Journal of Microbiology (2014) Vol. 52, No. 3, pp. 235–242
DOI 10.1007/s12275-014-4083-3

REVIEW

When a Virus is not a Parasite: The Beneficial Effects of Prophages on Bacterial Fitness

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(Received Feb 8, 2014 / Accepted Feb 10, 2014)

Most organisms on the planet have viruses that infect them. Viral infection may lead to cell death, or to a symbiotic relationship where the genomes of both virus and host replicate together. In the symbiotic state, both virus and cell potentially experience increased fitness as a result of the other. The viruses that infect bacteria, called bacteriophages (or phages), well exemplify the symbiotic relationships that can develop between viruses and their host. In this review, we will discuss the many ways that prophages, which are phage genomes integrated into the genomes of their hosts, influence bacterial behavior and virulence.

Keywords: prophages, superinfection exclusion, virulence, CRISPR-Cas, toxins, mutualism

Introduction

Contact between a phage particle and a receptive host leads to injection of the phage genome into the host cell. At this point, a phage may follow the lytic life cycle, in which the phage genome is replicated and packaged into phage particles, and the cell is ultimately lysed to release these particles. Alternately, the lysogenic cycle can be entered, in which the phage genome is integrated into the bacterial genome, or forms an extrachromosomal plasmid. The phage is then described as a prophage and the cell, a lysogen. Most prophage genes are repressed; thus, the lysogenic cell can survive and replicate without the production of phage particles or deleterious phage proteins. The sequencing of thousands of bacterial genomes has highlighted the fact that most harbor multiple prophages with some genomes being comprised of up to 20% prophage sequences (Canchaya *et al.*, 2003; Casjens, 2003). Remarkably, approximately 25% of phage genomes on earth exist in the form of prophages (Casjens, 2005). Given the prevalence of lysogeny, it is not surprising that phages carry a wide variety of genes that provide a fitness advantage to their host when expressed from the prophage. In fact, to truly appreciate the role of phages in bacterial physiology, phages must be regarded not only as bacterial parasites, but also as mutualists. A further crucial aspect of prophages is their ability to readily excise from their host genome, enter the lytic cycle, and infect other cells; thereby spreading their genes to other bacteria. The established capacity of phages to influence bacterial behaviour and disseminate potentially pathogenic genes has spurred great interest in investigations of prophage functions. In the sections below, we review many of these studies, particularly those focusing on the contributions of prophages to bacterial fitness, virulence and ecology (summarized in Table 1).

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General aspects of prophages

When a phage genome integrates into its host genome, most phage genes must be repressed to maintain normal cell viability. This general repression is achieved through the action of phage repressor proteins, and the mechanisms of action of many phage repressors have been studied in great detail (Dodd *et al.*, 2005). The action of the repressor proteins expressed by prophages leads to resistance to superinfection by the same phage. This "immunity" to subsequent infection by homologous phages is a hallmark of all lysogens. Prophage-mediated phenotypic changes that are not mediated simply through repressor activity have been referred to as "lysogenic conversion". To decipher the genetic basis for such phenotypes, two general features of a prophage need to be examined; a) the insertion site in the bacterial genome, and b) genes that may be expressed from an otherwise repressed prophage. Many phages, such as *Escherichia coli* phage Mu, integrate into the host genome at random positions through a transposition mechanism (Morgan *et al.*, 2002). These integration events can cause phenotypic changes by interrupting host genes. However, if the same phenotypes are observed in every lysogenic isolate of a given phage, prophage-encoded genes are likely responsible. Other phages, such as *E. coli* phage λ, always integrate at the same position in their host genome using a site-specific integrase (Kotewicz *et al.*, 1977). For these phages, the insertion event could cause a consistent phenotype, so this possibility must be investigated. In most cases, however, genes expressed from the prophage are responsible for lysogenic conversion phenomena.

Table 1. Summary of prophage-mediated phenotypes

Host	Prophage	Gene(s)	Effect	References
Superinfection Exclusion				
Escherichia coli	phi80, N15	*cor*	Inactivation of membrane receptor FhuA inhibits phage adsorption	Vostrov *et al.* (1996)
Pseudomonas aeruginosa	D3	*oac, wzy, iap*	Serotype conversion prevents phage adsorption	Newton *et al.* (2001)
Escherichia coli	HK97	*15*	Phage entry inhibited by inner membrane protein	Cumby *et al.* (2012b)
Salmonella Typhimurium	P22	*sieA*	Phage entry inhibited by inner membrane protein	Hofer *et al.* (1995)
Vibrio cholerae	K139	*glo*	Phage entry inhibited by periplasmic protein	Nesper *et al.* (1999)
Escherichia coli	P1	*sim*	Phage blocked downstream of adsorption	Maillou and Dreiseikelmann (1990)
Escherichia coli	T4	*imm*	Phage entry inhibited	Lu and Henning (1994)
Escherichia coli	T4	*sp*	Phage lysozyme activity inhibited	Lu and Henning (1994)
Lactococcus lactis	Tuc2009	*sie2009*	DNA injection inhibited	McGrath *et al.* (2002)
Streptococcus thermophilus	TP-J34	*ltp*	Phage entry inhibited by lipoprotein	Sun *et al.* (2006)
Streptococcus thermophilus	ΦSfi21	*orf203*	Unknown mechanism protects against heterologous phages	Bruttin *et al.* (1997)
Mycoplasma sp.	MAV1	*vir*	Phage entry inhibited by lipoprotein	Clapper *et al.* (2004)
Escherichia coli	λ	*rexA-rexB*	Superinfecting phage replication is inhibited through induced cell death	Shinedling *et al.* (1987)
Bacillus subtilis	SPβ	*nonA*	Superinfection induces *nonA* expression, aborting the infection	Yamamoto *et al.* (2014)
Pathogenesis				
Escherichia coli	λ	*lom*	Outer membrane protein increases adherance to epithelial cells	Vica Pacheco *et al.* (1997)
Escherichia coli	λ	*bor*	Lipoprotein improves survival in human serum	Barondess and Beckwith (1990)
Pseudomonas aeruginosa	FIZ15	Unknown	Lysogenic cells are more resistant to phagocytosis and human serum	Vaca-Pacheco *et al.* (1999)
Neisseria meningiditis	Unknown	*orf6*	Encoded TspB protein binds human immunoglobulin in biofilm matrix	Müller *et al.* (2013)
Vibrio cholerae	K139	*glo*	Required for virulence in mouse model of cholera	Reidl and Mekalanos (1995)
Vibrio cholerae	VPIΦ	*tcpA*	Filamentous phage encodes colonization factor and receptor for toxin producing phage, CTXΦ	Karaolis *et al.* (1999)
Vibrio cholerae	CTXΦ	*ctx*	Cholera toxin produced from a prophage	Waldor and Mekalanos (1996)
Escherichia coli	933W	*stx2*	Prophage induction via repressor cleavage produces Shiga-like toxin	Tyler *et al.* (2004)
Salmonella Typhimurium	SopEΦ	*sopE*	Prophage-encoded type III effector secreted into human cells	Mirold *et al.* (1999)
Salmonella Typhimurium	Gifsy-2	*sodC*	Prophage-encoded superoxide dismutase produced to neutralized reactive oxygen species	Figueroa-Bossi and Bossi (1999)
Hamiltonella defensa	APSE-3	*Unknown*	Aphid symbiotic bacterium protects host from parasitism through putative toxin	Oliver *et al.* (2009)
Streptococcus mitis	SM1	*pblA/B*	Phage structural proteins and phage lysins allow the bacterium to bind to platelets	Mitchell *et al.* (2007), Seo *et al.* (2010)
Streptococcus pyogenes	H4489A	*hylP*	Phage-associated hyaluronidase facilitates infection through capsule and is produced from prophage	Benchetrit *et al.* (1977)
CRISPR-Cas				
Pseudomonas aeruginosa	DMS3	*42*	An interaction between the CRISPR-Cas system and gene 42 inhibits biofilm production	Zegans *et al.* (2009), Cady and O"Toole (2011)
Pseudomonas aeruginosa	JBD30	*35*	Anti-CRISPR protein inhibits the CRISPR-Cas system during infection and lysogeny	Bondy-Denomy *et al.* (2013)
Vibrio cholerae	ICPI	CRISPR-Cas	A phage-encoded CRISPR-Cas system inhibits an anti-phage island during infection	Seed *et al.* (2013)
Clostridium difficile	Many	CRISPR-Cas	Prophages possess transcriptionally active CRISPR loci	Soutourina *et al.* (2013)
Metagenome	Metagenome	CRISPR-Cas	Metagenomic analysis of the human gut revealed prophage-encoded CRISPR-Cas systems	Minot *et al.* (2011, 2013)
Microbial Ecology				
Unknown	Unknown	Ab resistance	Ab treatment of mice caused the production of phages carrying various Ab resistance genes	Modi *et al.* (2013)
Bacteroides cellulosilyticus	Unamed	IG region	Prophage provides fitness advantage to host in murine gut	Reyes *et al.* (2013)
Cyanobacteria	Unamed	Metabolic	Prophage-encoded genes provide metabolic and photosynthetic capacity	Rohwer and Thurber (2009)
Bacillus anthracis	Many	σ factors	Prophage-mediated control of sporulation and exopolysaccharide production	Schuch and Fischetti (2009)
Escherichia coli	9 prophages	Unknown	Deletion of nine prophages compromised host fitness	Wang *et al.* (2010)
Pseudomonas aeruginosa	5 prophages	Unknown	Multiple prophages present in epidemic strain which play a role in virulence	Winstanley *et al.* (2009)
Enterococcus faecalis	Many	*pblA/B*	Prophages induced from clinical isolates encode PblA/B, platelet binding proteins	Yasmin *et al.* (2010)
Flavobacterium psychrophilum	Many	-	Eighty percent of a collection of strains contained a prophage related to phage 6H	Castillo *et al.* (2013)
Unknown	Many	-	Mitomycin C treatment of unculturable bacteria in soil revealed many lysogenic strains	Ghosh *et al.* (2008)

Abberviations: Ab, Antibiotic; IG, Intergenic

Prophage-expressed genes are usually not essential for the phage life cycle and often comprise more recently acquired genetic elements with the characteristic AT-richness of foreign DNA (Juhala *et al.*, 2000). These elements can increase host fitness and independent transcriptional promoters and terminators often control their expression. These elements are often recognized as extra genes when comparing closely related phage genomes. Thus, they have been referred to as "morons" to indicate that "when one is present in the genome there is more DNA than when it is not present" (Juhala *et al.*, 2000). Many of the phenotypic alterations caused by prophages are mediated by genes contained within moron elements (Cumby *et al.*, 2012a).

Effects of prophages on the cell envelope: Superinfection exclusion

The most intensively studied effect of prophages is their inhibition of other phages including themselves. This "superinfection exclusion" is expected to be highly adaptive since phages are by far the most abundant predators of bacteria [on average in most niches there are 10 phages for every bacterium (Suttle, 2005)]. Superinfection exclusion can be achieved through a wide variety of mechanisms, but most involve alterations to the cell surface or other cell envelope components. For example, the prophage-expressed *cor* gene of *E. coli* phages Φ80 and N15 blocks the cell surface adsorption of superinfecting phages T1, Φ80 and N15 (Vostrov *et al.*, 1996). The ferrichrome uptake protein FhuA is the receptor for all of these phages. The replication of many other phages that require FhuA as their receptor is also blocked by expression of the *cor* gene (Uc-Mass *et al.*, 2004). In addition, the uptake of ferrichrome, which is mediated by FhuA, is inhibited by *cor* expression. It was concluded that Cor blocks phage infection by inactivating FhuA (Uc-Mass *et al.*, 2004). The *Pseudomonas aeruginosa* phage D3 prophage blocks superinfection through an entirely different mechanism. A three-gene operon expressed from this prophage modifies the O-antigen of the cell surface lipopolysaccharide (LPS) of its host, thus changing its serotype. Since the replication of many phages requires specific binding to the O-antigen, this change in serotype prevents superinfection by phages including phage D3 itself (Newton *et al.*, 2001).

Following the adsorption of a superinfecting phage, the phage genome must be injected into the host. This step is a common target for prophage-mediated superinfection exclusion. For example, prophage-expressed gene *15* of *E. coli* phage HK97 produces a small likely inner membrane protein that inhibits superinfection by HK97. The product of gene *15*, gp15, prevents DNA entry into the cytoplasm, but does not block the replication of other phages using the same cell surface receptor as HK97. Thus, HK97 inhibition by gp15 must occur at a step after surface adsorption, likely via an interaction with the tail tube or tape measure proteins of the superinfecting phage (Cumby *et al.*, 2012b). Similarly, the *Salmonella* Typhimurium prophage P22 produces SieA, an inner membrane protein that blocks DNA entry of phage P22 without affecting its cell surface adsorption (Hofer *et al.*, 1995). *Vibrio cholerae* prophage K139 expresses Glo, a periplasmic protein which also appears to inhibit phage genome entry (Nesper *et al.*, 1999). The *sim* system of the *E. coli* P1 prophage also inhibits superinfection by self and other phages through a poorly characterized process operating downstream of adsorption (Maillou and Dreiseikelmann, 1990). These mechanisms are not unique to prophages, as the well studied lytic phage T4 produces the Imm protein which blocks DNA entry as well as the protein Sp which inhibits activity of the phage lysozyme (Lu and Henning, 1994). This is presumably a way of preventing superinfection when a lytic cycle is already underway, to ensure the fidelity of this process.

Superinfection exclusion systems have also been well studied in Gram-positive species, with much of the work aimed at resisting phage infection in the dairy industry. Many of these systems also block the DNA entry step. The Tuc2009 prophage-encoded moron gene, *sie2009*, encodes a protein that associates with the *Lactococcus lactis* cell membrane and blocks the DNA injection step of superinfecting phages (McGrath *et al.*, 2002). Distinct exclusion systems inhibiting DNA entry have also been found in other *L. lactis* prophages, such as two systems that act against the 936 phage group (Mahony *et al.*, 2008). The *Streptococcus thermophilus* TP-J34 prophage expresses the *ltp* gene, which encodes a lipoprotein that can block TP-J34 DNA entry as well as other members of the lactococcal 936 phage group (Sun *et al.*, 2006). Phage ΦSfi21, which infects *S. thermophilus*, possesses *orf203*, a gene that leads to resistance to superinfection by heterologous phages (Bruttin *et al.*, 1997). Interestingly, this gene is found in the same genomic position (i.e., next to the integrase gene) as the *V. cholerae* phage K139 *glo* and *L. lactis* phage Tuc2009 *sie2009* genes discussed above. Phages infecting *Mycoplasma* sp. (a genus that lacks peptidoglycan but is related to Gram positive bacteria) demonstrate similar superinfection exclusion effects. The prophage-expressed *vir* gene of phage MAV1 produces a lipoprotein localized to the outer surface of the cell membrane that blocks entry of MAV1 (Clapper *et al.*, 2004).

In summary, there are a wide variety of prophage-produced proteins both in Gram-negative and positive organisms that block phage replication by altering components of the cell envelope. Interestingly, most of these proteins do not block adsorption of phage particles to the cell surface, but inhibit the subsequent step of DNA entry. This phenomenon could indicate that there are generally fewer means by which phages can inject their DNA through the cell membrane than there are for adsorption to the cell surface. Thus, blocking DNA entry can potentially inhibit more phages than blocking one surface receptor.

Effects of prophages on the cell envelope: Increasing pathogenicity

A number of prophage-expressed genes affect bacterial pathogenicity. One of the first prophage-expressed genes identified was *lom* of *E. coli* phage λ, which encodes an outer membrane protein (Reeve and Shaw, 1979). The Lom protein increases the ability of λ lysogens to adhere to human buccal epithelial cells (Vica Pacheco *et al.*, 1997). The *bor* gene, which is also expressed from a λ prophage, encodes a lipoprotein

that improves the survival of lysogenic cells in animal serum (Barondess and Beckwith, 1990). Similar effects have been observed in *P. aeruginosa*, where prophage FIZ15 converts strain PAO1 to being more resistant to phagocytosis and human serum, while increasing adherence to epithelial cells (Vaca-Pacheco *et al.*, 1999). A protein called TspB, which is expressed from prophages in invasive *Neisseria meningiditis* strains, is present on the bacterial cell surface and binds to human IgG, leading to formation of large bacterial aggregates in a biofilm (Müller *et al.*, 2013). This reaction may protect the bacteria from immune responses.

In some cases, the alteration of bacterial pathogenic properties caused by prophage-expressed genes may be related to phage superinfection exclusion. Both of these phenomena are often the result of bacterial cell envelope alterations; thus, changes affecting one property could certainly affect the other. For example, the *P. aeruginosa* prophage FIZ15 mentioned above also causes cells to become resistant to phage D3 and likely alters the O-antigen in a similar manner as does the phage D3 prophage (see section above)(Vaca-Pacheco *et al.*, 1999). Consistent with this theme, the *glo* gene, which mediates superinfection exclusion by *V. cholerae* phage K139, is also required for full virulence in a mouse model of cholera infection (Reidl and Mekalanos, 1995). These examples suggest that resistance to phage superinfection may confer two benefits for a converted bacterial host: first, the ability to survive longer in the environment by fending of phage infection; and second, changes to the cell envelope that resist phage may have beneficial effects with respect to bacterial virulence. Although papers often focus on only one such aspect of conversion, it seems possible that any prophage-induced envelope modification could display a phenotype in both contexts if the appropriate phage and virulence assays were used.

Other types of prophage-induced phage inhibition

One of the longest known and most extensively studied prophage-induced superinfection exclusion systems is the *rexA-rexB* system of *E. coli* phage λ. These genes are expressed in λ lysogens in the same operon as the *cI* gene encoding the λ repressor protein, and they prevent replication of phage T4 rII and other phage mutants (Shinedling *et al.*, 1987). The Rex proteins do not prevent phage DNA from entering the cell, but do cause a severe drop in membrane potential during phage DNA replication, which leads to a drop in ATP levels, and eventual "altruistic" cell death. The *rex* genes have a number of other intriguing effects, such as inducing stationary phase-like properties and inhibiting toxin-antitoxin systems (Engelberg-Kulka *et al.*, 1998; Slavcev and Hayes, 2003). In *Bacillus subtilis*, when cells with an SPβ prophage are infected with phage SP10, the prophage gene *nonA* is induced. Expression of *nonA* during the late stages of phage infection inhibits growth of *B. subtilis* and blocks the synthesis of virion proteins, suggesting that this process aborts the infection (Yamamoto *et al.*, 2014). Abortive infection systems operating in a similar manner are common in *L. lactis*, but many are present on plasmids rather than prophages (Chopin *et al.*, 2005). A variety of other inhibition mechanisms have been observed for prophage-expressed proteins (Snyder, 1995; Samson *et al.*, 2013).

Prophage-encoded virulence factors

Many bacterial pathogens rely on prophage-encoded genes for toxin production. In some cases, the presence of a single toxin gene acquired via phage is the difference between a harmless and harmful bacterium. The mobility of phage-borne toxin genes makes them particularly dangerous and facilitates the emergence of novel pathogens. Famously, phages encoding a Shiga-like toxin were identified when *E. coli* K12 acquired the ability to produce the toxin after being lysogenized with phages from the highly virulent *E. coli* O157:H7 strain (O'Brien *et al.*, 1984). The Shiga-like toxin gene is not a traditionally defined moron since prophage induction was required for production of the toxin (Tyler *et al.*, 2004), explaining the clinical observation that treatment with antibiotics capable of causing phage induction exacerbated infections (Wong *et al.*, 2000). *Vibrio cholerae* was converted from a harmless water-dwelling bacterium to a significant pathogen upon the acquisition of prophage VPIΦ, which encodes the toxin co-regulated pilus (TCP). This protein is a colonization factor in humans, and also served as a receptor for subsequent phage CTXΦ infection (Karaolis *et al.*, 1999). Upon lysogeny, the cholera toxin is produced from the CTXΦ prophage (Waldor and Mekalanos, 1996). Type III secretion systems in some organisms have acquired effectors via a prophage. In *Salmonella* Typhimurium, for example, phage SopEΦ provides the secreted effector gene *sopE* (Mirold *et al.*, 1999), and the Gifsy-2 prophage provides the superoxide dismutase *sodC* which is required for virulence (Figueroa-Bossi and Bossi, 1999). Many other prophage-encoded toxins have been discovered, such as the cytotoxin from *P. aeruginosa* (Nakayama *et al.*, 1999), diphtheria toxin from *Corynebacterium diphtheriae* (Freeman, 1951), and the *Clostridium botulinum* neurotoxin (Eklund *et al.*, 1971). For reviews on this subject see Boyd and Brüssow (2002) and Brüssow *et al.* (2004).

Prophage-encoded toxins are not always detrimental to the eukaryotic host of a bacterium. For example, the gamma-proteobacteria symbiont *Hamiltonella defensa* protects its aphid host from attack by a parasitoid wasp. This protection is dependent on an *H. defensa* prophage-encoded toxin (Oliver *et al.*, 2009). Interestingly, the prophage is spontaneously lost when cultured in lab conditions, which leads to reduced aphid reproductive capacity (Weldon *et al.*, 2013). The widespread symbiotic bacterium *Wolbachia* is estimated to be present in 66% of all arthropod species and many isolates harbor prophage WO. *Wolbachia* is found as a parasite or a mutualist in many different organisms with putative toxin genes in WO being identified that could contribute to these various interactions (Kent and Bordenstein, 2010).

In addition to traditional toxins, which appear to serve no role in the actual production and dissemination of phage particles, some phage proteins act in both virulence and in the virion. Proteins PblA and PblB of *Streptococcus mitis* are encoded on a prophage and promote binding of the bacterium

to human platelets. Similar to the Shiga-like toxin, phage induction increases the levels of PblA and PblB protein, but these proteins are also present in the phage virion (Bensing *et al.*, 2001). Interestingly the phage lysin and holin were also required for the platelet binding activity and it was later shown that cytoplasmic PblA/B are released from the bacterium due to lysin and holin activity, allowing PblA/B to bind to the platelets (Mitchell *et al.*, 2007). It was later shown that the lysin protein itself can also interact with fibrinogen in the platelets (Seo *et al.*, 2010), thus ascribing multiple roles to the phage proteins. The capsule produced by group A streptococci consists of hyaluronic acid, and hyaluronidase is detectable in purified phages, presumably to allow phage penetration through the capsule during infection. Interestingly, lysates from some temperate phages infecting this organism have been found to contain very high levels of hyaluronidase, much of it not being phage associated (Benchetrit *et al.*, 1977). In addition to its role during infection, this overproduction of hyaluronidase may help the dissemination of the streptococci through hyaluronic acid-containing human tissue. These somewhat unorthodox examples of prophage-encoded virulence traits demonstrate the unpredictability of new phenotypes that emerge upon prophage acquisition.

Prophages and CRISPR-Cas systems

The maintained integrity of an injected phage genome is necessary for a successful infection. By analogy to eukaryotes, bacteria possess innate and adaptive immune systems that can detect such infections. An innate response like restriction enzymes detect a combination of sequence motifs and base modifications while CRISPR-Cas adaptive immune systems uses an RNAi-like mechanism to recognize foreign DNA. Both of these systems ultimately cleave phage DNA, terminating the infection. Phages have many ways of evading restriction enzymes during infection, which are not prophage based and will not be discussed here [for a review, see (Labrie *et al.*, 2010)]. The clustered regularly interspaced short palindromic repeats (CRISPR), along with the CRISPR associated genes (*cas*) comprise a system whereby small guide RNAs with identity to invading genomes use complementary base pairing to direct a protein-mediated cleavage event (Brouns *et al.*, 2008; Garneau *et al.*, 2010). CRISPR-Cas systems are present in ~90% of archaea and ~50% of bacteria (Bhaya *et al.*, 2011). The CRISPR locus houses sequences called spacers that match previously encountered phage genomes and the transcription of this locus leads to the production of small targeting CRISPR RNAs. Interestingly, new DNA can be added to this locus during phage infection when the CRISPR-Cas system acquires a small piece of the phage genome (called the protospacer) and incorporates it into the CRISPR array (Barrangou *et al.*, 2007). This adaptation will then guide the production of new crRNAs and will target a phage with this sequence in the future. The abundance of CRISPR-Cas systems and their established role in preventing both phage and plasmid propagation have led to extensive study and practical applications of these systems in various organisms [for a review, see Barrangou (2013)]. Most notably, the recent adaptation of the CRISPR-Cas system as a novel eukaryotic genome editing tool has the potential to revolutionize biology, biotechnology, and medicine (Mali *et al.*, 2013; Wang *et al.*, 2014).

While restriction enzymes do not target self DNA (i.e. a prophage) once it has received the appropriate protective modifications, CRISPR-Cas systems have no such method for preventing the cleavage of a self prophage protospacer (Edgar and Qimron, 2010). Therefore, the presence of prophages with sequences matching their host CRISPR locus are not commonly seen (Stern *et al.*, 2010); however, point mutations can accumulate in the phage protospacer, which are sufficient to evade targeting (Barrangou *et al.*, 2007). Mutations to the *cas* genes which render the CRISPR-Cas system inactive could also explain the co-existence of matching spacer/protospacer sequences (Jiang *et al.*, 2013). In the absence of mutations, however, the CRISPR-Cas system could actually prevent the acquisition of beneficial prophages (Nozawa *et al.*, 2011). In *P. aeruginosa,* phages use anti-CRISPR proteins to allow infection of a host with an active CRISPR-Cas system (Bondy-Denomy *et al.*, 2013). Anti-CRISPR proteins were discovered on different temperate phage genomes and homologs of anti-CRISPR proteins were also found in other mobile elements in *Pseudomonas* sp. isolates. This suggests that anti-CRISPRs enable the successful transfer of foreign DNA into a host, thus bypassing the CRISPR-Cas system. Although anti-CRISPRs function during lytic infection, phage integration into the bacterial genome is also promoted, generating a genome containing a matching spacer/protospacer pair. Continued expression of the anti-CRISPR from the prophage ensures the ongoing stability of this pair and may allow the prophage to provide other fitness benefits. Paradoxically, the CRISPR-Cas system of the host is rendered inactive, which actually facilitates superinfection by phages that would have otherwise been inhibited by a functioning CRISPR-Cas system (Bondy-Denomy *et al.*, 2013). Also in *P. aeruginosa*, it has been observed that a DMS3 prophage requires a functioning CRISPR-Cas system to inhibit the biofilm formation and swarming motility of this organism (Zegans *et al.*, 2009). This effect was mapped to an interaction between the CRISPR-Cas system and the prophage at a region with five mismatches between the spacer and protospacer (Cady and O'Toole, 2011), demonstrating a novel type of prophage-mediated phenotypic change which requires the CRISPR-Cas system. When these mismatches were corrected, the CRISPR-Cas system then targeted the phage genome in a canonical manner, preventing infection and lysogeny (Cady *et al.*, 2012).

Vibrio cholerae strains possess a phage inducible chromosomal island (PICI)-like element that functions to prevent phage infection. Interestingly, *V. cholerae* phage ICP1 encodes an entire CRISPR-Cas system with spacers that match the PICI-like element. Upon phage infection, the island excises from the genome and circularizes to limit a productive phage infection. The phage-encoded CRISPR-Cas system neutralizes the activity of that island and can even acquire new spacers (Seed *et al.*, 2013). Thus, the CRISPR-Cas system is not exclusively an anti-phage mechanism and can, in fact, aid in phage replication or potentially, in lysogeny. Bacteria can also acquire a CRISPR-Cas system from a new prophage, as demonstrated in a strain of *Clostridium difficile* with three transcriptionally active, prophage-encoded CRISPR loci

(Soutourina *et al.*, 2013). This could essentially be viewed as a novel prophage-mediated superinfection exclusion mechanism like those outlined above. Further, metagenomic data have identified human gut microbes with prophage encoded CRISPR-Cas systems containing spacers which have matches to the co-existing virome, suggesting a putative role in superinfection exclusion in the human gut (Minot *et al.*, 2011, 2013).

Prophage ecology

Advances in metagenomics and sequencing technology have led to the conclusion that not only are phages abundant, but their genetic and protein diversity is massive (Pedulla *et al.*, 2003; Jacobs-Sera *et al.*, 2012). Additionally, the role phages play in human health and their contributions to the gut microbiome are starting to be investigated. For example, antibiotic treatment in a mouse model led to enrichment of temperate phages with the ability to transfer antibiotic resistance genes of all classes, not just to resist the challenge antibiotic (Modi *et al.*, 2013). In another study, an artificial microbial community comprised of common human gut microbiome members was used to colonize a mouse. Many of these organisms possessed prophages which were frequently induced *in vivo*, and through the mutagenesis of one strain, *Bacteroides cellulosilyticus* WH2, a strong fitness advantage was shown to be provided by a prophage in this strain (Reyes *et al.*, 2013). Outside of the traditional examples of prophage-mediated changes (i.e. toxin production, increased virulence and superinfection exclusion), other prophage-controlled traits are becoming apparent due to metagenomic studies. For example, prophages in marine organisms significantly expand the metabolic capacities of their host and cyanobacteria phages carry many key photosynthesis genes (Rohwer and Thurber, 2009).

Few studies have addressed the effects of multiple different prophages within a single bacterial strain, but with the current capability to sequence many phage genomes and conduct rapid phenotype profiling, such studies should yield insightful data. One such study was undertaken in *Bacillus anthracis* where individual prophages were introduced into a strain of interest. Among the observations were the ability of a prophage to block or promote sporulation, induce exopolysaccharide production, and increase long term *B. anthracis* survival in soil and in an earth worm intestine (Schuch and Fischetti, 2009). A phage-encoded sigma factor was identified, which could explain some phenotypes. Using an inverse approach, all of the 9 prophages in an *E. coli* strain were deleted and significant fitness loss was observed with increased sensitivity to antibiotics, and osmotic and oxidative stress (Wang *et al.*, 2010). Furthermore, the Liverpool Epidemic Strain of *P. aeruginosa* caused a significant outbreak in a children's cystic fibrosis unit. Upon genome sequencing, the strain was found to have 5 prophages and mutagenesis of genes in different prophages impaired virulence in a rat infection model, suggesting an important link between novel virulence phenotypes and prophage acquisition (Winstanley *et al.*, 2009).

The characterization of the abundance, distribution, and induction potential of natural prophage populations prvides insight about how encoded genes will spread. In *Enterococcus faecalis*, 47 bacteremia isolates were induced with various agents, revealing 34 phages of 4 different groups. Sequencing of 8 phages revealed homologs of the *S. mitis* platelet binding proteins (PblA/B) in each phage. Interestingly, lysogens made with each of the 8 phages showed differential survival in a *Galleria* wax moth larvae infection model (Yasmin *et al.*, 2010). Similar observations of prophage abundance have been made in *Flavobacterium psychrophilum* where prophages similar to the sequenced phage 6H were found in a 80% of a collection of 49 strains (Castillo *et al.*, 2013). Finally, using novel *in situ* phage induction and collection methods, uncultured soil bacteria were induced with mitomycin C, revealing that ~80% of the strains were lysogenic (Ghosh *et al.*, 2008).

Conclusion

Genomic and metagenomic studies have highlighted the prevalence of phages in the environment and prophages within bacterial genomes. Importantly, it is now becoming clear that the interplay between phages and bacteria within the human microbiome have a significant impact on human health. The variety of studies reviewed here demonstrate the multiple and complex mechanisms by which prophages can influence the behaviour and pathogenicity of bacterial species. Thus, it is clear that further work in this area will be crucial for our understanding of the human microbiome and for developing effective new anti-bacterial therapies.

Acknowledgements

Phage and CRISPR-Cas research in the Davidson laboratory is currently funded by the Canadian Institute of Health Research MOP-130482 and previously by CIHR grant XNE86943.

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