A Fourth Dimension in the Bacterial Defence Against Bacteriophage Infections – Transcriptional Regulation of Horizontally-Acquired Genes

Microbiology
Matthew Dorman
Junior Sophister

The systems used by bacteria to resist infection by bacteriophages are diverse and well-studied. Three broad categories of destructive anti-phage strategies have been characterised; restriction-modification, CRISPR/Cas-mediated immunity, and abortive infection. Each system is used by bacteria to destroy phage genomes upon their injection into the cell, depriving a phage of the opportunity to undergo lytic replication, which would eventually kill its bacterial host. However, each of these systems is time-sensitive, and if an infected bacterium does not defend itself quickly, phage genes will be transcribed, and the cell will begin to manufacture phage particles. Time is therefore an important commodity to infected bacteria. Since the transcription of phage genes is essential for a phage to hijack bacterial systems and to replicate, repressing the transcription of phage genes may "buy" bacteria time for their other anti-phage systems to act. Phages have co-opted and hijacked bacterial transcriptional regulation, suggesting that reciprocal phage/host co-evolution is occurring at the level of transcriptional control, and that the silencing of bacteriophage genes by bacterial repressors has contributed to the process of bacterial speciation. Therefore, the temporal control of phage gene transcription can be considered to be as important as restriction-modification and CRISPR/Cas in the bacterial defence against phage attack.

Introduction

Approximately 10^{20} bacteria are believed to exist on Earth, in almost every conceivable environment, and are believed to be part of one of the most successful and prolific evolutionary taxa (Whitman *et al*. 1998). These prokaryotes are preyed upon by viruses, termed bacteriophages, or "phages". Phages are obligate intracellular parasites, like all viruses, and are typically plentiful in any environment in which bacteria exist. In the oceans, for instance, phages outnumber bacteria 10:1 (Bergh *et al*. 1989, Angly *et al*. 2006). Phages infect bacteria by injecting their chromosomes into the cell. If a phage is "lytic", upon injection, genes encoded by these chromosomes are transcribed, translated, and eventually produce the components necessary to manufacture new virus particles. New phages enter the environment by lysing their host cell, killing the bacterium (Ackermann & DuBow 1987). A second life cycle, the lysogenic cycle, exists for "temperate" viruses such as bacteriophage lambda - these phages can infect a cell but may refrain from entering the lytic cycle until they receive suitable inductive stimuli such as exposure to DNA-damaging ultraviolet light or mitomycin C (Ackermann & DuBow 1987, Weinbauer & Suttle 1999).

Fierce competition exists between phage and bacterial populations. Phages must infect bacteria to replicate. However, if all of the individuals in a bacterial population are infected and subsequently lyse, the remaining phage particles have no host to infect and can no longer propagate. A similar scenario results if a bacterial population evolves total resistance to an infectious phage. Here, the virus loses all potential hosts, and cannot survive. Phages must therefore defend against bacterial anti-phage systems, and bacteria must reciprocally mutate these systems to "stay ahead" of phage survival strategies. This model led to the suggestion that bacteria and phage co-evolve according to the Red Queen Hypothesis (Stern & Sorek 2011), in which phages and bacteria constantly mutate and evolve in order to ensure their respective survival (Van Valen 1973).

Three main systems have been identified by which bacteria defend against attack by phages (Labrie *et al*. 2010, Samson *et al*. 2013, Westra *et al*. 2014). A summary of each system is

provided in Table 1. Two of these systems, restriction-modification (R-M) and CRISPR/Cas-mediated immunity, defend individual cells against bacteriophage predators. The third strategy, abortive infection (Abi), refers to a form of bacterial suicide in response to infection by a phage. However, Abi does not allow single bacterial cells to resist a phage infection; rather, it causes an infected cell to lyse, denying the phage access to replicative machinery (Labrie *et al*. 2010). This has been characterised as an altruistic action by infected cells, designed to ensure the survival of the bacterial population as a whole by depleting the environment of viable infectious phages (Blower *et al*. 2012).

Table 1: Summary of commonly-characterised bacterial anti-phage strategies. *An overview of some characteristics of three commonly-cited bacterial defences against phage infection. Examples of Type II CRISPR/Cas and R-M systems are described. REase: restriction endonuclease. MTase: methyltransferase. crRNA: CRISPR-RNA. Cas: CRISPR-associated gene protein product. TA: Toxin-antitoxin. (Data derived in part from Labrie et al. 2010, Blower et al. 2012, Westra et al. 2012, Samson et al. 2013, Samson & Moineau 2013, Westra et al. 2014).*

	Restriction-modification (R-M)	**CRISPR/ Cas-mediated immunity**	**Abortive infection (Abi)**
Defends:	Single bacterium.	Single bacterium.	Bacterial population (altruistic).
Recognition basis:	DNA epigenetics and sequence.	DNA or RNA sequence.	Varies.
Molecules involved:	REase, MTase, may require specificity subunit (Type I R-M only).	Mature crRNA, Cas endonuclease, crRNA-maturation proteins.	Varies. May involve TA system.

Phage escape strategies:	Modified phage DNA epigenetics or sequences.	Phage-encoded CRISPR-silencing transcription factor, phage-encoded anti-CRISPR locus.	Various. Phage-encoded antitoxins (for TA system).
Example system:	HindII / MTase II (*Haemophilus influenzae*).	Type II Cas9 (*Streptococcus thermophilus*).	ToxIN (*Pectobacterium atrosepticum*).
Example applications in molecular biology:	Restriction enzymes; DNA methylases; restriction mapping of genomes.	Gene disruption by site-specific editing; gene silencing; transcriptional activation.	Engineering of phage-resistant bacteria in fermentation cultures.

Many excellent reviews and descriptions of these anti-phage systems exist. However, a consideration of current opinions in this field suggests that a fourth system, the control of phage gene expression, may not be considered to contribute to bacterial anti-phage defence to the same extent as R-M or CRISPR/Cas immunity. This review argues that the temporal control of phage gene transcription can be considered an important fourth strategy used by bacteria to resist phage infections.

Restriction-modification and CRISPR/Cas - two bacterial anti-phage survival strategies

One way by which a bacterium can defend itself against infection by phages is to use endonucleases to destroy phage chromosomes before they can be transcribed. However, these enzymes must be capable of discriminating between "foreign" phage genomes and bacterial DNA, lest the cell's chromosome or plasmids be damaged accidentally. Consequently, bacteria have evolved at least two

systems by which they can determine the provenance of a molecule of DNA. One of these, R-M, discriminates between "self" and "non-self" DNA on the basis of sequence and epigenetic modifications (Figure 1). Endonucleases exist in bacteria which recognise specific DNA sequences, but will only cleave DNA if the target has a specific pattern of modification, usually involving methylation (Landy *et al*. 1974, Old *et al*. 1975, Roszczyk & Goodgal 1975). Host DNA sequences are specifically modified by *S*-adenosyl methionine-dependent enzymes. In theory, phage-derived DNA will have a different methylation pattern to that of host DNA, causing it to be recognised as non-self, and to be digested by an endonuclease. These endonucleases and methylases are often referred to as R-M pairs, and it is from these pairs that Type II restriction endonucleases, now commonly used in research laboratories for purposes such as restriction digests and gene cloning, are routinely isolated (Smith & Welcox 1970, Roy & Smith 1973a, Roy & Smith 1973b, Old *et al*. 1975, Roszczyk & Goodgal 1975, Loenen *et al*. 2013)

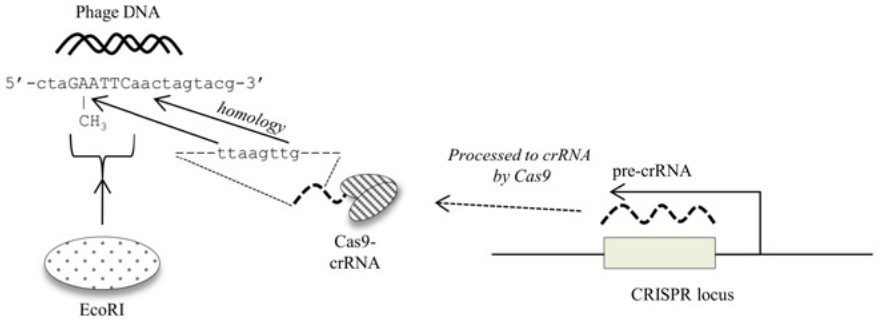

Figure 1: *Simplified overview of the activities of Type II REase-mediated and Type II CRISPR/Cas-mediated restriction of phage DNA.*

Type II REase and Type II Cas9-dependent CRISPR systems are depicted recognising their respective targets on a phage chromosome. REases recognise specific sequences in DNA, and determine if the sequence is foreign on the basis of the epigenetic markers harboured by the foreign sequence such as the methylation pattern of the foreign DNA molecule (Labrie *et al*. 2010). In this example, EcoRI's recognition sequence (capitalised) is methylated,

inhibiting EcoRI-digestion of this nucleic acid. In contrast, CRISPR loci on the bacterial chromosome are transcribed into mRNA, or pre-crRNA, which is then processed by the Cas9 endonuclease into short crRNA sequences. crRNA complexes with Cas9 direct the endonuclease to homologous regions of foreign DNA molecules, the RNA-DNA hybridisation will bring Cas9 into close proximity with the DNA causing cleavage of the phage genome (Barrangou & Marraffini 2014).

Recent studies have revealed that bacteria can also use RNA guidance to specifically target endonucleases to foreign genome sequences. Just as R-M systems rely on a methylase and an endonuclease, so too do these sequence-specific systems rely on two components; regions of clustered, regularly interspersed short palindromic repeats on the bacterial chromosome (CRISPR loci), and CRISPR-associated, or *cas* genes. Our current model of how these so-called CRISPR/Cas systems defend against phage attack is as follows: CRISPR loci comprise short regions of sequences derived from phages, plasmids, or other horizontally-acquired nucleic acids (Bolotin *et al*. 2005, Mojica *et al*. 2005, Pourcel *et al*. 2005). These loci are transcribed into a long mRNA, which is then cleaved into shorter CRISPR-RNA (crRNA) molecules by *cas*-encoded proteins, which associate with endonucleases encoded by *cas* genes (Sontheimer 2010, Westra *et al*. 2012, Marraffini &, Samson *et al*. 2013, Gasiunas *et al*. 2014). crRNA molecules are homologous to the sequence of foreign DNA from which they were derived, and hybridise with that sequence if it is present in the cell. This brings the crRNA-associated Cas endonuclease into close proximity with the foreign DNA, causing it to be degraded (Figure 1). However, the true power of CRISPR/Cas immunity lies in its ability to be expanded dynamically as a form of bacterial adaptive immunity. This involves the integration of a protospacer, a DNA sequence derived from foreign nucleic acids flanked by protospacer-adjacent motifs, or PAMs, into the ends of CRISPR loci. Once a protospacer is integrated, the bacterium is able to produce crRNAs homologous to the protospacer sequence, targeting Cas nucleases to that sequence and defending the cell, and all of its daughter cells, against re-infection by that DNA molecule (Barrangou *et al*. 2007, Barrangou & Marraffini 2014).

The Silencing of Horizontally-Acquired Genes Defends Bacteria Against Phage Attack

R-M and CRISPR/Cas are well-defined bacterial anti-phage strategies, but they are time-sensitive. In order for a phage to successfully propagate within a bacterium, it must first have its genes translated by the cell's own molecular machinery. Successful defence against phage infection therefore requires that phage genomes be degraded before they are transcribed. Inhibiting phage gene transcription presents an opportunity to prevent a phage from taking over the bacterium – silencing phage genes will prevent the unwanted synthesis of phage proteins, until such time as R-M or CRISPR/Cas nucleases can degrade the phage genome.

One system which bacteria use to delay the expression of phage genes involves the histone-like nucleoid-structuring protein (H-NS). This protein is a global regulator of bacterial gene expression in Gram-negative bacteria (Dorman 2004, Grainger *et al*. 2006, Lucchini *et al*. 2006, Navarre *et al*. 2006). H-NS forms multimers, which bind to AT-rich regions of DNA and spatially loop DNA, similar to those formed by the *lac* operon repressor, LacI (Lewis *et al*. 1996, Shin *et al*. 2005). These structures deny RNA polymerase access to promoters, preventing transcription; hence, H-NS is referred to as a transcriptional repressor (Schroder & Wagner 2002). The affinity of H-NS for DNA of specific base composition is significant. Since DNA which is imported horizontally into bacteria is often of a different AT content to the host's chromosome, it is conceivable that host and foreign DNA could be discriminated on the basis of nucleotide composition.

It has been observed that DNA which has a high AT content relative to the infected bacterium's own chromosome experiences selective gene silencing by H-NS (Lucchini *et al*. 2006). This observation suggests that bacteria have evolved mechanisms to determine the provenance of DNA on the basis of its nucleotide composition. This has significant implications for the evolution of bacterial species. For instance, *Salmonella enterica* serovar Typhimurium is believed to be a descendant of *Escherichia coli*. In addition to a genome which is similar to that of *E. coli*, *S.* Typhimurium possesses at least five "pathogenicity islands", regions of DNA of

a higher AT composition to the rest of the cell chromosome, and which are specific targets for repression by H-NS (Ochman *et al*. 2000, Oshima *et al*. 2006, Navarre *et al*. 2006). It is hypothesised that some of these islands were acquired by *Salmonella* from the virulence plasmid in *Shigella flexneri*, another closely-related bacterial species, through a horizontal gene transfer event which may have involved transduction *via* a phage (Groisman & Ochman 1997, Ochman *et al*. 2000). The specific silencing of horizontally-acquired DNA is believed to have accelerated the evolution of *Salmonella* by silencing laterally-acquired genes, the uncontrolled expression of which may negatively affect cell fitness. Strains of H-NS-deficient *Salmonella* suffer from decreased fitness relative to wild-type *Salmonella*, due in part to the uncontrolled expression of pathogenicity genes. These mutants increase their relative fitness back to wild-type levels by acquiring loss-of function mutations in their pathogenicity island genes, or by mutating an H-NS paralogue, StpA, to restore H-NS-like repressive function to the cell (Ali *et al*. 2014). This suggests that uncontrolled expression of genes known to be phage-derived negatively affects *Salmonella* fitness, and in order for an H-NS-deficient cell to regain wild-type fitness, it must halt transcription of laterally acquired genes, either by silencing the transcription of these genes (*via* StpA) or by disrupting the sequences of these genes. This implies that transcriptional silencing can be applied to bacteriophage genomes upon their injection into the cell, to prevent the synthesis of phage proteins which may negatively impact on cell fitness (Skennerton *et al*. 2011).

Phage gene silencing by H-NS is complicated by the fact that CRISPR loci, which must be transcribed in order for the CRISPR/Cas immunity system to operate, are produced from laterally-acquired DNA sequences and are therefore AT-rich. Consequently, transcription of CRISPR loci is inhibited by H-NS, a hypothesis which is supported by the observation that an *hns E. coli* mutant overexpresses CRISPR/Cas system components (Pougach *et al*. 2010, Westra *et al*. 2010, Yosef *et al*. 2011). In addition, the LeuO anti-repressor, known to alleviate the effect of H-NS repression (Stoebel *et al*. 2008), has been shown to induce CRISPR/Cas synthesis (Westra *et al*. 2010). It can be concluded that phage genomes, as well as CRISPR/Cas systems, are under tight, dynamic, and complex

transcriptional control.

Strategies Employed by Phage to Defeat Bacterial Transcriptional Regulation

Although phage genes and CRISPR/Cas systems are under careful transcriptional regulation, a number of strategies have been described by which phages can co-opt these control systems, to such an extent that H-NS silencing of phage genes has been described as a bacterial "Achilles' Heel" (Skennerton *et al*. 2011). For instance, bacteriophages have been described which contain *hns*-like genes in their genomes (Doyle *et al*. 2007, Skennerton *et al*. 2011). These phages therefore encode proteins which are likely to silence the transcription of their genes. Although this may seem unhelpful to the phage, these proteins also act to silence transcription of AT-rich CRISPR loci and *cas* genes on the bacterial chromosome, rendering CRISPR/Cas useless for the resistance of a phage infection (Skennerton *et al*. 2011). This suggests that phages have evolved mechanisms to evade anti-phage systems by simply repressing their transcription.

Other recent studies further increase the complexity of phage/host transcriptional regulation. For example, bacteriophage T7 encodes the 5.5 protein, which has been demonstrated to bind to the H-NS oligomerisation domain. 5.5 protein binding inhibits the formation of H-NS multimers and reduces the effect of H-NS-mediated gene silencing (H-NS repression is dependent upon oligomerisation), increasing the probability that T7 genes will be transcribed in a host cell (Ali *et al*. 2011). Similarly, T4 bacteriophage possesses the Arn protein which is believed to sequester H-NS from the bacterial cytosol. One Arn domain mimics the 3-D structure and the chemistry of AT-rich H-NS nucleic acid binding sites, and causes H-NS to specifically bind to Arn. Once sequestered by Arn, H-NS has no repressive activity, and this depletion of active H-NS means that T4 genes will not experience H-NS-mediated repression (Ho *et al*. 2014). The effects of selected phage anti-H-NS-silencing strategies are summarised in Figure 2. Plainly, phage and bacterium have evolved to interact with one another at the level of transcription, although the downstream effects of these interactions on global

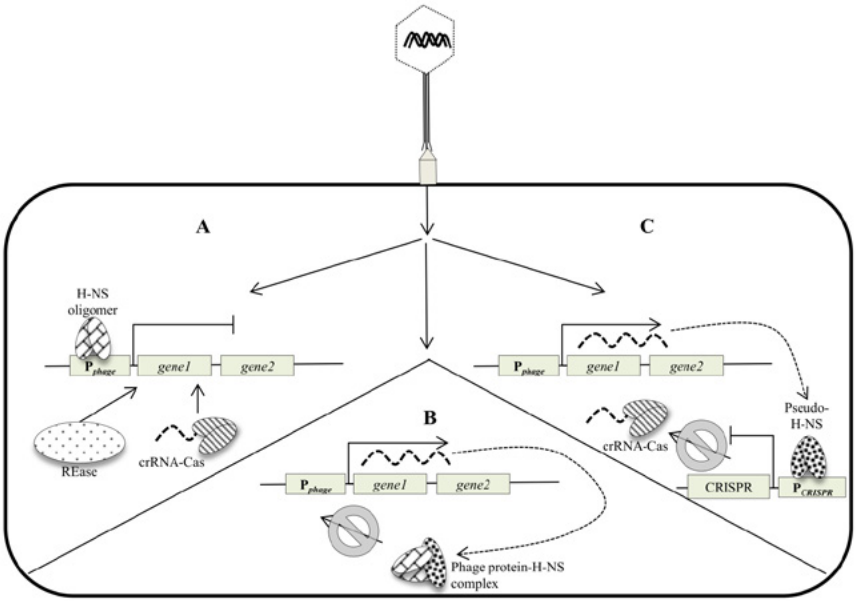

Figure 2: Summary of three potential transcriptional interactions that may take place between phage and bacterium. *Phage DNA is injected into the bacterial cytosol through the cell membrane. A: Transcription from phage promoters is repressed by H-NS (Lucchini et al. 2006), allowing the bacterium time to destroy the invading genome using REases and crRNA-guided Cas endonucleases. B: The phage genome may contain a gene encoding a protein which is biochemically and physically analogous to DNA regions to which H-NS can bind. If this protein is synthesised, phage-encoded proteins will sequester H-NS, reducing the repressive effect on the transcription of all other phage genes (Ho et al. 2014). C: The phage genome may also encode an H-NS orthologue, which is capable of binding to bacterial promoters associated with bacterial CRISPR loci. Upon binding, this pseudo-H-NS molecule represses the transcription of CRISPR loci, inhibiting the ability of the bacterium to mount a CRISPR/Cas-mediated defence against the phage genome (Ali et al. 2011).*

Conclusions

Bacteria possess several systems to resist a phage infection. R-M and CRISPR/Cas immunity allow cells to identify a foreign nucleic acid on the basis of its sequence and its epigenetic modifications. However, time is a dimension of significance in considering these resistances. Given sufficient time and opportunity, it is plausible

to expect that a single infected cell could defend itself against a phage using these endonuclease-based strategies. Upon injection of a phage genome into a bacterium, the cell must have time for its R-M or guided Cas endonucleases to reach the phage molecule, recognise it as non-self, and to destroy it, all before phage genes can be transcribed by bacteria RNA polymerase. Since the beginning of phage gene transcription will eventually cause bacterial lysis, it is logical that delaying the transcription of phage genes using a repressor such as H-NS would increase the time that the cell has to resist the phage infection. Since H-NS is constitutively expressed in the cell (Kröger *et al*. 2013), its inherent ability to delay phage gene transcription may allow the cell time to upregulate transcription of CRISPR/Cas or REase genes in response to an infection.

Phage/host co-evolution is known to have been driven by interactions through R-M and CRISPR/Cas immunities (Westra *et al*. 2012, Samson & Moineau 2013, Westra *et al*. 2014), but there is evidence that co-evolution has also occurred at the level of transcriptional control. Phages appear to have co-opted bacterial transcriptional repressors to prevent the transcription of CRISPR loci into RNA. Phages are also capable of sequestering bacterial repressors to ensure the transcription of their own genes, which may have a secondary effect on the bacterial transcriptome. Evidently, bacteria need time to resist a phage infection, and transcriptional repression of phage genes may facilitate this. It is therefore in the interest of phages to inhibit the activity of bacterial repressors, ensuring the successful and rapid synthesis of phage-encoded proteins. Hence, the role played by transcriptional regulation in maximising the time available to bacteria to destroy phage DNA should be considered a bacterial strategy for resisting phage attack in the same context as R-M, Abi, and CRISPR/Cas.

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