the activity of the host endonuclease RNase E (Goodrich and Steege, 1999; Kokoska and Steege, 1998). In addition to these proteins, Ff infection also results in a stress response; the well known sensitisation of infected cells to bile salts among other agents (Boeke *et al*., 1982; Roy and Mitra, 1970). Furthermore, a deregulated phage gene expression (especially overexpression of certain genes in the absence of assembly) may cause cell death (Model and Russel, 1988; Pratt *et al*., 1966; Schwartz and Zinder, 1968). Stressful situations like these are often triggers for stress responses; however, they are difficult to measure because the conditions cause severe effects on cell survival. Nevertheless, the insertion of phage protein pIV into the cell membrane during phage assembly has been shown to give rise to the expression of an *E. coli* phage shock protein; PspA (Brissette *et al*., 1990; Brissette *et al*., 1991). This expression represents the only previously known stress response of *E. coli* related to the expression of a single phage encoded gene. However, new technologies, such as oligo-nucleotide microarrays (Selinger *et al*., 2000), have made it possible to explore the entire transcriptional profile of infected bacteria to look for host proteins affected by the presence of filamentous phage. This was the aim of the study in PAPER IV.

6.1 GLOBAL TRANSCRIPTIONAL ANALYSIS OF PHAGE INFECTED *E. COLI*

In the study presented in PAPER IV, commercially available oligo-nucleotide microarrays (genechips), containing sequences complementary to all known open reading frames of *E. coli* as well as those of intergenic regions, control phage M13 and plasmid genes, were used. We chose to compare the transcriptional profiles of bacteria infected with M13 bacteriophage for 2 or 20 minutes to uninfected bacteria. The time points were carefully chosen to detect transcriptional changes that arise early after infection. Phage transcripts are normally detected after 2 minutes (Blumer and Steege, 1984; Steege, 2000) and the major phage mRNAs are generated sequentially over a 10 minute period after infection (Blumer and Steege, 1984; Smits *et al*., 1980). The first release of phage progeny appears 15-20 minutes after infection (Blumer and Steege, 1984; Hofschneider and Preuss, 1963). Thus, at this early phase post infection one can expect the level of adaptation within the infected cells to be the greatest.

In PAPER IV, Table 1, the up-regulation of phage transcripts, known to be present *in vivo* after 2 minutes (Blumer and Steege, 1984; Goodrich and Steege, 1999), is reported. After 20 minutes, the remaining phage mRNAs were detected (except gene VI mRNA), with many of the phage transcripts being abundant, as can be seen from the level of expression (PAPER IV; Table 1, Signal Intensity). Thus, the microarray technology was able to accurately detect transcriptional changes occurring in a fraction of the sampled cells. However, it was not until after 20 minutes of infection that we detected any significant regulation of the host genes.

6.2 REGULATION OF HOST GENES BY FF PHAGE INFECTION

Several host genes were found to be regulated by 20 minutes of phage infection (PAPER IV, Table 1 and 2). Of the up-regulated genes (PAPER IV; Table 1), two (*rraA* and *rof*) are involved in transcription processing (Lee *et al*., 2003; Pichoff *et al*., 1998). The products of these genes bind to two different proteins, the host endonuclease RNase E and the transcription termination factor Rho, respectively, that are both involved in the processing of phage transcripts. Thus, RraA and Rof may represent two novel proteins of functional importance for phage transcription, although this remains to be

tested. Phage infection also up-regulated two genes (*nagE* and *dhaK*) involved in carbohydrate-dependent phosphotransfer of the high-energy carrier molecule phosphoenolpyruvate (PEP), during the initial steps of cellular energy generation (Gutknecht *et al*., 2001; Plumbridge, 2001). This might suggest that the infected cells had an increased demand for energy compared to uninfected cells. The *pspA* gene was not found to be up-regulated in this study (PAPER IV), which would be expected from previous studies (Brissette *et al*., 1990; Brissette *et al*., 1991). The reason for this disagreement is not known, but could be related to different growth phases of the cells. Furthermore, two genes (*kefA* and *b2431*) were up-regulated, whose products do not have any previously known link to phage infection. MscK (encoded by *kefA*) is a mechanosensitive channel protein involved in cation efflux and the product of *b2431* is of unknown function.

Naturally, a number of host genes were down-regulated after 20 minutes of phage infection (PAPER IV; Table 2). All six of the down-regulated genes belong to the same regulatory network of genes normally expressed to combat conditions of low pH; the acid resistance (AR) genes (Gajiwala and Burley, 2000; Hersh *et al*., 1996; Pratt and Silhavy, 1998; Tucker *et al*., 2002). It is not clear how these results should be interpreted at present, but we decided to confirm the down-regulation of these AR genes on protein level. Therefore it was tested whether phage-infected cells were less tolerant to conditions of low pH, by way of AR assays. These assays activate each of the three existing AR systems separately (Castanie-Cornet *et al*., 1999). We found that two of the three AR systems tested were severely affected by phage infection (PAPER IV; Fig 1), and that the six down-regulated genes are part of these two AR systems. Thus, it seems like phage infection makes infected cells more sensitive to harmful conditions, in line with previous reports (Roy and Mitra, 1970), but to determine which stage in the phage life cycle is causing these effects, further studies are required.

Taken together, the use of microarray technology identified a total of twelve *E. coli* host genes potentially regulated by Ff phage infection, and the expression of the major phage mRNA species could be correlated to the *in vivo* pattern of expression. In addition, functional assays demonstrated the down-regulation on protein level of the genes identified by the microarray

analysis. However, it is currently difficult to evaluate the impact of these findings on display technology, until the functional relationships have been thoroughly investigated.

7 CONCLUDING REMARKS

In this thesis, I have provided the background information and rationales for the studies presented in PAPER I-IV. In addition, I have aimed to place the results of the investigations into the context of filamentous phage biology and (phage) display technology.

For the results presented in PAPER I, the possible evolution of the technology can be discussed. Selective infection have the capability of being a highly competitive selection tool to identify interacting pairs of high affinity (PAPER I). However, in its current design, it is not permitting cloning and analyses of cDNA, which is desirable for the cloning of full-length genes, to identify genes of unknown function. In such a case, there are variants of selective infection already existing that are compatible with cDNA display (on the N1N2-fusion), called *in vivo* selective infection (Krebber *et al*., 1995) and DIRE (Gramatikoff *et al*., 1994), for optional use. Alternatively, the feasibility to display peptide epitopes fused to the pilin subunits of the F-pilus (Malmborg *et al*., 1997; Rondot *et al*., 1998) suggests that a combination of traditional polypeptide display on phage and cDNA encoded display on the bacteria can be achieved. Such an approach would have the benefit of preserving the link between phenotype and genotype after productive infection of the cell (Malmborg *et al*., 1997). Similarly, one can envisage another approach that resembles the yeast two-hybrid system (Chien *et al*., 1991; Fields and Song, 1989), by display on the other receptor molecule of phage infection, TolA, and potentially in combination with non-infectious display phage, in which N1 is replaced by the displayed polypeptide. However, as demonstrated by the results in PAPER III, the TolA protein seems very sensitive to alterations in its sequence. Furthermore, conventional cell-display on *E. coli* has so far mainly involved display on the flexible loops of outer membrane porins (Francisco and Georgiou, 1994; Samuelson *et al*., 2002). Therefore, care must be taken to avoid compromising the membrane integrity function of TolA in such a system.

Furthermore, the findings from PAPER II and III in combination with recently published findings and reports concerning the roles of pilus assembly (Bidlack and Silverman, 2004; Frost *et al*., 1994) and the domain linking of

phage pIII (Bennett and Rakonjac, 2004), have generated a discussion concerning the mechanism of Ff phage infection. In PAPER II we discovered interactions that may be of mechanistic importance, and in PAPER III we observed that certain residues within the TolA C-terminal region have a high functional impact, leading to the identification of TolA molecules lacking membrane integrity function but preserving the phage receptor function. These latter results arise due to the different mechanisms involved in TolA-mediated phage infection and preservation of membrane integrity, as discussed in section 5.4

The work presented in PAPER IV may have identified additional *E. coli* proteins affected by phage infection. More specifically, the identification of two host candidates for phage transcription processing (RraA and Rof) could provide novel information about the regulation of phage gene expression. Additionally, the down-regulation of a number of general stress response genes are of interest to determine the cause for the decreased growth rate and increased sensitivity of phage infected cells. To follow up the results from PAPER IV, verification of up- or down-regulation by an independent method e.g. northern blotting or real-time PCR and subsequent analyses of the functional relationships by the study of *rraA* and *rof* mutants could be performed.

In a more general view, one of the interesting aspects of phage biology is that phage are known to carry key virulence factors (or beneficial factors), which result in the emergence of new bacterial pathogens (or fitness) as the phage infect new hosts (Brüssow *et al*., 2004). Examples of this are the transfer of photosynthesis genes to marine living cyanobacteria (Lindell *et al*., 2004), accounting for the production of oxygen from the ocean, and the transfer of toxin genes for cholera (Waldor and Mekalanos, 1996), shiga (Plunkett *et al*., 1999) and diphtheria (Matsuda and Barksdale, 1967), causing health problems and even death world wide. The transfer of the cholera toxin gene from the CTXϕ phage (a filamentous phage) to *Vibrio cholerae*, requires the protein products of the *tolQRA* genes (Heilpern and Waldor, 2000). Therefore, studies of the function of the TolA protein and its molecular interactions during the process of phage infection may prove helpful in the understanding of how bacterial cell populations acquire pathogenicity.

8 POPULÄRVETENSKAPLIG SAMMANFATTNING

Det finns ett stort behov av molekyler eller reagenser för korrekt och snabb diagnos och/eller behandling av sjukdom. På laboratorier används särskilda verktyg för att ta fram sådana reagenser. Verktygen utgörs ibland av levande organismer, som t ex bakterier och virus, och kallas bioteknologiska verktyg. Ett av dessa verktyg baseras på användandet av en speciell typ av trådlikt bakterievirus (fag). Om vi kan lära oss mer om samspelet mellan bakterie och fag, skulle detta verktyg kunna utvecklas så att processen att finna de eftersökta molekylerna för diagnos och terapi effektiviseras. I den här avhandlingen, som bygger på fyra vetenskapliga originalartiklar, har jag beskrivit den trådlika fagens biologi samt undersökt möjligheterna att utveckla den teknologi som heter "fag-display". Detta har jag gjort dels genom att utvärdera en variant av fag-display-teknologin som benämns "selektiv infektion" och dels genom att studera fagens mekanism för infektion av bakterien.

Mina resultat visar att verktyget "selektiv infektion" har egenskaper som gör det lämpligt för att hitta proteinlika molekyler som binder starkt till målmolekyler (exempelvis en markör för en sjukdom), något man med traditionell fag-display-teknologi inte alltid klarar av att göra. De övriga studierna har gällt fagens infektionsförlopp, som jag har studerat m h a tre olika metoder. I en studie dissekerade jag de två proteiner som är inblandade i infektionsförloppet. Det ena proteinet (pIII) härrör från fagen och det andra (TolA) är ett bakterieprotein. Resultaten visade på flertalet interaktioner mellan olika delar av de två proteinerna, varav några varit hittills okända. I en annan studie introducerades mutationer i bakterieproteinet TolA, varefter den kvarvarande funktionen i mutanterna mättes. De flesta mutanterna som isolerades hade behållit funktionen som fag-receptor men förlorat sin huvudsakliga funktion; att hålla samman cellmembranet. Därmed visades att TolA:s olika funktioner grundar sig i helt olika mekanismer. Slutligen studerades hur bakteriecellens genreglering förändrades i samband med fag-infektion. För detta användes mikromatriser som kan mäta genuttryck i hela organismer. Mikromatrisanalysen gav indikationer på att fagen påverkat uttrycket av vissa bakteriella gener som dittills inte satts i samband med fag-infektion. Generellt kan studier om fagens infektionsmekanism bidra till att öka förståelsen av de processer där bakterier fångar upp nya genetiska element och omvandlas till sjukdomsframkallande bakterier.

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