Bacteriophages and pathogenicity: more than just providing a toxin?

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Abstract

An increasing number of pathogenicity factors are discovered to be carried by bacteriophages. This review considers the bacteriophage-bacterium interaction and its relation to disease processes. We discuss the search for new bacteriophage-associated pathogenicity factors with emphasis on recent advances brought by the use of genomic sequence data and the techniques of genomic epidemiology.

Keywords: Neisseria meningitidis, bacteriophage, genetics, evolution, pathogenesis

1. Introduction and historical perspective

Horizontally mobile elements, including plasmids, transposons, genetic islands, and bacteriophages are responsible for the transfer of new functions to a bacterial cell and are recognised as important agents in bacterial evolution. To be maintained as part of the genetic content of the bacterium the genetic unit must either encode a mechanism by which its inactivation kills the cell (*eg*, restriction-modification or post-segregational killing systems), or must provide a benefit to the organism. Typically this involves coding for resistance to antibiotics, detoxification of heavy metals, acquisition and utilisation of certain nutrients, evasion of predators or colonisation of specific environments. In the case of human disease, the most important factors are colonisation, acquisition of nutrients, evasion of the innate and adaptive immune response, and pathogenicity factors. This review will discuss the involvement of bacteriophages in the transfer of pathogenicity factors.

Numerous examples have been brought to light of modification of bacterial virulence by bacteriophages. The first demonstration was that of Freeman [1], who found that avirulent strains of *Corynebacterium diphtheriae* infected with bacteriophage (now known as corynephage beta; Fig. 1) yielded virulent lysogens that produced diphtheria toxin. Later work showed that the gene was carried by the bacteriophage, the expression of which was intimately linked with genetic regulation (the Fur homologue, DtxR) in the host bacterial cell. Zabriskie demonstrated that the filtrable agent described by Frobisher in 1927, inducing production of streptococcal pyrogenic exotoxin (scarlatina toxin) by non-toxinogenic streptococci, was a temperate bacteriophage [2]. A further example is furnished by Shiga toxin-producing (enterohaemorrhagic) *Escherichia coli*, where systemic circulation of toxin may lead to renal failure and haemolytic uraemic syndrome (HUS). In this case, toxinogenicity was shown to be transferable between strains by a bacteriophage vector [3]. More recently Waldor and colleagues, [4] demonstrated that the cholera toxin was encoded on a filamentous bacteriophage, CTX-phi.

2. The place of the bacteriophage in the biology of the microbe. Regulation of virulence and phage particle production.

The case of CTX-phi highlights many of the important themes in bacteriophage-carried pathogenesis factors. The major bacterial determinants of choleric disease are the pili (composed of subunits known as TCP = toxin-coregulated pilin) which permit colonisation of the human intestine, and the secreted toxin, CTX, which is responsible for the life-threatening diarrhoea. The pili are encoded as part of a pathogenicity island of about 40 kb, VPI [5] and, in addition to mediating adhesion to enterocytes, serve as receptors for infection by the bacteriophage CTX-phi. This bacteriophage, resembles phage Ff (Ff is the collective designation for the nearly identical coliphages f1, fd and M13) in size and genetic organisation, but has toxin genes in place of the bacteriophage secretin, co-opting part of the bacterial type II secretion system for its release from the bacterial cell. Unlike Ff, the bacteriophage is carried as a prophage integrated into the bacterial chromosome. The production of bacteriophage particles is dependent on a particular, tandem arrangement of bacteriophage elements [6], and is maintained at a low level by the repressor protein (reviewed by [7]). The virulence factors, TCP and CTX, are part of the Tox regulon, a two-component regulatory system integral to the bacteria which, in response to external signals, directly activates expression of the toxin genes and, via ToxT (encoded on VPI), activates both the toxin genes and the TCP pilus operon. Other VPI-encoded factors link the production of toxin and flagellar motility with quorum sensing. This example shows how bacteriophages specifying pathogenicity phenotypes can be integrated into the genetic regulatory circuits of the host bacterium. This fine-tuning of the expression of phenotypes carried by the lysogenic bacteriophage is important to minimise the deleterious effects of virus-carriage and to optimise the advantage (competitive or in terms of colonisation of new environments) imparted by the bacteriophage pathogenicity genes.

Many pathogenesis factors are carried on bacteriophages related to coliphage lambda or on other temperate bacteriophages that switch from a lysogenic state to a lytic cycle. The expression of bacteriophage-associated pathogenicity genes may again be controlled by promoter regions responsive to elements of the host's genetic regulatory systems but is also strongly affected by induction of the bacteriophage, which in this case leads to death of the bacterial host. Bacteriophage replication increases the copy number of bacteriophage-associated pathogenesis genes, and also causes cell lysis, which results in release of large quantities of intracellular toxin. Thus, in the case of *Corynebacterium diphtheriae*, toxin production is derepressed under low iron conditions, as mentioned above, and is greatly increased after induction of the bacteriophage [8]. Similarly the genes *stx* in Shiga toxin-producing (enterohaemorrhagic) *Escherichia coli* are situated among genes controlled by the late promoter and are therefore cotranscribed with the lysis genes of the bacteriophage. Shiga toxin 1 (Stx1) is also regulated by iron concentration, whereas Stx2 is not [9]. These bacteriophages are also responsive to elements of the bacterial SOS system, DNA damage, leading to cleavage of the bacteriophage repressor by RecA binding ssDNA (This is a common response of bacteriophages, including CTX-phi). These regulatory systems are relevant in the context of human disease. Pathogens are likely to encounter iron-limited conditions imposed by the human organism as a defence against invading bacteria. In addition DNA damage may result from contact with bactericidal molecules produced by immune cells. Indeed, Broudy and colleagues [10] have shown that the temperate bacteriophage carrying the streptococcal pyrogenic exotoxin C was induced during co-culture of *Streptococcus pyogenes* with human pharyngeal cells, resulting in the release of large quantities of the toxin. Similarly interaction of STEC with neutrophils or with hydrogen peroxide (a neutrophil bactericidal product) induced both production of bacteriophage particles and secretion of Shiga toxin [11]. Equally, induction of toxin production has been

seen to result from the use of DNA-damaging antibiotics ([12] and references therein), leading to questions about their use in cases of gastroenteritis.

3. Survival of the bacteriophage, survival of the host bacterium

Since induction of the SOS response implies a high probability of death of host bacterium or of genetic damage to the prophage, there is an advantage in attempting to produce phage particles. In this case the survival of the phage, the fate of the bacteria or the human host is of no relevance, though the disease symptoms, diarrhoea for example, may help to disseminate the bacteriophage, within or without the bacteria. On the other hand, accessory pathogenesis genes carried by the bacteriophage are usually transcriptionally autonomous, and any benefit that they may bring to the bacterium is at least partially independent of the host bacteriophage. Hence mutations which inactivate genes involved in bacteriophage replication but leave the pathogenesis (or immunity) genes intact will be advantageous for the bacterium. Indeed prophage remnants are commonly found in genome sequences, and for example the majority of the lambdoid prophages in STEC are non-inducible. Another, interesting example are the R- and F-type pyocins of *Pseudomonas aeruginosa*, which appear to have evolved from the tail fibres of bacteriophages [13], the target in this case being competing bacteria. Thus genes transferred horizontally by bacteriophages, if they provide an advantage to the lysogen in a particular environment will tend over time to become dissociated from the bacteriophage functions and will end by being "appropriated" as part of the bacterial core chromosome, as in the model of the role of "pathogenicity islands" in the evolution of pathogens [14]. In keeping with this notion, the *stx* genes in certain strains of *Shigella* are encoded within a degraded prophage [15]. Striking exceptions to this are the bacteriophages of *Streptotoccus pyogenes*, which carry a variety of pathogenicity factors, for example the superantigen streptococcal pyrogenic exotoxin. In one serotype M3 strain, all pathogenicity factor-encoding prophages were inducible [16], suggesting a strong selection for mobility of these elements.

Whether a virus kills the bacterium by lysis, or is secreted without causing cell death, a time will come when the local population of bacteria is either killed or resistant. The bacteriophage particles, generally very resistant in the environment, may encounter a new susceptible population, but lysogeny provides a second means to promote survival. The propensity of the bacteria for transmission to a new human habitat will not only lead to an increased number of prophage genomes as the bacteria colonise the new host, but will also bring the bacteriophage into contact with new, potentially susceptible populations of bacteria. During the time spent integrated in the bacterial chromosome however, the bacteriophage is dependent on the host cell and carriage of the bacteriophage will add a metabolic burden to these bacteria and/or kill a proportion by lysis, potentially rendering the bacteriophage-bacterium association less competitive. This effect will be counteracted if the bacteriophage brings some form of selective advantage to the lysogenised bacterium. Thus, the inherent advantages of being a lysogen are immunity to lysis by infection with the same or similar bacteriophages and killing of competing bacteria by phage particles released from a small fraction of the lysogens. A further advantage may be achieved when the bacteriophage carries additional genes of benefit to the bacterium.

4. Functions carried by bacteriophages

Analysis of the first studied of the lambdoid bacteriophages, lambda itself, has indeed identified such beneficial genes among the "accessory" sequences, dispensable for phage growth and comprising a sizeable proportion of the 48.5 kilobase genome. The Bor and Lom gene products, homologous to other chromosomally coded bacterial pathogenicity factors, are

involved respectively in adhesion and in resistance to the host immune system. Another pair of genes *rexA* and *rexB* provides some immunity to a variety of unrelated bacteriophages. Hence the "original" lambda encodes factors which promote survival of the lysogen. Comparative virus genomics has uncovered many other such accessory genes, present in a subset of related viruses [17]. These include genes for the Shiga toxin, superoxide dismutase and SopE, an effector injected by the type III secretion system of *Salmonella*.

The list of pathogenesis determinants carried by bacteriophages continues to grow. Various surface proteins promoting adhesion are virus encoded. Bacteriophage-associated invasins include, as mentioned above, effectors important in uptake into mucosal cells, and also enzymes which cause degradation of the extracellular matrix to allow dissemination of the bacteria from the focus of infection in invasive streptococcal and staphylococcal disease. A group of bacteriophages in the enterobacteria code for enzymes which, interacting with the host cell biosynthetic machinery, alter the antigenic structure of the O side chain of the LPS, hence modifying interactions with human cells and providing a mechanism of avoidance of the immune response. Certainly the most well-known of the bacteriophage-carried pathogenicity factors are the exotoxins: diphtheria, Shiga and cholera toxins, certain of the botulinum toxins (predominantly associated with avian botulism rather than with foodborne intoxications) and a plethora of staphylococcal and streptococcal toxins involved in invasive disease. Indeed the importance of bacteriophages in the evolution of bacterial virulence is exemplified by the case of *Streptococcus pyogenes*, where an accumulation and reassortment of virus-encoded pathogenicity factors is probably responsible for the emergence of particularly virulent clones causing highly invasive disease [18].

An interesting alternative means by which bacteriophages may contribute to virulence is afforded by the recent example of the involvement of *Pseudomonas aeruginosa* filamentous bacteriophage Pf4 in the formation of biofilms [19]. Here, localised activation of the prophage (or spontaneous mutation to resistance to the bacteriophage repressor system) causes a decrease in growth rate in affected cells, and also to regions of cell death in the biofilm. The regions of cell death correspond to the voids characteristic of the three-dimensional structure of pseudomonal (and certain other) biofilms. It is tempting to speculate that this behaviour is adaptative in the context of the biofilm, participating in the genesis of the open structure increasing nutrient transport, and allowing dissemination of colonising bacteria (These regions of cell death nevertheless contain viable cells which are released from the biofilm). It is known that *P. aeruginosa* grows as a biofilm in the lungs of persons suffering from cystic fibrosis, and so it is possible that this filamentous phage plays a role in the pathogenesis. For further discussion of bacteriophage-carried pathogenicity factors, the reader may consult a number of recent reviews, for example [9,20,21].

5. Discovery of new bacteriophage-carried pathogenesis determinants

The discovery of the association of these pathogenicity factors with bacteriophages has involved different methods, depending on the historical context. For example, the discovery that the diphtheria toxin was bacteriophage-encoded proceeded, from the discovery of Freeman and Morse that toxin production was due to lysogeny by bacteriophage beta, through the production of mutants, genetic and physical maps of the bacteriophage [22], to sequencing of the *tox* gene [23] which opened up the possibility of the engineering of immunotoxins, for example. In an inverse procedure, based on the observation of tandem arrays of CTX operons in *V. cholerae* [6,24], Waldor and Mekalanos discovered that the toxin genes were part of a filamentous bacteriophage, and that the capacity to produce cholera toxin was transferable between strains. Advances in sequencing techniques and in particular the advent of genome sequencing allow the relatively facile identification of prophage sequences in genomes.

Nevertheless, identification is complicated by the fact that some conjugative transposon-like elements contain bacteriophage-like integrases, as do integrons and pathogenicity islands. Again, nucleotide sequence homologies may more or less strongly suggest that bacteriophage genes are involved in pathogenesis, but the large number of open reading frames (ORF) of undetermined function in bacteriophages [17] leaves room for speculation concerning the possible involvement in pathogenesis of ORFs lacking homology to proteins of known function.

Pathogenicity factors carried by bacteriophages are then a subset of genetically mobile pathogenicity factors and new, phage-encoded pathogenicity factors are amenable to detection by standard techniques. These include random transposon mutagenesis, *in vitro* expression technology, and systematic gene inactivation, using tissue culture or animal models of pathogenesis. In a recent example, Gonzalez and colleagues used signature-tagged mutagenesis to identify factors necessary for pathogenesis of *Escherichia coli* serotype K1 in the infant rat. Investigation of one of the pathogenicity factors so identified showed that it was part of a region with similarities to filamentous bacteriophages [25], and indeed bacteriophage particles were found to be secreted into the supernatant. By a search of available genomic sequences, a similar sequence was found in the clone orientalis of *Yersinia pestis* (enterobacteriaceae) responsible for the current plague pandemic.

Thus, biological assays screening banks of mutants produced, for example, by random transposon mutagenesis, signature-tagged mutagenesis provide powerful methods of detection of pathogenesis-associated genes. However, production and screening of the bank is very labour-intensive. This is a particular problem for in cases where there is no animal model that adequately represents the disease as it occurs in humans, and where a search for pathogenicity factors therefore necessitates screening in several assays representing different stages of the disease process. The advent of the "genomic era" provides a wealth of information permitting the choice of candidate genes, for example on the basis of similarity to known pathogenesis factors. On the other hand, the large quantity of information demands that methods be developed to find the most relevant candidates. Apart from the possible errors of attribution of function based on frequently partial homologies, many predicted genes are without homology to genes of known function. The choice of potential pathogenicity factors therefore remains a considerable task. Comparative genomics provides a method of narrowing down the range of possible candidates by choosing genes specifically present in a population expressing a particular pathogenicity phenotype. Whole genome DNA arrays allow the detection of the presence or absence of each gene in the genome in a range of isolates of varying pathogenic potential. This method will not detect relatively small genetic differences, but will reveal the presence or absence of all but the smallest genes and will certainly bring to light pathogenicity islands and bacteriophages.

6. Molecular epidemiological solutions: the case of *Neisseria meningitidis*

Such approaches have been used to search for new pathogenicity factors associated with certain disease phenotypes. A comparison of strains of *Vibrio cholerae* associated with epidemic versus non-epidemic disease brought to light several groups of genes including a type III secretion system [26]. Analysis of isolates of *Helicobacter pylori* associated with different outcomes of infection [27] showed great variability in gene content, and the authors were able to identify several putative pathogenesis determinants. *Neisseria meningitidis* is another bacterial species with differences in pathogenic potential between individual clones. Colonisation by the meningococcus usually leads to an asymptomatic infection of the nasopharyngeal mucosa, but in a small proportion of cases the bacteria cross the epithelium, gain access to the bloodstream and disseminate to cause systemic disease which is typically

characterised by a cerebrospinal meningitis. The reasons for which some meningococcal strains cause life-threatening disease remain unclear. Disease is a multifactorial process, depending on characteristics of both the host and the infecting bacteria. Several pathogenicity factors are well known, for example the adhesive pili which permit the attachment to the nasopharyngeal epithelium, the polysaccharide capsule which affords protection against the immune system and allows survival in the blood, and the iron-acquisition systems based on the transferrin- and lactoferrin-binding proteins. However these factors are widely distributed among meningococci irrespective of their pathogenicity and can not by themselves explain the differences in pathogenic potential between isolates of this species. It is likely that the capacity to cause disease is determined by the combination of a number of pathogenicity factors, and that some surely remain to be discovered. Comparison of strains of different pathogenic potential by the use of whole genome DNA arrays is an attractive strategy to accomplish this. The use of molecular genetic techniques such as Multi-Locus Sequence Typing [28] confirmed earlier observations that disease-causing isolates were restricted to a small number of the many clonal groups - the so-called "hyperinvasive" lineages - and hence permits the choice of isolates corresponding to meningococci of defined pathogenic potential for use in DNA array experiments.

N. meningitidis is a naturally-transformable species; genetic change is predominantly due to horizontal transfer between different strains, and the population structure of the meningococcus is web-like rather than tree-like (Fig. 2). Nevertheless successful clones arise and spread within the human population, where they survive for a matter of years before herd immunity leads to their being replaced by new strains. Some of these clones have relatively high pathogenic potential (measured as attack rate, or case/carrier ratio) and will give rise to epidemics of meningococcal disease. Others are probably non-pathogenic, while the majority of groups comprise bacteria of different degrees of pathogenicity, or too few isolates have been collected to allow an estimation of their potential to cause disease. Hence we can compare the genetic complement of isolates from several of the so called "hyperinvasive" clonal groups with that of isolates from clonal groups that have been associated only with asymptomatic carriage, which we may call "non-invasive" groups (presuming them to be incapable of causing disease). Indeed a whole genome DNA array comparison of 48 strains belonging to clonal groups of high pathogenicity with 19 strains belonging to clonal groups never having caused disease [29] brought to light a genetic island of about 8 kb that was specifically present in the isolates belonging to pathogenic clonal groups (Fig. 3). Blast comparisons against the public databases revealed similarity of the first ORF with the phage replication proteins of phages CTX and Ff. This, combined with a similar genetic organisation suggested that the genetic island corresponds to an integrated bacteriophage genome. Figure 3 summarises the similarities between the meningococcal prophage, the archetypal coliphage Ff and other filamentous bacteriophages recently associated with pathogenicity.

The presence of the prophage in a number of unrelated hyperinvasive lineages and its absence from a number of non-invasive lineages suggests a causal relationship (the probability that the results were due to chance alone was estimated to be 0.7%). However, the demonstrated association is with the hyperinvasive epidemiologial groups rather than with the disease itself. In addition, if the prophage were associated with another, hypothetical, gene that increased the pathogenic potential of the meningococcus, a large proportion of bacteria belonging to the same clonal complex would contain both the prophage and the pathogenicity gene and would have a higher attack rate independent of the effect of the prophage. However, *N. meningitidis* being a naturally transformable bacterium, gene transfer will occur horizontally as well as vertically (clonally). Furthermore a bacteriophage, being an infectious

DNA molecule has evolved to pass horizontally between individual organisms. If the prophage contributes to pathogenicity, bacteria carrying it will tend to cause disease more frequently than do bacteria without the element, though the effect may not be striking (since the most virulent organisms cause disease in only a small proportion of those people who are colonised).

The DNA array analysis was therefore followed by a larger epidemiological analysis of 193 strains taken from a study of carried strains in the Czech Republic [30]. The isolates were classified according to three characteristics: having or not having caused disease, possessing or not possessing the prophage (defined by PCR and/or Southern blot analysis) and belonging to or not belonging to a recognised hyperinvasive clonal complex. As discussed above, each of these variables affects the other two, but multivariate analysis permits an estimation of the strength of association of each of the pairs in the absence of an effect from the third. Hence the effect of the presence of the prophage on the attack rate of the meningococci is highly significant ($p < 0.001$) even taking into account the effect of the clonal relationships within the population studied. Meningococci carrying the prophage are more likely to cause disease.

Hence the prophage is apparently a pathogenicity factor for *Neisseria meningitidis*. However, once the prophage had been identified, similarity searches demonstrated little homology to known virulence factors (ORF1 was homologous to the CTX-phi phage replication protein, ORF8 showed some similarity to the phage assembly protein Zot and ORF9 is related to transposases). This contrasts with the case of bacteriophage CTX-phi, which carries a defined toxin, and that of the *E. coli* / *Yersinia* bacteriophage, which was identified after sequencing the flanking region of *puvA*, whose inactivation led to reduced virulence in an animal model. The question remains then as to the advantage brought by the prophage to the host cell, and how this causes the bacteria to be more pathogenic. By analogy with CTX-phi, or the *E. coli* (and *Y. pestis*) bacteriophage, the genes *ORF6* and *ORF9* of the meningococcal prophage would be the most attractive candidates (see Fig. 3), but in the absence of experimental evidence any of the genes might have an effect on the interaction of the bacteria with the host. The membrane proteins will come into contact with the host cells and immune effectors, while it is possible that the transposase ORF9 serves to maintain a high level of genetic fluidity, which may be beneficial to the clonal group as a whole.

Experiments attempting to demonstrate a possible effect of the bacteriophage on meningococcal disease have not so far been conclusive. Indeed deletion of the entire prophage did not have any effect in laboratory models of meningococcal pathogenesis [29], suggesting that the effect of the bacteriophage is either subtle, or not detectable by the tests used. It is certain that the balance between commensalism and pathogenicity is delicate in the case of *Neisseria meningitidis*. A small increase in resistance to the human's immune defence system, induced by the bacteriophage, giving the meningococci a competitive edge in the habitat of the nasopharyngeal mucosa might tip the balance from a relationship of commensalism to one of a pathogen-host conflict.

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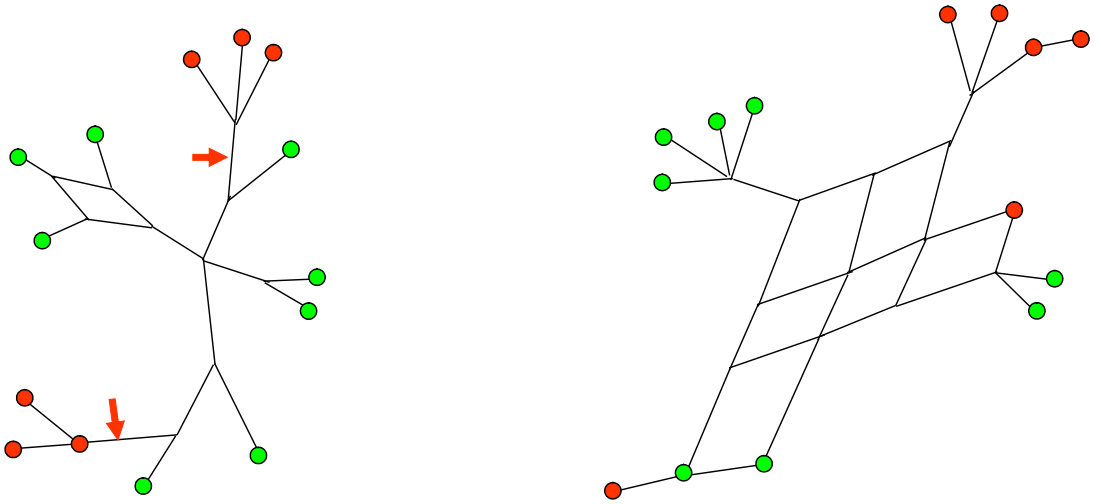
Fig. 1. Corynephage beta released from toxinogenic *C. diptheriae* are capable of transforming non-pathogenic strains to toxin-releasing strains which are virulent in animal models. Reprinted with permission from [1].



a) Predominantly clonal phylogeny **b) Phylogeny characterised by horisontal transfer**

Fig. 2. Schematic phylogenies of (a) a predominantly clonal bacterium (eg. *Escherichia coli*) and (b) a bacterium undergoing frequent horisontal transfer (eg. the naturally-transformable *Neisseria meningitidis*) as would be produced by split decomposition analysis of multi-enzyme sequence data. Isolates of higher pathogenic potential (red) are readily identified in the clonal phylogeny as clades, related by descent from a common, pathogenic ancestor. The most economical interpretation is the acquisition of one or more pathogenicity factors (arrows) by the common ancestor. In the case of the transformable species the genetic information, including the phylogenetic markers, of one isolate may come from more than one "parent", complicating the interpretation of the phylogeny. However, clonal expansion of certain genotypes will nevertheless lead to distinguishable groups of related bacteria (for example, at the top of the figure).

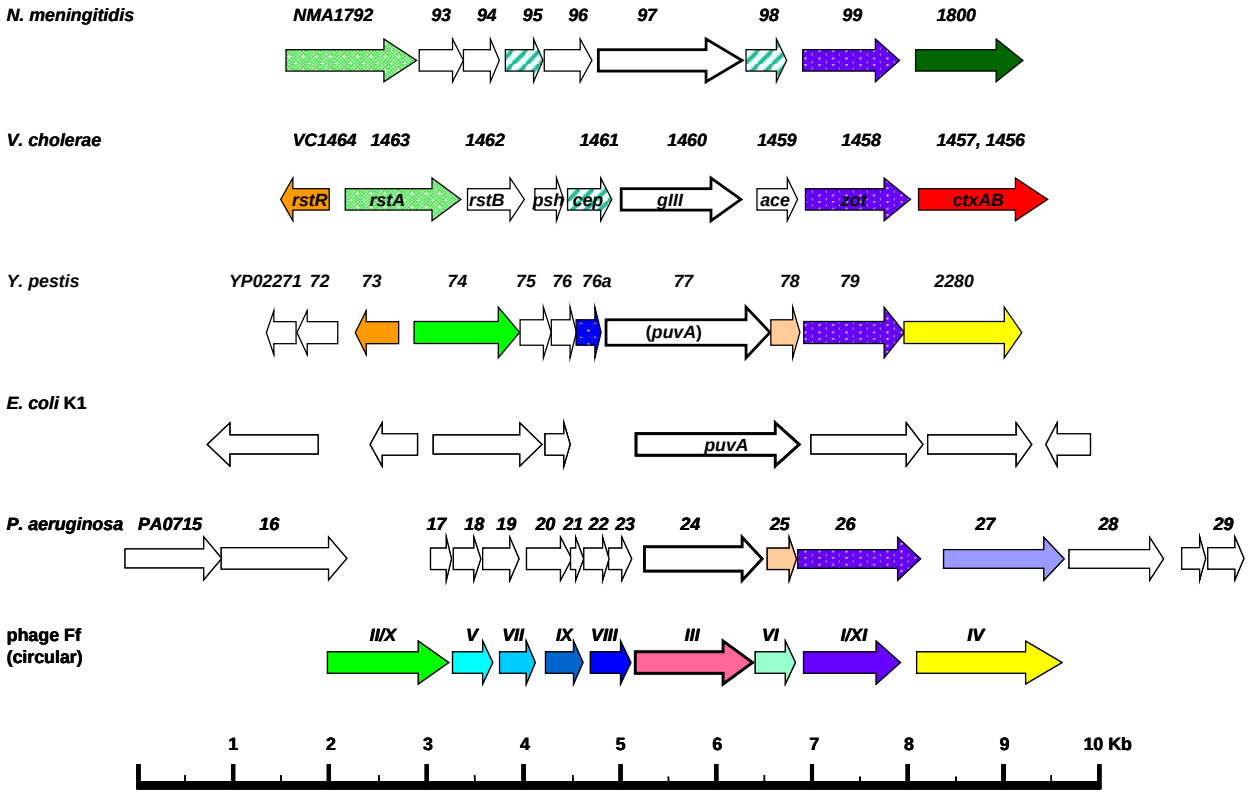


Fig. 3. Organisation of genes in the prophage form of filamentous bacteriophages which have been associated with pathogenesis and comparison with coliphage Ff. Homologous genes (having predicted protein products showing similarity by BlastP) are similarly coloured. Striped and stippled colouring indicates low homology. Green are phage replication proteins, orange are repressors, gene I/XI of coliphage Ff (violet) is involved in the export of bacteriophage particles. ORFs in *N. meningitidis*, *Y. pestis* and *P. aeruginosa* are as predicted by the genome sequencing projects; those of *V. cholerae* are modified with reference to [7]. The sequence of the prophage from *E. coli* was not available.

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