"Big Things in Small Packages: The genetics of filamentous phage and effects on fitness of their host"

Anne Mai-Prochnow[1,#], Janice Gee Kay Hui[1], Staffan Kjelleberg[1,2], Jasna Rakonjac[3], Diane McDougald[1,2] and Scott A. Rice[1,2*]

[1] The Centre for Marine Bio-Innovation and the School of Biotechnology and Biomolecular Sciences, The University of New South Wales Australia

[2] The Singapore Centre on Environmental Life Sciences Engineering and The School of Biological Sciences, Nanyang Technological University Singapore

[3] Institute of Fundamental Sciences, Massey University, Palmerston North, New Zealand

[#] Present address: CSIRO Materials Science and Engineering, PO Box 218, Lindfield NSW 2070, Australia

Running head: Filamentous phage and effects on fitness of their host

Key words: *Inoviridae*, *Inovirus*, filamentous phage, M13, Ff, CTX phage, bacteriophage, *E. coli*, *Pseudomonas*, *Vibrio cholerae*, Biotechnology

One sentence summary: It is becoming increasingly apparent that the genus Inovirus, or filamentous phage, significantly influence bacterial behaviours including virulence, stress adaptation and biofilm formation, demonstrating that these phage exert a significant influence on their bacterial host despite their relatively simple genomes.

Abstract

This review synthesises recent and past observations on filamentous phage and describes how these phage contribute to host phentoypes. For example, the CTXφ phage of *Vibrio cholerae*, encodes the cholera toxin genes, responsible for causing the epidemic disease, cholera. The CTXφ phage can transduce non-toxigenic strains, converting them into toxigenic strains, contributing to the emergence of new pathogenic strains. Other effects of filamentous phage include horizontal gene transfer, biofilm development, motility, metal resistance and the formation of host morphotypic variants, important for the biofilm stress resistance. These phage infect a wide range of Gram-negative bacteria, including deep-sea, pressure adapted bacteria. Many filamentous phage integrate into the host genome as prophage. In some cases, filamentous phage encode their own integrase genes to facilitate this process, while others rely on host-encoded genes. These differences are mediated by different sets of 'core' and 'accessory' genes, with the latter group accounting for some of the mechanisms that alter the host behaviours in unique ways. It is increasingly clear that despite their relatively small genomes, these phage exert signficant influence on their hosts and ultimately alter the fitness and other behaviours of their hosts.

Introduction

It is clear that bacteriophage have a significant role in the ecology of microbial communities, biotechnology and molecular biology. Phage include viruses with double- and single-stranded DNA (dsDNA, ssDNA), as well as double- and single-stranded RNA (dsRNA, ssRNA). The majority of known phage are tailed (over 95 %), which may be a reflection of the ease of isolation and identification due to their bacteriolytic activity that results in plaque formation on bacterial lawns. The remaining 5 % of phage display a broad range of morphologies, e.g. filamentous, cubic or pleomorphic.

Here, we will focus on the filamentous phage (Inovirus), which have ssDNA genomes packaged into filament-like virions. These bacteriophage were initially identified in *Escherichia coli* in the early 1960s, represented by F-pilus-specific closely related phage f1, fd and M13 (Loeb, 1960, Hofschneider, 1963, Marvin & Hoffmann-Berling, 1963). These three phage were independently isolated from the USA and European sewage systems, however, they are 98.5% identical in their nucleotide sequence and have over the years been used interchangeably as well as in combination in studies of Ff biology and molecular biology applications. Of these, M13, is probably the best known and was one of the first cloning vectors developed for molecular biology. The CTXφ phage of *Vibrio cholerae* is equally well known and the best described example where horizontal gene transfer of the phage, which encodes cholera toxin (CT), can convert nontoxigenic strains into highly virulent pathogens (Davis & Waldor, 2003, Faruque & Mekalonos, 2014). Filamentous phage from a range of Gram-negative bacteria have subsequently been described, including the *Pseudomonas* Pf phage (Kirov*, et al.*, 2007, Klockgether*, et al.*, 2010, Woo*, et al.*, 2012), *Xanthanomonas* Cf phage (Kuo*, et al.*, 1994), *E. coli* IKe, If1 and If2 phage (Meynell & Lawn, 1968, Khatoon*, et al.*, 1972), *Neisseria* Ngo and Nf phage (Bille*, et al.*, 2005, Kawai*, et al.*, 2006, Piekarowicz*, et al.*, 2006, Piekarowicz*, et al.*, 2014), *Shewanella* SW1 phage (Jian*, et al.*, 2012, Jian*, et al.*, 2013) and the *Ralstonia* RSM phage (Yamada*, et al.*, 2007). The goal of this review is to provide a historical and contextual insight into studies of filamentous phage. In addition, this review will attempt to convey a sense that despite this vast body of knowledge on phage, even well-characterized phage such as Ff continue to reveal new information, demonstrating that in contrast to their small size, they have significant impacts on the evolution and behavior of their bacterial hosts.

Filamentous phage and their distribution

Phage from the Genus *Inovirus* (family *Inoviridae*) (Day, 2011) are characterized by their long and thin filamentous shape (6 - 8 nm in diameter and 800 - 2000 nm in length) and a circular ssDNA

genome (Frost, 1993, Webster, 1996, Russel & Model, 2006, Rakonjac, *et al.*, 2011). The filament is composed of several thousand major coat protein subunits arranged in a helical array around a ssDNA core, with a few copies of the minor coat proteins at each end. The size of the phage is a function of the size of the genome, which ranges from 4 to 12 Kbp (Rakonjac, *et al.*, 2011). Filamentous phage are mostly carried by Gram-negative bacteria, although there are two examples of filamentous phage found in Gram-positive bacteria, B5 from *Propionibacterium freudenreichii* and CAK1 from *Clostridium acetobutylicum* (Kim & Blaschek, 1991, Chopin, *et al.*, 2002).

The classification of viruses remains a complex issue given the lack of universally conserved genes and features. As a consequence, viruses have largely been classified based on physical features, genome structure and host range. This is also true for the filamentous phage, which were initially divided into two groups that were distinguished by the symmetries of the helically arrayed coat protein, as determined by X-ray fibre diffraction (Marvin, *et al.*, 1974). The size and conformation of coat proteins, as well as the overall distribution of intensity of the X-ray fibre diffraction patterns are similar in the two different classes virion structures. However, class I diffraction patterns have some additional meridional reflections, due to a more complex symmetry, five-start helix and two-fold screw axis (C_5S_2 symmetry), whereas class II filamentous phage have simple one-start helix with 5.4 subunits per turn ($C_1S_{5.4}$) (Marvin, 1998). Details of phage structure along the filament as well as the structures of individual subunits of the major coat protein have been solved, however little is known about the structure of the ends of the filament. Detailed structural data have been presented in a recent review by Marvin *et al.* (2014) and will therefore not be discussed here. Class I phage include the well-studied *E. coli* phage Ff (M13, f1 and fd), IKe and If1, (Marvin & Hohn, 1969) while class II consists of the *Pseudomonas* filamentous phage (Pf1) (Hill, *et al.*, 1991). However, the structures of newly discovered filamentous phage have not been routinely analysed by X-ray fibre diffraction, hence they cannot be classified based on the symmetry.

An alternative method to classify or distinguish the filamentous phage is based on phage particle length, which is directly correlated to size of the phage genome (Marvin & Hohn, 1969). In this scheme, the Inoviruses represented by the Ff phage, have maximum lengths of around 870 nm, while the other genus, proposed as Dolichoinovirus (dolicho means long or narrow), includes those that are up to 1.3 μm long, e.g. the Pf phage. Both classification methods appear to result in similar groupings of the phage where Ff phage are separate from the Pf phage. However, since X-ray diffraction is not commonly used to characterize phage, it is not clear if differences in X-ray diffraction are closely tied to differences in phage particle length or differences in the major coat protein structures. More recently, the International Committee on Taxonomy of Viruses has classified the ssDNA rod- or filament-like phage into the family *Inoviridae* with two genera

identified, *Inovirus* and *Plectrovirus* (Day, 2011). Distinguishing features include width, which is approximately 6 nm for *Inovirus* and approximately 15 nm for the *Plectrovirus*. The ratio of the length of the virion to genome size for the *Plectrovirus* is several-fold smaller than for the *Inovirus*, the former appear morphologically as rods and latter as filaments. The diameter and the length differences are due to different packing and structure of ssDNA within the virion between the two genera. Interestingly, *Plectrovirus* prophages are present in many copies throughout chromosomes of their bacterial hosts; possibly due to replication by transposition (Sha*, et al.*, 2000). The host range of the second *Inoviridae* genus, the rod-shaped *Plectrovirus*, is limited to the cell-wall less intracellular bacteria (mollicutes or mycoplasmas) of animals and plants (Day, 2011). For the purposes of this review, we will focus on the genus *Inovirus*, or filamentous phage.

In addition to morphology, *Inovirus* classification is based on genomic organization rather than on nucleotide or amino acid homology, due to the fact that the genes and proteins encoded by the phage are not well conserved across host species. This is exemplified by only 13% amino acid identity between the major capsid proteins of Ff and Pf1 (Table 1). However, the order of many of the core genes, their sizes and membrane topology (predicted reliably from positions of hydrophobic transmembrane helices) tend to be a conserved feature and hence can be used to putatively identify filamentous phage genes. For convention, we will use the nomenclature of Ff (M13, f1 and fd) phage genes and proteins where possible (Table 1). For example, the major coat protein, pVIII or CoaB, is usually between 44 and 86 amino acids in length and is encoded by a gene located in the first half of the genome (from the origin of replication), directly upstream of a gene encoding adsorption protein, pIII (described in more detail below). Additionally, physical properties of the viral particles have been used to describe filamentous phage, including resistance to nucleases with a concomitant sensitivity to proteases (e.g. Nagarse, ficin, subtilisin and papain), sonication, SDS and chloroform treatment (Marvin & Hoffmann-Berling, 1963, Salivar*, et al.*, 1964, Williams & Fenwick, 1967, Minamishima*, et al.*, 1968).

A notable characteristic of filamentous phage is their ability to replicate without killing the host. There are two types of filamentous phage, those that integrate in the host chromosome and non-integrative filamentous phage such as Ff (Rakonjac*, et al.*, 2011), which replicate exclusively as extrachromosomal elements or episomes. Both the integrative and non-integrative filamentous phage meet the criteria defined for 'true lysogens' (Delbrock, 1946), however in contrast to true lysogens, the filamentous phage commonly continually shed viral particles without host cell death, even when inserted into the bacterial genome as a prophage. The chromosomally-inserted filamentous prophage of *V. cholerae* (e.g. VGJφ and CTXφ) and φRSM1 of *Ralstonia solanacearum* can excise from the genome without killing of the host (McLeod*, et al.*, 2005,

Askora, *et al.*, 2011, Das, *et al.*, 2011). In these respects, the bacteria infected permanently with filamentous phage represent an intermediate case where they carry the phage genome either integrated into the genome or episomally, but do not meet the strict definition of a lysogen. To avoid confusion with the term lysogeny, we will use the term "stable infection" to describe the scenario where the phage is either present in the host genome as a prophage or that replicates episomally.

Infection and replication cycles

Filamentous phage infection begins at the cell surface when the virion attaches to the host cell. The large phage-encoded adhesion protein, pIII, determines the specificity of this process by interacting with host surface receptors, which are typically pili or fimbriae. Binding of the virion to the receptor causes the retraction of the pili through the outer membrane, drawing the virion into the host cell periplasm where it interacts with the secondary receptor, TolA. The TolA membrane protein of *E. coli* belongs to a transmembrane complex TolQRA, which is essential for the entry of Ff phage into the host cytoplasm (Reichmann & Holliger, 1997). As the DNA crosses the inner membrane, the sheath of coat proteins is removed and individual pVIII subunits are inserted into the inner membrane to expose the viral ssDNA for replication. The ssDNA phage genome serves as a template for synthesis of the complementary (negative) strand via host RNA and DNA polymerases and DNA gyrase, forming double-stranded circular supercoiled form, called the replicative form (RF) (Higashitani, *et al.*, 1997). Once inside the host bacterial cell, the phage ssDNA can either directly insert into the host genome after conversion to dsDNA (e.g. CTXΦ) or first convert into the RF from, then insert (e.g. VGJΦ) to form a prophage. Alternatively, they can replicate exclusively as an episome (e.g. Ff). In all cases, virions are formed from ssDNA that is produced from double-stranded template, either the RF or prophage, by replication of viral DNA initiated by a rolling-circle mechanism from the positive strand origin of replication, resulting complete phage genome in a form of (positive strand) circular ssDNA. The phage-encoded ssDNA-binding protein, pV, coats the newly synthesized ssDNA, forming a ssDNA-pV complex. An exposed hairpin loop, called packaging signal, targets the ssDNA-pV complex to the assembly sites that are located in the inner-membrane. The assembly sites are composed of phage-encoded proteins pI/pXI, pVII and pIX for packaging into the virions (Russel & Model, 1989) (Fig. 1). The assembly machinery traverses the cell envelope and is composed of the inner membrane complex of pI and pXI, and an outer membrane protein (Feng, *et al.*, 1997, Feng, *et al.*, 1999, Haigh & Webster, 1999, Marciano, *et al.*, 2001).

Genome organization and function

Core genome

The best studied of the filamentous phage are the F pilus-specific *E. coli* phage known as Ff (f1, M13 and fd) phage. The core genome corresponding to genes of Ff, contains up to 11 genes clustered into three groups, coding for replication, assembly and structural genes (Fig. 1). Genomic and metagenomic analyses of bacteria and bacteriophage revealed variations in the core genes in filamentous prophage and free filamentous phage, as described below. Nevertheless, the "core" genes, whether encoded by the phage or prophage genomes, can be defined as a gene set that is required for a complete replication cycle in Gram-negative hosts, comprising infection of the host bacterium, replication and assembly/secretion.

Genes gII, gX, and gV encode proteins that assist in the replication of the RF and prepare newly synthesized ssDNA for assembly (Table 1) (Ray, 1978). Genes gVII, gIX, gVIII, gIII and gVI encode structural proteins that make up the phage particle (Grant*, et al.*, 1981). Gene gVIII encodes the major coat protein. Thousands of the pVIII subunit form a shaft of the filament that that envelops the ssDNA. Genes gVII and gIX encode two small coat proteins located on one tip of the phage particle (Endemann & Model, 1995), and are the first proteins secreted during assembly of the phage particle (Lopez & Webster, 1983). Genes gIII and gVI encode two minor proteins located at the opposite end of the virion filament from pVII and pIX. The pIII and pVI minor coat proteins mediate binding to the host cell receptors and entry during infection (Gailus & Rasched, 1994) as well as release from the host at the end of assembly (Rakonjac*, et al.*, 1999).

Genes gI, gIX and gIV encode proteins that form a trans-envelope complex essential for assembly and secretion the filamentous phage particle as described above (Feng, *et al.*, 1999). Specifically, pI and pXI form an inner membrane complex that is the site of phage assembly; pI has an ATP-binding Walker motif that is required for its function (Russel, 1991). pIV is an outer membrane protein, which forms a large gated channel (made up of 14 identical subunits) for the growing phage particle to pass through (Marciano*, et al.*, 1999, Marciano*, et al.*, 2001, Spagnuolo*, et al.*, 2010). This protein belongs to the secretin family of proteins that serve as outer membrane channels in type II and type III secretion systems and the type IV pilus assembly system found in many Gram-negative bacteria. Loss of either pI, pXI or pIV in Ff phage was shown to prevent assembly (Russel, 1995). Interestingly, a pIV homologue is missing from the genomes of a number of filamentous phage of Gram-negative bacteria, such as the CTXϕ phage, the RSM1 of *R. solanacearum* and Cf1 of *Xanthomonas campestris*. In some instances, the function of pIV is

fulfilled by chromosomally encoded secretins that are normally either part of the host type II secretion system, as has been shown for the *V. cholerae* CTXϕ phage (described below), or type IV secretion system as in *Neisseria meningitidis* MDAφ (Bille*, et al.*, 2005). For example, *V. cholerae* encodes a type II secretion system secretin *epsD*, otherwise mediating secretion of the CtxAB toxin, which functionally substitutes for the phage-encoded gene (Davis*, et al.*, 2000). Filamentous phage that infect Gram-positive bacteria (which do not contain an outer membrane) assemble in the absence of phage- or host-encoded secretin (Chopin*, et al.*, 2002). If pIV (or a host-encoded secretin) fulfills an unknown essential function in phage assembly, in addition to serving as an exit port through the outer membrane, this other assembly function may either be taken over by the extracellular domains of pI or by an as yet unidentified host-encoded protein.

It should be noted that four phage-encoded genes are described as being virulence factors in *V. cholerae* i.e. the zonula occludens toxin (Zot), the core-encoded pilin (Cep), the accessory cholera enterotoxin (ACE) and a protein with unknown virulence function (OrfU) (Johnson*, et al.*, 1993, Waldor & Mekalanos, 1996). However, comparison of the filamentous phage core genome and the CTXϕ phage reveals similar arrangements of the toxin-related genes *cep*, *orfU*, *ace* and *zot* with the core filamentous phage genes VIII, III, VI and I; the phage functions of the proteins encoded by *cep* and *orfU* genes were determined, respectively, as the major coat protein and the anti-receptor (Waldor & Mekalanos, 1996, Heilpern & Waldor, 2003). The *zot* gene has homology to a family of nucleoside triphosphate-binding proteins, including the gene I (gI) products of other filamentous phage (Koonin, 1992). As described above, the gI protein of filamentous phage plays a role as an inner membrane component of the trans-envelope phage assembly complex, (Feng*, et al.*, 1997, Haigh & Webster, 1999). In the absence of CT enterotoxin, *V. cholerae* is still capable of causing diarrhea due to expression of the Zot toxin that increases the permeability of the small intestinal mucosa by affecting the structure of the intracellular tight junctions, zonula occludens (hence the derivation of the name Zot) (Fasano*, et al.*, 1991). Interestingly, the effect of Zot is specific for intestinal cells and has been shown to play no role in lung infection in mice (Fullner*, et al.*, 2002). To the best of our knowledge, the role of pI in binding to epithelial cells as a colonization or virulence factor has not been tested for other filamentous phage. Closer comparison of gI and *zot* indicates that they share significant homology at the 5' end of the gene. However, *zot* has an additional 441 bp at the 3' end that has been linked to the toxin activity of the protein which would suggest that gI homologues that contain the extra 3' toxin domain could play a role in pathogenesis or association with a eukaryotic host (Baudry*, et al.*, 1992). Since gI and *zot* are related and since *zot* encodes an epithelial-cell-binding domain, we will refer to this gene as *zot* for the *Vibrio* phage

and gI for all others lacking this domain and will avoid use of the term 'Zot-like' which is often used to refer to gI homologues.

Accessory genes

In addition to the core genes described above, some filamentous phage carry additional genes that may be unique, or that may be conserved in other, but not all, filamentous phage. We have termed these accessory genes. While many of these genes have no demonstrable function or homology to known genes, some accessory genes have important roles; involvement in integration into the host genome, virulence (toxins) or interaction of the phage with its host. Most filamentous phage have sequences called phage attachment site (*attP*) that are often homologues of specific bacterial DNA sequences that flank the attachment site (named universally *attB*), allowing for integration into the host chromosome. For example, the CTXϕ phage of *V. cholerae* integrates into the *dif* site (recombination site at the chromosomal terminus of replication) (Huber & Waldor, 2002). The CTXϕ genome contains an inverted repeat of two incomplete *dif* sequences which, within the ssDNA genome, anneal to each other to form a hairpin structure that is a functional *attP* site (Das*, et al.*, 2011). Other integration sites have also been described, such as tRNA Gly locus of Pf4 and Pf5 from *Pseudomonas* (Mooij*, et al.*, 2007, Rice*, et al.*, 2009). Integration into these sites is dependent on the phage encoding a homologous region.

Some filamentous phage encode their own integrase, recombinase or transposase genes, including some of the filamentous phage of *Xanthomonas* (Cf1t) (Shieh*, et al.*, 1991)*, Pseudomonas* (Pf1 and Pf4)*, Xylella fastidiosa* (M23 Φ-Lf) (Chen*, et al.*, 2010), *Vibrio* (VSK) and *Ralstonia* (ΦRSM) (Askora*, et al.*, 2009) (Fig. 2). Interestingly, the *Pseudomonas* Pf3 phage lacks an integrase-encoding gene while this function is encoded by other *Pseudomonas* Pf phage, indicating that the Pf3 phage DNA integration mechanism is significantly different from the other Pf phage (described in more detail below). For CTXϕ of *V. cholerae* and other phage that integrate into the chromosome and do not encode an integrase, the insertion of the phage into the host chromosome is mediated by the host-encoded site-specific recombinases, XerC/XerD (McLeod & Waldor, 2004, Askora*, et al.*, 2012), while others rely on a host-encoded transposase (Bille*, et al.*, 2005, Kawai*, et al.*, 2005, Kawai*, et al.*, 2006). This limited distribution of integrase genes amongst phage of the *Inovirus* genus would suggest that the integrase genes are either recently acquired by these phage, or that the integrase genes have been lost in most of the other lineages of filamentous phage in favour of using a host recombinase system. It is interesting to note that phage encoding an integrase tend to have larger genome sizes in comparison to those that do not encode such enzymes. Some host- and phage-encoded integrases can mediate both excision and integration, which may

facilitate the spread of prophage genomes amongst bacterial genera or across families (Askora*, et al.*, 2011, Das*, et al.*, 2011).

Filamentous phage are well-suited to the horizontal exchange of DNA and it is not surprising that some filamentous phage carry virulence factors as part of the accessory gene set. The best understood is cholera toxin, encoded by the *ctxAB* operon within the CTXϕ filamentous phage genome that infects *V. cholerae.* Cholera toxin is an ADP-ribosylating enzyme that causes the characteristic, voluminous rice water stool of cholera (Fig. 2). The distribution of cholera toxin in *V. cholerae* strains is described below. While the φRSM3 phage of *R. solanacearum* does not encode a virulence factor *per se*, it does modify the virulence of its host. This is accomplished by the activity of the phage-encoded gene, ORF15, which modifies expression of the virulence regulators, *phcA* and *phcB* (described below) (Addy*, et al.*, 2012). Thus, phage-encoded genes can influence host virulence indirectly by adding a layer of regulatory control to existing host virulence genes.

Other unique genes identified in filamentous phage genomes have been hypothesized to contribute to interactions between the phage and its host. The Pf4 filamentous phage encodes several genes unique to its genome, including a reverse transcriptase (RT), ABC transporter ATPase, toxin-antitoxin (TA) system and a putative repressor or immunity gene (Rice*, et al.*, 2009). The putative RT and ABC transporter with an ATP binding domain are located at the 5' end of the genome, which may suggest a role in phage replication. In retroviruses, the RT is a multifunctional enzyme required for cDNA synthesis that uses the viral RNA genome as a template (Goff, 1990). While there are no known retroviruses of bacteria, RT has been shown to be encoded in retron elements that are involved in the synthesis of unusual multi-copy, ssDNA extrachromosomal elements (msDNA) (Rice & Lampson, 1996). The Pf4-encoded RT has amino acid motifs indicative of bacterial RTs (e.g. the RYADD box in domain five), but lacks the characteristic 'VTG' sequence in domain seven, suggesting that this RT is not associated with msDNA production (Rice & Lampson, 1996). The role of this putative reverse transcriptase is unclear, given that the filamentous phage genomes are composed of DNA rather than RNA; the latter, not the former, being a template for the RT. However, this polymerase could potentially use ssDNA as template to synthesize a negative strand replication primer, a function that is carried out by host RNA polymerase in Ff phage (Higashitani*, et al.*, 1997). This novel function, if experimentally tested, would be unique to Pf4 phage as it is the only example to date of an *Inovirus* encoding a RT.

The ABC transporter proteins, a homologue of which is encoded by the Pf4 phage genome, are associated with a range of different functions, including DNA replication, protein degradation,

membrane fusion, antibiotic efflux, signal transduction pathways and chemoreceptors (Tam & Saier, 1993, Ogura & Wilkinson, 2001). There are no current reports on the function of this gene in Pf4 and hence, it is unclear if it provides a selective advantage to the Pf4-containing *P. aeruginosa*. Neither the RT nor the ATPase-encoding genes are present in the closely related Pf6 filamentous phage nor are they found in *P. aeruginosa* PAO1 strain chromosome, further suggesting they are not essential for the function of the Pf4 phage (Tay, 2008).

The TA systems often affect cell viability and have been shown to influence motility, biofilm formation, quorum sensing, plasmid or episome maintenance and persistence (Gerdes, 2000, Gerdes*, et al.*, 2005, Fozo*, et al.*, 2008). There are five major types of bacterial TA systems based on the nature and the mechanism of action the antitoxin (Goeders & Van Melderen, 2014). In recent biofilm studies, a TA system was shown to play a role in biofilm formation and the switch between planktonic and sessile lifestyles in *P. aeruginosa* (Wang & Wood, 2011). The *parE-phd* TA system found in the *P. aeruginosa* Pf4 phage is a type II toxin-antitoxin pair that targets DNA gyrase. To date, the Pf4 phage is the only filamentous phage described with a functional TA system in its accessory gene set. Disturbance of the toxin-antitoxin equilibrium in biofilm microcolonies could lead to cell death (Webb*, et al.*, 2004) and the lack of the TA system in the Pf4 deletion mutant may explain why it does not undergo cell death during biofilm development (Rice*, et al.*, 2009).

Several filamentous prophage carry transcriptional repressors, which have various phage-specific functions. The repressor gene in λ is responsible for repressing the lytic cycle, thereby maintaining lysogeny (Oppenheim*, et al.*, 2005). The repressor can interfere with RNA-RNA and DNA-protein interactions that regulate lysogenic conversion (Cheng*, et al.*, 1999). Repressor genes have also been identified in the SW1 filamentous phage of *Shewanella piezotolerans* (Wang*, et al.*, 2007) and in the CTXϕ phage of *V. cholerae* (Waldor*, et al.*, 1997). The RstR repressor of CTXϕ phage regulates replication of the phage by repressing the *rstA* promoter, which controls the expression of all the CTXϕ phage genes required for phage production, thereby maintaining the non-productive prophage in the host chromosome (Quinones*, et al.*, 2005). In the tailed lysogenic phage of *E. coli*, such as λ, the repressors confer immunity to the lysogen against lytic phage superinfection. *P. aeruginosa* PAO1 chromosome encodes two repressors, both with homology to that of the P2 phage, however their functions in cell physiology are yet to be determined. Interestingly, deep sequencing of PAO1 biofilm dispersal cell populations demonstrated that one of these two putative repressor genes accumulates mutations at a disproportionately high rate relative other host genes in this population (McElroy*, et al.*, 2014). The repressors identified in filamentous phage genomes have almost no homology to each other at the nucleotide or amino acid level. The highest similarity

was observed between the *Xanthomonas* Cf1 and *Ralstonia* RSM phage repressors, at less than 20% amino acid similarity. This low level of similarity, also noted above for the pVIII protein, highlights the difficulty in identification of the filamentous phage genes and proteins by homology searches. The *Ralstonia* RSM phage repressor has been shown to play a role in host virulence (Addy*, et al.*, 2012), whilst the repressor of Cf1 filamentous phage of *Xanthomonas* has been identified to be an important component of the immunity system of the host against phage infection (Shieh*, et al.*, 1991, Cheng*, et al.*, 1999, Cheng*, et al.*, 2009).

Phylogenetic relationships

The evolutionary relatedness of filamentous phage that infect different bacterial species, e.g. *E. coli* vs. *V. cholerae*, is apparent through the conserved order of genes in the genome as mentioned above. The conservation of gene order (the synteny) and function argues against convergent evolution of morphologically similar phage. However, individual genes often show low overall amino acid identities, ranging from 8% to 17% when compared to the Ff phage (Table 1), which can makes identification of the genes by homology analysis difficult. The lack of homology could suggest low selection pressure on the maintenance of sequence integrity. Alternatively, a low sequence homology in the core gene set may be a consequence of extensive horizontal gene transfer among phage infecting distantly related bacterial species, resulting in mosaic genomes. This process, in conjunction with the subsequent selection for mutants that fit with the host codon bias or that allow functional integration (interactions) between the proteins encoded by the poly-origin core genome, would ultimately contribute to the overall low primary sequence conservation. Differences in the assortment and sequence of accessory genes among filamentous phage are likely to be a consequence of integration into different sites in the host chromosome and excision by imperfect recombination, thereby mobilising host genes adjacent to the phage integration site (*attB*).

To investigate the phylogenetic relationship between common filamentous phage, phylogenetic trees were generated based on core and accessory genes. Using the 'Phylogeny.fr' platform (Dereeper*, et al.*, 2008) a tree was assembled from the major coat protein pVIII (CoaB), that determines the structure of the virion coat (Fig. 3). This gene is found in all filamentous phage and is the most abundant protein, making it a good gene for comparison. The pVIII subunit is largely an α-helix and is made up of approximately 50 amino acid residues. While different phage can have quite different pVIII sequences, the resulting virion structures are similar. Moreover, all pVIII subunits have a similar acidic N-terminal region, a stretch forming an amphipatic helix, continuing into a hydrophobic helix followed by basic residues near the C terminus (Pederson*, et al.*, 2001). In

most phage (e.g. Pf1, Ff) the pVIII subunit is synthesized with an N-terminal signal sequence which is later removed by a signal peptidase (Marvin, 1998).

The organisation of the phylogenetic tree based on this protein largely follows the expected 16S rRNA gene-based phylogenetic tree of the host at the genus level (Fig. 3A) with some exceptions. The major coat proteins from the Enterobacteria phage group closely together, indicating a high similarity and common origin. Phage that infect the *Vibrio* spp. cluster together, with the exception of the *Vibrio parahaemolyticus* phage KXV237, and three *V. cholerae* phage, VSK, fs1 and CTXϕ. The major coat protein sequence of the *V. parahaemolyticus* phage KXV237 was previously reported to be different from that of the filamentous phage lvpf5 that infects the same species (Nakasone*, et al.*, 1999), but was similar to that of *V. cholerae* phage fs1 and *P. aeruginosa* phage Pf1 (Nasu*, et al.*, 2000). This may suggest that either pVIII of a *V. parahaemolyticus* filamentous phage was acquired by the KXV237 phage or that this phage has jumped the host species barrier from one of the more closely related hosts, *Ralstonia* or *Pseudomonas*, into *V. parahaemolyticus*. More detailed analysis would be needed to determine which of these possibilities is correct.

Phage from the Pseudomonad group appear to be more closely related to each other than to the phage of *Vibrio spp.*, with the exception of Pseudomonas phage Pf3. The Pf3 phage has a genome size and organization similar to the Enterobacteria phage Ff (except for a different order for genes I and IV) (Luiten*, et al.*, 1985). In contrast to Pf1, Pf4 and Pf5, the Pf3 major coat protein is not synthesized with an N-terminal signal sequence and thus is significantly shorter relative to the major coat protein of other Pf phage (Luiten*, et al.*, 1983). This difference may explain the phylogenetic divergence of the Pf3 coat protein relative to the other Pf *Pseudomonas* phage, despite the amino acid sequence conservation within the mature portion of the protein. *Xanthomonas* phage Lf and *Xylella* phage Lf are closely related as shown by their neighbouring position in the tree (Moreira*, et al.*, 2005). The *Xanthomonas* phage Xf appears to be of similar origin to the *Pseudomonas* Pf1, Pf4 and Pf5 phage.

The pI proteins are essential for the assembly of the phage, however in some they can also have a role in the interaction of the host bacterium with the eukaryotic host (Fig. 3B). Their position in the phage genome appears to be conserved, the size of the proteins range from 242 – 461 amino acids and all have a nucleotide triphosphate-binding site. A translational product from an internal start codon within gene I is pXI. Protein XI has an N-terminal membrane-anchor but lacks the NTP-binding domain and is essential for the Ff phage assembly. A complex of pI and pXI form the inner membrane component of a trans-envelope complex (Haigh & Webster, 1999). It is not clear whether pXI is produced in other filamentous phage.

In the full-length product (pI or Zot), the cytoplasmic NTP-binding N-terminal domain is conserved across all filamentous phage. The larger variant of the Zot protein was originally identified in, and was primarily associated with, toxigenic *V. cholerae* strains (Fasano*, et al.*, 1991, Baudry*, et al.*, 1992, Bakhshi*, et al.*, 2008). The Zot protein is encoded by the CTXϕ and the filamentous phage of *V. cholerae*, *Pseudomonas* (Koonin, 1992, Johnson*, et al.*, 1993, Mooij*, et al.*, 2007, Rice*, et al.*, 2009) and *Stenotrophomonas maltophilia* (Hagemann*, et al.*, 2006). In contrast, Ike, I2-2, M13 and If1 from *Enterobacteria* and phage PE226 from *R. solanacearum* (Murugaiyan*, et al.*, 2011) do not contain the zot toxin-specific domain. Overall, relative conservation and presence of distinct grouping makes pI/Zot a good candidate for the study of phylogenetic relationships and possible gene transfer between phage. A tree was therefore assembled based on protein pI/Zot. This analysis of pI suggests that the evolutionary history of the phage genomes follows that of the hosts, although there are some clear exceptions. There are two main groups of *Vibrio* Zot protein homologues. The Zot homologues from *Vibrio* phage VSK, Fs1, VGJ, VEJ, VS12 and VF33 cluster together on the phylogenetic tree, distinct from VFO4K68, VFO3K6, KSF-1 and the CTXϕ Zot homologues, suggesting that the toxin may have been gained independently by the members of these two groups. This is also supported by the observation that the CTXϕ does not cluster with the other *Vibrio* phage based on the pVIII analysis. Interestingly, in the pI - Zot phylogenetic tree, the pI proteins from phage infecting *P. aeruginosa* cluster closely with the Zot protein from CTXϕ. Koonin *et al.* (1992) suggested that both Zot and pI proteins have a similar transmembrane topology. It has been proposed that Zot proteins have evolved from Pf1-like bacteriophage, because the Zot sequence is most closely related to the pI protein from *Pseudomonas* bacteriophage, but more distantly related to pI proteins from other filamentous phage (Koonin, 1992, Di Pierro*, et al.*, 2001). This is also evident from the phylogenetic tree (Fig. 3B).

All pI homologues from Enterobacteria phage lack the putative toxin module at the C-terminal end and cluster together in the tree, indicating that they are closely related. Based on the complete amino acid sequence, pI from the two *S. maltophilia* phage, SHI and SMA9 (Hagemann*, et al.*, 2006), cluster with *Vibrio* and Enterobacteria phage, respectively, suggesting that although SHI and SMA9 infect the same host species, their respective pI homologues have separate origins. Interestingly, the pI of SHI and SMA9 both carry the C-terminal toxin domain found in the Pf and CTXϕ phage. This further supports that this gene was either acquired by SMA9 through horizontal gene transfer from a distantly related phage, e.g. a *Vibrio* or *Pseudomonas* phage, or that the SMA9 phage was acquired by *S. maltophila* from another bacterial host.

Effects of filamentous phage on the host

Enterobacteriaceae phage

It has been shown that after phage infection with Ff phage, *E. coli* experiences envelope stress due to the presence of the large trans-envelope channel and LPS damage, coupled to a hugely increased membrane protein production and translocation, resulting in slowed growth and formation of turbid "plaques" on bacterial lawns; furthermore, the colonies derived from Ff phage-infected cells have changed morphology they are small and transparent (Chen*, et al.*, 2009) (Table 2). In part, slow growth can be attributed to over-expression of gI upon infection and the very existence of a trans-envelope assembly-secretion system, which compromises the integrity of bacterial envelope. For example, it was shown that expression of pI alone in *E. coli* inhibited host protein and RNA expression (Horabin & Webster, 1986). Indeed, the authors also indicated that over-production of pI eventually resulted in host cell death (Horabin & Webster, 1986, Russel, 1995, Horabin & Webster, 1988). This is thought to be due to membrane insertion and loss of the membrane potential (Horabin & Webster, 1988). Thioredoxin, which is involved in detoxification of reactive oxygen species, is also required for Ike, f1 and fd (but not M13) phage assembly in its reduced form independently of its general function as a cofactor for reductases (Russel & Model, 1986). While it has been proposed that the amino terminal end of pI, in the cytoplasm, may interact with thioredoxin (Horabin & Webster, 1988), the observed *E. coli* growth inhibition effect mediated by pI production was independent of thioredoxin. Therefore it is unlikely that the growth effects of pI are associated with oxidative stress responses. Conversely, it has been shown that during phage infection with the exception of gII, inactivation of any single phage gene results in the host cell death (Hohn*, et al.*, 1971). The mechanism behind this cell death is not clear, but it has been observed by electron microscopy that the cells accumulate christae-like invaginations of the inner membrane and become packed-full of the ssDNA-pV complex (Schwartz & Zinder, 1968). This is similar to the observation that inhibition of phage release (by application of antibodies that cross-link the hundreds of phage filaments emanating from bacterial surface or conditions that lead to abortive infection) also results in cell death of *E. coli* (Pratt*, et al.*, 1966, Marvin & Hohn, 1969). While this has not been shown for other filamentous phage, it seems likely that disruption of the balance between phage synthesis and assembly/secretion in highly productive species may be detrimental to the host.

The host-encoded protein that has been shown to accumulate to the highest level upon the Ff phage infection in *E. coli* is the aptly named phage shock protein, PspA (Brissette*, et al.*, 1990, Russel & Kazmierczak, 1993). The *pspA* gene is part of the *psp* regulon (*pspFABCDE* and the unlinked gene

pspG) and is highly induced in response to overproduction of pIV and those secretins that are partially mislocalised into the inner membrane. The inner membrane proteins PspB and C have been shown to sense the stress and release the repression of the *psp* regulon transcription. When stress reaches a critical threshold, a conformational shift in the cytoplasmic domain of PspC uncovers the cytoplasmic domain of PspB, which is then able to sequester the negative regulator PspA away from the transcriptional activator, PspF (Maxson & Darwin, 2006, Gueguen*, et al.*, 2009, Joly*, et al.*, 2010). Expression of the *psp* regulon is induced in response to a range of stressors in addition to phage infection and production of secretins, including the heat stress and the ethanol exposure. Given that many of the phage assembly proteins are inserted into the cell membranes, it is tempting to speculate that *pspA* induction occurs in response to general membrane stress (Joly*, et al.*, 2010). Thus, while phage production is not lethal to the host, it could easily be perceived as a membrane-stressing event, due to the reported secretin mislocalization to the inner membrane, pI toxicity in the inner membrane and overproduction of membrane-targeted pVIII, with a possibility of energy loss due to some dissipation of membrane potential. Interestingly, *P. aeruginosa* does not possess the *psp* regulon, hence it must have different mechanism for coping with the stress of filamentous phage infection (Seo*, et al.*, 2009).

In contrast to Ff, the filamentous phage of *Ralstonia*, *Vibrio* and *Pseudomonas*, have phenotypically remarkable effects on the host, including changes in capsule production, motility, virulence factor expression and biofilm development and will be described in detail in the following sections.

Ralstonia *phage*

R. solanacearum is a soil-borne Gram-negative bacterium that is the causative agent of bacterial wilt in many important crops. Several filamentous phage from *R. solanacearum* are well-characterized and sequenced, including φRSS1, φRSM1, ϕRSM3 ϕRSM4 and PE226. Similar prophage are found in the genomes of various strains of *R. solanacearum*, *Ralstonia pickettii* and *Burkholderia pseudomallei*. The ϕRSM3 and ϕRSM4 phage are closely related to, but differ from, ϕRSM1. The nucleotide sequence of ϕRSM3 is highly conserved relative to ϕRSM1 with the exception of an unknown protein encoded by ORF2 and an adsorption protein responsible for host range determination, encoded by ORF9, which based on its position in the genome could be a gIII homologue (Askora*, et al.*, 2009). Several of the filamentous phage of the *Ralstonia* genus were shown to be important for pathogenicity of the host bacterium (Table 2). Interestingly, harbouring different filamentous phage can either increase or decrease virulence of *R. solanacearum* towards plants, but this effect was observed to be specific for each phage type.

It has been shown that infection of *R. solanacearum* by φRSS1 leads to an altered physiological state and behavior of the bacteria by changing the expression of virulence factors, extracellular polysaccharide (EPS) production and twitching motility. In phage-infected bacterial hosts the global virulence regulator, *phcA,* was found to be induced early and at low cell densities, leading to increased co-regulated EPS synthesis and twitching motility through increased number of functional type IV pili responsible for this type of motility. It was further speculated that phage particles emanating from the assembly sites on the bacterial surface change the cell's hydrophobicity leading to high local cell densities. The enhanced virulence of φRSS1-infected *R. solanacearum* leads to early wilting of tomato plants compared to the non-phage carrying control (Addy*, et al.*, 2012).

Interestingly, strains of *R. solanacearum* that are sensitive to infection by φRSS1 were resistant to infection by another filamentous phage, φRSM1, and vice versa. This would suggest that these phage share common immunity systems that can prevent infection, although the mechanism by which protection is mediated has not been described. This cross-protection from infection is somewhat unexpected since it has been shown that φRSS1 and φRSM1 differ significantly in genome size (6.6 Kbp for φRSS1 and for 9.9 Kbp for φRSM1) and sequence, and they target different *R. solanacearum* strains as their hosts (Yamada*, et al.*, 2007). Cross-protection from infection is also intriguing since phylogenetic analysis based on pVIII suggests that φRSS1 is divergent from ϕRSM1 and ϕRSM3. One possible explanation for the cross-protection is that the low expression of pIII from the prophage may block the periplasmic receptor, TolA, and/or cause retraction of the pilus that serves as a primary receptor for infection, as has been observed for the Ff phage (Boeke*, et al.*, 1982).

The different phage also have quite different effects on their host bacterium. The ϕRSM1- and ϕRSM3- carrying strains have reduced virulence in tomato plants, in contrast to φRSS1-carrying strains, which have increased virulence in the same disease model. A decreased virulence of φRSM3-infected cells was attributed to several factors, including reduced twitching motility and reduced expression of type IV pili, lower levels of β-1,4-endoglucanase activity and extracellular polysaccharides, and reduced expression of some virulence genes (*egl*, *pehC*, *phcA*, *phcB*, *pilT* and *hrpB*) (Addy*, et al.*, 2012). The φRSM3 phage carries a repressor gene (ORF15), which acts on the host-encoded regulators of virulence, *phcA* and *phcB*. Deletion of the ORF15 from the φRSM3 phage genome restores the virulence and levels of PhcA and PhcB in the host bacterium, comparable to the phage-negative cells (Addy*, et al.*, 2012). In this way, the phage appears to influence regulation of one of the key virulence mediators of the host bacterium. Phage such as φRSS1, ϕRSM1 and ϕRSM3 display opposite effects on the virulence of their host bacterium, which is intriguing and it remains to be determined if this is in part due to the individual genetic

capacity of the host or if the phage carry specific determinants that differ that would account for these differences in effects.

In a study aimed at characterizing bacteriophage from *R. solanacearum,* phage PE226 was identified as having a wide range of host strains. This phage has a genome of 5,475 bp and its gI gene shares high homology in the N-terminal region to *zot* from *V. cholerae* and gI from *Pseudomonas* Pf1 phage (Murugaiyan*, et al.*, 2011). Sequence analysis indicates that the gI of PE226 lacks the C-terminal domain associated with binding to epithelial cells. The lack of the Zot-type C-terminal domain most likely also explains why these phage cluster together with the *Vibrio* phage K68, K6 and KSF-1 based on the pI-Zot analysis (Fig. 3B).

Filamentous phage are also present in other *Ralstonia* species. The *R. pickettii* strain 12J was originally isolated from a copper-contaminated lake sediment and is adapted to growth at high levels of copper. This strain was found to contain a filamentous phage that was hypothesized to be important for horizontal gene transfer of a region containing genes encoding for a range of metal-resistance proteins. These include a gene encoding for a blue copper domain protein, one mercury resistance operon, two iron permease-encoding genes, three complete *copABCD* operons, five *czc* genes, five genes encoding RND efflux transporters seven genes encoding the metal-translocating P-type ATPases and eight genes encoding the heavy metal signal/sensor proteins (Yang*, et al.*, 2010). The 12J phage genome sequence is partially syntenic to that of the *R. solanacearum* phage PE226 (Murugaiyan*, et al.*, 2011). With respect to organisation within the phage genome, nine ORFs of these two phage are similar in sequence, while 6 ORFs (ORFs 2 – 7) have identical organisation. Both phage encode a pI/Zot protein where the conserved N-terminal region shows homology to the Zot family protein domain, but lack the Zot-like C-terminal domain (Murugaiyan*, et al.*, 2011). Its role in virulence remains to be elucidated.

Vibrio cholerae *phage*

The CTXϕ phage of *V. cholerae,* which encodes the CtxA and CtxB, subunits of CT, has been well studied, primarily because of its remarkable effect on the host virulence (Table 2). As a result, much of what is known about the filamentous phage, in conjunction with fd, Ff and M13, is based on CTXϕ. In this section, we will describe some of the key aspects of CTXϕ biology and direct the reader to a number of reviews that focus explicitly on CTXϕ (Faruque & Mekalanos, 2003, McLeod*, et al.*, 2005, Faruque & Mekalonos, 2014). *V. cholerae* is a common inhabitant of marine and estuarine habitats. Interestingly, the majority of *V. cholerae* strains are non-toxigenic (do not

possess the CT genes). However, strains that acquire the CTXϕ phage become toxigenic, and such strains, including the Classical and El Tor biotype O1 strains and O139 strains (also called Bengal strains) are associated with epidemics. Indeed, it has been shown that toxigenic El Tor strains can transfer the CTXϕ phage into non-toxigenic, environmental strains, highlighting the role that these phage play in the conversion of non toxigenic strains into pathogens (Choi, *et al.*, 2010). Integration of the CTXϕ genome into *V. cholerae* chromosome is dependent on a functional chromosomal *dif* site. Interestingly, in strains of *V. cholerae* that have a defective *dif* site, integration of a secondary or helper filamentous phage genome, TLC-Knϕ1, contributes the functional phage *dif* sequence through XerCD-specific recombination, to restore a complete chromosomal *dif* site, correcting the defect in cell division and permitting integration of the CTXϕ (Hassan, *et al.*, 2010).

Carriage of the CTXϕ phage and hence, CT, is thought to allow for rapid amplification of *V. cholerae* in the host and dissemination as a consequence of intense diarrhea. Thus, the phage plays an important role in the virulence and dissemination of *V. cholerae*. Infection of *V. cholerae* by the CTXϕ phage is dependent on the host expression of the toxin coregulated pilus, TCP (Waldor & Mekalanos, 1996). Interestingly, this surface receptor is itself encoded by another filamentous phage, VPIϕ; it should be noted that this is a different class of filamentous phage. VPIϕ is suggested to replicate as an extra-chromosomal element and to have a ssDNA genome of approximately 40 Kbp, encoding a putative transposase as well as integrase genes (Karaolis, *et al.*, 1999). However, the determination of whether VPIϕ is a true phage remains unresolved, as it has been suggested that this genetic region does not produce active phage particles (Karaolis, *et al.*, 1999).

The accessory cholera toxin (ACE) of *V. cholerae* has also been suggested to play an important role in infection and diarrhea in the human host. Exposure of intestinal cells to this protein was associated with membrane depolarization as well as fluid secretion (Trucksis, *et al.*, 1993). This gene is located upstream of the *zot* and the two may be transcriptionally linked (Trucksis, *et al.*, 1993). The ACE protein is a homologue of the minor virion protein pVI of other filamentous phage, is an integral membrane protein predicted using the TMHMM algorithm (Krogh, *et al.*, 2001) to have three transmembrane helices prior to incorporation in to the virion. It functions in concert with pIII to release the virion from the bacterial cells at the end of assembly and presumably to facilitate entry of the phage into the host. Since the pVI/ACE protein is present in the virion, even though it is mostly hydrophobic and covered by pIII (Endemann & Model, 1995), it is potentially partially exposed on the surface at the C-terminus (Hufton, *et al.*, 1999). Therefore, unlike Zot, which is shielded from the environment and epithelial cells by the outer membrane,

pVI/ACE could potentially interact with the mammalian host cells to mediate toxicity, although this remains to be experimentally demonstrated. In addition to the putative interactions of the Zot and/or the ACE proteins with the mammalian host, it has also been shown that some phage genes could be expressed in mammalian cells, independent of the bacterial host (Merril, *et al.*, 1971, Bentancor, *et al.*, 2013, Lengeling, *et al.*, 2013), highlighting that the phage-bacteria-host relationship still holds some mysteries to be unraveled. Thus, it would be of particular interest to investigate how these proteins, when applied in a purified form, from the bacterial membrane or viral extracts, interact with mammalian cells. For example, even though the toxin domain of Zot is located in the periplasm, it would be interesting to explore whether this protein could become surface exposed upon cell lysis. Additionally, where the CTXϕ phage penetrate mammalian cells, it is unclear whether this is mediated by a receptor-ligand interaction, as is the case for phage binding to its bacterial host. Similarly, it would be of interest to determine if pVI/ACE homologues from phage other than CTXϕ have similar effects when infecting a mammalian host.

Another question that remains unexplored is the fate of the CTXϕ and toxigenic hosts once out in the environment as they are rarely detected and it has been suggested that the phage therefore does not confer increased environmental fitness to *V. cholerae*, in contrast to its role in colonization of the human host. Some evidence for the loss of the CTXϕ phage from *V. cholerae* was recently reported and may account for the low prevalence of the CTXϕ phage in environmental strains. Kamruzzanman *et al*. (2014) demonstrated that superinfection of CTXϕ-carrying *V. cholerae* results in excision of the phage from the genome and ultimately loss of CTXϕ, as it is not able to reintegrate. This process is mediated by the activity of an antirepressor, RstC, encoded by the adjacent prophage RSϕ that antagonizes the function of RstR of CTXϕ, which is responsible for maintaining CTXϕ in the lysogenic state (Kamruzzaman, *et al.*, 2014). This RSϕ-mediated effect was observed to occur in the intestine of infected mice and ultimately resulted in the recovery of CTXϕ-negative *V. cholerae* isolates. In this way, environmental strains acquire the CTXϕ phage by transduction, which may be a rare event in nature, infect a host, become superinfected by the RSϕ phage and ultimately lose the phage and are distributed back into the environment through the stools of the infected host.

Neisseria phages

Several filamentous phage have been described for the genus *Neisseria*, including Ngoφ6-9 (Piekarowicz, *et al*., 2006), MDA (Bille, *et al.*, 2005) and Nf (Kawai, *et al.*, 2006) (Table 2).

Subtypes of these phage are found in *N. meningitidis* and *N. gonorrhoeae.* Nf phage carry their own transposases for integration, which are involved in phage rearrangements (Kawai*, et al.*, 2006). The MDA phage element from *N. meningitidis* has been studied in detail (Bille*, et al.*, 2005) and has high organizational similarity to other filamentous phage with all core genes present (Bille*, et al.*, 2005). The ORF 8 has similarity to the Zot toxin from *V. cholera* CTXϕ. Similar to CTXϕ, the MDA element uses a chromosomally encoded secretin (PilQ) for its secretion. While the mechanism of infection remains to be elucidated, it was speculated that the MDA island is spread by transformation of chromosomal NDA fragments derived from the lysed *N. meningitidis* cells, in addition to putative receptor-mediated binding and entry of the phage ssDNA genome into the host cell. Integration of the MDA-containing chromosomal fragments acquired by transformation into the recipient's chromosome would in this case be mediated by homologous recombination via the flanking homologous bacterial sequences, rather than through the site-specific (non-homologous) recombination like other *dif*-integrating prophage (Bille*, et al.*, 2005). The occurrence of the MDA island in *N. meningitidis* isolates was correlated with invasiveness of disease causing strains. This may indicate that the MDA phage plays a role in increasing the ability of *N. meningitidis* to invade mammalian cells and if so, the phage would represent an additional virulence determinant. It was also shown that multiple MDA islands can exist within a single *N. meningitides* genome, for example in strains MC58 and FAM18 (Bille*, et al.*, 2005). While it is uncommon to have multiple copies of the same phage type integrated into the host genome, there is a clear precedence for this in *V. cholerae* (which encodes two copies of CTXϕ, described below) and *P. aeruginosa* PAO1 (e.g. Pf4 and Pf6). It was recently reported that a hybrid Ngoϕ6 phage, from *N. gonorrheae,* could infect, replicate and produce phage particles in a range of Gram negative bacteria, including *E. coli*, *Pseudomonas sp.*, *Haemophilus influenza*, and *Paracoccous methylutens* in addition to *Neiserria sicca* (Piekarowicz*, et al.*, 2014). It was further shown that infection and replication was not dependent on the large adhesin (Piekarowicz*, et al.*, 2014), pIII, which is normally required for binding to the host cell receptor and that dictates host cell specificity (Heilpern & Waldor, 2003).

Shewanella *phage*

The first filamentous phage isolated from the deep-sea environment is SW1 that infects *Shewanella piezotolerans* WP3 (Wang*, et al.*, 2007) (Table 2). SW1 shares significant similarities in genome organization with M13 and CTXϕ and its key genes are induced at low temperatures (Wang*, et al.*, 2007, Jian*, et al.*, 2012). SW1 phage was found to contribute to the fitness of its host by regulating genes important for flagellum production (Jian*, et al.*, 2013). Lateral flagella are necessary for

swarming motility which in turn is vital for the host bacterium for the acquisition of nutrients from the deep-sea sediment (Xavier*, et al.*, 2011).

Xanthomonas *phage*

The plant pathogen, *X. campestris,* harbors several different types of phage, including the filamentous phage Cf1t and Cf1c (Kuo*, et al.*, 1991) (Table 2). Phage Cf1c and Cf1t are approximately 7.3 Kbp in size and encode 12 open reading frames, including a site-specific integrase. *Xanthomonas* phage have been shown to play a role in genome rearrangement and strain differentiation as well as affecting growth of the host (Varani*, et al.*, 2013). While Cf1t has very little effect on the growth of the host, Cf1c was found to drastically reduce the growth rate of infected *X. campestris* (Kuo*, et al.*, 1991). Infection with Cf1tv, the superinfective form of Cf1t, leads to the formation of small colony variants and almost all infected cells are killed after 28 h (Kuo*, et al.*, 1994). The formation of small colony variants upon superinfection has also been reported for *P. aeruginosa* when infected by Pf4 (see below) and it is possible that superinfection by filamentous phage selects for SCVs, although the mechanism and selective advantage for this response is not currently understood.

Yersinia phage

YpfΦ (Table 2) is a filamentous phage that infects *Yersinia pestis*, the causative agent of plague. Genome organization of this phage is similar to other filamentous phage; it is comprised of the three genome modules necessary for production of infectious virions. This phage replicates through an extrachromosomal RF, but can also integrate into the chromosomal *dif* site (Chouikha*, et al.*, 2010). Interestingly, deletion of YpfΦ from the host results in alteration of pathogenicity in mice, although it had no effect in the classical flea-borne transmission of *Y. pestis* (Derbise, et al., 2007). It has been suggested that the acquisition of YpfΦ played a major role in the evolution of the highly virulent plague bacterium, because the avirulent ancestor *Y. pseudotuberculosis* does not contain the phage. Moreover, the maintenance of the phage in all pathogenic sublines despite its *in vitro* instability suggests that it was advantageous (for example by increasing its pathogenicity) for the bacterium to maintain the phage (Derbise*, et al.*, 2007).

Pf phage of *Pseudomonas aeruginosa*

P. aeruginosa Pf phage were first described by Takeya and Amako (1966) (Takeya & Amako, 1966) who characterized the plaque morphology as less than 1 mm in diameter, and indicated that the phage (Pf1) had a very limited host range, infecting only one strain, PAK, out of the nearly 50 isolates tested. The Pf4 phage does not form plaques on the wild-type PAO1 carrying the Pf4 prophage, but readily forms plaques when the prophage has been deleted (Rice*, et al.*, 2009). The Pf phage were determined to be twice the size of previously identified Ff (Bradley, 1973). Tests of a range of clinical and laboratory *P. aeruginosa* isolates indicate that most of the strains tested produce filamentous phage particles and hence, it is likely that most *P. aeruginosa* strains, and PAO1 sublines in particular, carry Pf phage (Kirov*, et al.*, 2007, Klockgether*, et al.*, 2010, Woo*, et al.*, 2012). Subsequent Pf phage were identified and given sequential names, Pf1 - Pf6. All of the *P. aeruginosa* filamentous phage harbor the core genes as well as an integrase and a putative repressor C homologue. The primary distinguishing features are in the accessory genes carried by each phage and the number and sizes of these are responsible for the differences in the size of the genome and corresponding phage particle sizes.

The source of the Pf1 phage isolated by Takeya and Amako (Takeya & Amako, 1966) was not identified by the authors; however, Pf2, which they also identified, was isolated from *P. aeruginosa* strain P28 (Minamishima*, et al.*, 1968). Cross-reactivity of Pf1 antibodies with Pf2, indicated that the two phage are serologically related, while neither reacted to antibodies against fd. These authors further demonstrated that infection of strain K (*P. aeruginosa* PAK) with either Pf1 or Pf2, yielding phage-producing colonies, suggesting that the Pf phage could stably infect the *P. aeruginosa* host. The Pf1 phage (ATCC 25lO-Bl) genome sequence was reported in 1991 (Hill*, et al.*, 1991) and was shown to be 7,349 nucleotides in length. Comparison with the previously sequenced Pf3 phage indicated that the genome organization of Pf1 and Pf3 was conserved but they shared little identity at the nucleotide or protein level. The Pf3 phage has a G + C content of 45 % (Luiten*, et al.*, 1985) which is considerably different from the *P. aeruginosa* and the Pf1 genomes which are 67 (Stover*, et al.*, 2000) and 61 % G + C (Hill*, et al.*, 1991) respectively. These results suggest that Pf3 is distinct from the other Pf phage, in agreement with the phylogenetic analyses, which in fact suggest that the Pf3 is more closely related to the CTXϕ and VSK phage of *V. cholerae* than to other Pf phage (Fig. 3A). Altogether, these findings suggest that the Pf3 phage was horizontally acquired by *P. aeruginosa* and may have originated from another species.

Several reports investigated the host ranges of Pf phage and compared the genomes and structures of the Pf phage particles or their capsid proteins (as indicated above), however there are few reports

733 on the biology of these phage. Of the Pf phage, Pf4 is best understood, in part because Pf4 is one of
734 the two filamentous phage gene clusters found in the genome of *P. aeruginosa* PAO1, arguably one
735 of the most commonly studied strains of *P. aeruginosa*. In contrast, the host-origin for Pf1 is
736 unknown and the P28 strain producing Pf2 is not commonly described in the literature. Similarly,
737 the natural host for Pf3 is not defined.

738 The Pf4 genome was shown to contain one of the most highly induced sets of genes during biofilm
739 growth relative to planktonic growth and similarly, these genes were found to be highly upregulated
740 when grown under anaerobic conditions in the presence of nitrate (Platt*, et al.*, 2008). It was
741 subsequently shown that the Pf4 phage was associated with cell death within microcolonies during
742 biofilm development (Table 2). Further studies demonstrated that the cell death phenomenon was
743 linked to a genetic change in the phage particles, where they adopted a superinfective phenotype.
744 Here, superinfective is defined as the ability to cause plaques on a normally insensitive host,
745 containing the Pf4 integrated into the chromosome as a prophage. The superinfective phage is
746 primarily detected during continuous biofilm cultivation system and is not normally isolated from
747 planktonic cultures or batch biofilm systems (Tay, 2008). Interestingly, the superinfective Pf4
748 phage can induce the formation of morphotypic variants, which are observed when the cells are
749 plated onto solid agar subsequent to the shift to the super-infectious Pf4 release. For example, *P.*
750 *aeruginosa* clones isolated from the superinfective Pf4-phage-releasing stage of the continuous
751 biofilm were observed to form small colony variants, mucoid variants and wrinkly variants at a
752 much higher frequency relative to the clones derived from the biofilm releasing the wild-type Pf4
753 (Hui, 2014).

754 Like Pf4, the Pf6 phage of PAO1 can also develop a superinfective form and this can induce
755 morphotypic variant formation (Tay, 2008), In contrast to the Pf4 or Pf6, another filamentous
756 phage, Pf5 of *P. aeruginosa* PA14, does not appear to induce the formation of morphotypic variants
757 under the same continual biofilm conditions (Mooij*, et al.*, 2007). The Pf5 encodes three unique
758 genes at the 5' end of the genome and lacks homologues of Pf4 genes encoding putative ABC
759 transporter, RT and TA. Thus the accessory gene sets of Pf4, Pf5 and Pf6 are distinct from each
760 other and therefore it is unlikely that the *P. aeruginosa* morphotypic switch, as the one caused by
761 Pf4 and Pf6, is related to the accessory genes. This in turn suggests that there are biological
762 differences in the interaction of the different Pf phage with their hosts or that there is an absolute
763 requirement for the phage to be superinfective in order to initiate the formation or selection for
764 morphotypic variants. The mechanisms for this remain to be elucidated to develop a fundamental
765 understanding of the diverse Pf phage and their effects on *Pseudomonas*.

766 The identification of superinfective forms of the Pf4 and Pf6 filamentous phage demonstrates the
767 two major hurdles in identifying filamentous phage and understanding their effect on the
768 corresponding bacterial host. First, detection of filamentous phage requires a sensitive host, which
769 may be difficult to find, considering that the host bacterium has to be prophage-free and contain a
770 cognate receptor. Second, even when a filamentous phage is identified, the major phenotypic
771 effects on the host, that may have a major clinical relevance, may only be observed under certain
772 laboratory growth conditions and may therefore be missed under the standard culture conditions.
773 The formation of morphotypic variants is clinically important because of the appearance of
774 morphotypic variants in the sputum of chronically infected cystic fibrosis patients as well as the
775 detection of phage particles in the sputum.

776 The Pf4 phage also confers additional virulence-related phenotypes on PAO1. For example, the
777 strain from which the Pf4 prophage was deleted using recombinant DNA approach is less virulent
778 in a mouse model of acute lung infection and also forms biofilms that are less stable than the wild-
779 type biofilm when challenged with the surfactant, SDS (Rice*, et al.*, 2009). There is currently no
780 direct explanation for either phenotype as the Pf4 phage does not encode obvious virulence factors
781 nor is the mechanism of surfactant stress resistance clear. The Pf4 encodes a TA system, but there
782 is no evidence to date that these putative addiction systems are directly toxic to mammalian cells, so
783 that their removal from the PAO1 genome would decrease virulence. Other potential toxic proteins
784 could be the pI of Pf4, which shows significant homology to the Zot toxin of *V. cholerae* and, as
785 indicated above, the two may be related. In *V. cholerae*, this protein has been linked to binding to
786 the tight gap junctions in the intestine, thus facilitating infection and virulence. However, the *V.*
787 *cholerae* (and presumably PAO1) Zot protein does not bind to the tight junctions of the lung
788 epithelia, and there is currently no evidence that this protein serves a similar function in *P.*
789 *aeruginosa*. Alternatively, virulence and biofilm stability may be related to the role of the phage in
790 biofilm development, specifically the formation of colony variants and biofilm cell death, as
791 morphotypic variants have increased stress resistance.

792 The cell death observed during biofilm formation could result in the release of DNA (eDNA),
793 which is incorporated into the biofilm matrix and has been shown to play an important role in
794 biofilm development (Whitchurch*, et al.*, 2002). It should be noted that the role of eDNA in biofilm
795 development is typically associated with the early stages of development and hence it is not clear if
796 the phage, via cell lysis, plays a similar role in the later stages, e.g. after microcolonies are already
797 fully formed. While there are no current reports in the literature, it is tempting to speculate that the
798 long, thin filamentous phage, which form bundles when viewed by TEM, act as a structural
799 component of the biofilm matrix, perhaps by forming bridges between cells, the polysaccharides

and eDNA that are abundant within the biofilm. Finally, there remains a possibility that the phage genes are also involved in regulating the PAO1 host genes, opening avenues for future exploration.

Pf6 was initially detected by plaque formation in the Pf4 deletion strain (Tay, 2008) and was subsequently identified through whole genome sequencing of different *P. aeruginosa* sub-lines (Klockgether*, et al.*, 2010). In the latter report, this phage was given the name RGP42 and we suggest it should be subsequently referred to as Pf6 in-line with the current nomenclature. Pf6 is distinguished from the remainder of the Pf phage by the presence, in addition to the core genes, of two genes encoding two putative protein kinases. Pf4 and Pf6 genomes are inserted into two different loci in the PAO1 genome, in tRNA genes at positions PA0729.1 and PA4673.1. The presence of the Pf6 as a second prophage in addition to Pf4 in the same genome was somewhat surprising given that the two phage are closely related. *V. cholerae* also carries two copies of the CTXϕ phage, which are normally present as tandem repeats in El Tor and O139 strains. Phage production is relatively high in these strains and loss of one of the repeat elements results in low or no phage production (Davis*, et al.*, 2000). However, in the Classical strains, the two CTXϕ prophage are separately inserted into a different chromosome (*V. cholerae* has two chromosomes) (Davis*, et al.*, 2000). As noted above, the genes of the Pf4 phage were observed to be the most highly induced during biofilm development in PAO1. Given that the genes of Pf6, which are annotated in the originally sequenced PAO1 genome, were not monitored (Stover*, et al.*, 2000, Klockgether*, et al.*, 2010), it remains uncertain as to whether the observed induction is a combination of the two phage clusters or was specifically due to expression of the Pf4 phage.

Regulation of Pf phage

For λ phage, it is clear that the host-encoded proteins, such as RecA and LexA, play important roles in the control of the lytic-lysogenic switch. Surprisingly, there are few studies directly focused on such regulators for Pf phage and most observations come from global analyses focused on *P. aeruginosa* for other reasons. When the Pf1 genome was sequenced, it was noted that there was a well-conserved Ntr-dependent promoter at the 5' end of gene VIII (also known as PA0723, the *coaB* gene for PAO1) (Hill*, et al.*, 1991), a gene that is strongly expressed during phage replication. The authors also concluded from their analysis that most of the Pf1 promoters are likely to be Ntr-dependent (Hill*, et al.*, 1991). The implication of this is that phage expression is regulated by the alternative sigma factor RpoN, which is typically active under conditions of nitrogen limitation as well as under anaerobic conditions in *P. aeruginosa*. In line with this suggestion, biofilms formed by a PAO1 *rpoN* mutant failed to undergo cell death during biofilm formation, suggesting that the

Pf4 gene expression may be under the control of the RpoN (Webb*, et al.*, 2003). The lack of cell death could be a consequence of reduced or no expression of the type IV pili, which are the primary receptor for the superinfective Pf4 phage and whose expression is, at least in part, dependent on RpoN (Ishimoto & Lory, 1989). Based on this observation, the hypothesis would be that the biofilm microcolonies experience reduced or oxygen depleted conditions, inducing the denitrification pathway, under control of RpoN, which also induces the expression of type IV pili along with the phage and thus, reinfection can occur.

It has been demonstrated that TolA, the essential receptor of filamentous phage for the Ff and CTXϕ infection, was upregulated four-fold during *P. aeruginosa* biofilm development (Whiteley*, et al.*, 2001). Given the almost universal role of TolA as the secondary phage receptor, it is likely that biofilm growth results in conditions that favor phage reinfection. It was previously suggested that the *P. aeruginosa* pili serve as the Pf phage receptors (Bradley, 1973), although it was not confirmed until much later that the type IV pili were indeed the receptors. It was proposed that phage are produced at the poles of *P. aeruginosa* (Bradley, 1973) where the type IV pili are assembled, although the significance of this is not currently understood. The co-localization of the assembly points at the poles for the Pf phage and the type IV pili could be a reflection of the fact that the type IV pilus assembly system secretin PilQ (a homologue of the filamentous phage pIV) (Hobbs & Mattick, 1993) could be used for the Pf4 assembly, as is the case with the MDAφ of *N. meningitidis* (Bille*, et al.*, 2005). However, the Pf4 genome encodes for its own secretin (pIV) and should not depend on PilQ for assembly.

MvaT and MvaU are homologues of DNA binding proteins in the HNS family. Deletion of both genes resulted in increased Pf4 RF production, but this increase was not observed in the single deletion mutants (Li*, et al.*, 2009). The double mutant also produced phage particles that were able to form plaques on the wild-type PAO1 host, suggesting these were superinfective Pf4 mutants. Interestingly, the superinfective phage production, once induced in the double mutant, culture, could not be repressed by overproduction of MvaT and MvaU (Li*, et al.*, 2009). The lack of suppression by MvaTU complementation can be reconciled by mutations in the prophage genome that resulted in the superinfective Pf4, which is no longer repressed by MvaT and MvaU. The induction of superinfective Pf4 is further supported by the observation that the double *mvaT-mvaU* mutations are typically lethal upon induction of superinfective Pf4 phage and the lethality is suppressed by the second-site mutations in the Pf4 prophage or genes encoding the type IV pilus components that prevent, respectively, the Pf4 phage production or infection (Castang & Dove, 2012).

It was reported that superinfection in *P. aeruginosa* is regulated by BfmR, part of a two-component signal transduction pathway (Petrova & Sauer, 2011). Surfactant treatment led to an induction of *bmfR* expression, suggesting that membrane perturbing stresses may induce BmfR, ultimately reducing the amount of phage produced. BmfR was also demonstrated to regulate the expression of a chromosomal anti-toxin gene, *phdA*. When PhdA levels are high, there is a reduction in phage production and decreased biofilm cell death and when low, there is increased phage production and cell lysis in the biofilm (Petrova & Sauer, 2011). This is particularly interesting in light of the observation that the Pf4 phage itself encodes a *phd* homologue that is coupled to a putative toxin gene, *parE* (Webb*, et al.*, 2003). The *phdA* identified by Petrova and Sauer (2011) and the *phd* of the Pf4 prophage genome are independent loci and the significance of the strain carrying two copies of the *phd*, is currently not known.

Another study showed that the primary oxidative stress response protein, OxyR, binds to a sequence within a small open reading frame, *repC*, in the Pf4 prophage genome. RepC has homology to immunity proteins of other phage such as P2 (Wei*, et al.*, 2012). This would suggest that oxidative stress may in part control induction or expression of the Pf4 phage. Thioredoxin was also shown to interact with OxyR in these experiments (Wei*, et al.*, 2012). What is particularly interesting about this observation is that thioredoxin is recruited to the phage assembly site, although it has been shown that its oxygen scavenging properties were not essential for phage production and that the reduced form of the thioredoxin is the active form required for phage assembly (Russel, 1991). This again suggests that there is considerably more to the control of phage production and superinfection than what is currently known.

Biotechnology and applications of filamentous phage

Original applications of Ff (M13, f1 and fd) bacteriophage were originally used as cloning vectors for sequencing and *in vitro* oligonucleotide-directed mutagenesis (Sanger*, et al.*, 1980, Kunkel*, et al.*, 1991, Messing, 1991). In addition, Ff phage, most notably M13, have been used as cloning vectors, called phagemids. Upon infection of cells with a helper phage, phagemids replicate using the phage origin of replication, producing copious amounts of ssDNA which is packaged into filamentous phage-like particles (Russel*, et al.*, 1986, Vieira & Messing, 1987).

The replicative features of the Ff phage have more recently been exploited for use in phage display, a combinatorial technology for identification of rare desirable variants of antibodies, proteins or short peptides in large libraries (Zwick*, et al.*, 1998, Rodi & Makowski, 1999, Bradbury & Marks,

2004). The key to this technology is a physical link between the protein displayed on the surface of the virion and its encapsulated coding sequence (Smith, 1985). The protein-to-coding-sequence link allows amplification of a very small number of proteins or protein variants that are enriched for by binding to the "bait" or a ligand, so that one binder in a library billions of non-binders can be identified. In principle, any bacteriophage can be converted into a display particle. However, because of the small genome size, ease of manipulation by recombinant DNA methods and exceptional stability of the virions to a broad range of pH and temperatures (the latter allowing a variety of binding and elution conditions), the Ff filamentous phage are far more frequently used in phage display technology than tailed phage such as λ and T7.

The Ff phage and phagemid vectors used in phage display are designed for constructing translational fusions to one or more virion proteins that are used as display "platforms" (Smith, 1985). All Ff virion proteins have been used as a platform for display, but most commonly used are the minor protein pIII or the major coat protein pVIII. Examples of multiple proteins being displayed at two different ends and along the filament, using two or more virion proteins as platforms, have also been reported (Huang*, et al.*, 2005, Hess*, et al.*, 2012).

In addition to peptide and antibody libraries, cDNA libraries displayed on filamentous bacteriophage have been constructed and used for identification of interacting proteins (Di Niro*, et al.*, 2010). High-throughput sequencing combined with limited affinity-screening has been used to identify a "landscape" of numerous binding variants in a phage display library, rather than a few high-affinity interacting proteins (Dias-Neto*, et al.*, 2009, Di Niro*, et al.*, 2010).

Phage display has been used in bacteriology and vaccine development, to identify bacterial proteins that bind to targets of interest or to identify suitable vaccine targets, through construction and screening of bacterial shot-gun genomic phage display libraries (Mullen*, et al.*, 2006). For example, this approach was used to identify a cell-surface-associated agglutinin, RapA, from *Rhizobium leguminosarum* (Ausmees*, et al.*, 2001) and adhesins of *Borrelia burgdorferi* (Antonara*, et al.*, 2007). Recently, a selective display of bacterial surface and secreted proteins has been used to characterize this group of bacterial proteins and to identify immunodominant antigens, respectively, in *Lactobacillus rhamnosus* and *Mycobacterium tuberculosis* (Jankovic*, et al.*, 2007, Liu*, et al.*, 2011). This approach was expanded to a metagenome scale, in combination with next-generation sequencing, to identify and display surface and secreted proteins in a microbial community (Ciric*, et al.*, 2014). The Ff virion is an excellent antigen carrier for immunization (van Houten*, et al.*, 2010); the clone banks or libraries of phage-displayed bacterial surface and secreted proteins can therefore be used to facilitate identification of immunodominant antigens, whereas

931 individual antigen-displaying phage or phagemid clones can be amplified and used directly for
932 immunisation.

933 Filamentous phage, other than Ff, have not been used in phage display technology as yet. The site-
934 specific XerCD-dependent integration of the CTXφ into into *V. cholera*e *dif* sequence at the
935 chromosomal replication termini has inspired construction of a chromosome-integrating vector. A
936 CTXφ *dif*-like *attP* site in this vector mediates single-copy integration into the single chromosomal
937 *dif* site in *P. aeruginosa* (Hoang*, et al.*, 2000).

938 Most recently developed applications of phage display technology cross into nanotechnology.
939 Through screening of peptide libraries, peptides were selected that can nucleate nanocrystal
940 assembly of metals (Huang*, et al.*, 2005), semiconductors, paramagnetic aloys (FePt, CoPt; (Mao*, et*
941 *al.*, 2004) electrode ($FePO_4$) (Lee*, et al.*, 2009) and light-harvesting complexes (Dang*, et al.*, 2013).
942 Thousands of the major coat protein subunits displaying nanocrystal-nucleating peptides served as a
943 scaffold for assembly of nanowires (Mao*, et al.*, 2004), while display of distinct tag-binding
944 peptides at the asymmetrical ends of the filament allow assembly of individual filaments into more
945 complex nanostructures, such as nanorings and branched structures (Waites*, et al.*, 1991, Huang*, et*
946 *al.*, 2005, Hess*, et al.*, 2012).

947 The fibrous nature of filamentous phage allows their electrospinning into microfibers. Furthermore,
948 the liquid-crystalline state of the phage at high concentrations ($>10^{12}$ per mL), including the ability
949 to transition between different liquid-crystalline forms, or to form colloidal membranes that can
950 assume controllable shapes (Sanchez*, et al.*, 2012, Sharma*, et al.*, 2014), are opening new
951 opportunities for applications in tissue engineering (Chung*, et al.*, 2011) and colorimetric sensors
952 (Oh*, et al.*, 2014). A curious, but widespread application of the filamentous phage as liquid crystals
953 is their use as an ordering medium for the elucidation of macromolecule structures by Nuclear
954 Magnetic Resonance (Hansen*, et al.*, 1989). The property of the phage liquid crystals to be aligned
955 in strong magnetic fields facilitates alignment of DNA, RNA and many proteins, allowing structural
956 analysis of aligned proteins by dipolar coupling. Pf1 appears to be the preferred filamentous phage
957 in this regard due to a low overall curvature of the filament (Zweckstetter & Bax, 2001).

958

959 ## Future challenges

960 Filamentous phage were described and characterized in the 1960s (Marvin & Hohn, 1969) but have
961 recently received renewed attention. After reviewing current literature, it is clear that our
962 understanding of filamentous phage is rapidly growing but that the effects of these phage on their
963 bacterial host are still underappreciated. Effects range from influencing virulence (Waldor &

964 Mekalanos, 1996, Waldor & Mekalanos, 1996, Addy*, et al.*, 2012, Addy*, et al.*, 2012), biofilm
965 formation (Rice*, et al.*, 2009) and regulating swarming motility (Jian*, et al.*, 2013). To further
966 advance our understanding, it is necessary to revisit the effects of various phage on their respective
967 bacterial hosts.

968 Analysis of the genome structure and organization, phylogenetic relationships as well as the
969 lifestyle of several phage, suggest that two common features of filamentous phage are of note.
970 Firstly, the relationship of filamentous phage with their bacterial hosts is universally characterized
971 by the stable carriage and production of phage particles by the bacterial host and thus represents a
972 stable infective state. The second characteristic feature is the presence or absence of a phage-
973 encoded transcriptional repressor that has the key role in initiating phage replication, assembly and
974 release and its role in the filamentous phage relationship with the bacterium as well as relationship
975 of phage-carrying bacterium with its plant or animal host. The phage transcriptional repressor may
976 play a significant role in the lifestyle of the host bacterium, as its presence in the phage genome has
977 been linked to increase in bacterial virulence (Yamada, 2013). Coupled with further studies of
978 phage-bacteria interactions, the presence or absence of repressor-encoding genes may be useful in
979 predicting aspects of the phage life-cycle or its effects on the bacterial host that would have a
980 consequence on the pathogenicity of the bacterium and in turn would influence consideration of a
981 filamentous phage for use in the pathogen control.

982 Some studies on *Pseudomonas* and *Ralstonia* phage have highlighted the phenomenon of
983 filamentous phage superinfection, where the normally resistant bacterial host (containing the
984 prophage integrated into its genome) nevertheless supports the infection, replication and plaque
985 formation (Rice*, et al.*, 2009, Yamada, 2013, Askora*, et al.*, 2014). Superinfection has particular
986 importance for the lifestyle and virulence of the host bacterium. For the filamentous phage,
987 superinfection is required for plaque formation on a lawn of a stably infected host, therefore this
988 state of infectivity overcomes the resistance of the lysogen and allows identification of both the
989 phage and the host. The mechanism of superinfection is still unclear and will be further elucidated
990 by future studies, in particular because of its dramatic effect on virulence and bacterial physiology
991 of both *Pseudomonas* and *Ralstonia*. In particular, the role of inactivation of a phage-encoded
992 repressor, proposed to be involved in acquiring the superinfective state, needs to be investigated.

993 Because of their non-lytic lifestyle and ease of genetic manipulation, filamentous phage are used in
994 a variety of applications, including phage display technology (Devlin*, et al.*, 1990, Clackson*, et al.*,
995 1991), assembly of nanostructures (Mao*, et al.*, 2004) and synthesis of biosensors (Lee*, et al.*,
996 2013). However, other applications are feasible, for example, their use in phage therapy of bacterial

997 infectious diseases. It was already shown that an M13 vector genetically engineered to suppress the
998 SOS DNA repair response can enhance stress-induced killing of bacteria, including antibiotic
999 resistant cells, biofilm and persister cells (Lu & Collins, 2009). During their normal lifecycle
1000 filamentous phage do not lyse or otherwise kill the host bacterium. However, mutations in specific
1001 genes in the phage genome that prevent assembly and secretion of progeny phage lead to death of
1002 the host bacterium (Pratt*, et al.*, 1966, Marvin & Hohn, 1969). Thus, a strategy of de-regulating
1003 phage gene expression in such a way that it results in decreased virulence, growth inhibition and/or
1004 killing of the host, may be utilized to engineer filamentous phage for applications in therapy of
1005 diseases caused by pathogenic bacteria.

1006

1007 ## Conclusions

1008 The filamentous phage have been studied for some fifty years and have played an important role in
1009 the development of molecular biology technology as well as our understanding of gene regulation.
1010 These phage, which do not normally kill their host, are widely distributed in the Gram-negative
1011 bacteria,. Despite having relatively simple genomes, it is increasingly apparent that they can have
1012 high impact on the physiology, adaptation and virulence of their host bacteria. As novel
1013 filamentous phage are being constantly discovered, it becomes apparent that, besides the core genes
1014 that are common to all, each newly discovered phage contains a distinct and novel set of accessory
1015 genes, as well as novel variations to the modes of relationships with their hosts. This variety adds
1016 to growing evidence that filamentous phage are important mediators of horizontal gene transfer,
1017 resulting in novel filamentous phage variants, novel virulent strains of pathogenic bacteria and
1018 novel impacts on physiology of their hosts. We submit therefore that there is yet a great deal to be
1019 discovered about this group of phage and their contribution to biology, physiology and
1020 pathogenicity of their host bacteria.

1021

1022 ## Acknowledgements

1023 Funding support (SR and DM) was provided by the Australian Research Council (DP110104525)
1024 and the Centre for Marine Bio-Innovation. JGKH was supported by an Australian Post-Graduate
1025 Award. Support to the JR laboratory was provided by an Anonymous Donor, the Royal Society of
1026 New Zealand Marsden Council Grant MAU210, Massey University Research Fund, Palmerston
1027 North Medical Research Fund, Lottery Health Board and Grant 280289 and New Zealand
1028 Foundation for Research and Technology contract C03X0701. Funding was also provided by the

1029 NRF and Ministry of Education Singapore under its Research Centre of Excellence Programme. We
1030 are indebted to Marjorie Russel for her insightful comments that helped improve this manuscript.
1031 The authors state that they have no conflict of interest.

1032

References

Addy HS, Askora A, Kawasaki T, Fujie M & Yamada T (2012) Loss of virulence of the phytopathogen *Ralstonia solanacearum* through infection by phiRSM filamentous phages. *Phytopathology* **102**: 469-477.

Addy HS, Askora A, Kawasaki T, Fujie M & Yamada T (2012) The filamentous phage varphiRSS1 enhances virulence of phytopathogenic *Ralstonia solanacearum* on tomato. *Phytopathology* **102**: 244-251.

Antonara S, Chafel RM, LaFrance M & Coburn J (2007) *Borrelia burgdorfer*i adhesins identified using in vivo phage display. *Mol Microbiol* **66**: 262-276.

Askora A, Abdel-Haliem ME & Yamada T (2012) Site-specific recombination systems in filamentous phages. *Mol Genet Genomics* **287**: 525-530.

Askora A, Kawasaki T, Fujie M & Yamada T (2011) Resolvase-like serine recombinase mediates integration/excision in the bacteriophage [phi]RSM. *J Biosci Bioen* **111**: 109-116.

Askora A, Kawasaki T, Fujie M & Yamada T (2014) Insights into the diversity of φRSM phages infecting strains of the phytopathogen *Ralstonia solanacearum* complex: regulation and evolution. *Mol Gen Genom* 1-10.

Askora A, Kawasaki T, Usami S, Fujie M & Yamada T (2009) Host recognition and integration of filamentous phage ϕRSM in the phytopathogen, *Ralstonia solanacearum*. *Virology* **384**: 69-76.

Askora A, Kawasaki T, Usami S, Fujie M & Yamada T (2009) Host recognition and integration of filamentous phage phiRSM in the phytopathogen, *Ralstonia solanacearum*. *Virology* **384**: 69-76.

Ausmees N, Jacobsson K & Lindberg M (2001) A unipolarly located, cell-surface-associated agglutinin, RapA, belongs to a family of Rhizobium-adhering proteins (Rap) in *Rhizobium leguminosarum bv. trifolii*. *Microbiology* **147**: 549-559.

Bakhshi B, Pourshafie MR, Navabakbar F & Tavakoli A (2008) Genomic organisation of the CTX element among toxigenic *Vibrio cholerae* isolates. *Clin Microbiol Infect* **14**: 562-568.

Baudry B, Fasano A, Ketley J & Kaper JB (1992) Cloning of a gene (zot) encoding a new toxin produced by *Vibrio cholerae*. *Infect Immun* **60**: 428-434.

Bayer ME & Bayer MH (1986) Effects of bacteriophage fd infection on *Escherichia coli* HB11 envelope: a morphological and biochemical study. *J Virol* **57**: 258-266.

Bentancor LV, Mejias MP, Pinto A*, et al.* (2013) Promoter sequence of shiga toxin 2 (Stx2) is recognized *in vivo*, leading to production of biologically active Stx2. *Mbio* **4**: 501-513.

Bille E, Zahar JR, Perrin A*, et al.* (2005) A chromosomally integrated bacteriophage in invasive meningococci. *J Exp Med* **201**: 1905-1913.

Boeke J, Model P & Zinder N (1982) Effects of bacteriophage f1 gene III protein on the host cell membrane. *Mol Gen Genet* **186**: 185-192.

Bradbury ARM & Marks JD (2004) Antibodies from phage antibody libraries. *J Immunol Meth* **290**: 29-49.

Bradley DE (1973) Length of filamentous *Pseudomonas aeruginosa* bacteriophage Pf. *J Gen Virol* **20**: 249-251.

Brissette JL, Russel M, Weiner L & Model P (1990) Phage shock protein, a stress protein of *Escherichia coli*. *Proc Natl Acad Sci USA* **87**: 862-866.

Campos J, Martinez E, Izquierdo Y & Fando R (2010) VEJ{phi}, a novel filamentous phage of *Vibrio cholerae* able to transduce the cholera toxin genes. *Microbiology* **156**: 108-115.

Campos J, Martinez E, Suzarte E*, et al.* (2003) VGJ phi, a novel filamentous phage of *Vibrio cholerae,* integrates into the same chromosomal site as CTX phi. *J Bacteriol* **185**: 5685-5696.

Castang S & Dove SL (2012) Basis for the essentiality of H-NS family members in *Pseudomonas aeruginosa*. *J Bacteriol* **194**: 5101-5109.

Chen J & Civerolo EL (2008) Morphological evidence for phages in *Xylella fastidiosa*. *Virol J* **5**: 75.

1083 Chen J, Xie G, Han S, Chertkov O, Sims D & Civerolo EL (2010) Whole genome sequences of two
1084 *Xylella fastidiosa* strains (M12 and M23) causing almond leaf scorch disease in California. *J*
1085 *Bacteriol* **192**: 4534.
1086 Chen Y-Y, Wu C-C, Hsu J-L, Peng H-L, Chang H-Y & Yew T-R (2009) Surface rigidity change of
1087 *Escherichia coli* after filamentous bacteriophage infection. *Langmuir* **25**: 4607-4614.
1088 Cheng CM, Wang HJ, Bau HJ & Kuo TT (1999) The primary immunity determinant in modulating
1089 the lysogenic immunity of the filamentous bacteriophage cf. *J Mol Bio* **287**: 867-876.
1090 Cheng JL, Zhou X, Chou TF, Ghosh B, Liu BL & Wagner CR (2009) Identification of the amino
1091 acid-AZT-phosphoramidase by affinity T7 phage display selection. *Bioorg Med Chem Lett* **19**:
1092 6379-6381.
1093 Choi S, Dunams D & Jiang SC (2010) Transfer of cholera toxin genes from O1 to non-O1/O139
1094 strains by vibriophages from California coastal waters. *J Appl Microbiol* **108**: 1015-1022.
1095 Chopin MC, Rouault A, Ehrlich SD & Gautier M (2002) Filamentous phage active on the gram-
1096 positive bacterium *Propionibacterium freudenreichii*. *J Bacteriol* **184**: 2030-2033.
1097 Chouikha I, Charrier L, Filali S, Derbise A & Carniel E (2010) Insights into the infective properties
1098 of YpfPhi, the *Yersinia pestis* filamentous phage. *Virology* **407**: 43-52.
1099 Chung WJ, Oh JW, Kwak K*, et al.* (2011) Biomimetic self-templating supramolecular structures.
1100 *Nature* **478**: 364-368.
1101 Ciric M, Moon CD, Leahy SC*, et al.* (2014) Metasecretome-selective phage display approach for
1102 mining the functional potential of a rumen microbial community. *BMC Genomics* **in press**.
1103 Clackson T, Hoogenboom HR, Griffiths AD & Winter G (1991) Making antibody fragments using
1104 phage display libraries. *Nature* **352**: 624-628.
1105 Crowther RA (1980) Structure of bacteriophage Pf1. *Nature* **286**: 440-441.
1106 Dang X, Qi J, Klug MT*, et al.* (2013) Tunable localized surface plasmon-enabled broadband light-
1107 harvesting enhancement for high-efficiency panchromatic dye-sensitized solar cells. *Nano Lett* **13**:
1108 637-642.
1109 Das B, Bischerour J & Barre FX (2011) VGJφ integration and excision mechanisms contribute to
1110 the genetic diversity of *Vibrio cholerae* epidemic strains. *Proc Natl Acad Sci USA* **108**: 2516-2521.
1111 Davis BM & Waldor MK (2003) Filamentous phages linked to virulence of *Vibrio cholerae*. *Curr*
1112 *Opin Microbiol* **6**: 35-42.
1113 Davis BM, Moyer KE, Boyd EF & Waldor MK (2000) CTX prophages in classical biotype *Vibrio*
1114 *cholerae*: Functional phage genes but dysfunctional phage genomes. *J Bacteriol* **182**: 6992-6998.
1115 Davis BM, Lawson EH, Sandkvist M, Ali A, Sozhamannan S & Waldor MK (2000) Convergence
1116 of the secretory pathways for cholera toxin and the filamentous phage, CTXϕ. *Science* **288**: 333-
1117 335.
1118 Day LA (2011) *Family Inoviridae*. Elsevier Academic press, San Diego.
1119 Delbrock M (1946) Bacterial viruses or bacteriophages. *Biolp Revp* **21**: 30-40.
1120 Derbise A, Chenal-Francisque V, Pouillot F*, et al.* (2007) A horizontally acquired filamentous
1121 phage contributes to the pathogenicity of the plague bacillus. *Mol Microbiol* **63**: 1145-1157.
1122 Dereeper A, Guignon V, Blanc G*, et al.* (2008) Phylogeny.fr: robust phylogenetic analysis for the
1123 non-specialist. *Nucleic Acids Res* **36**: W465-469.
1124 Devlin JJ, Panganiban LC & Devlin PE (1990) Random peptide libraries: a source of specific
1125 protein binding molecules. *Science* **249**: 404-406.
1126 Di Niro R, Sulic AM, Mignone F*, et al.* (2010) Rapid interactome profiling by massive sequencing.
1127 *Nucleic Acids Research* **38**. e110.
1128 Di Pierro M, Lu R, Uzzau S*, et al.* (2001) Zonula occludens toxin structure-function analysis:
1129 Identification of the fragment biologically active on the tight junctions and of the zonulin receptor
1130 binding domain. *J Biol Chem* **276**: 19160-19165.
1131 Dias-Neto E, Nunes DN, Giordano RJ*, et al.* (2009) Next-generation phage display: Integrating and
1132 comparing available molecular tools to enable cost-effective high-throughput analysis. *Plos One* **4**.
1133 e8338.

1134 Endemann H & Model P (1995) Location of filamentous phage minor coat proteins in phage and in
1135 infected cells. *J Mol Biol* **250**: 496-506.
1136 Faruque SM & Mekalanos JJ (2003) Pathogenicity islands and phages in *Vibrio cholerae* evolution.
1137 *Trends Microbiol* **11**: 505-510.
1138 Faruque SM & Mekalonos JJ (2014) Phage-bacterial interactions in the evolution of toxigenic
1139 *Vibrio cholerae*. *Virulence* **3**: 556-565.
1140 Fasano A, Baudry B, Pumplin DW, Wasserman SS, Tall BD & Ketley JM (1991) *Vibrio cholerae*
1141 produces a second enterotoxin, which affects intestinal tight junctions. *Proc Natl Acad Sci USA* **88**:
1142 5242-5246.
1143 Fasano A, Baudry B, Pumplin DW, Wasserman SS, Tall BD, Ketley JM & Kaper JB (1991) *Vibrio*
1144 *cholerae* produces a second enterotoxin, which affects intestinal tight junctions. *Proc Natl Acad Sci*
1145 *USA* **88**: 5242-5246.
1146 Feng JN, Russel M & Model P (1997) A permeabilized cell system that assembles filamentous
1147 bacteriophage. *Proc Natl Acad Sci USA* **94**: 4068-4073.
1148 Feng JN, Model P & Russel M (1999) A trans-envelope protein complex needed for filamentous
1149 phage assembly and export. *Mol Microbiol* **34**: 745-755.
1150 Fozo EM, Hemm MR & Storz G (2008) Small toxic proteins and the antisense RNAs that repress
1151 them. *Microbiol Mol Biol Rev* **72**: 579-589.
1152 Frost LS (1993) Bacterial conjugation. *Conjugative pili and pilus-specific phages.,*(Clewell DB,
1153 ed.), pp. 189-221. Plenum Press, New York.
1154 Fullner KJ, Boucher JC, Hanes MA*, et al.* (2002) The contribution of accessory toxins of *Vibrio*
1155 *cholerae* O1 El Tor to the proinflammatory response in a murine pulmonary cholera model. *J. Exp.*
1156 *Med.* **195**: 1455-1462.
1157 Gailus V & Rasched I (1994) The adsorption protein of bacteriophage fd and its neighbour minor
1158 coat protein build a structural entity. *Eur J Biochem* **222**: 927-931.
1159 Gerdes K (2000) Toxin-Antitoxin modules may regulate synthesis of macromolecules during
1160 nutritional stress. *J Bacteriol* **182**: 561-572.
1161 Gerdes K, Christensen SK & Lobner-Olesen A (2005) Prokaryotic toxin-antitoxin stress response
1162 loci. *Nat Rev Microbiol* **3**: 371-382.
1163 Goeders N & Van Melderen L (2014) Toxin-antitoxin systems as multilevel interaction systems.
1164 *Toxins* **6**: 304-324.
1165 Goff SP (1990) Retroviral reverse transcriptase: synthesis, structure and function. *J Acq Imm Def*
1166 *Synd* **3**: 817-831.
1167 Grant RA, Lin TC, Koningsberg V & Webster RE (1981) Structure of the filamentous
1168 bacteriophage f1. *J Biol Chem* **256**: 539-546.
1169 Gueguen E, Savitzky DC & Darwin AJ (2009) Analysis of the *Yersinia enterocolitica* PspBC
1170 proteins defines functional domains, essential amino acids and new roles within the phage-shock-
1171 protein response. *Mol Microbiol* **74**: 619-633.
1172 Hagemann M, Hasse D & Berg G (2006) Detection of a phage genome carrying a zonula occludens
1173 like toxin gene (*zot*) in clinical isolates of *Stenotrophomonas maltophilia*. *Arch Microbiol* **185**: 449-
1174 458.
1175 Haigh NG & Webster RE (1999) The pI and pXI assembly proteins serve separate and essential
1176 roles in filamentous phage assembly. *J Mol Biol* **293**: 1017-1027.
1177 Hansen MR, Rance M & Pardi A (1989) Observation of long-range 1H-1H distances in solution by
1178 dipolar coupling interactions. *J Am Chem Soc* **120**: 11210-11211.
1179 Hassan F, Kamruzzaman M, Mekalanos JJ & Faruque SM (2010) Satellite phage TLC[phgr]
1180 enables toxigenic conversion by CTX phage through dif site alteration. *Nature* **467440**: 982-985.
1181 Heilpern AJ & Waldor MK (2003) pIIICTX, a predicted CTXφ minor coat protein, can expand the
1182 host range of coliphage fd to include *Vibrio cholerae*. *J Bacteriol* **185**: 1037-1044.
1183 Hess GT, Cragnolini JJ, Popp MW*, et al.* (2012) M13 bacteriophage display framework that allows
1184 sortase-mediated modification of surface-accessible phage proteins. *Bioconjug Chem* **23**: 1478-
1185 1487.

1186 Higashitani A, Higashitani N & Horiuchi K (1997) Minus-strand origin of filamentous phage versus
1187 transcriptional promoters in recognition of RNA polymerase. *Proc Natl Acad Sci USA* **94**: 2909-
1188 2914.
1189 Hill DF, Short NJ, Perham RN & Petersen GB (1991) DNA sequence of the filamentous
1190 bacteriophage Pf1. *J Mol Biol* **218**: 349-364.
1191 Hoang TT, Kutchma AJ, Becher A & Schweizer HP (2000) Integration-proficient plasmids for
1192 *Pseudomonas aeruginosa*: Site-specific integration and use for engineering of reporter and
1193 expression strains. *Plasmid* **43**: 59-72.
1194 Hobbs M & Mattick JS (1993) Common components in the assembly of type-4 fimbriae, DNA
1195 transfer systems, filamentous phage and protein-secretion apparatus - a general system for the
1196 formation of surface-associated protein complexes. *Mol Microbiol* **10**: 233-243.
1197 Hofschneider PH (1963) Untersuchungen uber kleine *E. coli* K 12 bakteriophagen 1 und 2
1198 Mitteilung. *Zeitschrift Fur Naturforschung Part B-Chemie Biochemie Biophysik Biologie Und*
1199 *Verwandten Gebiete* **B 18**: 203-&.
1200 Hohn B, Schutz HV & Marvin DA (1971) Filamentous bacterial viruses. II. Killing of bacteria by
1201 abortive infection with Fd. *J Mol Biol* **56**: 155-165.
1202 Horabin JI & Webster RE (1986) Morphogenesis of F1 filamentous bacteriophage - increased
1203 expression of gene-I inhibits bacterial-growth. *J Mol Biol* **188**: 403-413.
1204 Horabin JI & Webster RE (1988) An amino-acid sequence which directs membrane insertion causes
1205 loss of membrane-potential. *J Biol Chem* **263**: 11575-11583.
1206 Huang Y, Chiang CY, Lee SK, Gao Y, Hu EL, De Yoreo J & Belcher AM (2005) Programmable
1207 assembly of nanoarchitectures using genetically engineered viruses. *Nano Letters* **5**: 1429-1434.
1208 Huber KE & Waldor MK (2002) Filamentous phage integration requires the host recombinases
1209 XerC and XerD. *Nature* **417**: 656-659.
1210 Hufton SE, Moerkerk PT, Meulemans EV, de Bruine A, Arends JW & Hoogenboom HR (1999)
1211 Phage display of cDNA repertoires: the pVI display system and its applications for the selection of
1212 immunogenic ligands. *J Immunol Methods* **231**: 39-51.
1213 Hui JGK (2014) The genes and factors that drive the conversion of the *Pseudomonas aeruginosa*
1214 Pf4 prophage into the superinfective form. Thesis, University of New South Wales, Sydney,
1215 Australia.
1216 Ikema M & Honma Y (1998) A novel filamentous phage, fs-2, of *Vibrio cholerae* O139.
1217 *Microbiology* **144**: 1901-1906.
1218 Ishimoto KS & Lory S (1989) Formation of pilin in *Pseudomonas aeruginosa* requires the
1219 alternative sigma-factor (Rpon) of RNA-polymerase. *Proc Natl Acad Sci USA* **86**: 1954-1957.
1220 Iyer R, Darby V & Holland IB (1976) Changes in membrane proteins of *Escherichia coli* K12
1221 mediated by bacteriophage IKe-specific plasmids. *Biochim Biophys Acta* **453**: 311-318.
1222 Jankovic D, Collett MA, Lubbers MW & Rakonjac J (2007) Direct selection and phage display of a
1223 Gram-positive secretome. *Genome Biology* **8**. R266.
1224 Jian H, Xiao X & Wang F (2013) Role of filamentous phage SW1 in regulating the lateral flagella
1225 of *Shewanella piezotolerans* strain WP3 at low temperatures. *Appl Environ Microbiol* **79**: 7101-
1226 7109.
1227 Jian H, Xiao X & Wang F (2013) Role of filamentous phage SW1 in regulating the lateral flagella
1228 of *Shewanella piezotolerans* strain WP3 at low temperatures. *Appl Environl Microbiol* **79**: 7101-
1229 7109.
1230 Jian H, Xu J, Xiao X & Wang F (2012) Dynamic modulation of DNA replication and gene
1231 transcription in deep-sea filamentous phage SW1 in response to changes of host growth and
1232 temperature. *PLoS One* **7**: e41578.
1233 Johnson JA, Morris JGJ & Kaper JB (1993) Gene encoding zonula occludens toxin (*zot*) does not
1234 occur independently from cholera entertoxin genes (*ctx*) in *Vibrio cholerae*. *J Clin Microbiol* **31**:
1235 732-733.
1236 Joly N, Engl C, Jovanovic G*, et al.* (2010) Managing membrane stress: the phage shock protein
1237 (Psp) response, from molecular mechanisms to physiology. *FEMS Microbiol Rev* **34**: 797-827.

1238 Kamruzzaman M, Robins WP, Bari SM, Nahar S, Mekalanos JJ & Faruque SM (2014) RS1
1239 satellite phage promotes diversity of toxigenic *Vibrio cholerae* by driving CTX prophage loss and
1240 elimination of lysogenic immunity. *Infect Immun* **82**: 3636-3643.
1241 Kar S, Ghosh RK, Ghosh AN & Ghosh A (1996) Integration of the DNA of a novel filamentous
1242 bacteriophage VSK from *Vibrio cholerae* 0139 into the host chromosomal DNA. *FEMS Microbiol*
1243 *Lett* **145**: 17-22.
1244 Karaolis DKR, Somara S, Maneval DR, Johnson JA & Kaper JB (1999) A bacteriophage encoding
1245 a pathogenicity island, a type-IV pilus and a phage receptor in cholera bacteria. *Nature* **399**: 375-
1246 379.
1247 Karlsson F, Malmborg-Hager AC, Albrekt AS & Borrebaeck CA (2005) Genome-wide comparison
1248 of phage M13-infected vs. uninfected *Escherichia coli*. *Can J Microbiol* **51**: 29-35.
1249 Kawai M, Uchiyama I & Kobayashi I (2005) Genome comparison in silico in *Neisseria* suggests
1250 integration of filamentous bacteriophages by their own transposase. *DNA Res* **12**: 389-401.
1251 Kawasaki T, Nagata S, Fujiwara A, Satsuma H, Fujie M, Usami S & Yamada T (2007) Genomic
1252 characterization of the filamentous integrative bacteriophages phi RSSI and phi RSMI, which infect
1253 *Ralstonia solanacearum*. *J Bacteriol* **189**: 5792-5802.
1254 Khatoon H, Iyer RV & Iyer VN (1972) A new filamentous bacteriophage with sex-factor
1255 specificity. *Virology* **48**: 145-155.
1256 Kim AY & Blaschek HP (1991) Isolation and characterization of a filamentous viruslike particle
1257 from *Clostridium acetobutylicum* NCIB 6444. *J Bacteriol* **173**: 530-535.
1258 Kirov SM, Webb JS, O'May CY, Reid DW, Woo JKK, Rice SA & Kjelleberg S (2007) Biofilm
1259 differentiation and dispersal in mucoid *Pseudomonas aeruginosa* isolates from patients with cystic
1260 fibrosis. *Microbiology* **153**: 3264-3274.
1261 Klockgether J, Munder A, Neugebauer J*, et al.* (2010) Genome diversity of *Pseudomonas*
1262 *aeruginosa* PAO1 laboratory strains. *J Bacteriol* **192**: 1113-1121.
1263 Koonin EV (1992) The second cholera toxin, Zot, and its plasmid-encoded and phage-encoded
1264 homologues constitute a group of putative ATPases with an altered purine NTP-binding motif.
1265 *FEBS Lett* **312**: 3-6.
1266 Krogh A, Larsson B, von Heijne G & Sonnhammer EL (2001) Predicting transmembrane protein
1267 topology with a hidden Markov model: application to complete genomes. *J Mol Biol* **305**: 567-580.
1268 Kunkel TA, Bebenek K & Mcclary J (1991) Efficient site-directed mutagenesis using uracil-
1269 containing DNA. *Meth Enzymol* **204**: 125-139.
1270 Kuo MY, Yang MK, Chen WP & Kuo TT (2000) High-frequency interconversion of turbid and
1271 clear plaque strains of bacteriophage f1 and associated host cell death. *Can J Microbiol* **46**: 841-
1272 847.
1273 Kuo T-T, Tan M-S, Su M-T & Yang M-K (1991) Complete nucleotide sequence of filamentous
1274 phage Cf1c from *Xanthomonas campestris pv. citri*. *Nucleic Acids Research* **19**: 2498.
1275 Kuo TT, Chiang CC, Chen SY, Lin JH & Kuo JL (1994) A long lytic cycle in filamentous phage
1276 Cf1tv infecting *Xanthomonas campestris* pv. citri. *Arch Virol* **135**: 253-264.
1277 Lee JW, Song J, Hwang MP & Lee KH (2013) Nanoscale bacteriophage biosensors beyond phage
1278 display. *Int J Nanomedicine* **8**: 3917-3925.
1279 Lee YJ, Yi H, Kim WJ*, et al.* (2009) Fabricating genetically engineered high-power lithium-ion
1280 batteries using multiple virus genes. *Science* **324**: 1051-1055.
1281 Lengeling A, Mahajan A & Gally DL (2013) Bacteriophages as pathogens and immune
1282 modulators? *mBio* **4**. e00868.
1283 Li CR, Wally H, Miller SJ & Lu CD (2009) The multifaceted proteins MvaT and MvaU, members
1284 of the H-NS family, control arginine metabolism, pyocyanin synthesis, and prophage activation in
1285 *Pseudomonas aeruginosa* PAO1. *J Bacteriol* **191**: 6211-6218.
1286 Li M, Kotetishvili M, Chen Y & Sozhamannan S (2003) Comparative genomic analyses of the
1287 vibrio pathogenicity island and cholera toxin prophage regions in nonepidemic serogroup strains of
1288 *Vibrio cholerae*. *Appl Environ Microbiol* **69**: 1728-1738.

1289 Lin JY, Wu CC & Kue TT (1971) Amino acid analysis of the coat protein of the filamentous
1290 bacterial virus xf from *Xanthomonas oryzae*. *Virology* **45**: 38-41.
1291 Liu SS, Han WY, Sun CJ*, et al.* (2011) Subtractive screening with the *Mycobacterium tuberculosis*
1292 surface protein phage display library. *Tuberculosis* **91**: 579-586.
1293 Loeb T (1960) Isolation of a bacteriophage specific for the F+ and Hfr mating types of *Escherichia*
1294 *coli* K-12. *Science* **131**: 932-933.
1295 Lopez J & Webster RE (1983) Morphogenesis of filamentous bacteriophage-F1 - orientation of
1296 extrusion and production of polyphage. *Virology* **127**: 177-193.
1297 Lu TK & Collins JJ (2009) Engineered bacteriophage targeting gene networks as adjuvants for
1298 antibiotic therapy. *Proc Natl Acad Sci USA* **106**: 4629-4634.
1299 Luiten RG, Putterman DG, Schoenmakers JG, Konings RN & Day LA (1985) Nucleotide sequence
1300 of the genome of Pf3, an IncP-1 plasmid-specific filamentous bacteriophage of *Pseudomonas*
1301 *aeruginosa*. *J Virol* **56**: 268-276.
1302 Luiten RGM, Schoenmakers JGG & Konings RNH (1983) The major coat protein gene of the
1303 filamentous *Pseudomonas aeruginosa* phage-pf3 - absence of an n-terminal leader signal sequence.
1304 *Nuc Acids Res* **11**: 8073-8085.
1305 Mao CB, Solis DJ, Reiss BD*, et al.* (2004) Virus-based toolkit for the directed synthesis of
1306 magnetic and semiconducting nanowires. *Science* **303**: 213-217.
1307 Marciano DK, Russel M & Simon SM (1999) An aqueous channel for filamentous phage export.
1308 *Science* **284**: 1516-1519.
1309 Marciano DK, Russel M & Simon SM (2001) Assembling filamentous phage occlude pIV channels.
1310 *Proc Natl Acad Sci USA* **98**: 9359-9364.
1311 Marvin DA (1998) Filamentous phage structure, infection and assembly. *Curr Opin Struct Biol* **8**:
1312 150-158.
1313 Marvin DA & Hoffmann-Berling H (1963) Physical and chemical properties of two new small
1314 bacteriophages. *Nature* **197**: 517-518.
1315 Marvin DA & Hohn B (1969) Filamentous bacterial viruses. *Bacteriol Rev* **33**: 172-209.
1316 Marvin DA, Pigram WJ, Wiseman RL, Wachtel EJ & Marvin FJ (1974) Filamentous bacterial
1317 viruses. XIL. Molecular architecture of the class I (fd, If1, IKe) virion. *J Mol Biol* **88**: 581-598.
1318 Maxson ME & Darwin AJ (2006) PspB and PspC of *Yersinia enterocolitica* are dual function
1319 proteins: regulators and effectors of the phage-shock-protein response. *Mol Microbiol* **59**: 1610-
1320 1623.
1321 McElroy K, Hui J, Woo JKK*, et al.* (2014) Strain-specific, parallel evolution drives short-term
1322 diversification during *Pseudomonas aeruginosa* biofilm formation. *Proc Natl Acad Sci USA* **111**:
1323 E1419-1427.
1324 McLeod SM & Waldor MK (2004) Characterization of XerC- and XerD-dependent CTX phage
1325 integration in *Vibrio cholerae*. *Mol Microbiol* **54**: 935-947.
1326 McLeod SM, Kimsey HH, Davis BM & Waldor MK (2005) CTX and *Vibrio cholerae*: exploring a
1327 newly recognized type of phage-host cell relationship. *Mol Microbiol* **57**: 347-356.
1328 Merril CR, Geier MR & Petricci JC (1971) Bacterial virus gene expression in human cells. *Nature*
1329 **233**: 398-400.
1330 Messing J (1991) Cloning in M13-phage or how to use biology at its best. *Gene* **100**: 3-12.
1331 Meynell GG & Lawn AM (1968) Filamentous phages specific for the I Sex Factor. *Nature* **217**:
1332 1184-1186.
1333 Minamishima Y, Takeya K, Ohnishi Y & Amako K (1968) Physicochemical and biological
1334 properties of fibrous Pseudomonas bacteriophages. *J Virol* **2**: 208-213.
1335 Mooij MJ, Drenkard E, Llamas MA, Broucke-Grauls CMJEV, Savelkoul PHM, Ausubel FM &
1336 Bitter W (2007) Characterization of the integrated filamentous phage Pf5 and its involvement in
1337 small-colony formation. *Microbiology* **153**: 1790-1798.
1338 Mooij MJ, Drenkard E, Llamas MA, Vandenbroucke-Grauls CMJE, Savelkoul PHM, Ausubel FM
1339 & Bitter W (2007) Characterization of the integrated filamentous phage Pf5 and its involvement in
1340 small-colony formation. *Microbiology* **153**: 1790-1798.

1341 Moreira LM, De Souza RF, Digiampietri LA, Da Silva AC & Setubal JC (2005) Comparative
1342 analyses of Xanthomonas and Xylella complete genomes. *OMICS* **9**: 43-76.
1343 Mullen LM, Nair SP, Ward JM, Rycroft AN & Henderson B (2006) Phage display in the study of
1344 infectious diseases. *Trends Microbiol* **14**: 141-147.
1345 Murugaiyan S, Bae JY, Wu J*, et al.* (2011) Characterization of filamentous bacteriophage PE226
1346 infecting *Ralstonia solanacearum* strains. *J Appl Microbiol* **110**: 296-303.
1347 Nakasone N, Honma Y, Toma C, Yamashiro T & Iwanaga M (1998) Filamentous phage fs1 of
1348 *Vibrio cholerae* O139. *Microbiol Immunol* **42**: 237-239.
1349 Nakasone N, Ikema M, Higa N, Yamashiro T & Iwanaga M (1999) A filamentous phage of *Vibrio*
1350 *parahaemolyticus* O3:K6 isolated in Laos. *Microbiol Immunol* **43**: 385-388.
1351 Nasu H, Iida T, Sugahara T*, et al.* (2000) A filamentous phage associated with recent pandemic
1352 *Vibrio parahaemolyticus* O3:K6 strains. *J Clin Microbiol* **38**: 2156-2161.
1353 Nguyen DT, Nguyen BM, Tran HH*, et al.* (2008) Filamentous vibriophage fs2 encoding the rstC
1354 gene integrates into the same chromosomal region as the CTX phage. *FEMS Microbiol Lett* **284**:
1355 225-230.
1356 Ogura T & Wilkinson AJ (2001) AAA+ superfamily ATPase: common structure-diverse function.
1357 *Genes to Cells* **6**: 575-597.
1358 Oh J-W, Chung W-J, Heo K*, et al.* (2014) Biomimetic virus-based colourimetric sensors. *Nat*
1359 *Commun* **5**.
1360 Oppenheim AB, Kobiler O, Stavans J, Court DL & Adhya S (2005) Switches in bacteriophage
1361 lambda development. *Ann Rev Gen* **39**: 409-429.
1362 Pederson DM, Welsh LC, Marvin DA, Sampson M, Perham RN, Yu M & Slater MR (2001) The
1363 protein capsid of filamentous bacteriophage PH75 from *Thermus thermophilus*. *J Mol Biol* **309**:
1364 401-421.
1365 Peeters BP, Peters RM, Schoenmakers JG & Konings RN (1985) Nucleotide sequence and genetic
1366 organization of the genome of the N-specific filamentous bacteriophage IKe. Comparison with the
1367 genome of the F-specific filamentous phages M13, fd and f1. *J Mol Biol* **181**: 27-39.
1368 Peterson C, Winter WT, Dalack GW & Day LA (1982) Structure of the filamentous bacteriophage,
1369 Pf3, by X-ray fiber diffraction. *J Mol Biol* **162**: 877-881.
1370 Petrova OE & Sauer K (2011) SagS contributes to the motile-sessile switch and acts in concert with
1371 BfiSR to enable *Pseudomonas aeruginosa* biofilm formation. *J Bacteriol* **193**: 6614-6628.
1372 Piekarowicz A, Majchrzak M, Klyz A & Adamczyk-Poplawska M (2006) Analysis of the
1373 filamentous bacteriophage genomes integrated into *Neisseria gonorrhoeae* FA1090 chromosome.
1374 *Pol J Microbiol* **55**: 251-260.
1375 Piekarowicz A, Kłyż A, Majchrzak M*, et al.* (2014) *Neisseria gonorrhoeae* filamentous phage
1376 NgoΦ6 is capable of infecting a variety of gram-negative bacteria. *J Virol* **88**: 1002-1010.
1377 Platt MD, Schurr MJ, Sauer K*, et al.* (2008) Proteomic, microarray, and signature-tagged
1378 mutagenesis analyses of anaerobic *Pseudomonas aeruginosa* at pH 6.5, likely representing chronic,
1379 late-stage cystic fibrosis airway conditions. *J Bacteriol* **190**: 2739-2758.
1380 Pratt D, Tzagoloff H & Erdahl WS (1966) Conditional lethal mutants of the small filamentous
1381 coliphage M13. I. Isolation, complementation, cell killing, time of cistron action. *Virology* **30**: 397-
1382 410.
1383 Quinones M, Kinsey HH & Waldor MK (2005) LexA cleavage is required for CTX propahge
1384 induction. *Molecular Cell* **17**: 291-300.
1385 Rakonjac J, Feng JN & Model P (1999) Filamentous phage are released from the bacterial
1386 membrane by a two-step mechanism involving a short C-terminal fragment of pIII. *J Mol Biol* **289**:
1387 1253-1265.
1388 Rakonjac K, Bennett NJ, Spagnuolo J, Gagic D & Russel M (2011) Filamentous bacteriophage:
1389 biology, phage display and nanotechnology applications. *Curr Issues Mol Biol* **13**: 51-76.
1390 Ray DS (1978) Single-stranded DNA phages. *In viva replication of filamentous phage DNA.,*
1391 (Denhardt DT, Dressler D & Ray DS, eds.), pp. 325-339. Cold Spring Harbor Laboratory, New
1392 York.

1393 Reichmann L & Holliger P (1997) The C-terminal domain of TolA is the coreceptor for filamentous
1394 phage infection of *E. coli. Cell* **90**: 351-360.
1395 Rice SA & Lampson BC (1996) Bacterial reverse transcriptase and msDNA. *Virus Genes* **11**: 95-
1396 104.
1397 Rice SA, Tan CH, Mikkelsen PJ*, et al.* (2009) The biofilm life cycle and virulence of *Pseudomonas*
1398 *aeruginosa* are dependent on a filamentous prophage. *ISME J* **3**: 271-282.
1399 Rodi DJ & Makowski L (1999) Phage-display technology - finding a needle in a vast molecular
1400 haystack. *Curr Opin Biotechnol* **10**: 87-93.
1401 Roy A & Mitra S (1970) Susceptibility of *E. coli* K-12 to actinomycinD after infection with phage-
1402 M13. *Nature* **228**: 365-366.
1403 Roy A & Mitra S (1970) Increased fragility of *Escherichia coli* after infection with bacteriphage
1404 M13. *J Virol* **6**: 333-339.
1405 Russel M (1991) Filamentous phage assembly. *Mol Microbiol* **5**: 1607-1613.
1406 Russel M (1995) Moving through the membrane with filamentous phages. *Trends Microbiol* **3**: 223-
1407 228.
1408 Russel M & Model P (1986) The role of thioredoxin in filamentous phage assembly - construction,
1409 isolation, and characterization of mutant thioredoxins. *J Biol Chem* **261**: 4997-5005.
1410 Russel M & Model P (1989) Genetic analysis of the filamentous bacteriophage packaging signal
1411 and of the proteins that interact with it. *Virology* **63**: 3284-3295.
1412 Russel M & Kazmierczak B (1993) Analysis of the structure and subcellular location of filamentous
1413 phage-Piv. *J Bacteriol* **175**: 3998-4007.
1414 Russel M & Model P (2006) Filamentous phage. *The bacteriophages,*(Calendar RC, ed.), pp. 146-
1415 160. Oxford University Press Inc., New York.
1416 Russel M, Kidd S & Kelley MR (1986) An improved filamentous helper phage for generating
1417 single-stranded plasmid DNA. *Gene* **45**: 333-338.
1418 Salivar WO, Tzagoloff H & Pratt D (1964) Some physical-chemical and biological properties of the
1419 rod-shaped coliphage M13. *Virology* **24**: 359-371.
1420 Sanchez T, Chen DTN, DeCamp SJ, Heymann M & Dogic Z (2012) Spontaneous motion in
1421 hierarchically assembled active matter. *Nature* **491**: 431-434.
1422 Sanger F, Coulson AR, Barrell BG, Smith AJH & Roe BA (1980) Cloning in single-stranded
1423 bacteriophage as an aid to rapid DNA sequencing. *J Mol Biol* **143**: 161-178.
1424 Schwartz FM & Zinder ND (1968) Morphological changes in *Escherichia coli* infected with the
1425 DNA bacteriophage fl. *Virology* **34**: 352-355.
1426 Seo J, Brencic A & Darwin AJ (2009) Analysis of secretin-induced stress in *Pseudomonas*
1427 *aeruginosa* suggests prevention rather than response and identifies a novel protein involved in
1428 secretin function. *J Bacteriol* **191**: 898-908.
1429 Sha Y, Melcher U, Davis RE & Fletcher J (2000) Common elements of spiroplasma plectroviruses
1430 revealed by nucleotide sequence of SVTS2. *Virus Genes* **20**: 47-56.
1431 Sharma P, Ward A, Gibaud T, Hagan MF & Dogic Z (2014) Hierarchical organization of chiral
1432 rafts in colloidal membranes. *Nature* **513**: 77-80.
1433 Shieh GJ, Charng YC, Yang BC, Jenn T, Bau HJ & Kuo TT (1991) Identification and nucleotide
1434 sequence analysis of an open reading frame involved in high-frequency conversion of turbid to
1435 clear plaque mutants of filamentous phage Cf1t. *Virology* **185**: 316-322.
1436 Shieh GJ, Charng YC, Yang BC, Jenntu, Bau HJ & Kuo TT (1991) Identification and nucleotide-
1437 sequence analysis of an open reading frame involved in high-frequency conversion of turbid to
1438 clear plaque mutants of filamentous phage Cf1t. *Virology* **185**: 316-322.
1439 Smith GP (1985) Filamentous fusion phage - novel expression vectors that display cloned antigens
1440 on the virion surface. *Science* **228**: 1315-1317.
1441 Snell DT & Offord RE (1972) The amino acid sequence of the B-protein of bacteriophage ZJ-2.
1442 *Biochem J* **127**: 167-178.
1443 Soding J (2005) Protein homology detection by HMM-HMM comparison. *Bioinformatics* **21**: 951-
1444 960.

Spagnuolo J, Opalka N, Wen WX*, et al.* (2010) Identification of the gate regions in the primary structure of the secretin pIV. *Mol Microbiol* **76**: 133-150.
Stassen AP, Schoenmakers EF, Yu M, Schoenmakers JG & Konings RN (1992) Nucleotide sequence of the genome of the filamentous bacteriophage I2-2: module evolution of the filamentous phage genome. *J Mol Evol* **34**: 141-152.
Stover CK, Pham XQ, Erwin AL*, et al.* (2000) Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature* **406**: 959-964.
Takeya K & Amako K (1966) A rod-shaped Pseudomonas phage. *Virology* **28**: 163-165.
Tam R & Saier MHJ (1993) Structural, functional and evolutionary relationships among extracellular solute-binding receptors of bacteria. *Microbiol Rev* **57**: 320-346.
Tay M (2008) Identification of the secondary phage in *Pseudomonas aeruginosa* and determination of its role in biofilm development. Thesis, University of New South Wales, Sydney, Australia.
Trucksis M, Galen JE, Michalski J, Fasano A & Kaper JB (1993) Accessory cholera enterotoxin (Ace), the 3rd toxin of a *Vibrio-Cholerae* virulence cassette. *Proc Natl Acad Sci USA* **90**: 5267-5271.
Tseng YH, Lo MC, Lin KC, Pan CC & Chang RY (1990) Characterization of filamentous bacteriophage phi Lf from *Xanthomonas campestris* pv. campestris. *J Gen Virol* **71 (Pt 8)**: 1881-1884.
van Houten NE, Henry KA, Smith GP & Scott JK (2010) Engineering filamentous phage carriers to improve focusing of antibody responses against peptides. *Vaccine* **28**: 2174-2185.
Varani AM, Monteiro-Vitorello CB, Nakaya HI & Van Sluys MA (2013) The role of prophage in plant-pathogenic bacteria. *Annu Rev Phytopathol* **51**: 429-451.
Vieira J & Messing J (1987) Production of single-stranded plasmid DNA. *Meth Enzymol* **153**: 3-11.
Waites KB, Canupp KC & DeVivo MJ (1991) Efficacy and tolerance of norfloxacinin treatment of complicated urinary tract infection in outpatients with neurogenic bladder secondary to spinal cord injury. *Urology* **38**: 589-596.
Waldor MK & Mekalanos JJ (1996) Lysogenic conversion by a filamentous phage encoding cholera toxin. *Science* **272**: 1910-1914.
Waldor MK, Rubin EJ, Pearson GD, Kimsey HH & Mekalanos JJ (1997) Regulation, replication, and integration functions of the *Vibrio cholerae* CTX are encoded by region RS2. *Mol Microbiol* **24**: 917-826.
Wang F, Wang FP, Li Q & Xian X (2007) A novel filamentous phage from the deep-sea bacterium *Shewanella piezotolerans* WP3 is induced at low temperature. *J Bacteriol* **189**: 7151-7153.
Wang X & Wood TK (2011) Toxin-antitoxin systems influence biofilm and persister cell formation and the general stress response. *Appl Environ Microbioly* **77**: 5577-5583.
Webb JS, Lau M & Kjelleberg S (2004) Bacteriophage and phenotypic variation in *Pseudomonas aeruginosa* biofilm development. *J Bacteriol* **186**: 8066-8073.
Webb JS, Thompson LS, James S*, et al.* (2003) Cell death in *Pseudomonas aeruginosa* biofilm development. *J Bacteriol* **185**: 4585-4592.
Webster RE (1996) Phage display of peptides and proteins. *Biology of the filamentous bacteriophage.,*(Kay BK, Winter J & McCafferty J, eds.), pp. 1-20. Academic Press, New York.
Wei Q, Phu NLM, Dotsch A*, et al.* (2012) Global regulation of gene expression by OxyR in an important human opportunistic pathogen. *Nuc Acids Resh* **40**: 4320-4333.
Whitchurch CB, Tolker-Nielsen T, Ragas PC & Mattick JS (2002) Extracellular DNA required for bacterial biofilm formation. *Science* **295**: 1487-1487.
Whiteley M, Bangera MG, Bumgarner RE, Parsek MR, Teitzel GM, Lory S & Greenberg EP (2001) Gene expression in *Pseudomonas aeruginosa* biofilms. *Nature* **413**: 860-864.
Williams PG & Fenwick ML (1967) Degradation of the filamentous phage ZJ/2 by sodium dodecylsulphate. *Nature* **214**: 712-713.
Woo JKK, Webb JS, Kirov SM, Kjelleberg S & Rice SA (2012) Biofilm dispersal cells of a cystic fibrosis *Pseudomonas aeruginosa* isolate exhibit variability in functional traits likely to contribute to persistent infection. *FEMS Immunol Med Microbiol* **66**: 251-264.

1497 Xavier JB, Kim W & Foster KR (2011) A molecular mechanism that stabilizes cooperative
1498 secretions in *Pseudomonas aeruginosa*. *Mol Microbiol* **79**: 166-179.
1499 Xue H, Xu Y, Boucher Y & Polz MF (2012) High frequency of a novel filamentous phage, VCY
1500 phi, within an environmental *Vibrio cholerae* population. *Appl Environ Microbiol* **78**: 28-33.
1501 Yamada T (2013) Filamentous phages of *Ralstonia solanacearum*: double-edged swords for
1502 pathogenic bacteria. *Front Microbiol* **4**: 325.
1503 Yamada T, Kawasaki T, Nagata S, Fujiwara A, Usami S & Fujie M (2007) New bacteriophages that
1504 infect the phytopathogen *Ralstonia solanacearum*. *Microbiology* **153**: 2630-2639.
1505 Yang F, Pecina DA, Kelly SD, Kim SH, Kemner KM, Long DT & Marsh TL (2010)
1506 Biosequestration via cooperative binding of copper by *Ralstonia pickettii*. *Environ Technol* **31**:
1507 1045-1060.
1508 Zweckstetter M & Bax A (2001) Characterization of molecular alignment in aqueous suspensions
1509 of Pf1 bacteriophage. *J Biomol NMR* **20**: 365-377.
1510 Zwick MB, Shen JQ & Scott J (1998) Phage-displayed peptide libraries. *Cur Opin Biotechnol* **9**:
1511 427-436.

1512

1513

1514 Table 1. Ff filamentous phage proteins and putative homologues. Identity values were calculated
1515 using *clustalo* (Soding, 2005).

Protein name	Function	Homologues
pI (G1P) **Virion assembly-export protein**	**Morphogenesis – Phage Assembly** • Inner membrane component of the trans-envelope assembly/secretion system. • Interacts with pIV (G4P).	Enterobacteria phage f1 (P03657) 99.7% identity[a] Enterobacteria phage IKe **(P03658)** 50.1 % identity *Xanthomonas* phage **(O55247)** 14.4% identity Zot toxin *Vibrio cholerae* **(P38442)** 15.5% identity Zot-like *Pseudomonas* phage Pf4 (Q9I5K2) 13.6% identity
pII (G2P) **Replication protein**	**Replication - Endonuclease** • Plays an essential role in viral DNA replication (the positive strand synthesis). • Cleaves the dsDNA replicative form I (RFI) and after binding, generates the dsDNA replicative form II (RFII). • Joins the ends of the displaced strand to generate a circular single-stranded molecule ready to be packed into a virion.	Enterobacteria phage f1**(P69546)** Enterobacteria phage Ike **(P03660)** *Pseudomonas* phage Pf3 **(P03627)** *Xanthomonas* phage ΦLf **(Q38617)**
pIII (G3P) **Attachment protein**	**Structural - Minor Virion Protein, Coat protein A - Adsorption** • Plays essential roles both in the entry of the viral genome into the bacterial host and in the release from the host membrane, as well as forming the pIII-pVI virion cap. • Mediates adsorption of the phage to its primary receptor (F-pilus) during initiation and secondary receptor (domain III of TolA protein). • Mediates the release of the membrane-anchored virion from the cell via its C-terminal domain. • Interacts with pVI (G6P), pVIII (G8P) and host TolA	Enterobacteria phage f1 **(P69169)** 99.8 % identity Enterobacteria phage Ike **(P03663)** 17.4% identity *Pseudomonas* phage Pf1 **(P25129)** 16.1% identity *Pseudomonas* phage Pf3 **(P03624)** 14.6% identity *Xanthomonas* phage ΦLf **(Q37972)** 15.9% identiy *Pseudomonas* phage Pf4 **(Q9I5K4)** 17.1% identity ORF9 *Ralstonia* phage Rsm1 **(A0JC13)** 12.4% identity
pIV (G4P)	**Morphogenesis - Phage Assembly and Virion Export**	Enterobacteria phage f1 **(P03666)**

Virion assembly-export protein	• Acts in the assembly and export of the bacteriophage by forming a gated channel across the host outer membrane. • Interacts with pI (G1P).	Enterobacteria phage Ike (**P03667**)
pV (G5P) **DNA binding protein**	**Replication – ssDNA binding** • Binds to ssDNA in a highly cooperative manner without pronounced sequence specificity. • Prevents the conversion into the double-stranded replicative form during synthesis of the single-stranded (progeny) viral DNA. • Displaced by the capsid protein pVIII (G8P) during phage assembly at the inner bacterial membrane.	Enterobacteria phage f1 (**P69543**) Enterobacteria phage Ike (**P03670**) *Pseudomonas* phage Pf1 (**P03671**) *Pseudomonas* phage Pf3 (**P03672**) *Xanthomonas* phage ΦLf (**P68676**)
pVI (G6P) **Minor virion protein**	**Structural – Minor Virion Protein, Coat Protein D** • Plays essential roles in the release of virions from the host membrane. • Formation of the G3P-G6P complex is essential for correct termination of filamentous phage assembly and formation (structure) of the pIII-pVI virion cap.	Enterobacteria phage f1 (**P69531**) Enterobacteria phage Ike (**P03674**) *Pseudomonas* phage Pf1 (**Q38066**) *Pseudomonas* phage Pf3 (**P03625**) PA0725 *Pseudomonas* Pf4 (**Q9I5K3**) *Xanthomonas* phage ΦLf (**O55246**) Ace *V. cholerae* phage CTX (**Q7BBA3**) ORF10 *Ralstonia* phage Rsm1 (**A0JC05**)
pVII (G7P) **Minor virion protein**	**Structural – Minor Virion Protein, Coat protein C** • Initiates with pIX (G9P) the virion concomitant assembly-export process by interacting with the packaging signal of the viral genome.	Enterobacteria phage f1 (**P69534**) Enterobacteria phage Ike (**P03676**) *Xanthomonas* phage ΦLf (**P68672**)
G8P **Major capsid protein**	**Structural Major Virion Protein – Coat protein B** • Assembles to form a helical filament-like capsid, wrapping up the viral genomic DNA.	Enterobacteria phage f1 (**P69540**) 98.6% identity Enterobacteria phage Ike (**P03620**) 35.4% identity *Pseudomonas* phage Pf1 (**P03621**) 17.0% identity *Pseudomonas* phage Pf3 (**P03623**) 8.3% identity *Thermus* phage PH75 (**P82889**) 14.3% identity *Xanthomonas* phage ΦLf (**P68674**) 8.8% identity *Xanthomonas* phage Xf (**P03622**) 9.6% identity

pIX (G9P) **Minor virion protein**	**Structural – Minor Virion Protein - Coat protein C** • Initiates with pVII (G7P) the virion assembly-export process, by interacting with the packaging signal of the viral genome.	Enterobacteria phage f1 (**P69537)** Enterobacteria phage Ike (**P03678)** *Xanthomonas* phage ΦLf (**P68670)**
pX (G10P) **Replication-associated protein**	**Replication** • Translational product from an internal start codon within gene II; identical to the C-terminal domain of pII (G2P) • Binds to double-stranded DNA and prevents hydrolysis by nucleases. • Inhibitor of DNA replication.	Enterobacteria phage f1(**P69546)** Enterobacteria phage Ike (**P03660)** *Pseudomonas* phage Pf3 (**P03627)** *Xanthomonas* phage ΦLf (**Q38617)**
pXI (G1P) **Virion assembly-export protein**	• Translational product from an internal start codon within gene I. • Required for phage assembly. • Part of a trans-membrane complex with pI and pIV to protect pI from cleavage by endogenous proteases.	Enterobacteria phage f1 (P03657) Enterobacteria phage IKe (**P03658)** *Xanthomonas* phage (**O55247)** *V. cholerae* **(P38442)** *Pseudomonas* phage Pf4 **(Q9I5K2)**

[a], percent amino acid identity compared to the M13 homologue

1519 Table 2. The effect of filamentous phage on their bacterial host.

Phage Name	Bacterial host	• Effects of phage infection on the host	References
M13 f1 fd	Enterobacteria	• Lenghtens generation time, results in small and transparent colonies. • Induces the phage shock protein response, presumably through membrane stress due to mistargeting of pIV secretin to the inner membrane. • Impaired function of the oxidative and the glutamate-dependent acid resistance systems • Higher susceptibility to actinomycin D • Increased fragility • Affects cell membrane lipids	(Roy & Mitra, 1970, Karlsson, *et al.*, 2005) (Bayer & Bayer, 1986) (Joly *et al*. 2010)
If1	Enterobacteria	• Induces small colonies and host cell death	(Kuo, *et al.*, 2000)
I2-2	Enterobacteria	• Not known	(Stassen, *et al.*, 1992)
IKe	Enterobacteria	• Changes membrane proteins in *E. coli* K12	(Iyer, *et al.*, 1976, Peeters, *et al.*, 1985)
ZJ-2	Enterobacteria	• Not known	(Snell & Offord, 1972)
Pf1	*Pseudomonas*	• Suggested to be important for gene transfer or exclusion of other strains in PAO1 biofilms	(Crowther, 1980, Hill, *et al.*, 1991, Whiteley, *et al.*, 2001)
Pf3	*Pseudomonas*	• Not known	(Peterson, *et al.*, 1982)
Pf4	*Pseudomonas*	• Induces biofilm cell death, biofilm dispersal, small colony variants • Increases host virulence	(Webb, *et al.*, 2004, Rice, *et al.*, 2009)
Pf5	*Pseudomonas*	• Shown to not be involved in small colony variant formation	(Mooij, *et al.*, 2007)
Pf6	*Pseudomonas*	• Not known	(Tay, 2008)
CTXΦ	*Vibrio*	• Phage carries cholera toxin genes and thus is important for	(Waldor &

		pathogenicity	Mekalanos, 1996, Davis & Waldor, 2003)
VSK	*Vibrio*	• Not known	(Kar, *et al.*, 1996)
VEJΦ	*Vibrio*	• Can horizontally transmit cholera toxin	(Campos, *et al.*, 2010)
VGJΦ	*Vibrio*	• Not known	(Campos, *et al.*, 2003)
fs1	*Vibrio*	• Not known	(Nakasone, *et al.*, 1998)
fs2	*Vibrio*	• Reduces fimbrial production	(Ikema & Honma, 1998, Nguyen, *et al.*, 2008)
VCY-Φ	*Vibrio*	• Not known	(Xue, *et al.*, 2012)
KXV237	*Vibrio*	• Not known	(Nasu, *et al.*, 2000)
VPIΦ	*Vibrio*	• Encodes vibrio pathogenicity island	(Li, *et al.*, 2003)
RSS1	*Ralstonia*	• Enhances virulence • Increased EPS synthesis and twitching motility (through enhanced PilA and type IV pilin production) when phage is present • early expression of phcA (global virulence regulator) • surface-associated phage proteins may change the cell surface nature (hydrophobicity) to give high local cell densities	(Kawasaki, *et al.*, 2007, Addy, *et al.*, 2012)
RSM1	*Ralstonia*	• Enhances bacterial cell aggregation and reduce host virulence	(Kawasaki, *et al.*, 2007)
RSM3	*Ralstonia*	• Enhances bacterial cell aggregation and reduce host virulence	(Addy, *et al.*, 2012)
p12J	*Ralstonia*	• Phage harbours zot-like toxin	(Yang, *et al.*, 2010)
PE226	*Ralstonia*	• Phage harbours zot-like toxin	(Murugaiyan, *et al.*, 2011)

Xf	*Xanthomonas*	• Not known	(Lin*, et al.*, 1971)
Φ-Lf	*Xanthomonas*	• Not known	(Tseng*, et al.*, 1990)
Cf1c	*Xanthomonas*	• Reduces host growth rate	(Kuo*, et al.*, 1991)
YPf	*Yersina*	• Contributes to pathogenicity • Confers protection against superinfection	(Derbise*, et al.*, 2007, Chouikha*, et al.*, 2010)
M23 Φ-Lf	*Xylella*	• Not known	(Chen & Civerolo, 2008)
PH75	*Thermus*	• Not known	(Pederson*, et al.*, 2001)
ΦB5	*Propionibacterium*	• Not known	(Chopin*, et al.*, 2002)
ΦSMA9	*Stenotrophomonas*	• Not known	(Hagemann*, et al.*, 2006)
SW1	*Shewanella*	• Induces lateral flagella genes and enhances swarming	(Jian*, et al.*, 2013)
NgoΦ	*Neisseria*	• not known	(Piekarowicz*, et al.*, 2006, Piekarowicz*, et al.*, 2014)
MDA	*Neisseria*	• Correlates with invasivenes of host	(Bille*, et al.*, 2005)
Nf	*Neisseria*	• not known	(Kawai*, et al.*, 2006)

Figure Legends

Figure 1. Genes and genome organisation of filamentous phage. A. The genes are grouped based on function and colour-coded accordingly. The Replication genes are shown in red. Genes encoding virion Structural proteins are shown in yellow, pink, purple and blue. The Assembly and Secretion genes are in green. The same colour scheme is used to identify relevant proteins that comprise the mature phage particle (B).

Figure 2. Comparison of filamentous phage genomes. M13 is presented as the type phage for the group 1 Inovirus with the standard gene notations of gI to gX. The genes are coloured according to function, where red indicates replication genes, blue represents structural genes and green arrows represent the assembly and secretion genes. White boxes indicate genes that are unique for each phage. The orientation of the ORFs is indicated by the arrows. Note that the genomes and genes are not drawn to scale.

Figure 3. Phylogenetic relationships of the filamentous phage. Phylogenetic trees were generated using the phylogeny.fr platform (Dereeper*, et al.*, 2008). A) Analysis using the major coat protein, CoaB or pVIII. B) Analysis using Zot or pI proteins.