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# Filamentous Bacteriophage: Biology, Phage Display and Nanotechnology Applications

**Jasna Rakonjac[1]*, Nicholas J. Bennett[1§], Julian Spagnuolo[1], Dragana Gagic[1†] and Marjorie Russel[2]**

[1]Institute of Molecular BioSciences, Massey University, Palmerston North, New Zealand
[2]The Rockefeller University, New York, NY, USA
[†]Current address: AgResearch Grasslands, Palmerston North, New Zealand
[§]Current address: Department of Microbiology and Immunology, McGill University, Montreal, Quebec, Canada

**Abstract**
Filamentous bacteriophage, long and thin filaments that are secreted from the host cells without killing them, have been an antithesis to the standard view of head-and-tail bacterial killing machines. Episomally replicating filamentous phage Ff of *Escherichia coli* provide the majority of information about the principles and mechanisms of filamentous phage infection, episomal replication and assembly. Chromosomally-integrated "temperate" filamentous phage have complex replication and integration, which are currently under active investigation. The latter are directly or indirectly implicated in diseases caused by bacterial pathogens *Vibrio cholerae*, *Pseudomonas aeruginosa* and *Neisseria meningitidis*. In the first half of the review, both the Ff and temperate phage are described and compared. A large section of the review is devoted to an overview of phage display technology and its applications in nanotechnology.

## Introduction

The archetypal bacteriophage has a head and a tail; it injects its linear double-stranded DNA, blocks the host's vital processes, replicates and assembles new virions in the cytoplasm, ultimately lysing the host cell in order to release the progeny. Some dramatically different bacteriophage, however, both in the terms of morphology and lifestyle, exist in Nature. One such group are phage of the genus Inovirus, commonly called filamentous bacteriophage – long, thin filaments containing a circular single-stranded DNA genome. Even more radically different than their morphology is their relationship to the host – filamentous phage reproduce without killing the host, and some even take part in the host's "social" life – i.e. the dynamics of biofilms, as has been recently reported for *Pseudomonas aeruginosa* phage Pf4 (Rice et al., 2009). Filamentous bacteriophage are widespread among Gram-negative bacteria, infecting a wide range of genera including *Escherichia*, *Salmonella*, *Pseudomonas*, *Xanthomonas*, *Vibrio*, *Thermus* and *Neisseria* (Table 1; Russel and Model, 2006). There are only two published reports of filamentous phage that infect Gram-positive organisms (Chopin et al., 2002; Kim and Blaschek, 1991). In total around 60 different filamentous phage have been described to date (International Committee on Taxonomy of Viruses., 2005), but their number is likely much larger, given that many replicate too poorly to affect the host and hence their detection by observing plaques on host cell lawns is not possible (Table 1).

The filamentous phage of *Escherichia coli* are the most productive phage in Nature, giving rise to titers of up to $10^{13}$ per mL of culture. This productivity is coupled to slowing down the host's growth and leads to formation of turbid plaques on bacterial lawns. Among *E. coli* filamentous phage, the best-studied and most-exploited group are the F pilus-specific phage or Ff, known as f1, M13 and fd (Hoffman-Berling et al., 1963; Hofschneider, 1963; Loeb, 1960), 98.5% identical in their DNA sequence (ICTVdB, 2008; International Committee on Taxonomy of Viruses., 2005). These three phage have interchangeably been featured, not only as a model system in understanding membrane transactions, but also in combinatorial technologies, whose most influential product are therapeutic recombinant antibodies (Bradbury and Marks, 2004) and the most unexpected - a lithium-powered nano-battery (Lee et al., 2009). Members of a large group of temperate filamentous bacteriophage are directly or indirectly implicated in pathogenesis of *Vibrio cholerae*, *Neisseria meningitidis* and *Pseudomonas aeruginosa* (Bille et al., 2008; Waldor and Mekalanos, 1996; Webb et al., 2004).

## The virion

The overwhelming majority of what we know about the filamentous phage virion structure comes from Ff phage of *E. coli* and Pf1 of *P. aeruginosa*. Unless otherwise stated, this review will be referring to Ff phage (f1, fd and M13). The simplicity of the virion filament is matched by the small number of proteins that form it (Figure 1). The filament tube is formed by thousands of helically arranged copies of pVIII, a small protein of only 50 amino acids. The ends of the filament are built by two different pairs of proteins – pVI-pIX and pIII-pVI.

### *Major coat protein pVIII*

The major coat protein pVIII is an integral inner membrane protein prior to assembly into the virion. PVIII of Ff and *P. aeruginosa* Pf3 have been used extensively to investigate the mechanism of protein insertion into phospholipid bilayers; it was in fact the vehicle for discovery and characterization of bacterial protein YidC that mediates insertion of small hydrophobic proteins into the inner membrane in a Sec translocon-independent manner (Rohrer and Kuhn, 1990; Samuelson et al., 2000; Serek et al., 2004). Interestingly, pVIII of Ff contains a signal sequence whereas Pf3 pVIII does not; yet they use the same mechanism for insertion into the inner membrane. A C-terminal hydrophobic transmembrane helix anchors pVIII in the inner membrane prior to assembly (Papavoine et al., 1998; Russel and Model, 1982), with the N-terminal portion in the periplasm and the C-terminal tail in the cytoplasm (Vos et al., 2009; Wickner, 1975). A number of physico-chemical methods for the investigation of membrane protein dynamics have been

*Corresponding author: Email: j.rakonjac@massey.ac.nz

Table 1. Filamentous phage

| Name[a] | Organism | Temperate | Plaque forming | Titer | Virulence associated | Virulence factor | Reference |
|---|---|---|---|---|---|---|---|
| f1 | *Escherichia coli* | No | Yes | High | No | N/A | (Loeb, 1960) |
| Fd | *Escherichia coli* | No | Yes | High | No | N/A | (Marvin and Hoffmann-Berling, 1963) |
| M13 | *Escherichia coli* | No | Yes | High | No | N/A | (Hofschneider, 1963) |
| IKe | *Escherichia coli* | No | Yes | High | No | N/A | (Khatoon et al., 1972) |
| If1 | *Escherichia coli* | No | Yes | High | No | N/A | (Meynell and Lawn, 1968) |
| If2 | *Escherichia coli* | No | Yes | High | No | N/A | (Meynell and Lawn, 1968) |
| MDA | *Neisseria meningitidis* | Yes | Not indicated | Not indicated | Yes[c] | None | (Bille et al., 2005) |
| Pf1 | *Pseudomonas aeruginosa* | No | Yes | High | No | N/A | (Takeya and Amako, 1966) |
| Pf4 | *Pseudomonas aeruginosa* | Yes | Yes | Medium | Yes | None | (Webb et al., 2004) |
| Pf5 | *Pseudomonas aeruginosa* | Yes | Not indicated | Low | Yes | None | (Mooij et al., 2007) |
| CUS1 | *Escherichia coli* O18:K1:H7 | Yes | No | Low | Yes | Unknown | (Gonzalez et al., 2002) |
| CUS2 | *Yersinia pestis* biovar *orientalis* | Yes | No | Low | Yes | Unknown | (Gonzalez et al., 2002) |
| CTXφ | *Vibrio cholerae* | Yes | No | Low | Yes | CTXαβ | (Waldor and Mekalanos, 1996) |
| F237 | *Vibrio parahaemolyticus* O3:K3 | No | Yes | Not Indicated | Yes | Unknown | (Nasu et al., 2000) |
| Lf | *Xanthomonas campestris pv. campestris* | No | Yes | High | No | N/A | (Tseng et al., 1990) |
| CAK1 | *Clostridium acetobutylicum*[b] | No | Not Indicated | Not Indicated | No | N/A | (Kim and Blaschek, 1991) |
| B5 | *Propionibacterium freudenreichii*[b] | No | Yes | Not Indicated | No | N/A | (Chopin et al., 2002) |

[a]This is not a complete list of known filamentous phage, but rather examples of the various filamentous phage lifestyles.
[b]Gram-positive bacteria
[c]Statistically significant association with infection in young adults (Bille et al., 2008)

tested on Ff pVIII as a model (Hemminga et al., 2010) and more recently solid-state NMR has been used to resolve the structure of pVIII of Ff and *P. aeruginosa* phage Pf1 in membranes and the virions (Goldbourt et al., 2010; Opella et al., 2008; Park et al., 2010).

In the virion, the negatively charged N-terminal 4-5 residues are disordered. The remaining part of pVIII is a slightly curved α helix, at a small angle to the virion axis. In Ff pVIII, the α helix is amphipathic down to the 20th residue and then hydrophobic to residue 39, ending with a 10-residue positively charged helix that interacts with encapsulated DNA (Figure 1A; Marvin et al., 2006). This hydrophobicity and charge arrangement is the conserved in pVIII of other filamentous phage (Marvin, 1998). Thousands of pVIII subunits are held together through hydrophobic interactions, in a helical arrangement that is reminiscent of

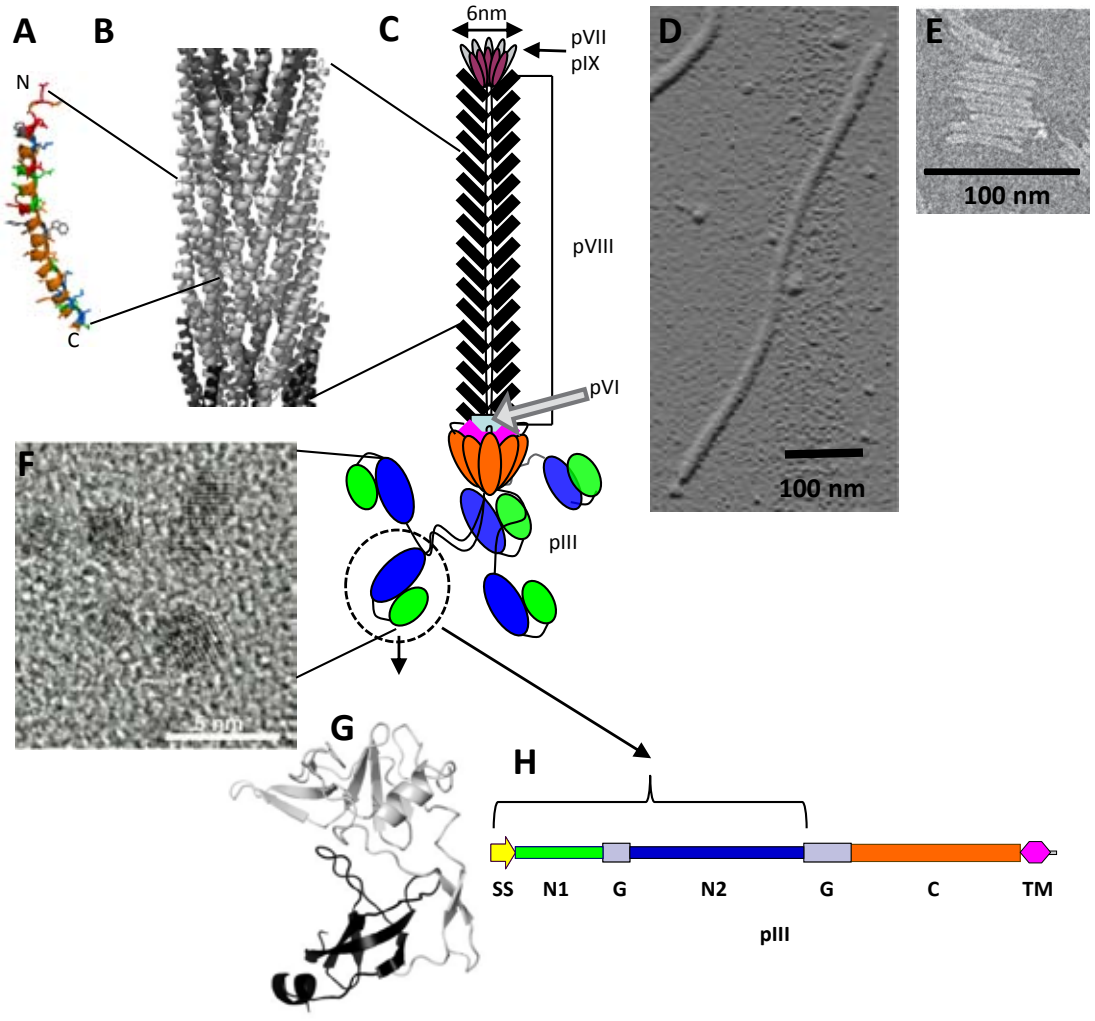


**Figure 1. The Ff virion. A.** Model of a pVIII monomer showing hydrophobic residues (green), hydrophilic residues (orange), positively charged residues (blue), and negatively charged residues (red). N and C termini of pVIII are indicated. **B.** Ribbon representation of the pVIII arrangement within the filamentous phage capsid. PVIII within the capsid forms a shingle-like array of helices. **C.** Schematic representation of the virion. **D.** Atomic force microscope image of Ff virion (M. Russel and P. Model, sample prepared by J. Rakonjac). **E.** Electron micrograph of microphage (kindly provided by James Conway and Dalaver H. Anjum, microphage sample prepared by Bennett and Rakonjac, unpublished). **F.** High-resolution TEM lattice fringe images of five ZnS nanocrystals at the pIII-end of a single Ff virion. From Lee et al. (2002), reprinted with permission from AAAS. **G.** Ribbon representation of the N1 domain (dark gray) and the N2 domain (light gray) of pIII. The high glycine linker sequence located between the C-terminus of the N1 domain and the N-terminus of the N2 domain is flexible and hence is not visible in this structure. **H.** Domain organization of pIII preprotein. SS, signal sequence, N1, N2, C, domains of pIII; G, glycine-rich linkers; TM, transmembrane helix.

The images of the pVIII subunit (A), the capsid (B), and the N1-N2 domains of pIII (G) were derived from coordinates of the RCSB PDB database accession numbers 2cOw (Marvin et al., 2006) and 1g3p (Lubkowski et al., 1998), respectively, using PyMOL (DeLano, 2006).

snake-skin scales (Figure 1B). The structure of pVIII and the virion is conserved among analyzed filamentous phage (Day et al., 1988; Marvin, 1998); however there are two different classes of symmetries of the helical virion. In *E. coli* phage Ff, IKe and If1,(class I), pVIII subunits are arranged on a five-start helix with 2-fold screw axis ($C_5S_2$ symmetry) of about 32 Å pitch, with five subunits in each "ring" of the virion tube, related by a 5-fold rotation axis (Caspar and Makowski, 1981; Marvin et al., 2006). In the phage of Pseudomonas and Xanthomonas (class II) pVIII subunits are arranged on a simple one-start helix of ~ 15 Å pitch, with 5.4 subunits per turn ($C_1S_{5.4}$; Caspar and Makowski, 1981; Goldbourt et al., 2007).

The set ratio of subunits per nucleotide in Ff is 0.42± 0.01 (Newman et al., 1977) whereas in Pf1 it is exactly 1 (unity; Wiseman and Day, 1977). The DNA conformations in the two classes differ greatly although both DNA helices are right-handed. In Ff, the bases are stacked at the center with the phosphates on the outside interacting with the capsid; the bases from opposing sides of the single-stranded DNA circle are H-bonded to one another, but only about 25% of the H-bonding can be of the Watson-Crick type (Day et al., 1988). In Pf1, the two sides of the single-stranded DNA circle are so highly stretched and twisted that the sugar phosphate back-bones are in contact with each other at the center and the bases are exposed on the outside for interaction with the capsid; the bases are so widely separated from each that H-bonding is not possible (Liu and Day, 1994; Tsuboi et al., 2010). Other filamentous phage have yet other DNA conformations.

Given that the length of a virion is determined by the size of the DNA and its particular conformation as maintained by the capsid, inserting or deleting DNA is a simple way to control the length of the virion. While the wild-type Ff phage is normally ~900 nm long, the shortest virion that can be assembled is only 50 nm in length (Figure 1E; see below in the "Ff replication" section; Specthrie et al., 1992). Aside from the size of packaged DNA, the length of the virion

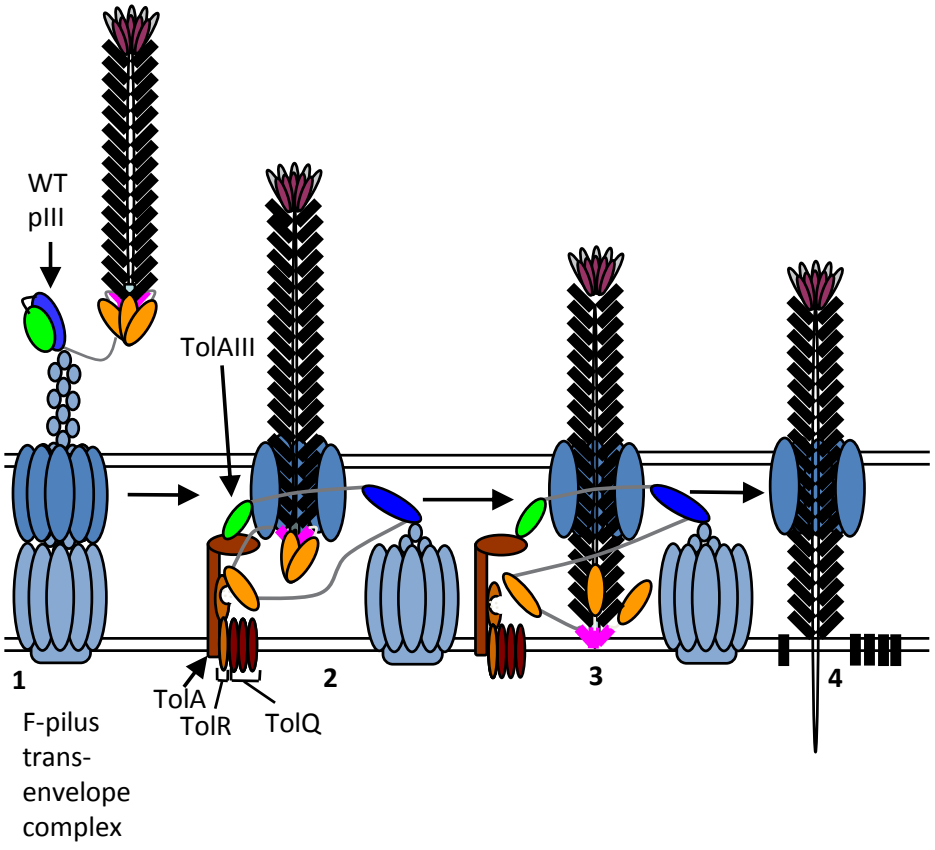


**Figure 2. Model of Ff phage infection**. (1) Binding of N2 domain (dark-blue oval) to the tip of the F-pilus (light-blue circles) and pilus retraction. (2) Binding of N1 domain (bright-green oval) to TolA III domain (brown oval). (3) "Opening" of the C-domain and insertion of the C-terminal hydrophobic helix into the inner membrane. (4) Entry of phage DNA into the cytoplasm and integration of the major coat protein pVIII into the inner membrane. Steps 1 and 4 are based on published findings, whereas steps 2 and 3 are speculative. Symbols: OM, outer membrane; IM, inner membrane. pIII N1 domain, dark-blue oval; pIII N2 domain, bright-green oval; pIII C domain, orange oval; pIII C-terminal hydrophobic helix (membrane anchor), pink rectangle; pIII glycine linkers, gray lines; major coat protein pVIII, black rectangles; pVII, gray ovals, pIX, purple ovals; TolA and TolRQ, brown shapes; F-pilus, and the trans-envelope pilus assembly/retraction system, light-blue. The phage contains 5 copies of pIII, but for simplicity only one full-length pIII is shown. However, this is consistent with experimental data: N1N2 and C domain operate "*in cis*" and fewer than five functional copies are sufficient for infection (Bennett and Rakonjac, 2006).

depends on the relative efficiency of initiation or termination to elongation. In Ff mutants of the minor proteins that initiate (pVII/pIX) or terminate (pIII/ pVI) assembly, extremely long virions that carry sequentially packaged genomes are produced, more twenty-fold in length compared to wild-type virions (Lopez and Webster, 1983; Rakonjac and Model, 1998).

*Minor virion proteins: pVII, pIX, pIII, pVI*

In contrast to the detailed knowledge of pVIII structure and packing along the filament, no structural information is available for the two caps. Proteins pVII and pIX are incorporated into the virion at the initiation step of assembly and are the first to be extruded from the cell (Fig 1C; (Endemann and Model, 1995; Grant et al., 1980). Both are small hydrophobic proteins of only 32 (pVII) and 33 (pIX) amino acids; they are inner membrane proteins prior to assembly, but in Ff phage they do not contain a signal sequence and are thought to spontaneously insert into the membrane (Endemann and Model, 1995). The structure of these two proteins has not been solved and their arrangement in the virion has not been determined. Genetic analysis showed that the residues near the C-terminus are involved in interactions with the packaging signal, a DNA hairpin that targets phage genome for packaging (Russel and Model, 1989). The sole clue about the accessibility of these two proteins is their ability to display peptides and proteins fused to their N-termini. For both proteins, once an addition to the N-terminus has been made, a signal sequence is required for successful incorporation of these chimeric proteins into the virion and display on the surface of the virion (Gao et al., 1997; Gao et al., 2002; Huang et al., 2005).

Proteins pIII and pVI are added to the virion at the end of assembly. They form a distal ”cap” of the filament and at the same time release the virion from the cell (Rakonjac et al., 1999; Rakonjac and Model, 1998). These two proteins are required for the structural stability of the virion and also for termination of assembly. In addition, pIII mediates entry of the phage into the host cell (Figure 2). Both pIII and pVI are integral membrane proteins (Boeke and Model, 1982; Endemann and Model, 1995).

The structure of the pIII-pVI complex in the virion cap is not known. Direct evidence for the number of pIII subunits per virion has been provided recently as a by-product of a nanotechnology application, in which ZnS nanocrystals were nucleated by N-terminally displayed peptides (Lee et al., 2002). The TEM of the nanocrystal-decorated virion tip shows five ZnS nanocrystals associated with the tip of the virion, corresponding to five copies of pIII (Figure 1F). Since pVI and pIII are equimolar in the virion cap (Grant et al., 1980), there must be five pVI subunits per virion. Thus the distal cap maintains the fivefold axial symmetry of the pVIII arrangement along the virion filament.

PVI is a 112-residue, mostly hydrophobic protein. It is integral membrane protein prior to assembly into the virion (Endemann and Model, 1995), predicted by the TMHMM2.0 (Krogh et al., 2001) to contain three transmembrane α helices, with the N terminus in the periplasm and the C terminus in the cytoplasm. At 406 amino acids in length (424 residues including signal sequence), pIII is distinctly larger than the other four virion proteins. PIII is composed of three domains (N1, N2 and C) separated by long glycine-rich linkers (Figure 1C,G,H). Prior to assembly into the virion, pIII is targeted to the inner membrane by its N-terminal signal sequence and anchored in the phospholipid bilayer by a C-terminal hydrophobic transmembrane α helix, in a SecYEG and SecA-dependent manner (Boeke and Model, 1982). Given the C-terminal position of the membrane anchor, most of pIII is localized in the periplasm prior to assembly into the virion; only five C-terminal residues are located in the cytoplasm (Davis et al., 1985; Davis and Model, 1985).

The N1 and N2 domains of pIII interact with the host receptors; the structure of these two domains has been determined using X-ray crystallography and NMR (Figure 1G; (Holliger et al., 1999; Lubkowski et al., 1998). The three-dimensional structure of the C domain, which is required for termination of phage assembly, formation of a detergent-resistant virion cap and for late steps in phage infection, is yet to be determined (Bennett and Rakonjac, 2006; Rakonjac et al., 1999). An antibody specific for the C-terminal 10 residues of pIII cannot bind to pIII when it is in the virion (Rakonjac, unpublished). Therefore, this C-terminus must be buried within the virion cap, which is composed of the pIII C-domain and pVI.

**Life cycle**

Filamentous phage adhere to either of two major life styles – exclusively episomally replicating phage or temperate phage that are chromosomally integrated, but can be induced to start episomal replication; Table 1). Episomally replicating filamentous phage (e.g. Ff and Pf1) produce large numbers of progeny phage, reaching titers of up to $10^{13}$ per mL of culture. In contrast, many chromosomally integrated phage are very low producers, releasing only 1 phage per 10-100 cells under the inducing conditions (Davis et al., 2002). However, this is not the rule; temperate phage of Xanthomonas can produce $10^{11}$ phage per mL of culture upon switching to a poorly understood “virulent” mode of growth (Kuo et al., 1994). The replication and gene expression of episomally replicating filamentous phage (e.g. Ff or Pf1), once they infect the host cell, proceeds unabated; their genomes do not encode regulatory proteins. In contrast, gene expression and replication of chromosomally integrated filamentous phage is tightly controlled. These phage encode transcriptional regulators whose role is to inhibit transcription of the replication protein and downstream virion genes (Waldor and Friedman, 2005).

*Infection*

The primary receptors for filamentous phage are pili, long filamentous structures on the surface of bacterial cells. Three types of pili can serve as primary receptors, including conjugative F, N (or I) and type IV pili (Campos et al., 2003b; Deng et al., 1999; Endemann et al., 1992; Holland et al., 2006; Jouravleva et al., 1998; Waldor et al., 1997; Yang et al., 2004). The secondary receptor for phage as diverse as *E, coli* Ff and *V. cholerae* CTXφ is the TolQRA complex of inner membrane proteins, highly conserved in Gram-negative bacteria (Click and Webster, 1997; Heilpern and Waldor, 2000). TolQRA belongs to a larger trans-envelope Tol-Pal complex involved in cell division and maintenance of cell envelope integrity (Cascales et al., 2007; Gerding et al., 2007).

The largest virion protein, pIII, described in the previous section, mediates infection of the host (Figure 2). Its two N-terminal domains bind to the primary and secondary receptors and its C-domain is involved in virion uncoating and DNA entry into the host cell cytoplasm (Bennett and Rakonjac, 2006; Deng and Perham, 2002; Reichmann and Holliger, 1997). pIII is the most diverse virion protein among filamentous phage, often with no significant homology between the counterparts from distant phage. This is puzzling, given that distantly related phage can use highly conserved ToQRA complex for pIII-mediated infection and entry. The gene (gIII) can nevertheless be identified based on its size and position in the genome.

The organization and order of the two N-terminal receptor-binding domains can differ even between closely related phage. For example, pIII of *E. coli* phage IKe, which binds to the N (or I) pili, has no glycine-rich linker between the two N-terminal domains, and the order of the two domains is switched relative to Ff phage (Endemann et al., 1992). In Ff phage, the N2 domain binds to the primary receptor, the tip of the F (or conjugative) pilus, whereas the N1 domain binds the periplasmic domain III of TolA; the opposite is true for IKe pIII. Binding to the F pilus induces a conformational change by cis-trans isomerization of $Pro_{213}$ within the N2 domain (Eckert et al., 2007). This releases the N1 domain from the N2 domain, exposing the TolA binding site on the N1 domain (Eckert et al., 2007; Lubkowski et al., 1999; Reichmann and Holliger, 1997). If the F pilus or the F-pilus-binding domain of pIII are absent, the infection efficiency decreases by several orders of magnitude, but is not completely abolished; in contrast, TolQRA and the cognate pIII domain are absolutely required for infection (Click and Webster, 1997, 1998; Reichmann and Holliger, 1997; Russel et al., 1988).

A common characteristic of pili that serve as primary receptors for filamentous phage is the ability to retract towards the cell surface, bringing the filamentous phage close to the secondary receptor located in the periplasm (Lawley et al., 2003; Maier, 2005). It had been thought that the retraction of the F pilus was induced binding of the Ff phage, however recent evidence shows that the F pilus undergoes spontaneous oscillatory extension and retraction cycles (Clarke et al., 2008). The events that follow pilus retraction and allow pIII to gain access to TolA are unknown, simply because the mechanics of the F pilus conjugative machine is poorly understood. Pilus retraction, followed by a set of unknown events, somehow ushers pIII (and presumably the virion cap) across/through the outer membrane and into the periplasm, where the N1 domain can interact with the periplasmic domain of TolA (Lubkowski et al., 1999; Reichmann and Holliger, 1997). The post-receptor binding steps of infection are also unknown. All three proteins of the TolQRA complex, and a functional pIII C-domain covalently linked to the N1N2 domains, are absolutely required for phage infection, which ultimately results in entry of the phage ssDNA into the cytoplasm and integration of the major coat protein into the inner membrane (Bennett and Rakonjac, 2006; Click and Webster, 1997; Smilowitz, 1974; Trenkner et al., 1967). C domain of pIII is predicted to be α-helical; three C-terminal helices (two amphipathic and the third, hydrophobic anchor) are required for phage entry (Bennett et al., 2011). The organization of α helices in the C domain resembles α-helical pore-forming toxins; this is supported by threading using the algorithm I-TASSER (Zhang, 2008), which selected the membrane insertion domain (domain T), of diphtheria toxin as the top ranked template for modeling of the C domain (Choe et al., 1992).

Interestingly, expression of pIII N2 domain alone in $F^+$ *E. coli* abolishes conjugation as well as infection with another F-pilus-specific phage (small ssRNA phage f2 or MS2) that binds along the sides of the pilus. These two phenotypes are suggestive of pIII-mediated pilus assembly inhibition (Boeke et al., 1982). Given that the F-pilus is up to 4 μm in length, inhibition of its assembly by the N2 domain is strategically very important to securely dock the virion to the host cell envelope and make the infection irreversible.

A number of filamentous phage use retractable type IV and conjugative N (or I) pili for infection, but little is known about their receptor-pIII interactions. Interestingly, the type IV pili are evolutionarily unrelated to the conjugative pili (including F and N) and completely different in the mode of their assembly and in morphology, hence the pIII-pilus interactions in phage that utilize them as primary receptors are likely very different from those of Ff. An approach using chimeric pIII molecules showed that the primary receptor-binding domain (N2) of pIII from phage IF1 and CTXφ allow f1 to infect the I-pilus containing *E. coli* and TCP pilus-containing *V. cholerae*, respectively (Heilpern and Waldor, 2003; Lorenz et al., 2011). The efficiency of infection, mediated by chimeric pIII proteins, however, decreases by several orders of magnitude if the distance between the three domains (N1, N2 and C) is changed by inserting additional domains (Heilpern and Waldor, 2003; Marzari et al., 1997). Despite very low N1 domain conservation, these phage use the conserved TolA protein as a secondary receptor, and this appears to be sufficient to trigger $pIII_{Ff}$ C domain-mediated entry.

*Replication*

Following entry into the host cell, the ssDNA genome of episomally replicating and temperate phage have two different fates, the former entering replication and the latter integrating into the chromosome. Interestingly, both of these processes rely on the enzymes that normally act on double-stranded DNA: RNA polymerase for replication of the negative strand and XerC recombinase for chromosomal integration. In both of these situations, locally folded ssDNA forms quasi-double-stranded binding sites for the appropriate proteins: -35 and -10 boxes for RNA polymerase (Higashitani et al., 1997) and *dif* sites for the site-specific XerCD recombinase (Val et al., 2005).

Ff replication

The life cycles of episomal filamentous phage are strikingly different from those of chromosomally integrated phage; the genomes of the former phage are also simpler, lacking sequences for host integration and regulation of gene expression (Figure 3; Russel and Model, 2006).

The circular Ff genome, 6,407 nucleotides in length, contains 9 genes, but produces 11 proteins, thanks to internal translational starts within two genes, pII and pI, which give rise to two additional proteins, pX and pXI, respectively. Phage proteins pII, pV and pX, involved in replication, remain in the cytoplasm, whereas all other proteins are targeted to the membranes (Endemann and

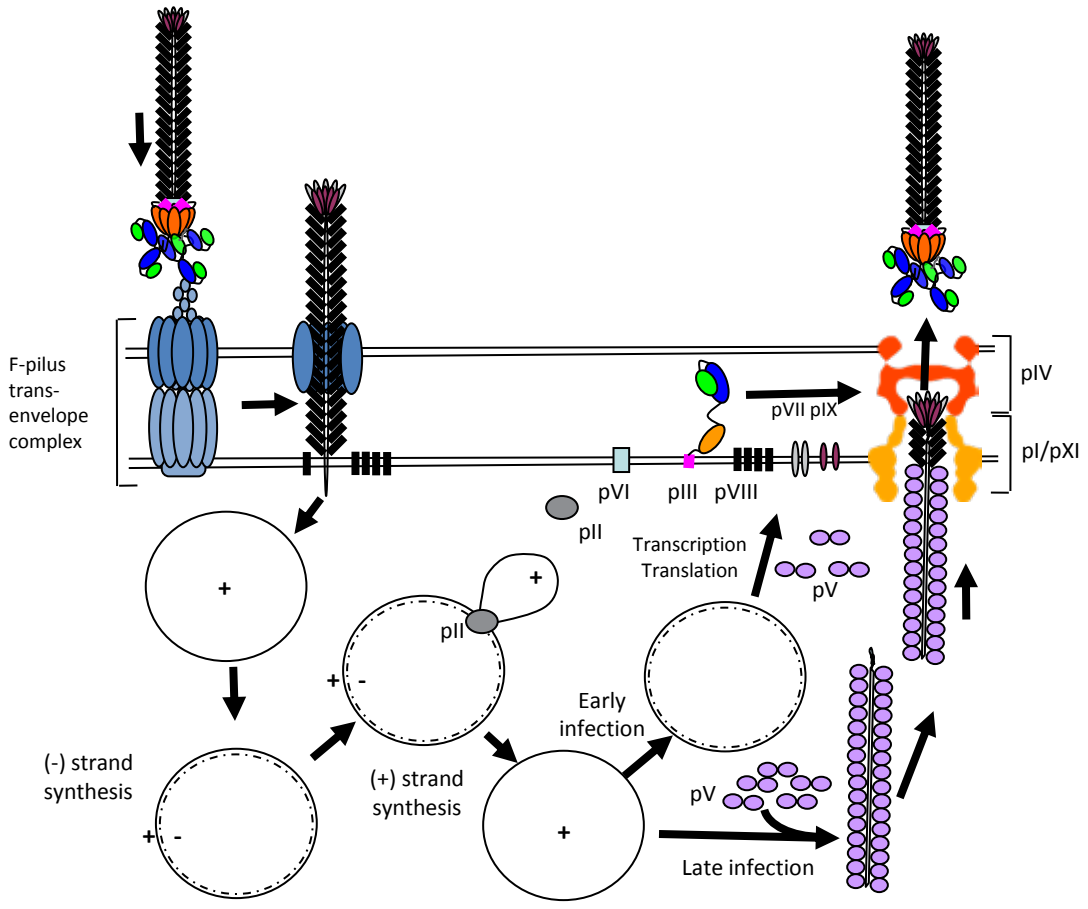


**Figure 3. The Ff phage life cycle.** Upon infection, the ssDNA (the + strand) enters into the cytoplasm, while the pVIII major coat protein integrates into the inner membrane. Synthesis of the negative (-) strand is initiated at the negative strand origin of replication by RNA polymerase, which generates an RNA primer and is then released from the template (Zenkin et al., 2006). Host DNA polymerase III uses this primer to replicate the complete negative strand. Positive strand synthesis is initiated by pII (gray circle), which creates a nick in the + strand of the dsDNA replicative for at the positive origin of replication. Supercoiling and formation of a stem-loop structure of the positive (+) origin of replication is required for this step (not shown in the figure). Rolling circle replication then ensues, one strand at a time. During the initial period of viral infection, new positive strands are used as templates for synthesis of negative strands, resulting in an increase in copy number of the dsDNA replicative form (RF). The RF serves as a template for production of phage proteins. Phage proteins II, V and X remain in cytoplasm and mediate genome replication and formation of the packaging substrate. Proteins pI, pIV and pXI form a transport complex spanning the inner and outer membrane (yellow and orange, respectively). Virion proteins pVII, pIX, pVIII, pVI, pIII are inserted into the membrane prior to their assembly into phage particles. Later in the infection, positive strands are coated by dimers of the phage encoded single-stranded DNA binding protein pV to form the packaging substrate and brought to the cell membrane assembly/export complex (pI/pXI and pIV) for assembly and export. The pIV silhouette is derived from determined cryo-EM structure (Opalka et al., 2003). The structure of the inner membrane complex (yellow silhouette) has not been determined; it is drawn based on the cryo-EM structure of the type III secretion system (Marlovits et al., 2004).

Model, 1995; Marciano et al., 1999; Rapoza and Webster, 1995). The positive and negative origins of replication, as well as the packaging signal or morphogenetic signal, are located in the intergenic region (between gIV and gII).

After entry into the cytoplasm, the Ff ssDNA genome positive (+) strand serves as a template to synthesize the negative (-) strand. This step is independent of phage proteins, and is initiated by host RNA polymerase. The negative origin, which forms two stem-loop structures resembling -35 and -10 promoter sequences, serves as a starting site for host RNA polymerase to synthesize a primer (Higashitani et al., 1997). Following binding to this promoter mimic, the polymerase synthesizes RNA on the ssDNA template, then stalls at a poly-G tract, backtracks and dissociates from the template, leaving an RNA primer of defined length hybridized to the template (Zenkin et al., 2006). Host DNA polymerase III then uses this primer to synthesize the (-) strand of DNA, yielding a double-stranded circle. The negative strand origin is not absolutely required for phage replication. An RNA primer for DNA replication can be synthesized at other locations in the genome, albeit with a lower efficiency (Kim et al., 1981).

The Ff phage replicate by a rolling-circle mechanism, one strand at a time (Figure 3). The dsDNA form of the

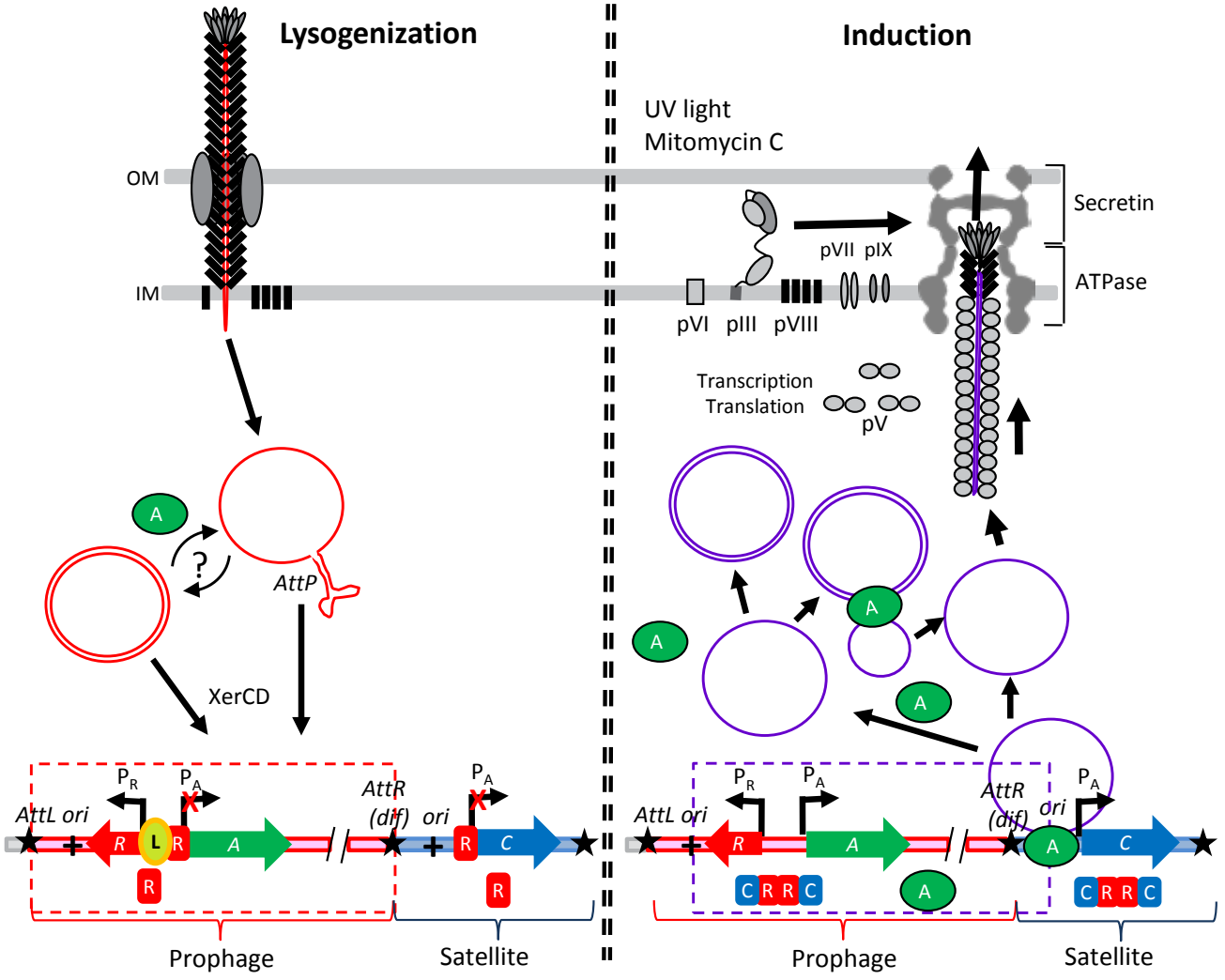


**Figure 4. Temperate filamentous phage life cycle.** The figure is based on the large body of work on *V. cholerae* filamentous bacteriophage CTXφ (Davis et al., 2002; Huber and Waldor, 2002; Kimsey and Waldor, 2004, 2009; McLeod et al., 2005; Moyer et al., 2001; Val et al., 2005).

**Left panel:** entry and lysogenization. After entry into the cytoplasm, ssDNA (red) can either serve as a template to synthesize the (-) strand, resulting in the RF form, or become a substrate for the XerCD recombinase which binds to a forked hairpin loop (*AttP*) and mediates site-specific recombination into the *dif* site located between the chromosomal DNA (gray) and an already inserted satellite phage, e.g. RS1φ or TLCφ (blue; Hassan et al., 2010). Given that ssDNA is the substrate for integration and that replication is required for lysogenization, it is likely that not only the (-) strand, but also the (+) strand replication occurs before integration. Replication and/or repair synthesizes the (-) strand of CTXφ, forming a prophage (pink) flanked by the modified dif site (*AttL*), reconstituted *dif* site (*AttR*), followed by the satellite or defective prophage (blue). Expression of negative regulator RstR (red, labeled "R") and its binding to the regulatory sequences between two divergent promoters ($P_R$ and $P_A$) in combination with chromosomally-encoded LexA (yellow, labeled "L"), inhibits transcription of the replication protein RstA (green, labeled "A"), genes encoding other phage proteins (not shown) and the positive regulator RstC encoded by the satellite (blue, labeled "C"), while stimulating its own expression.

**Right panel:** induction, replication and phage assembly. Upon induction of the SOS response by UV or Mitomycin C, LexA becomes degraded. As a consequence, the $P_A$ promoter transcription and production of the replicator (A), positive regulator (C) is and other prophage genes transcribed from this promoter are induced. C sequesters R, preventing $P_A$ promoter repression. Replication of prophage is initiated from (+) origin of replication (*ori*) which is located ~500 nucleotides downstream from the *AttL* site, and proceeds until it reaches the *ori* of the satellite, synthesizing new (+) strand and releasing the recircularized old (+) strand ssDNA, a combination of the prophage and satellite, between two (+) origins of replication. Therefore, the resulting replicon (purple) is not the same as the one that was integrated into the chromosome (red). The episomal replication ensues, producing the (+) ssDNA which is packaged into the virion and secreted out of the cell.

Ff phage genome is often referred to as the replicative form (RF), whereas the positive (+) strand ssDNA form of the genome is referred to as the infective form (IF). The replicative form serves as a template to synthesize the (+) strand and for transcription of the phage genes.

The (+) strand origin of replication is absolutely required for phage DNA replication and packaging. The product of the replication cycle initiated from this origin is the circular ssDNA that is packaged into the virion. PII, the replication protein, binds to a stem-loop structure in the positive origin formed in supercoiled RF, cleaves the positive strand at a set site and covalently binds to the 5' end (Asano et al., 1999; Greenstein et al., 1988; Horiuchi, 1997). The cleavage of the (+) strand by pII leaves a free 3' end that serves as a primer for host DNA polymerase III which synthesizes the new (+) strand (Meyer and Geider, 1982). The old (+) strand is displaced as the new one is synthesized. When a circle is complete, the covalently linked pII cuts the displaced (+) strand at the junction between the old and newly synthesized DNA and re-ligates the two ends together in a strand-transferase reaction that liberates pII (Asano et al., 1999). Early in infection the newly synthesized (+) strands are used as a template for (-) strand replication, increasing the copy number of dsDNA to about 50 per cell and in turn increasing the synthesis of phage-encoded proteins. Later in the infection when the concentration of phage proteins has increased, (+) strands are coated by ssDNA-binding protein pV, with the exception of a hairpin loop that serves as a packaging signal. The ssDNA-pV complex is a packaging substrate for phage assembly. pV has an additional regulatory role – it inhibits translation of pII (Michel and Zinder, 1989). This regulatory loop serves to coordinate ssDNA production and packaging.

The packaging or morphogenetic signal targets the ssDNA-pV complex to the pI/pXI/pIV phage export complex and assists the minor proteins pVII and pIX in identifying phage ssDNA genomes so that they can be packaged into virions and exported (Russel and Model, 1989). Only the packaging signal on the positive strand is recognized and packaged (Zinder and Horiuchi, 1985). It has been noted, however, that the export system packages ssDNA of Ff mutants that do not contain the packaging signal as well as unrelated plasmid ssDNA, albeit at a low efficiency (Russel and Model, 1989).

In Ff filamentous bacteriophage, some truncated virions are produced, often after about 40 passages of the phage through host cells in the absence of clonal (plaque) purification, when genomes containing spontaneous duplications of the replication origin tend to appear in the culture (La Farina et al., 1987). In these spontaneous double-origin genomes, (+) strand replication is initiated at the origin 1 (*ori1*); when the replication fork reaches the next positive origin 2 (*ori*2) the termination signal is recognized by pII, which makes another cut and then ligates the two ends of the (+) strand, to create a small genome spanning the segment between *ori1* and *ori*2. Given that virion length is determined by the size of packaged ssDNA, the resulting virions are relatively short. This small replicating segment interferes with full-length genome replication and packaging, the short virions becoming a significant fraction of the phage progeny. This property has been used to engineer a "microphage"-producing template – a plasmid containing the packaging signal flanked by two positive origins, of which the second functions only as a terminator. When cells containing this plasmid are infected with an interference-resistant helper phage (R474), the (+) strand replication between the two origins results in a 200-nucleotide "genome" that is packaged into a 50 nm-long particle (Figure 1E; Specthrie et al., 1992).

Temperate filamentous phage - chromosomal integration and replication

The genomes of temperate or lysogenic filamentous phage integrate into *dif* sites on the host chromosome (Figure 4); this event is dependent on the host site-specific *dif*-site binding recombinase, XerCD, which normally serves to resolve dimers of chromosomes (Huber and Waldor, 2002). The phage genome contains two inverted repeats of the *dif* site that form a forked stem-loop structure, thus reconstituting a double-stranded XerCD binding sequence in the ssDNA (Val et al., 2005). The action of XerCD recombinase on this secondary structure results in site-specific recombination and insertion of the phage genome into the *dif* site of the chromosome. In *V. cholerae*, duplicate CTXφ genomes are inserted into the same *dif* site, suggesting that at least one round of each (-) and (+) strand replication precedes integration (Davis and Waldor, 2000). Interestingly, the complex integrated element that contains the CTXφ genome, including *ctxAB* genes (encoding Cholera toxin, CtxAB), was likely generated during the evolution of two recent pandemic strains through successive integration events that include, besides CTXφ, two types of satellite filamentous phage, RS1φ and TLCφ (Hassan et al., 2010). Replication and assembly of these two satellite phage depend on "helper" phage - RS1φ on KSFφ or CTXφ, and TLCφ on fs2φ. *V. cholerae* strains containing successive lysogenization stages can be found in Nature, and these can also be recreated in the laboratory (Campos et al., 2003b).

After lysogenization, the integrated CTXφ phage genomes are inactive in replication and virion genes are not transcribed due to the presence of a regulatory circuit involving a combination of host, phage and satellite-encoded regulatory proteins (Figure 4; Davis et al., 2002; Kimsey and Waldor, 2009; Waldor and Friedman, 2005). The absence of virion gene expression, particularly lack of pIII (which in Ff phage blocks the TolQRA secondary receptor complex), likely allows super-infection to occur, making successive lysogenization events possible. In contrast to genes encoding proteins that are required in the viral life cycle, the "passenger" toxin genes (*ctxAB*) within CTXφ genome contain additional regulatory sequences that allow their independent regulation by a chromosomally-encoded environmentally-controlled *toxR* regulon (Childers and Klose, 2007).

The same set of stresses that induces the lytic cycle in the "standard" tailed temperate or lysogenic phage (e.g. λ) also induces replication in filamentous phage lysogens through a regulatory circuit that involves induction of SOS response and degradation of LexA protein (Quinones et al., 2005). The consequence of this induction, in contrast to that of tailed phage, is not genome excision and cell lysis. Rather, expression of a replicator protein (RstA in *V. cholerae* RS1φ and CTXφ) and a positive regulator (RstC in RS1φ) is induced, resulting in a positive feedback loop that sequesters the transcriptional inhibitor, RstR (Davis et al.,

2002). Upon accumulation of the replication protein (RstA), a functional equivalent of Ff pII, replication of the (+) strand ensues, using the integrated genome as a template. Not only (+) strand, but also double-stranded replicative form (RF) is isolated from the cells after induction, hence the replication occurs both using the chromosomally inserted and episomal DNA as templates (Figure 4; McLeod et al., 2005). In CTXφ, the genome is replicated between two origins of replication located in the intergenic sequences flanking the CTXφ coding region, one upstream (derived from the CTXφ genome) and one downstream (derived from the closely related sequentially integrated satellite phage RS1φ; Davis and Waldor, 2000; Moyer et al., 2001). *V. cholerae* strains containing a single chromosomally-integrated filamentous phage genome have never been found in natural isolates. Replication in an engineered strain containing a single CTXφ insertion (and therefore only a single positive origin of replication) only very rarely generated episomal replicons, which were smaller than complete genomes (Moyer et al., 2001). In this dead-end situation, a satellite phage, which could be generated by origin duplication, much like in Ff phage, could rescue efficient replication and virion production and result in observed composite lysogenic arrays in *V. cholerae*. In summary, the requirement of two sequentially integrated origins for induction of episomal replication and efficient virion production makes propagation and transmission of *V. cholerae* temperate filamentous bacteriophage a very social affair (Campos et al., 2003a; Hassan et al., 2010). Xanthomonas and Yersinia lysogenic filamentous phage, like those of Vibrio, insert into *dif* sites (Chouikha et al., 2010; Lin et al., 2001). In contrast, the Pf4 lysogenic filamentous phage of *P. aeruginosa* PA01 does not seem to use *dif* sites; the replicative form arises by recombination between two direct repeats that flank the Pf4 genome (Webb et al., 2004). Another peculiarity of Pf4 is that it encodes a putative reverse transcriptase, which is not found in any other lysogenic filamentous phage genome.

Some lysogenic phage, including CTXφ, are produced at very low levels after induction – up to $10^5$ per mL (Quinones et al., 2005). Others, like *Xanthomonas campestris* Cf16 and *P. aeruginosa* lysogenic phage Pf4, achieve much higher titers. These two phage have rather dynamic regulation of virion production, which seems to be tuned with the age of the cell lawn (Cf16) or the biofilm (Pf4); (Dai et al., 1987; Rice et al., 2009). *P. aeruginosa* PA01 growing in biofilms assemble and release Pf4 phage continually; however, on about day 4 of biofilm growth, the phage release intensifies (titer increases from $10^7$ to $10^9$ per mL). These new virions represent a different entity, named "superinfective" phage. These late-released "superinfective", but not the early-released "regular" phage form plaques on lawns of PA01 (which is a Pf4-lysogen and is resistant to superinfection because of active episomal replication). Both "regular" and "superinfective" phage variants can infect a strain containing a complete deletion of the Pf4 genome (Rice et al., 2009; Webb et al., 2004). The molecular basis of this fascinating phenomenon has not been investigated as yet.

*Assembly*

During replication, the newly synthesized circular ssDNA phage genome is covered with dimers of the phage-encoded ssDNA-binding protein, pV, which collapses the DNA into a rod (Figure 3). The only exposed segment of the genome is the packaging signal, a hairpin loop. For the assembly to be initiated, the packaging signal must interact with the assembly machinery. Replacement of the Ff packaging signal with a hairpin loop of different sequence prevents assembly, implying that a sequence-specific component binds to it (Russel and Model, 1989). Further, compensatory mutations for a defective packaging signal were mapped to pI, the inner membrane component of the assembly machinery, and pVII and pIX, the two minor proteins at the end of the virion at which the assembly starts (Russel and Model, 1989). All these data are consistent with a model in which packaging signal recognition by pVII, pIX and pI initiates the assembly. The assembly machine is composed of inner membrane ATPase/channel pI/pXI and outer membrane channel pIV, which form the phage assembly "complex" (Feng et al., 1999; Marciano et al., 1999).

The elongation phase of assembly ensues, during which the virion is assembled as it is extruded from the cells. As the ssDNA traverses the membrane, pV dissociates and is replaced by major coat protein pVIII. When the DNA is completely coated with pVIII, minor coat proteins pVI and pIII are added to the virion and this event results in the release of the assembled phage from the virion. If either of the two proteins is absent, the phage remain tethered to the membrane (Rakonjac et al., 1999; Rakonjac and Model, 1998). Interestingly, rather than stopping the assembly altogether, the filaments keep elongating by adding new genomes and pVIII monomers, resulting in a bizarre appearance of the infected cell in electron micrographs: after a 90 min infection 200-300 pili-like structures, several micrometers long, emanate from the cell surface.

Deletion analyses have shown that a 93 residue C-terminal fragment of pIII, containing one amphipathic helix and the C-terminal hydrophobic helix, is sufficient for release of the phage from the membrane. A shorter C-terminal fragment of 83 residues, lacking the amphipathic helix, incorporates into the filament, but cannot release it from the host cell. These results indicate that a certain minimal portion of the C domain is required for disruption of the growing filament and release from the cell (Rakonjac et al., 1999), however this fragment is shorter than the one required for phage entry (Bennett et al., 2011). The requirement for incorporation of pIII into the virion has been systematically investigated by alanine scanning mutagenesis, which showed that out of 150 residues of the C domain only 24 side-chains, located within the C-terminal 70 residues are required (Weiss et al., 2003). Considering that both pIII and pVI are integral membrane proteins, and that lipids are not found in the virion (Day et al., 1988; Hemminga et al., 2010), it remains puzzling how the assembly machinery mediates the release of these two proteins and the virion becomes excised from the membrane at the end of phage assembly.

Ff production does not kill the cell, but it is a highly coordinated process which coaxes the cell to synthesize and integrate an estimated $10^6$ copies of pVIII into the inner membrane per cell per generation. This large burden is relieved through secretion of the virion. If the secretion is blocked by mutating components of the assembly machinery (pI, pXI and pIV) or proteins involved in the initiation of phage assembly (pVII, pIX), the host cell accumulates extensive internal mitochondrial-cristae-like folded inner membrane structures and dies (Schwartz and Zinder, 1968). Mutation in the replication protein pII, that prevents synthesis of the

(+) strand, or sublethal concentrations of the translational inhibitor chloramphenicol rescue from killing (Pratt et al., 1966). Furthermore, a plasmid that expresses all phage proteins, but in which the origin of replication and packaging signal are replaced by a low copy number θ-replication *ori* (pA15) is not lethal to the host cells (Chasteen et al., 2006). These findings suggest that lowered production of pVIII in the absence of assembly is tolerated by the host cells.

*Secretion machinery*

The inner membrane component of the assembly machinery consists of two proteins, pI and pXI, the latter corresponding to a C-terminal portion of pI that is produced by translation from an internal ATG codon within gI. Both pI and pXI are required for assembly (Haigh and Webster, 1999; Rapoza and Webster, 1995). The pI protein (but not pXI) contains a putative ATP-binding domain necessary for assembly. The analysis of pI has been hampered by its high toxicity to the host cell. It is thought that pI can form a channel and it has been shown to have an ATPase activity (Feng, 2000; Horabin and Webster, 1988). A semi-permeable system has been used to show that the ATP is required for Ff assembly (Feng et al., 1997).

pIV, the outer membrane component of the assembly machinery forms a radially symmetrical 14-meric complex upon insertion into the membrane (Opalka et al., 2003). The pIV multimer forms a barrel-like structure with an outer diameter of about 13.5 nm and height of about 12 nm. The barrel is comprised of three “stacked” rings, an N-terminal ring (N-ring), middle ring (M-ring) and C-terminal ring (C-ring), with a discontinuous pore in the centre that is interrupted by a septum across the M-ring. The pore diameter ranges from 6 nm at the N-ring to 8.8 nm at the C-ring (Opalka et al., 2003). This channel is utilized by the elongating filamentous phage (with a diameter of about 6 nm) to cross the outer membrane (Marciano et al., 2001). N-terminal periplasmic portions of the pIV subunits interact with the inner membrane components of the assembly complex, pI/pXI (Daefler et al., 1997; Russel, 1993).

Interest in pIV was intensified by the discovery of homologues in Gram-negative bacteria. These homologues are collectively referred to as "secretins" (Bayan et al., 2006). The secretins are conserved components of otherwise diverse Gram-negative bacterial systems that export proteins and filamentous phage or assemble type IV pili (Figure 5A). Type II systems are utilized for secretion of hydrolytic enzymes and toxins into the extracellular environment (Johnson et al., 2006; Russel, 1998; Sandkvist, 2001). The related type IV pilus biogenesis system is involved in assembly and extrusion of type IV pili, twitching motility and uptake of DNA from the environment (Craig and Li, 2008; Pelicic, 2008). Type III secretion systems translocate proteins directly from the bacterial cytoplasm into eukaryotic cells. Acting together in a coordinated fashion, the translocated proteins manipulate signal transduction and cytoskeleton dynamics within eukaryotic cell, allowing invasion and intracellular growth of bacteria (Block et al., 2008; Coburn et al., 2007; Galan and Collmer, 1999; Moraes et al., 2008).

Characterization of purified wild-type pIV and a “leaky” point mutant by electrophysiology as well as by *in vivo* experiments established that pIV is a large (6 nm in diameter) gated channel that is blocked by the assembling phage filament (Marciano et al., 1999; Marciano et al., 2001). A simple septum-like occlusion that interrupts the continuity of the secretin channel has been identified in all of the secretins analyzed thus far by cryo-EM and single particle analysis except the type IV pilus biogenesis system secretin PilQ of *N. meningitidis*, which has a complex plug (Frye et al., 2006). The septum or plug is presumably the gate that maintains the channel in a closed state (Chami et al., 2005; Hodgkinson et al., 2009; Marlovits et al., 2004; Opalka et al., 2003; Reichow et al., 2010). We have recently identified two segments in the centre of the conserved C-terminal secretin family domain that likely form the septum: GATE1 (that spans 39 residues) and GATE2 (that spans 14 residues). GATE1 and GATE2 were identified by selecting for “leaky” mutants able to utilize maltooligosaccharides in the absence of maltoporin LamB. Three additional leaky mutations are located in the region that encodes the periplasmic N-terminal module proximal to the M-ring and could be involved in triggering gate opening (Spagnuolo et al., 2010).

A number of filamentous phage lack the gene for the outer membrane channel, posing the question of how they are extruded through the outer membrane. For two of these phage, CTXφ of *Vibrio cholerae* (Waldor and Mekalanos, 1996) and MDAφ of *Neisseria meningitidis* (Bille et al., 2005), it was found that each depend on a host-encoded secretin: CTXφ utilizes EpsD of the type II secretion system (Davis et al., 2000) and MDAφ utilizes PilQ of the type IV pilus assembly system (Bille et al., 2005). The “borrowed” secretin channel EpsD does not require inner membrane components of the type II secretion system in order to secrete CTXφ phage (Davis et al., 2000). Hence, EpsD of *V. cholerae* and presumably PilQ of *N. meningitidis* must function with two dissimilar inner membrane secretion components, those of the phage and the type II secretion system or the type IV pilus assembly systems. This is puzzling, given the strict specificity that is normally observed between the N-terminal domains of a secretin and the cognate inner membrane moiety of its trans-envelope secretion machinery. The inner membrane complexes of secretion machineries are believed to energize the transport and presumably to couple secretin channel opening to secretion (Clausen et al., 2009; Craig and Li, 2008; Johnson et al., 2006; Marlovits and Stebbins, 2010). The production of CTXφ phage is very low, about one per $10^3$-$10^4$ cells, suggesting that the “borrowed” secretin may function at very low efficiency.

**Host responses to secretins and filamentous phage infection**

During the highly productive Ff infection (about 1000 phage per cell per generation), accumulation of the major coat protein pVIII in the inner membrane, together with expression of a few hundred copies of trans-envelope assembly/secretion complexes results in a change in cellular phospholipid metabolism through inhibition of phosphatydilserine synthetase (Chamberlain and Webster, 1978), increased phosphorylation of several proteins including chaperone DnaK (Rieul et al., 1987) and induction of a stress response called the phage shock protein (Psp) response (Brissette et al., 1990; Darwin, 2005; Model et al., 1997).

Originally detected in filamentous phage f1-infected *E. coli* (Brissette et al., 1990), and later identified in a genetic screen for virulence factors in *Yersinia enerocolitica* (Darwin

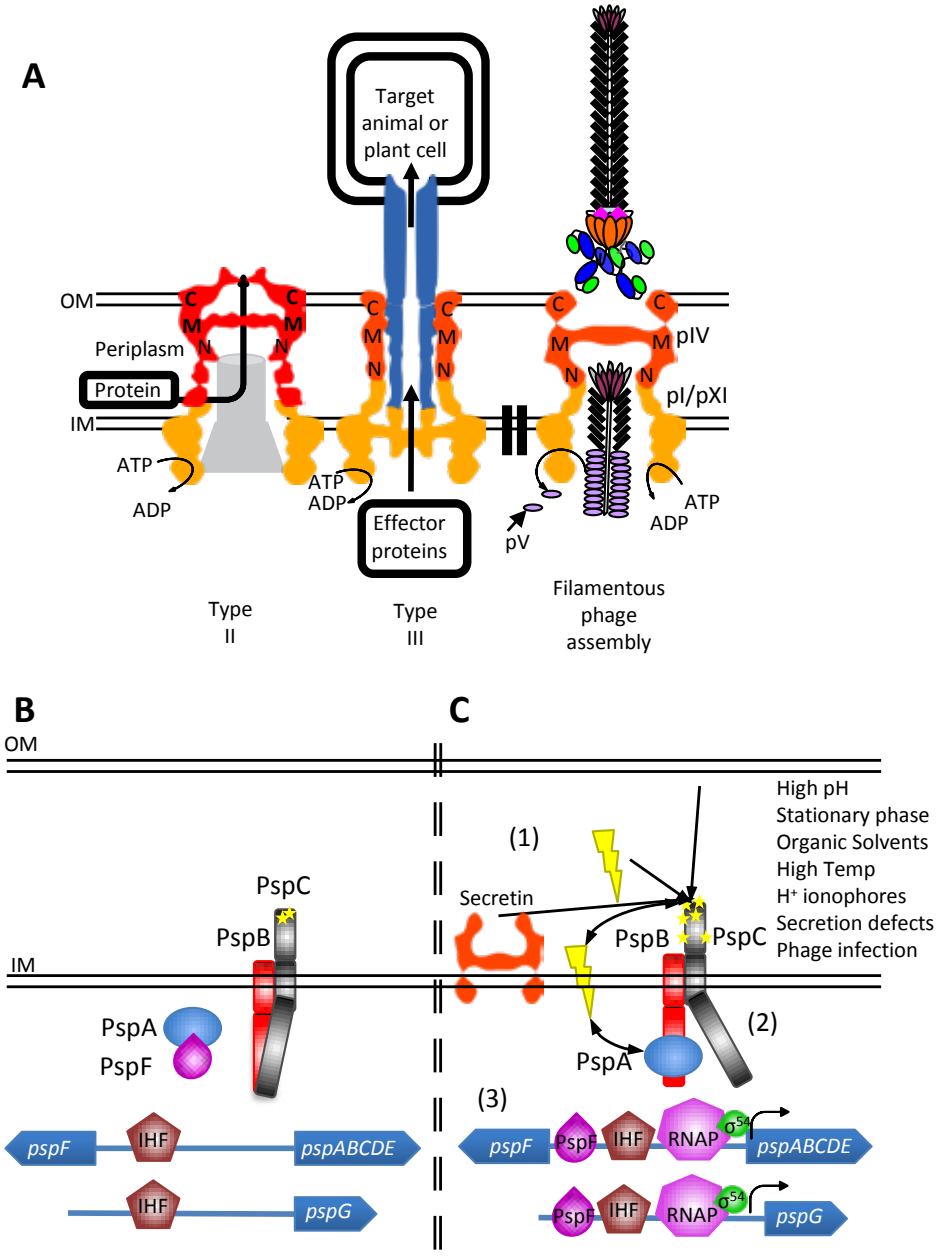


**Figure 5. Secretion systems and *psp* regulon induction. A.** Comparison of three secretion systems. IM components vary between systems and species, however some generalities exist. Export is driven by ATP hydrolysis, and each utilise a similar OM component called the secretin (shown in red). Type II secretion substrates enter the secretion apparatus in the periplasm and are exported to the extracellular milieu. Type III secretion substrates enter a molecular needle (blue) threaded through the centre of the secretion apparatus, and are injected directly into target cells. Filamentous phage assembly. The silhouettes of the inner membrane complexes, all drawn in yellow, are based on the cryo-EM structure of the type III secretion system (Marlovits et al., 2004). The structures of the inner membrane complexes of the type II secretion system and the phage assembly system have not been determined. The secretin channels (red silhouettes) were derived from the respective structures obtained by cryo-EM and single particle analysis (Marlovits et al., 2004; Opalka et al., 2003; Reichow et al., 2010). C, M and N denote the respective "rings" recognizable in the cryo-EM structures of secretins. **B and C**. Induction of the *p*hage *s*hock *p*rotein (*psp*) stress response regulon. **B.** In the absence of stress signals, the *psp* regulon activator PspF is inactive due to its interaction with PspA. **C.** The periplasmic domain of PspC detects stresses, inducing the expression of the *psp* regulon. Orange shape, pIV or other secretin inserted in the inner membrane; yellow shape, other stresses (listed below the outer membrane). When a stress threshold has been reached (1), a conformational shift in the cytoplasmic domain of PspC occurs, uncovering the binding domain of PspB. PspA then interacts with PspB (2), causing the release of response transcription factor PspF and subsequent transcription of the psp regulon (3); (Gueguen et al., 2009). IM, inner membrane; OM, outer membrane.

and Miller, 1999), the *psp* regulon has been found to encode 7 proteins (PspA-G). pspA-E genes constitute the *psp* operon; *pspF*, gene encoding the transcriptional activator of the operon, is located just upstream and is divergently transcribed, while PspG is not linked to the other *psp* genes (Figure 5B).

A wide range of stresses induces the Psp response. Upon induction, PspB and C have been shown to activate expression of the *psp* regulon by releasing PspF from a complex with PspA (Figure 5C; Gueguen et al., 2009; Joly et al., 2010; Jovanovic et al., 2009; Jovanovic et al., 2010; Maxson and Darwin, 2006). The intermediate events that transduce stress signals to PspB and PspC, however, are unclear and the mechanism of stress relief is not well understood (Darwin, 2005; Jovanovic et al., 2009; Jovanovic et al., 2006; Seo et al., 2007). The accumulation of PspA, the most massively-produced protein of the Psp response, requires hours, in contrast to minutes generally required for accumulation of proteins after induction of bacterial promoters. Given that fast *pspA* mRNA accumulation is observed (Spagnuolo et al., 2010), it is possible that post-translational regulation plays a role in the Psp response. More information about the activation of the CRISPR response (Perez-Rodriguez et al., 2011) as well as genome-wide changes in small RNAs, proteome and metabolome or other posttranscriptional and posttranslational processes will likely unlock the secrets of this stress response.

**Phage display technology**

Much of the acquired knowledge about Ff filamentous phage replication and structure has been exploited in phage display technology and derived applications in nanotechnology. In 1990/91 first phage display libraries of both peptides and antibodies were published (Barbas III et al., 1991; Clackson et al., 1991; Cwirla et al., 1990; Devlin et al., 1990; Marks et al., 1991; Scott and Smith, 1990), followed by an explosion of phage display use in protein and antibody engineering. Peptide libraries are mainly used to identify motifs to which important cell-signaling proteins bind (e.g. SH3 domain or BiP chaperone; Blond-Elguindi et al., 1993; Cheadle et al., 1994) or to select for agonists of antagonists of the peptide hormones for a receptor of interest (Lowman et al., 1991; Wrighton et al., 1996). Antibody libraries are used to select recombinant “monoclonal” antibodies that bind to potential therapeutic targets, such as antigens present on the surface of malignant cells (Schier et al., 1995). Besides these two major applications, phage display has also been used to select variants of proteins of interest with changed binding target or increased affinity to targets of interest (e.g. protease inhibitors with increased activity (Roberts et al., 1992) or transcription factors with changed DNA recognition sequence; Rebar and Pabo, 1994). The twenty first century brought the new use for Ff phage and phage display – as a toolkit for assembly of nanostructures (Mao et al., 2004).

*The principles of phage display*

The aim of all phage display library screenings is to find a rare variant in a library. Once constructed, the library must be screened to isolate the variant of interest. To do so, the phage first must be assembled and released from host cells in sufficient numbers to make it likely that a desired variant is present and can be selected. Success will depend on the affinity of the peptide for the ligand and the number of copies of the peptide per virion. For a library of $10^{10}$ variants, representation of every variant demands that, depending on the expected affinity of interaction, a larger number of particles be screened. This is not a problem, because Ff used in phage display technology are produced at concentrations (or titers) of up to$10^{13}$ per ml of culture. Increasing the culture volume and concentrating the phage particles further increases this number.

The basic method for affinity screening of the phage display libraries is often referred to as “biopanning” (Parmley and Smith, 1988). The ligand of interest is immobilized on a solid support, and the phage display library (in the form of purified virions) in solution is applied to it to allow binding of specific variants. Multiple rounds of washing are performed to eliminate the adherent non-binders, and what remains bound is eluted. If no specific eluent is available, low or high pH are commonly used to disrupt interactions of the displayed peptide with immobilized ligand. Because some unspecific binding of phage to the matrix surrounding the ligand occurs, at least three rounds of panning are typically required in order to amplify the binding variants and eliminate the “background” of non-specific binders. However, if several variants of binders are present in the library, the high-affinity variants will outcompete those with low affinity. This could be a problem if, for example, a cDNA library is screened in order to identify the interactome for a ligand of interest, or a peptide library is screened against multiple ligands. Recent reports combined high-throughput sequencing with one or two rounds of panning, to overcome the competition problem and identify a “landscape” of numerous binding variants in a phage display library (Di Niro et al., 2010; Dias-Neto et al., 2009). The readers are referred to phage display manuals for detailed protocols (Barbas III et al., 2001; Clackson and Lowman, 2004; Rodi et al., 2002).

Because of ease of manipulation and exceptional stability of the virions to a broad range of pH and temperatures, Ff filamentous phage continue to be far more frequently used in phage display technology than tailed phage λ and T7. Each of the five Ff virion proteins have been used as platforms for phage display, but most commonly used are the minor protein pIII (Figure 6B-E) and the major protein pVIII (Figure 6G,H). Both proteins in Ff phage have an essential N-terminally located signal sequence which is required for their targeting to the inner membrane. During translocation, the signal sequence is cleaved by signal peptidase, and the N terminus of the mature protein is localized in the periplasm. Therefore, to be displayed on surface, proteins need to be inserted, in frame, between the signal sequence and the mature portion of these two proteins.

Small hydrophobic proteins pVII and pIX (Figure 6F) can also serve as a display platform if a signal sequence is provided at the N-terminus of the insert. Moreover, two subunits of a heterodimeric protein can be displayed in pairs in pVII and pIX in the same host cell, resulting in display of the heterodimeric protein on the surface of the phage (Gao et al., 1999). pVI, the least frequently used protein in phage display, only displays proteins fused to its C-terminus (Jespers et al., 1996).

*PVIII display*

In pVIII display, the number of copies of the foreign insert per virion depends on the size of the displayed peptide. The cut-off length for unbiased display of peptides on every

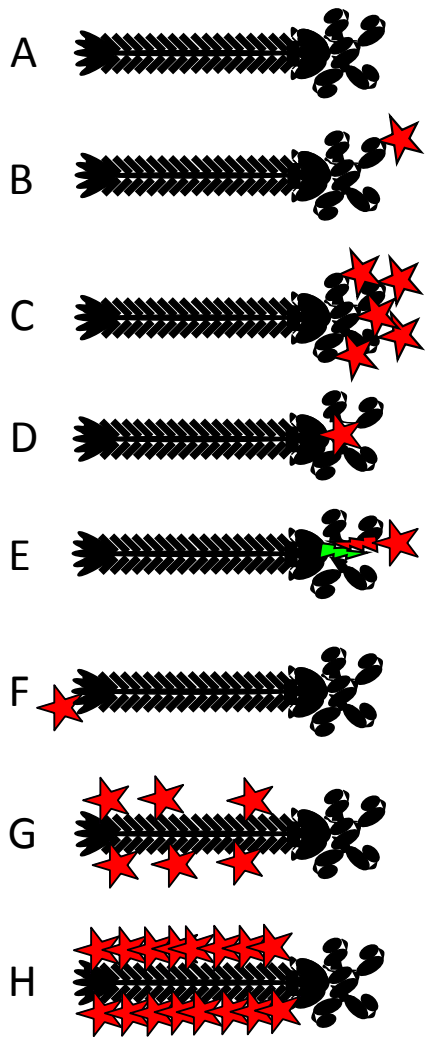


**Figure 6. Types of phage display. A**, Wild-type phage, **B-E**, types of pIII display: **B**, monovalent display, with fusions to the full length pIII; **C**, polyvalent display, fusion with the full length pIII; **D**, monovalent display, with fusion to the C domain of pIII; **E**, indirect phage display in which the green and red connecting shapes between the star-shaped displayed protein and truncated pIII represent the leucine zipper dimerization domains of transcription factors Fos and Jun; **F**, pVII and /or pIX display; **G**, mosaic pVIII display, in which wild-type pVIII is co-incorporated into the virion together with the pVIII fusion. **H**, uniform pVIII display, in which all pVIII subunits are fused to peptides.

pVIII copy is 6-7 residues (Iannolo et al., 1995). In order to display larger peptides and even entire proteins, a wild-type pVIII and display fusions have to be co-assembled into the phage. PVII has also been used for display of heterodimeric protein complexes, by expressing in the same cell two different pVIII fusions which include a cysteine-containing linker. Because pVIII assembles as a dimer, cysteines of the two linkers become covalently linked by an S-S bridge, thereby forming a dimer upon assembly into the virion (Zwick et al., 2000).

*PIII display*

Only the C domain is of pIII is required for assembly of the virion, hence this domain alone (Figure 6D) can be used in display as long as it is preceded by a signal sequence (Barbas III et al., 1991; Griffiths et al., 1993). Given that N1 and N2 domains of pIII are required for infectivity of the particles, if truncated pIII is used for display, a wild-type (full length) pIII must be provided in order to allow easy amplification of phage. As with pVIII, the inserted sequence must be in frame with the upstream signal sequence and downstream mature (or truncated) pIII.

The pIII fusions are most commonly expressed from phagemid vectors, which carry both plasmid and f1 origins of replication, the f1 packaging signal and an antibiotic resistance gene (Barbas III et al., 2001). The phagemid vectors carry a cloning restriction site between sequences encoding the signal sequence and mature full length or truncated pIII. This allows proteins encoded by the inserts to be displayed on the surface of the phage as a fusion with pIII. Phagemid DNA into which inserts have been cloned are introduced into $F^+$ cells, and upon helper phage infection, the phagemids replicate from the f1 origin, resulting in production of ssDNA that is assembled into the virions displaying the proteins encoded by the phagemids. These virions are usually called phagemid particles or transducing particles (TDP). To amplify a phagemid, the particles are mixed with $F^+$ host cells. The phagemid DNA is introduced into the cell by infection, resulting in expression of the antibiotic resistance encoded by the phagemid. In the absence of the helper phage, the phagemid replicates from the plasmid origin of replication.

The phagemid offers flexibility in that the pIII fusion for display can be expressed from an inducible promoter. This is desirable because many pIII fusions are relatively toxic to the host cell. Furthermore, phagemid vectors are generally smaller than the phage genome, thereby increasing the efficiency of transformation, which is the bottleneck limiting the diversity (or primary size) of phage display libraries. Phagemid phage display vectors also offer flexibility in the number of displayed copies of pIII per virion. If wild-type helper phage is used (Figure 6B), there is on average less than one displayed fusion per virion, due to preferential incorporation of wild-type pIII encoded by the helper phage. The frequency of display can be increased by using a helper phage with gene III amber ($gIII^{am}$) mutation in a suppressor strain. The amount of pIII expressed from the suppressed $gIII^{am}$ helper is lower than from the wild-type helper, favoring incorporation of phagemid-expressed pIII fusion (Oh et al., 2007). For display in all five pIII copies (e.g. when libraries are screened for low-affinity interacting partners; Fig 6D), helper phage that carry a deletion of gene III are used (de Wildt et al., 2002; Griffiths et al., 1993; Rakonjac et al., 1997). Recently, a helper plasmid has been constructed, eliminating the need for a helper phage in phage display (Chasteen et al., 2006).

*Phage display of antibodies*

Antibodies are irreplaceable reagents used in diagnostics and detection of trace amount of substances, from pesticides and hormones to viruses. In the early nineties, phage display technology empowered researchers to use *in vitro* phage affinity selection instead of immunization and hybridomas, to successfully isolate monoclonal recombinant

antibodies that recognize the target or antigen of interest. These initial reports showed that when expressed in *E. coli* and assembled into the Ff phage fused to pIII or pVIII, the variable domains of the heavy ($V_H$) and light ($V_L$ and $V_K$) chains can fold correctly and recognize the cognate antigen (Barbas III et al., 1991; Kang et al., 1991; McCafferty et al., 1990). Two types of display were designed, one in which the heavy and light chain variable domains were expressed as separate polypeptides from the same vector and became associated in the *E. coli* periplasm prior to phage assembly (Fab; Barbas III et al., 1991; Kang et al., 1991), and the other where $V_H$ and $V_L/V_K$ domains were expressed as a single polypeptide chain separated by a long flexible linker (scFv; Clackson et al., 1991). These first antibody libraries were amplified from B cell mRNA of immunized animals or volunteers. However, antibodies against human (self) target proteins, to which humans are naturally tolerant, are crucial targets for therapy. Human antibodies against human antigens have already been obtained by in vitro screening pre-immune phage display libraries of antibodies (Griffiths et al., 1993) and used for development of therapeutic recombinant antibodies (Chames et al., 2002; de Haard et al., 1999; Marks et al., 1991; Schier et al., 1996) and targeting gene delivery to specific human cells (Poul et al., 2000).

Initial screening of human pre-immune libraries yields low-affinity antibodies. However, the affinity can be improved by random mutagenesis of the antibody coding sequence in *E. coli*, or by shuffling variable segments of the selected low affinity antibody with a library of variants in order to find a combination with an increased affinity (Schier et al., 1996; Thompson et al., 1996). The other limitation of pre-immune phage display libraries is their primary size. This limitation was overcome by combining two libraries using *in vivo* recombination (Sblattero and Bradbury, 2000; Tsurushita et al., 1996; Waterhouse et al., 1993).

Once an antibody with a sufficiently high affinity for the antigen of interest is isolated, it can be expressed independently of the phage in high amounts for clinical use. Moreover, Ff virions displaying antibody variable chain can be used as detection devices or tracers. The scFv-pIII fusions that are displayed on the tip of the phage filament bind the antigen, whereas the filament, which contains over 2000 copies of pVIII, is used for detection of the bound antibody. Anti-pVIII antibodies or fluorophores conjugated to pVIII are used to “flag” the binding sites, thereby allowing immunohistological detection of the antigen (Li et al., 2010; Sobry et al., 2005; Willats et al., 1999; Zhou et al., 2002)

Besides use in diagnostics and therapy, antibodies generated by phage display are also used as catalysts. This application requires immunizing the experimental animal with an organic molecule that mimics the transition state intermediate in a chemical reaction. The antibodies are then displayed on the phage to select for clones that bind to the transition state intermediate and subsequently tested to determine whether they catalyze the reaction of interest (Baca et al., 1997; Fujii et al., 1998; Janda et al., 1997).

*Protein evolution by phage display*

Phage display has been a powerful tool for evolution of proteins in the laboratory, to create proteins with novel properties. This is achieved by creating a library of variants of a protein of interest, in which the residues required for binding to substrate or interacting partner are randomized. From that library, a variant binding to a new ligand can be selected. One of the most striking examples of protein evolution is that of zinc finger-containing transcription factors (Rebar and Pabo, 1994). These authors randomized the residues of the zinc finger motif that confer specificity for binding to a certain DNA sequence and then screened the library for binding to a different sequence, to obtain a new zinc finger motif with a changed specificity. Another pioneering example is creation of a variant human growth hormone with increased affinity for the receptor (Lowman et al., 1991). Numerous groups have focused on protease inhibitors, randomizing the protease binding sites in order to achieve increased affinity or changed specificity (Koiwa et al., 2001; Roberts et al., 1992).

Early in the history of phage display technology it was confirmed that the enzymes displayed on the surface of the phage are active (McCafferty et al., 1991). In the following years, sophisticated schemes have been developed to isolate, from randomized libraries, the enzymes that catalyze a reaction of interest. One such scheme is to covalently link a substrate to one copy of pIII and the enzyme to another copy of pIII in the same particle. Because of the proximity of the substrate, the variant carries out a single turnover reaction to generate a product. The selection scheme is designed so that the product is specifically captured by the solid phase, thereby capturing the enzyme-encoding phage (Forrer et al., 1999; Gao et al., 1997; Hansson et al., 1997; Soumillion et al., 1994).

*Phage display cDNA libraries*

Determination of interactions between cellular proteins is a key to understanding the essential cellular processes. The yeast two-hybrid system is most frequently used for this purpose (Fields and Song, 1989). However, the interactions in that system are identified *in vivo*. The advantage of phage display is that the interactions are selected *in vitro*, and that the interacting partner (or bait) can be any molecule or molecular complex.

For construction of phage display cDNA libraries, the necessity for the insert to be in frame with both upstream and downstream sequences and in the same orientation as the phage protein to which it is fused effectively decreases the size of the library by a factor of 18. These odds were improved by designing an indirect display system: the library was fused downstream of a dimerizing leucine zipper domain of the transcription factor Jun, and its interaction partner, Fos, was fused to pIII (Figure 6E). The leucine zipper was formed in the periplasm, allowing display of cDNA fusion proteins on the surface of the phage (Crameri et al., 1994). In this setup, fusion of the library inserts to the C-terminus of Fos eliminated the requirement for an in-frame joint downstream of the insert and increased the effective library size from 1/18 to 1/6. This system has been successfully used to identify allergen proteins: phage display cDNA libraries of biological materials that cause the allergy were screened, to identify the protein(s) that induce the allergic reaction. The baits in these screens were IgE antibodies of the patients (Crameri and Blaser, 1996). In another approach, a phage display phagemid vector was designed that contains a removable marker cassette (*lox* sites-flanked β lactamase gene) between the cloning site and gIII. Clones containing an open reading frame in correct

orientation and frame are initially fused to β lactamase, conferring resistance to ampicillin to transformed cells. Following selection on ampicillin, helper phage is used to generate the phagemid particles, which are then transfected into a Cre recombinase-expressing host to remove the β lactamase cassette by Cre-mediated recombination of *lox* sites, resulting in fusion of the inserted open reading frame with pIII (Zacchi et al., 2003). This method, in combination with two rounds of affinity selection and next generation sequencing, was recently used to identify "interactome", a set of proteins that interact with enzyme transglutaminase 2 (TG2), involved in the regulation of cell growth, differentiation and apoptosis (Di Niro et al., 2010).

Protein fusions displayed on the surface of Ff are targeted to the periplasmic compartment. This location is unfavorable for folding of many cytosolic proteins, particularly those carrying cysteines, because periplasmic chaperones such as DsbA catalyze formation of S-S bonds, which do not exist in the cytoplasm, and consequently proper folding of the protein is prevented (Bardwell et al., 1991). As the C terminus of pIII is in the cytoplasm, a vector has been constructed for the pIII C-terminal display of cytosolic proteins (Fuh and Sidhu, 2000). Another approach to avoid misfolding of cytosolic proteins in the periplasm is to replace the standard signal sequence with a Tat signal sequence. The Tat signal sequence allows translocation of folded protein domains through the inner membrane (Velappan et al., 2010). T7 and λ phage, which assemble in the cytosol and therefore eliminate the problem of protein misfolding associated with Ff display, are also used as vectors for the cDNA libraries. Proteins that bind to DNA or RNA sequences of interest, or to chromatin were identified by screening T7 and λ phage display cDNA libraries (Cicchini et al., 2002; Danner and Belasco, 2001; Wang et al., 2004).

*Phage display of bacterial proteins*

Phage display bacterial genomic shot-gun libraries have been used to identify bacterial proteins that bind to targets of interest. For example, this approach was used to identify and map cell-surface-associated agglutinin, RapA, from the *Rhizobium leguminosarum* genome (Ausmees et al., 2001). Recently, a *Borrelia burgdorferi* shot-gun phage display genomic library was used to screen for potential adhesins in the animal (*in vivo*), resulting in discovery of at least five new adhesion proteins (Antonara et al., 2007). Single-gene phage display libraries were utilized to map a fibronectin-binding motif of the protein FNZ of *Streptococcus equi*, the IgG- and β2-glycoprotein-I-binding domains of protein Sbi of *Staphylococcus aureus* and many others (Lindmark et al., 1996; Zhang et al., 1999). Recently, two methods for the selective display of bacterial surface and secreted proteins have been designed and used to systematically characterize this group of bacterial proteins. In both, the phagemid vector encodes the C-terminal domain of pIII, but not for the signal sequence. Thus, only the inserts that encode signal sequence-carrying proteins in frame with pIII are incorporated into the virion. Furthermore, in the absence of pIII from the helper phage (if a Δ*gene III* helper phage is used), termination of phage assembly and virion release depends on the presence of a signal sequence-containing insert fused in frame to pIII of the cloning vector. Affinity screening for a vector-encoded pIII-fused tag (Wall et al., 2003) or direct selection for virion release (Jankovic et al., 2007) were successfully used to obtain clone banks of surface and secreted proteins from Lactobacilli. These phage display clone banks allow functional analyses of individual surface and secreted proteins without a need for systematic cloning, expression and purification. Ff virion is an excellent hapten for immunization (van Houten et al., 2010); hence clone banks of bacterial surface proteins can be used to greatly facilitate screening of vaccine candidates.

*Random peptide libraries*

Finding peptide candidates or "leads" for design of organic compounds that bind to a target of interest has been revolutionized by phage display. In pioneering work, (Scott and Smith, 1990) constructed short peptide libraries and screened them for binding to a ligand of interest. Peptide libraries can vary in the length of the peptides they display; furthermore, they can be constrained by formation of a disulphide bond by including two flanking cysteine residues (Felici et al., 1993). A beautiful example of a successful library screen is isolation of a random peptide agonist analogous to erythropoietin, a polypeptide hormone that stimulates erythrocyte proliferation (Wrighton et al., 1996). A multivalent-display linear peptide library was first used to isolate low affinity binding peptides. Building on the consensus derived from this library, a new library of constrained peptides randomized at some of the residues was constructed, displayed at one copy per virion, and used to select a high affinity peptide.

Peptide display has been taken to a new level by applications in cell and tissue targeting as well as receptor-mediated gene delivery. This was achieved by *in situ* screening of phage display peptide libraries against cells and tissues to isolate specific cell or tissue binding peptides (Pasqualini and Ruoslahti, 1996). The tissue-binding peptides were isolated by injecting mice with the libraries and recovering the phage from specific tissues of interest, or from human breast cancer tissue xenotransplanted into mice. Such peptides were able to target the transplanted tumor for destruction by fusing the peptide to the cancer drug doxorubicin. In addition to tumor tissue binders, peptide ligands that specifically bind to the vasculature of particular organs such as prostate or kidney have been isolated (Arap et al., 1998). These peptides can be used for therapeutic purposes, for example by fusing them to cytokines or other drugs to concentrate them at the target tissue. This strategy aims to relieve systemic toxicity and/or increase the efficiency of delivery to the affected tissue. Phage for display of two different peptides on the same particle has been adapted for organ-specific delivery: tissue-specific peptide was displayed as a fusion to pIII (at the tip of the phage) while an avidin-binding peptide was displayed as a fusion to pVIII, in 100-200 copies along the phage filament, allowing visualization of phage that binds to the specific ligand via its terminally displayed peptide (Chen et al., 2004).

To adapt Ff phage for use in receptor-mediated gene delivery, libraries were screened to identify cell-specific internalization-mediating peptides (Burg et al., 2004; Larocca et al., 2002). The selected peptides were not only used for gene delivery, but also for marking or tracking the target cells by expression of phagemid-encoded reporter protein that was imported into the target cell nucleus along with the internalized phage. Finally, high density phage

display can be used to create diagnostic or tracking devices for biological threat agents, such as spores of the pathogen *Bacillus anthracis* (Williams et al., 2003).

*Bacteriophage as templates for inorganic nanostructures*

Over the last decade, a few research groups resorted to combinatorial libraries of peptides displayed on Ff phage to vastly extend the types of inorganic materials that can be templated by viruses, including bacteriophages. By screening phage or cell surface display libraries, peptides that bind metals (Au, Pt, Pd, Ag) semiconductor materials (GaAs, ZnS, CdS), paramagnetic materials (FePt, CoPt), electrode materials (e.g. $Co_3O_4$, $FePO_4$; (Lee et al., 2009), catalysts (Neltner et al., 2010), $SiO_2$, Zeolites, $CaCO_3$, carbon nanotubes and many others have been isolated (reviewed in Flynn et al., 2003; Kriplani and Kay, 2005; Petrenko, 2008; Sarikaya et al., 2003). Filamentous phage not only allowed library screening to isolate inorganic material-binding peptides, but also served as a template for assembly of the nanometer scale structures (Mao et al., 2004).

Organic-inorganic liquid crystal nanostructures

The tendency of Ff phage to align and form liquid crystalline sheets has been exploited to utilize them as building blocks in assembly of three-dimensional lattices and films. In this application, the Ff particles displaying peptides that bind inorganic molecules at the pIII end of the virion were highly concentrated and exposed to a magnetic field, which orients the particles due to the diamagnetic properties of pVIII major coat protein subunits. This results in formation of liquid crystalline layers of aligned viral filaments interspersed with the inorganic layers composed of the nanocrystals nucleated at the peptides displayed at the tips. When dried, these liquid crystal structures form a very stable film in which phage retain their infectivity for up to seven months (Lee et al., 2002).

The liquid crystalline nature of Ff can be exploited for forming fibers by wet spinning or electrospinning. Wet spinning is achieved by extruding Ff liquid crystals mixed with soluble polymer polyvinyl pyrolidone (PVP) through a capillary tube of 20 µm into a glutaraldehyde solution, which cross-links the PVP and Ff fibers. Depending on the peptides displayed on Ff, these fibers can be used for biomedical and tissue engineering or as templates for synthesis of electronic or optical materials. Moreover, Ff fibers produced by electrospinning can be used for nanofabrication or nanolithography (Lee and Belcher, 2004).

Nanowires and nanorings

High-density displayed nanocrystal-nucleating peptides along the filament of Ff have been used both as nucleators and templates for nanotube assembly. For example, exposure of phage displaying ZnS-nanocrystal-nucleating peptides to the ZnS solution at 0°C resulted in formation of a tube of nanocrystals wrapping the filament completely. Fast Fourier transformation showed that most of the nanocrystals assumed the same orientation relative to the filament (Mao et al., 2003). Furthermore, combination of two peptide specificities, for ZnS and CdS, nucleating the crystals along the filament, formed nanowires composed of both semiconductors (Mao et al., 2004).

Nanowires composed of virus and inorganic crystal can be transformed into inorganic nanowires by annealing, essentially heating to 350°C to eliminate the organic component, most importantly the nucleating peptides which prevent nanocrystals from fusing into a single-crystal nanowire. The resulting nanowires were 475-650 nm long,

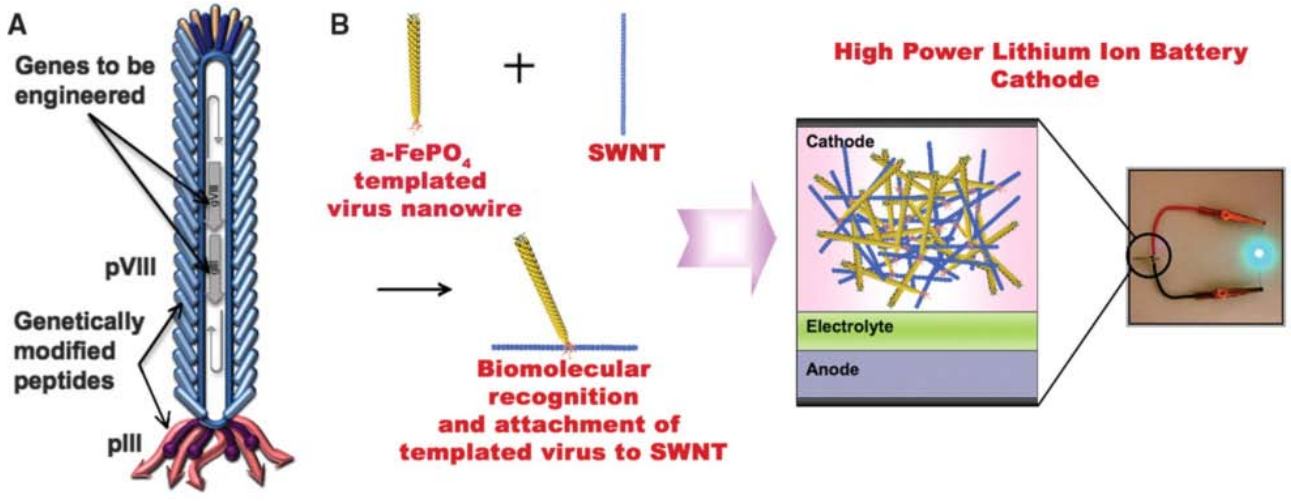


**Figure 7. Phage-templated electrode. A.** A schematic presentation of the multifunctional M13 virus with genetically engineered proteins. The pVIII is modified to serve as a template for a-$FePO_4$ growth; pIII is engineered to have a binding affinity for SWNTs. **B.** A schematic diagram for fabricating genetically engineered high-power lithium-ion battery cathodes using multifunctional viruses (pVIII-pIII system) and a photograph of the battery used to power a green LED. The biomolecular recognition and attachment to conducting SWNT networks make efficient electrical nanoscale wiring to the active nanomaterials, enabling high power performance. These hybrid materials were assembled as a positive electrode in a lithium-ion battery using lithium metal foil as a negative electrode to power a green LED. Active cathode materials loading was 3.21 mg/cm$^2$. The 2016 Coin Cell used, which is 2 cm in diameter and 1.6 mm thick (From Lee et al., 2009, reprinted with permission from AAAS).

with the diameter of 20 nm, retaining the same crystal orientation as prior to annealing (Mao et al., 2003). The same method was applied to the assembly of ferromagnetic CoPt and FePt monocrystalline nanowires, with potential application in ultrahigh-density recording media (Mao et al., 2004; Reiss et al., 2004).

Not only wires, but more complex shapes can be produced by adding different functionalities to the ends of the Ff filaments. To form filamentous phage rings, a phage that carried ligands for a different binding specificity at each end was constructed: a poly-histidine peptide that binds nickel ions was displayed at the pVII/pIX end of the phage, and a streptavidin-binding peptide was displayed at the opposite, pIII end of the filament; an adapter molecule with two binding functions, Ni-NTA and avidin, was used to join the filament ends (Nam et al., 2004).

In 2009 Ff phage had its debut in gadget-making (Lee et al., 2009). In this technological feat, a phage displaying peptide EEEE was displayed on pVIII, allowing assembly of an anhydrous $FePO_4$ nanowire along the filament, while peptide DMPRTTMSPPPR that binds single wall carbon nanotubes was displayed on pIII (on one of the filament ends). Assembling $FePO_4$ nanowires on a network made of Ff filaments and single wall nanotube hybrid nanostructures resulted in a high power lithium ion battery (Figure 7).

## Concluding remarks

Filamentous phage have an enormous impact on many aspects of biology, pathogenesis and physiology of their host bacteria. Many questions remain unanswered, from those that refer to all filamentous phage, such as the structure of the virion caps, mechanisms of infection and assembly and the mechanism and structure of the assembly machinery, to the questions concerning lysogenic phage, specifically their replication and the nature of “virulent” or “superinfectious” phage. Future research into the surprising variety of cooperative arrangements between filamentous phage and their hosts should help elucidate aspects of bacterial adaptation to their environment.

While phage display as a combinatorial method is now a gold standard for finding antibodies and peptides of a desired specificity, its role in nanotechnology is rapidly expanding; discoveries to come will be limited only by the imagination of researchers.

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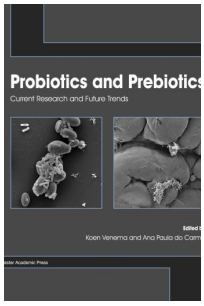


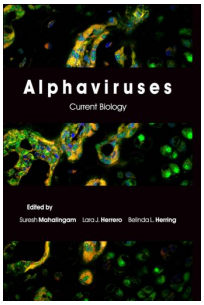

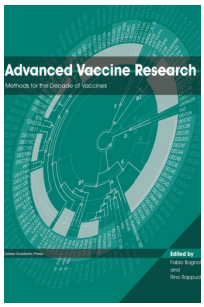


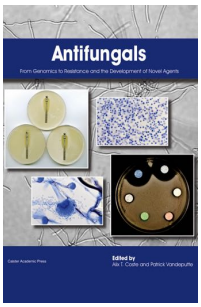


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