Cryptic Prophages as Targets For Drug Development

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ABSTRACT

Bacterial chromosomes may contain up to 20% phage DNA that encodes diverse proteins ranging from those for photosynthesis to those for autoimmunity; hence, phages contribute greatly to the metabolic potential of pathogens. Active prophages carrying genes encoding virulence factors and antibiotic resistance can be excised from the host chromosome to form active phages and are transmissible among different bacterial hosts upon SOS responses. Cryptic prophages are artifacts of mutagenesis in which lysogenic phage are captured in the bacterial chromosome: they may excise but they do not form active phage particles or lyse their captors. Hence, cryptic prophages are relatively permanent reservoirs of genes, many of which benefit pathogens, in ways we are just beginning to discern. Here we explore the role of active prophage- and cryptic prophage-derived proteins in terms of (i) virulence, (ii) antibiotic resistance, and (iii) antibiotic tolerance; antibiotic tolerance occurs as a result of the non-heritable phenotype of dormancy which is a result of activation of toxins of toxin/antitoxin loci that are frequently encoded in cryptic prophages. Therefore, cryptic prophages are promising targets for drug development.
INTRODUCTION

Bacteriophages and bacteria are the most abundant life forms on Earth. They also interact frequently, and each phage infection has the potential to introduce new genetic material into the bacterial host, thereby driving the evolution of bacteria. The introduction of novel genes by phages into the bacterial host can confer beneficial phenotypes that enable the exploitation of competitive environments (Canchaya et al 2003, Lawrence and Ochman 1998, Penadés et al 2015). For example, marine bacteriophage encode photosynthesis genes which may provide relief from intense sunlight in oceans for the phage and host (Mann et al 2003), as well as encode adaptive bacterial immune systems to provide immunity from competing phage known as clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) systems (Bellas et al 2015).

Among the beneficial genes, phages provide DNA for virulence, resistance, and tolerance to antibiotics as three major factors for pathogens during infection. Resistance involves genetic mutations that allow for growth in the presence of antibiotics whereas tolerance involves metabolic dormancy from the activation of toxins of toxin/antitoxin (TA) systems that allows pathogens to sleep through a course of antibiotic treatment (Wood 2016).

Treating pathogenic bacteria that evade antibiotics has become a global issue. This review will focus on the virulence, resistance, and tolerance genes carried by prophages, the antibiotics or other chemicals that can trigger the spread of these genes by prophage excision/integration, and also some preventative strategies to treat pathogens by targeting prophages. We also emphasize ways to avoid the adverse effects of triggering virulence dissemination by prophages.

Prophages are reservoirs of virulence genes

Bacteriophages provide one of the most efficient vehicles for moving DNA sequences (their own and the host’s DNA by mistake), via transduction, between bacterial cells. Horizontal transfer of genetic information by phages is much more prevalent than previously thought, and the environment plays a crucial role in the phage-mediated transfer of virulence genes (Penadés et al 2015). The relatively high numbers of phage \((10^{30} \text{ phage and approximate ratio of 10 phage to each bacterium})\) leads to frequent lytic and lysogenic phage infections \((10^{25} \text{ infections/sec})\) (Chibani-Chennoufi et al 2004). Unlike lytic phages, temperate phages are integrated into the bacterial genome and maintain a long-term lysogenic
relationship with their hosts (Figure 1). Lysogeny has a unique role within the bacterium-phage arms race in that it favors the development of a symbiotic relationship by providing an ecological window for the evolution of mutually-beneficial functions (Feiner et al 2015). Hence, these frequent phage infections provide ample opportunity to affect virulence.

There are major differences both between and within different bacterial species in their ability to cause infection. Opportunistic and pathogenic bacterial species include *Escherichia coli*, *Vibrio cholerae*, *Pseudomonas aeruginosa*, *Listeria* spp., *Salmonella enterica*, *Enterococcus faecalis*, *Streptococcus* spp., and *Staphylococcus* spp. A major driving force in the emergence and evolution of pathogenic isolates is the horizontal transfer and acquisition of virulence factors. Several mobile genetic elements (insertion sequences, plasmids, bacteriophages, and pathogenicity islands) have been implicated in the horizontal transfer of virulence genes; one of the most significant groups is the bacteriophages (reviewed in (Hastings et al 2004)).

Lysogenic conversion by prophages encoding toxins and other virulence determinants is the most ostensible contribution to bacterial pathogenesis (Brussow et al 2004). Many diseases are caused by toxins that are encoded by phages such as diphtheria, cholera, dysentery, botulism, food poisoning, scalded skin syndrome, necrotizing pneumonia or scarlet fever (Hacker and Kaper 2000). Among these, exotoxin production such as scarlatinal toxin, cholera toxin and Shiga toxin is the among the best documented virulence factors (Wagner and Waldor 2002). Bacteriophages can also alter host physiology to increase virulence at different stages of infection, including bacterial adhesion, colonization, invasion, resistance to host immune defenses, and transmissibility among human host (as reviewed by (Wagner and Waldor 2002)).

The contribution of phages to pathogenicity was first discovered in streptococci in 1927 when it was shown that nontoxic streptococci acquired the ability to produce scarlatinal toxin through the phages released by the toxic streptococci (Frobisher and Brown 1927). In the 1950’s, toxin-encoding bacteriophages of *Corynebacterium diphtheria* further linked phages to bacterial pathogenicity (Barksdale and Arden 1974, Freeman 1951, Groman 1953).

The cholera toxin of *Vibrio cholerae* illustrates well a case of how multiple phages contribute to bacterial pathogenicity (Brüssow and Hendrix 2002, Davis et al 2000, Karaolis et al 1998, Karaolis et al
Shiga toxin (Stx) is present in *Shigella dysenteriae* type 1 and shiga toxin producing *E. coli* (STEC). Two major classes of Stx are found in STEC, Stx1 and Stx2. The *stx* genes in *E. coli* strains are the central contributors to the virulence of enterohemorrhagic *E. coli* (EHEC), and EHEC infection can cause bloody diarrhea and can lead to hemolytic anemia, thrombocytopenia, renal failure, and even to death (Kaper et al 2004). Stx in *E. coli* O157:H7 is encoded as a late gene product by temperate bacteriophage integrated into the chromosome. Phage late genes, including *stx*, are silent in the lysogenic state, and Shiga toxin (Stx) production depends on the activation of the Stx prophage. Stress signals, including some induced by antibiotics, trigger the phage to enter the lytic cycle, and phage replication and Stx production occur concurrently (Kaper et al 2004).

Prophage also play a role in the virulence of *Pseudomonas aeruginosa*. *P. aeruginosa* is an important opportunistic pathogen with a broad host range (plants, invertebrates, and vertebrates) (Palleroni 1984), and *P. aeruginosa* is the most common cause of chronic lung infections in cystic fibrosis (CF) patients (Lyczak et al 2002). The Pf4 prophage is essential for several stages of the *P. aeruginosa* biofilm life cycle, and it significantly contributes to its virulence *in vivo* (Mai-Prochnow et al 2004, Rice et al 2009).

*Salmonella enterica* serovar Typhimurium harbors two functional prophages, Gifsy-1 and Gifsy-2 that contain virulence genes (Figueroa-Bossi and Bossi 1999). Prophage Gifsy-2 carries the *sodC* gene for a periplasmic [Cu, Zn]-superoxide dismutase involved with the defense against killing by macrophages (Farrant et al 1997). The removal of both prophages leads to a significant attenuation of virulence, and the curing bacteria for the Gifsy-2 prophage significantly reduces the ability of *S. enterica* serovar Typhimurium to establish a systemic infection in mice (Figueroa-Bossi and Bossi 1999).

In *Staphylococcus aureus*, expression of the phage encoded *sea, seg2, sek2* and *sak* toxins is greatly increased following prophage induction (Sumby and Waldor 2003). In *S. aureus*, *tst*, the gene that encodes toxic shock syndrome toxin, is carried by a 15 kb-long pathogenicity island (Hochhut and Waldor 1999). Prophage and prophage-like elements are also the major sources of variation between the genomes from *Streptococcus pyogenes* strains involved in two distinct pathologies, wound infections and rheumatic fever. The prophages encode several secreted proteins involved in the human–bacterium interaction, including the scarlet fever toxin.

The mammalian intestine is home to a dense community of bacteria and its associated bacteriophage,
which influence virulence. The Gram-positive bacterium *Enterococcus faecalis* is a natural inhabitant of the mammalian gastrointestinal tract and is commonly found in soil, sewage, water, and food, frequently through fecal contamination (Matos RC 2013). *E. faecalis* is an opportunistic pathogen that is a major cause of urinary tract infections, bacteremia and infective endocarditis, and *E. faecalis* V583 harbors a composite phage derived from two distinct chromosomally-encoded prophage elements. One prophage encodes the structural genes necessary for phage particle production and the other prophage is required for phage infection of susceptible host bacteria. *E. faecalis* V583 uses phage particles to establish and maintain dominance of its intestinal niche in the presence of closely-related competing strains. Recent studies of the human fecal virome show that temperate rather than lytic phages are long-term contributors to the microbial host phenotype through provision of adaptive genes (Reyes et al 2010).

**Prophages are reservoirs of antibiotic resistance genes**

Many microorganisms produce secondary metabolites with antimicrobial activities and release them into their natural habitats. These antibiotic-producing microorganisms are resistant to the antibiotics they produce, but for the non-resistant bacteria, they need to develop resistance mechanisms to ensure survival in these environments (Muniesa et al 2013). The presence of antibiotics in the environment may exert long-term selective pressure for the emergence and horizontal transmission of resistance mechanisms in the non-producing microorganisms. In recent years, the explosive spread of antibiotic-resistance determinants among pathogenic, commensal, and environmental bacteria has reached a global dimension. Prophages not only encode toxin genes for human pathogens but also carry genes that enhance the fitness of the bacterial cell in ecological niches (Hendrix 2003). For examples, prophage contain genes that provide protection from attack by other phages, such as restriction-modification systems (Vasu and Nagaraja 2013) and CRISPR/Cas systems (Deveau et al 2010). Growing evidence shows that phages also carry or transfer genes that participate in other cellular process such as inactivating antibiotics (Muniesa et al 2013). Erythromycin resistance methylases (Erm) confer resistance to three classes of clinically-important antibiotics (the macrolides, the lincosamides, and the streptogramins B), and are widespread in *Staphylococcus* and other bacterial species (Seppälä et al 1998). For example, in *Staphylococcus xylosus* isolated from bovine mastitis milk, a novel macrolide-lincosamide-streptogramin B resistance gene is located on a 53-kb prophage that is site-specifically integrated into the *S. xylosus*
chromosome (Wipf et al 2014). In Staphylococcus fleurettii, this gene is located in a genomic island which is site-specifically integrated into the housekeeping gene guaA, and exhibits the ability to circularize (Wipf et al 2015). Transfer of erythromycin resistance via prophages of clinically isolated Staphylococcus pyogenes was suggested to be responsible for the emergence of streptococci with multiple resistances in the clinical environment (Hyder and Streifled 1978). P1 bacteriophages lysogenize bacteria as independent plasmid-like elements, and a recent report shows that P1-like bacteriophage carrying SHV-2 extended-spectrum β-lactamase is present in clinical strains of E. coli (Billard-Pomares et al 2014). Acquired resistance to β-lactam antibiotics is conferred principally by β-lactamases and penicillin-binding proteins (Livermore and Woodford 2006). Two β-lactamase genes and one gene encoding a penicillin-binding protein have been detected in the bacteriophage DNA fraction of sewage, river water, and fecal waste from farmed animals, suggesting that bacteriophages can be environmental vectors for the horizontal transfer of antibiotic resistance genes (Colomer-Lluch and Muniesa 2011). Quinolone antibiotic resistance genes (qnrA and qnrS) have also been found in phage DNA isolated from urban wastewater and animal wastewater, suggesting that spreading genetic information via bacteriophages has gained importance in the resistance dissemination in environments (Colomer-Lluch et al 2014).

Prophages may also become trapped in the host genome due to mutation (Canchaya et al 2003); these inactive prophage elements are referred to as cryptic prophages (Figure 1), and they also play a role in antibiotic resistance and tolerance. For example, the E. coli K-12 genome has gained 1,600 kb of novel DNA (18%) since its divergence from Salmonella sp. 100 million years ago (Lawrence and Ochman 1998), and contains one active Lambda prophage and nine cryptic prophages (Blattner et al 1997). At least cryptic prophage rac is a phage fossil that is present in other E. coli strains having been acquired over 4.5 million years ago, which appears more ancient than the Lambda prophage (Perna et al 2001). These cryptic prophage are not inactive DNA remnants generated in the course of host evolution but are important for host fitness in terms of both antibiotic and stress resistance (Wang et al 2010). For example, by deleting all the cryptic prophage genes in E. coli (166 kb), it has been shown that that cryptic prophages contribute significantly to resistance to sub-lethal concentrations of quinolone and β-lactam antibiotics and that the prophages are beneficial for withstanding osmotic, oxidative, and acid stresses (Wang et al 2010).
Specifically, the nine cryptic prophages in *E. coli* K-12 contain 165 putative genes, and 50 of them are related to antibiotic resistance either by transcriptome studies or in whole genome screening tests (Kohanski et al 2007, Peter et al 2004, Walker et al 2004). As shown in Table 1, 17 cryptic prophage genes affect antibiotic resistance by survival tests or metabolic activity assays using deletion strains or using plasmids to express these prophage genes. In particular, the products of kilR in rac prophage and dicB in Qin prophage are responsible for inhibiting cell division and are important for resistance to nalidixic acid (a quinolone) and azlocillin (a β-lactam) (Wang et al 2010). YdaC encoded by rac was identified in the screen for antibiotic resistance using pooled plasmids from the ASKA library that showed increased resistance to erythromycin (Soo et al 2011), and we have confirmed this phenotype in survival assays using two different constructs to express ydaC (unpublished data). Rac-like prophage is transmissible to other *E. coli* strains (Asadulghani et al 2009), thus enabling it to spread these resistance genes.

In Gram-negative bacteria, antimicrobial agents must traverse both the outer membrane and plasma membrane to gain entry into the cell. One of many effective cellular resistance strategies involves the extrusion of the antimicrobial from the cell by transporters, which may be anchored in the inner membrane or reside in the trans-membrane space and which are also encoded by cryptic prophage. For example, the transporter ethidium multidrug resistance protein E (EmrE) in *E. coli* is a proton-dependent secondary transporter from cryptic prophage DLP12 (Verushalmi et al 1995). EmrE confers resistance to positively charged hydrophobic antibiotics such as tetracycline by actively expelling the drug (Viveiros et al 2005). Outer membrane protease OmpT from prophage DLP12 has been shown to increase resistance to streptomycin and chlorotetacycline (Li et al 2008). The impact of OmpT on *E. coli* resistance to urinary cationic peptides was investigated by testing an *ompT* knockout strain, and OmpT may help the host persist longer in the urinary tract by enabling it to resist the antimicrobial activity of urinary cationic peptides (Hui et al 2010).

In other species, many resistance genes are located on genomic islands, and there are several common features shared by cryptic prophage and genomic islands. Both of them harbor phage integrase or excisionase that directly regulate the integration or excision of these mobile elements. Another common feature is the presence of two perfect or near-perfect repeats at the borders of these mobile elements.
genetic elements, and they are used as site-specific recombination events during excision. P4 or P4-like integrase genes are normally adjacent to the tRNAs or tRNA modification genes, which serve as the phage attachments (Williams 2002). Mobile genetic elements that carry P4-like integrases are termed cryptic prophages in *E. coli* (e.g. CP4-6, CP4-44, CP4-57) but often are referred to as genomic islands or pathogenicity islands in other species such as *Salmonella* and *Shigella*. For example, the genomic island that carries resistance to ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline in *Salmonella enterica* Typhymurium phagetype DT104 is flanked by a near perfect 18-bp repeat and inserted in the *trmE* gene encoding a tRNA modification enzyme (Cabedo et al 1999). The resistance locus pathogenicity island in *Shigella* spp. mediates resistance to streptomycin, ampicillin, chloramphenicol, and tetracycline, and it can be excised from the chromosome via site-specific recombination mediated by the P4-related integrase (Turner et al 2004). We have shown in *E. coli* that excisionase AlpA in CP4-57 can lead to a complete removal of CP4-57 prophage (Wang et al 2009) and that excisionase XisR in rac and a modified host protein H-NS can lead to a complete removal of rac prophage (Hong et al 2010, Liu et al 2015). Therefore, the global dissemination of multiple antibiotic resistances harbored by mobile genetic islands in pathogenic bacteria seems to be closely related to the site-specific recombination events.

**Prophages are reservoirs of antibiotic tolerance genes**

In many cases, TA loci are closely linked to mobile genetic elements. For example, *V. cholera* has 13 TA pairs and all of them are clustered in the megaintegron on ChrII, the smaller of its two chromosomes (Budde et al 2007, Gerdes et al 2005). Also, type I, type II and type IV TA loci have been identified in the nine cryptic prophages of *E. coli* (**Table 1**), indicating TAs are overrepresented in *E. coli* prophages (~8%). The presence of these toxins is important in that activation of all toxins to date leads to a dramatic increase in persister cells (Chowdhury et al 2016a, Wood 2016). So these cryptic prophages not only provide the means for antibiotic resistance, but they also provide the means to make the cells dormant and more persistent.

The type I toxin/antitoxin pair RalR/RalA in *E. coli* rac cryptic prophage increases resistance to broad-spectrum fosfomycin (Guo et al 2014), and the underlying mechanism remains to be determined. RelE toxin of Type II TA RelB/RelE in *E. coli* prophage Qin is a sequence-specific endoribonuclease...
which blocks translation by cleavage of mRNAs (Christensen et al 2001, Neubauer et al 2009). Critically, RelE leads to high persister cell formation in the presence of high concentrations of ciprofloxacin, ampicillin, and tobramycin (Keren et al 2004, Koga et al 2011). Also, the toxin of the type II TA system RnlA/RnlB of the *E. coli* cryptic prophage CP4-57 causes inhibition of cell growth and rapid degradation of cellular mRNAs. The toxin of first recognized type IV TA pair, CbtA/CbeA in *E. coli* cryptic prophage CP4-44, not only inhibits cell growth, but alters cell shape by inhibiting the polymerization of cytoskeletal proteins FtsZ and MreB via direct protein-protein interaction (Masuda et al 2012). Moreover, this TA pair has related to resistance to norfloxacin, novobiocin, and spectinomycin (Tan et al 2011, Kohanski et al 2007, Masuda et al 2012). The only other two homologous TA loci of CbtA/CbeA also reside in prophages, YkfL/YafW on *E. coli* cryptic prophage CP4-6 and YpjF/YfjZ on *E. coli* cryptic prophage CP4-57 (Brown and Shaw 2003). Interestingly, YkfL/YafW is related to the resistance to bacteriocin colicin E3 (Walker et al 2004), and YpjF/YfjZ is related to resistance to novobiocin (Peter et al 2004). One of the most striking features of these P4-like cryptic prophages in *E. coli* is that they are pervasively mosaic, with different segments seem to have distinct evolutionary histories (Brussow et al 2004). The presence of three homologous TA loci in three P4-like prophages (CP4-6, CP4-44, and CP4-57) suggests that horizontal genetic exchange plays a dominant role in shaping these genome architectures.

**Antibiotics trigger prophage excision**

UV irradiation and mitomycin C (MMC) are classical agents that can efficiently induce prophage excision in lysogenic bacteria (Otsuji et al 1959). The SOS response is induced by UV radiation or MMC, and it can also be activated by antibiotics that inhibit DNA replication or inhibit DNA gyrase activities to produce single-strand DNA (ssDNA). During the course of repair of DNA damage, ssDNA is produced (e.g., DNA crosslinks produced as a result of MMC). Trimethoprim (dihydrofolate reductase inhibitor) is an example of an SOS-inducing antibiotic that inhibits DNA replication (Lewin and Amyes 1991). Fluoroquinolones, broad-spectrum antibiotics that inhibit bacterial DNA gyrase and topoisomerase activity, also lead to SOS responses by generating DNA double-strand breaks (Drlica and Zhao 1997).

The genes coding for Shiga toxins are silent in lysogenic bacteria, and prophage induction is necessary for their efficient expression and toxin production. Both toxins are usually encoded in the genomes of bacteriophages (Stx phages), and they can lysogenize *E. coli* strains, thereby allowing a
mechanism for toxin dissemination via transfer of bacteriophages (Huang et al 1987, O'Brien et al 1984).
Shiga toxin-producing EHEC (O157 Sakai) possesses 18 prophages that encode numerous genes related
to EHEC virulence, including those for Shiga toxins and two other potent cytotoxins (Hayashi et al 2001).
Nine out of the 18 prophages can be excised to form a circle by MMC-mediated induction, and three of
them are transferable to the non-pathogenic commensal *E. coli* strain K-12 and stably maintained in the
new host (Asadulghani et al 2009). The induction of Shiga toxin-converting prophages in EHEC also
occurs in the presence of norfloxacin and under oxidative stress (Łoś et al 2010). Hence, these cryptic
prophages have a high potential for disseminating virulence-related genes and other genetic traits to other
bacteria under stress conditions, and stress activates pathogenicity.

Increased virulence caused by increased Stx production has been related to stx prophage induction
both *in vitro* (Mühldorfer et al 1996) and *in vivo* (Zhang et al 2000). In particular, clinically-used
antibiotics known to trigger the SOS response, including ciprofloxacin, have been shown to enhance Stx
production (Mühldorfer et al 1996). The SOS response induced by MMC or fluoroquinolones causes
enhanced intra-intestinal transfer of Stx2 prophages *in vivo* (Zhang et al 2000). Prophages of *E. faecalis*
V583 excise from the bacterial chromosome in the presence of a fluoroquinolone, and are able to produce
active phage progeny (Matos RC 2013). The *S. typhimurium* functional prophages, *Gifsy*-1 and *Gifsy*-2,
can be induced by exposing bacteria to hydrogen peroxide (Figueroa-Bossi and Bossi 1999). Recent
studies have demonstrated that oxidative stress conditions may occur during colonization of the human
intestine by enteric bacteria (Kumar et al., 2007). Moreover, earlier studies on a clinical isolate of EHEC
suggested that hydrogen peroxide produced by human neutrophils, may increase the production of Stx2
(Wagner et al 2001).

Carbadox is a quinoxaline-di-N-oxide, and exposure of *Salmonella* sp. to carbadox induces prophages
that can transfer virulence and antibiotic resistance genes to susceptible bacterial hosts (Stanton et al
2008). Carbadox frequently induces generalized transducing phages in multidrug-resistant phage type
DT104 and DT120 isolates, resulting in the transfer of chromosomal and plasmid DNA that included
antibiotic resistance genes (Brunelle B W 2014). Metagenomics approaches were used to evaluate the
effect of two antibiotics in feed (carbadox and ASP250 [chlortetracycline, sulfamethazine, and penicillin])
on swine intestinal phage metagenomes (viromes), and the abundance of phage integrase-encoding genes
was significantly increased in the viromes of medicated swine over that in the viromes of non-medicated swine (Allen et al. 2011). Prophage-like VSH-1 was detected in *Brachyspira hyodysenteriae* cultures treated with mitomycin C, carbadox, metronidazole, and hydrogen peroxide. Carbadox- and metronidazole-induced VSH-1 particles transmitted tylosin and chloramphenicol resistance determinants between *B. hyodysenteriae* strains (Stanton et al. 2008).

As previously described for SOS induction by MMC, fluoroquinolone antibiotics, and trimethoprim (Goerke et al. 2006), β-lactams are also capable of triggering prophage induction in *S. aureus* lysogens. β-lactam-mediated phage induction also resulted in replication and high-frequency transfer of the staphylococcal pathogenicity islands, showing that such antibiotics may have the unintended consequence of promoting the spread of bacterial virulence factors (Maiques et al. 2006). β-lactam antibiotics are extracellular stimuli of the SOS response in *S. aureus* as well as in *E. coli* and demonstrate another case for horizontal dissemination of virulence factors.

Integrating conjugative elements (ICE) can also carry antibiotic resistance genes and recruit SOS responses to mobilize themselves from one bacterial genome to another by cell-to-cell contact (Hastings et al. 2004). Therapeutic agents such as ciprofloxacin and MMC promote the spread of antibiotic resistance genes carried on ICE in *V. cholerae* among a variety of Gram-negative species including *E. coli* (Beaber et al. 2004, Hochhut and Waldor 1999). "Drugs and phage"

Since MMC and other antibiotics trigger SOS responses that may contribute the augmentation of toxin production by inducing Stx prophage induction, the treatment of infections using antibiotics that result in DNA damage and phage induction may lead to unexpected adverse consequences. Stimulation of gene transfer following bacterial exposure to fluoroquinolones should be considered an adverse effect, and clinical decisions regarding antibiotic selection for infectious disease therapy should include this potential risk. Antibiotics that inhibit protein synthesis, such as chloramphenicol, tetracycline and streptomycin, do not induce SOS responses, and neither do agents that act upon the outer membrane (Hastings et al. 2004). The use of fosfomycin which is an inhibitor of cell-wall synthesis did not cause the intraintestinal transfer of Stx2 prophage transfer in mice (Zhang et al. 2000), and effectively reduced the risks of hemolytic-uremic syndrome (Takeda 1998). Thus, efforts should be made in developing new
compounds with antimicrobial activities targeting more specific cellular functions/components rather than DNA replication. For example, lassomycin is a newly identified antibiotic that exhibits potent bactericidal activity against both growing and dormant mycobacteria. It binds to a highly acidic region of the ClpC1 ATPase complex and markedly stimulates its ATPase activity without stimulating ClpP1P2-catalyzed protein breakdown, which is essential for viability of mycobacteria (Gavrish et al 2014). Another newly identified antibiotic, teixobactin, from uncultured bacteria, inhibits bacterial cell wall synthesis by binding to a highly conserved motif of lipid II (precursor of peptidoglycan) and lipid III (precursor of cell wall teichoic acid) (Ling et al 2015).

In contrast to active prophages that are triggered to excise by the DNA repair (SOS) response, cryptic prophages usually stay as stable residents on the host chromosome under adverse growing conditions including during the SOS response. For example, for the nine prophages in *E. coli* K-12, e14 was the only inducible prophage upon MMC treatment (Wang et al 2010). Among the eight cryptic prophages that do not excise with MMC, two prophages were induced to excise during *E. coli* biofilm formation, thus providing benefits for the population by creating a subpopulation of prophage-excised cells with different biofilm-related phenotypes (Liu et al 2015, Wang et al 2009). Furthermore, Pf4 prophage excision has been linked to both cell death and lysis for *P. aeruginosa* cells in biofilms as filamentous-like prophage excision increases diversity in dispersing cells as well as impacts biofilm architecture and virulence (Rice et al 2009, Webb et al 2003). It is well established that biofilm provides increased tolerance towards antibiotic treatment (Costerton et al 1995), thus prophage can also indirectly contribute to antibiotic tolerance by promoting biofilm formation.

Moreover, cryptic prophages carrying TA systems can be activated during stress (Yamaguchi and Inouye 2011). During oxidative stress and starvation, proteases such as Lon and ClpXP degrade unstable antitoxins and releases free toxins (Christensen et al 2004, Maisonneuve et al 2013, Wang et al 2011, Wang and Wood 2011). It has been suggested that activating toxins by deactivating antitoxins of TA systems would be beneficial in terms fighting pathogens (Chan et al 2015); however, this approach is short-sighted in that this approach will indubitably lead to an increase in the numbers of pathogen persister cells (Shapiro 2013) since proteins that reduce growth such as toxins increase persistence (Chowdhury et al 2016a). Perhaps the best approach then, to target pathogens that utilize TA systems like
those encoded by phages to form persister cells, is to utilize a combination of drugs with one used to kill growing cells and another one to kill persister cells. Examples of this approach of targeting both growing and dormant cells include combining rifampicin with the acyldepsipeptide ADEP4 (Conlon et al 2013) and by combining cefoperazone and doxycycline with daptomycin (Feng et al 2015). Other possible approaches include using either mitomycin C (Kwan et al 2015) (for infections where it does not lead to extracellular toxin production like Shiga toxins) or cisplatin (Chowdhury et al 2016b) to kill simultaneously both actively growing pathogens as well as their persister cells; both compounds kill active and persister cells by crosslinking their DNA, both have been shown to be broadly effective against pathogens such as *P. aeruginosa*, *EHEC*, *S. aureus*, and *Borrelia burgdorferi* (Chowdhury et al 2016b, Kwan et al 2015, Sharma et al 2015), and both are approved by the Food and Drug Administration for human use.

As an interesting use of TA systems from cryptic prophage as drugs, toxin RelE from cryptic prophage Qin causes apoptosis when it is produced in a human osteosarcoma cell line (Yamamoto et al 2002). Unfortunately, although toxins of TA systems have many of the same targets as antibiotics, they are active only intracellularly; i.e., they are not effective when added extracellularly but must be translocated to the cell interior. For example, the Hok toxin of the Hok/Sok TA system is not active with Gram positive or Gram negative bacteria unless it is electroporated into the cell (Pecota et al 2003).

Also, other non-TA components of prophage have potential as drugs. For example, recent progress has been made in HIV-1 therapy by directed evolution of a site specific recombinase that can recognize a 34-bp sequence flanking the majority of the integrated provirus HIV-1; this evolved recombinase can efficiently and precisely remove the integrated provirus from infected cells and is efficacious on clinical HIV-1 isolates *in vitro* and *in vivo* (Karpinski et al 2016). Thus, targeting the excisionase of prophage may be a promising approach for treating both viral and bacterial infections.

**PERSPECTIVES**

There is growing need to understand phage-host interactions and bacterial-host interactions in complex systems, such as among gut microbiota. Phages can regulate the microbiome using different strategies, such as killing competing bacteria to allow lysogenic bacteria to thrive in niches with limited nutrients, by encoding toxins or virulence factors that increase pathogenicity, by encoding genes that
increase antibiotic tolerance, and by functioning as vehicles for the horizontal transfer of genes among different species of bacteria. Clearly, the spread of antibiotic resistance among pathogenic bacteria has become a serious global issue for public health, and the role of phage in this process should not be neglected. Increasing evidence has shown that prophages of commensal and environmental bacteria are also reservoirs of antibiotic resistance and tolerance, and their roles in the dissemination of resistance and tolerance to pathogenic bacteria through horizontal gene transfer should be recognized. Prophages can also carry new families of virulence, resistance and tolerance genes. Prophages or prophage elements can be identified and annotated in the sequenced bacterial genomes through web servers such as PHAST (Zhou et al 2011), Prophage Finder (Bose and Barber 2006), IslandViewer (Dhillon et al 2015), and MobilomerFINDER (Ou et al 2007). In addition, the virome sequences that are present in publicly available databases (e.g., MG-RAST (Keegan KP et al 2016)) can also be mined for the presence of virulence, resistance and tolerance genes inside phages and prophages.

The human gut also contains large amounts of free viral particles, most of them bacteriophages probably released after spontaneous induction of prophages of lysogenic bacteria in the gut (Breitbart et al 2003). A recent study by Gordon’s group shows that temperate phages are prominent in fecal microbiota, and an in vivo mice study demonstrated the prophage induction in a fecal community occurs upon exiting the host (Penadés et al 2015). Moreover, host-associated bacteria often encounter various host-related stresses such as nutritional deprivation, oxidants, temperature upshifts, and low pH which can also trigger prophage excision. Therefore, the human microbiome and environmental microbiome projects that have been initiated throughout the world (Dubilier et al 2015), should strive to identify prophages and functional genes embedded in the prophages, given the prominent role of phage in virulence, antibiotic resistance, and antibiotic tolerance.

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Conflict of interest statement. None declared.
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Table 1. Summary of genes that participate in antibiotic resistance or tolerance in the cryptic prophages of *E. coli* K-12.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Prophage</th>
<th>Function</th>
<th>Antibiotics tested</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>ydaC</td>
<td>Rac</td>
<td>Putative double-strand break reduction protein</td>
<td>Erythromycin</td>
<td>(Soo et al 2011)</td>
</tr>
<tr>
<td>ralR</td>
<td>Rac</td>
<td>DNase, toxin of type I TA pair RalR/RalA</td>
<td>Fosfomycin</td>
<td>(Guo et al 2014)</td>
</tr>
<tr>
<td>kilR</td>
<td>Rac</td>
<td>Toxin, Ftsz inhibitor</td>
<td>Novobiocin; Bicyclomycin; Azlocillin</td>
<td>(Peter et al 2004, Sabina et al 2003)</td>
</tr>
<tr>
<td>relE</td>
<td>Qin</td>
<td>Toxin of TA pair RelE/RelB, Sequence-specific endoribonuclease</td>
<td>Cefotaxime; Ofloxacin; Tobramycin; Ciprofloxacin; Ampicillin</td>
<td>(Gottfredsen and Gerdes 1998, Keren et al 2004, Maisonneuve et al 2011)</td>
</tr>
<tr>
<td>dicB</td>
<td>Qin</td>
<td>Control of cell division</td>
<td>Azlocillin</td>
<td>(Wang et al 2010)</td>
</tr>
<tr>
<td>hokD</td>
<td>Qin</td>
<td>Small toxic membrane polypeptide</td>
<td>Kanamycin; Novobiocin</td>
<td>(Kohanski et al 2007, Peter et al 2004)</td>
</tr>
<tr>
<td>emrE</td>
<td>DLP12</td>
<td>Multidrug resistance pump</td>
<td>Methyl viologen; Tetracycline; Ethidium; Tetraphenylphosphonium</td>
<td>(Morimyo et al 1992, Yerushalmi et al 1995),</td>
</tr>
<tr>
<td>ompT</td>
<td>DLP12</td>
<td>Outer membrane protease</td>
<td>Streptomycin; Chlortetracycline</td>
<td>(Hui et al 2010, Li et al 2008)</td>
</tr>
<tr>
<td>yfdO</td>
<td>CPS-53</td>
<td>Uncharacterized protein</td>
<td>Lidoçane; Nalidixic acid</td>
<td>(Soo et al 2011)</td>
</tr>
<tr>
<td>rnlA</td>
<td>CP4-57</td>
<td>Toxin of RnlA/RnlB TA pair</td>
<td>Gentamicin</td>
<td>(Koga et al 2011)</td>
</tr>
<tr>
<td>yfiZ</td>
<td>CP4-57</td>
<td>Antitoxin of putative TA pair Ypfj-Yfjz</td>
<td>Novobiocin</td>
<td>(Peter et al 2004)</td>
</tr>
<tr>
<td>ypjF</td>
<td>CP4-57</td>
<td>Toxin of putative TA pair Ypfj-Yfjz</td>
<td>Novobiocin</td>
<td>(Peter et al 2004)</td>
</tr>
<tr>
<td>yeeU</td>
<td>CP4-44</td>
<td>Antitoxin of TA pair YeeU/YeeV, cytoskeleton bundling-enhancing factor A</td>
<td>Norfloxacin; Novobiocin</td>
<td>(Masuda et al 2012, Sabina et al 2003)</td>
</tr>
<tr>
<td>yeeV</td>
<td>CP4-4</td>
<td>Toxin of TA pair YeeU/YeeV, cytoskeleton binding toxin</td>
<td>Norfloxacin; Spectinomycin</td>
<td>(Masuda et al 2012)</td>
</tr>
<tr>
<td>ykfI</td>
<td>CP4-6</td>
<td>Toxin of putative TA pair YkfI-YafW</td>
<td>Colicin E3</td>
<td>(Walker et al 2004)</td>
</tr>
<tr>
<td>yafW</td>
<td>CP4-6</td>
<td>Antitoxin of putative TA pair YkfI-YafW</td>
<td>Colicin E3; Kasugamycin</td>
<td>(Walker et al 2004)</td>
</tr>
<tr>
<td>yagE</td>
<td>CP4-6</td>
<td>2-keto-3-deoxy gluconate (KDG) aldolase</td>
<td>Novobiocin; Norfloxacin; Ampicillin; Streptomycin</td>
<td>(Bhaskar V et al 2011, Peter et al 2004)</td>
</tr>
</tbody>
</table>
Figure 1. **Different types of phages and prophages.** Genes in cryptic prophages are abbreviated as *int*: integrase, *xis*: excisionase, RM: Restriction-modification system, and TA: Toxin-antitoxin systems.