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MINIREVIEW

Bacteriophage Orphan DNA Methyltransferases: Insights from Their Bacterial Origin, Function, and Occurrence

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Type II DNA methyltransferases (MTases) are enzymes found ubiquitously in the prokaryotic world, where they play important roles in several cellular processes, such as host protection and epigenetic regulation. Three classes of type II MTases have been identified thus far in bacteria which function in transferring a methyl group from *S*-adenosyl-L-methionine (SAM) to a target nucleotide base, forming N-6-methyladenine (class I), N-4-methylcytosine (class II), or C-5-methylcytosine (class III). Often, these MTases are associated with a cognate restriction endonuclease (REase) to form a restriction-modification (R-M) system protecting bacterial cells from invasion by foreign DNA. When MTases exist alone, which are then termed orphan MTases, they are believed to be mainly involved in regulatory activities in the bacterial cell. Genomes of various lytic and lysogenic phages have been shown to encode multi- and mono-specific orphan MTases that have the ability to confer protection from restriction endonucleases of their bacterial host(s). The ability of a phage to overcome R-M and other phage-targeting resistance systems can be detrimental to particular biotechnological processes such as dairy fermentations. Conversely, as phages may also be beneficial in certain areas such as phage therapy, phages with additional resistance to host defenses may prolong the effectiveness of the therapy. This minireview will focus on bacteriophage-encoded MTases, their prevalence and diversity, as well as their potential origin and function.

RESTRICTION-MODIFICATION SYSTEMS

Genes encoding restriction-modification (R-M) systems are present on approximately 90% of currently available bacterial and archaeal genome sequences (1). These systems can be encoded by genes on plasmids or chromosomes, and their general role is to recognize and target invading foreign DNA with restriction enzymes, while simultaneously protecting the host's DNA by methyltransferase (MTase) activity. Bacterial R-M systems have been comprehensively reviewed (2–4), and therefore, only the main characteristics of these R-M systems will be summarized here. Four types of R-M systems (I, II, III, and IV) are currently recognized (5), differing in the functional arrangement of the restriction endonuclease (REase) and methyltransferase activities, as well as the requirement for specificity subunits or additional cofactors (6). Typical type I R-M systems consist of three subunits, the S (specificity subunit), M (methyltransferase), and R (restriction endonuclease) subunits, where the S subunit determines the target recognition specificity of the system, while the M and R subunits are required for methylation activity and DNA restriction, respectively (7). Type II R-M systems are the most prevalent type and generally function as two individual proteins (8), where the REase cleaves the target DNA at defined positions within or close to their recognition site, while the MTase protects host DNA by methylation. Type III R-M systems are composed of the products of at least two genes, *res* and *mod*, where Mod binds to and methylates DNA, while Res functions in DNA restriction. Mod can function independently of Res; however, Mod is required for Res activity (9, 10). The type IV restriction systems differ from the other types in that the methyltransferase and endonuclease activities are combined in a single enzyme (11, 12), which exclusively cleaves modified DNA (methylated, glucosyl-hydroxymethylated, and hydroxymethylated) (5).

As this minireview focuses on orphan MTases, the majority of which belong to type II MTases, it is necessary to first define this type in more detail. Type II DNA MTases are enzymes found ubiquitously in the prokaryotic world and play important additional roles (other than in host protection from invading DNA) in several cellular processes, such as replication, transcription, and population evolution (13). Type II methyltransferases function by transferring a methyl group from *S*-adenosyl-L-methionine (SAM) to a target sequence, and these MTases can be divided into three functional classes. Two target the exocyclic nitrogen atoms in certain bases of double-stranded DNA at position 4 in cytosine or position 6 in adenine to generate N-4-methylcytosine (m4c) or N-6-methyladenine (m6a), respectively. The third class is the MTase targeting the carbon 5 position of cytosine to generate C-5-methylcytosine (m5c) (14). MTases are further subclassified based on the presence of conserved amino acid motifs, which represent the DNA binding domain, the target recognition domain (TRD) and the catalytic domain. The C-5-MTases are found to contain 10 conserved domains (designated I through to X) (15), whereas the exocyclic N-targeting MTases harbor nine conserved domains and can be subdivided into 6 groups, α, β, γ, ζ, δ, and ε based on the SAM binding site, active site, and TRD (16, 17). At the time of writing, REBASE listed 9,789 type II REases, and 13,787 putative type II MTases (http://rebase.neb.com/rebase/rebase.html). The large number of MTases relative to that of REases may be the result of the toxicity of the latter on bacterial

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cells, and as a result, the MTases may have been retained more freely. It should be noted that REases may not be identified as such by bioinformatics due to limited overall sequence similarity (18), and as mentioned above, MTases have additional roles other than R-M systems, such as cell cycle regulation (19). While R-M systems typically consist of a combination of REase and MTase, both have been found to exist independently as orphan genes in bacterial genomes, and the remainder of this minireview will focus on the orphan MTases.

BACTERIAL ORPHAN MTases

While this minireview is focused on highlighting orphan MTases in bacteriophage genomes, it is first important to understand the role that they play in bacterial cells. Mobile genetic elements, including plasmids, prophages, insertion sequence elements (ISs), and transposons, harbor and may mobilize methyltransferase-encoding genes (sometimes accompanied by a cognate restriction endonuclease-encoding gene) and as a result can facilitate their spread among bacterial genomes via plasmid uptake and exchange or through integration of lysogenic phages (20, 21). It has been demonstrated that the genetic material encoding R-M systems can become integrated into host chromosomes where it replicates along with the host genome and may block other "parasitic" DNA attempting to integrate/enter into the host (22, 23). The genes encoding R-M systems in bacteria may be challenged by newly introduced DNA (e.g., "incompatible" plasmids or a transducing fragment of homologous DNA), which may attempt to displace the genes encoding R-M systems (20). In such cases, R-M systems may behave "selfishly" if the incoming parasitic DNA gains access to the host cell. Displacement of the R-M systems in members of the populations may cause the cells to die, a process known as postsegregational killing, a similar mechanism of plasmid maintenance that occurs with toxin-antitoxin systems (24–26). The daughter cells are no longer protected due to a reduction in methylation activity, and their genetic material is subjected to cleavage by the still present REase (27). An example of this phenomenon is the chromosomally encoded BamHI R-M system of *Bacillus amyloliquefaciens*, which was shown to resist replacement by homologous recombination (28). Recent studies have demonstrated that the presence of an orphan MTase targeting the same DNA sequence as a resident R-M system may protect the host in the event of the displacement of such an R-M system (18). For example, when the genes encoding the R and M subunits of the type II EcoRII system are expressed from a plasmid in the absence of selective pressure, the resulting segregational loss of the plasmid was shown to lead to incomplete methylation of host DNA and to cell death due to the persistent activity of the EcoRII REase. These detrimental effects can be counteracted by expressing an orphan MTase with the same recognition site as the EcoRII REase. These findings suggest that such events occur naturally in bacterial strains and that it may drive the acquisition and maintenance of orphan MTases. It has been shown that the acquisition of R-M systems can occur by means of horizontal gene transfer (HGT) (29), for example by being present on insertion sequence (IS) elements (30), and as a large number of annotated genes on bacterial genomes encode orphan MTases, it has been proposed that these genes have also been acquired by HGT or are due to genetic decay of R-M systems in the host cell (31).

While MTases are most often described in the context of R-M systems, MTases can exist as orphan MTases without a cognate REase partner and as such have been shown to be involved in cell regulation, replication, DNA repair, and population evolution (19, 32, 33). Understanding the roles of orphan MTases in bacterial cells may reveal the function they play in phage genomes. For the DAM (DNA adenine MTase) enzyme of *Escherichia coli*, these roles include DNA mismatch repair, a process requiring discrimination between the parental DNA strand and newly synthesized DNA behind the replication fork (34). Due to the DAM methyltransferase, the parental DNA is already methylated prior to replication, while the newly replicated strand is not, allowing the mismatch repair protein, MutH, to distinguish between the (presumed) correct sequence of the parental strand and noncomplementary bases on the newly synthesized strand. The mismatch repair protein can then utilize the parental strand as a template to fix such replication errors (35). The frequency by which the DAM recognition site (GATC) occurs in the origin of replication of *E. coli* allows tight regulation of the cell cycle and consequently chromosomal replication. The hemimethylated DNA prevents the replication initiation protein DnaA from acting more than once on the replication origin, *oriC*, in a given cell cycle, a process known as sequestration (36). Several examples of the role of DAM in bacterial virulence have also been reviewed (37).

Initially identified in *Caulobacter crescentus*, the cell cycle regulator methyltransferase (CcrM) targets the recognition sequence GANTC (38, 39) in a nonprocessive manner (40), in contrast to earlier findings (39). The CcrM methylase is essential for the proper control of the life cycle of *Caulobacter*, as methylation of the *ori* directs initiation by DnaA (41). Following cell division, which produces two morphologically distinct cell types, the stalk cell (DNA replication allowed) and the swarmer cell (flagellum, no DNA replication allowed), the CcrM methylase is degraded, as methylation of the *ori* is not required until late in the cycle (42). In the swarmer cell, CtrA (cell cycle regulator) prevents chromosome replication; however, when the swarmer cell changes to a stalk cell, this regulatory protein becomes subject to degradation. Transcription of the gene specifying the CtrA regulator protein is dependent on two promoter sequences, one of which contains a GANTC site (43). When this site is methylated, transcription of *ctrA* is repressed, allowing timed and controlled synthesis of CcrM, as CtrA accumulation in the cell is required to transcribe *ccrM*. As DNA replication proceeds, the replication forks pass the CtrA locus whereby it becomes hemimethylated and is subsequently expressed under the control of the GcrA protein (41, 42).

BACTERIOPHAGE MTases, POSSIBLE FUNCTION, AND PREVALENCE

Currently, little is understood as to the function of orphan MTases when genes encoding them are present in the genomes of bacteriophages, but previous studies indicate that they may have functions that are similar to the functions of MTases found in bacterial cells. Well-studied phage-derived methyltransferases are those encoded by the *Escherichia coli*-infecting T-even bacteriophages. Previous *in vitro* studies have shown that phage T4 encodes a DAM MTase, which was found to target GATC sites (44) and protects phage DNA from restriction endonuclease recognizing this sequence (45). DAM activity is essential in the regulation of *E. coli* cellular functions (46), and while it does not appear to play a role in the lytic cycle of the T2 and T4 phage (47), it was found to play a role other than blocking host endonucleases in the *E. coli* temperate phage P1. P1 specifies a functional DAM enzyme (des-

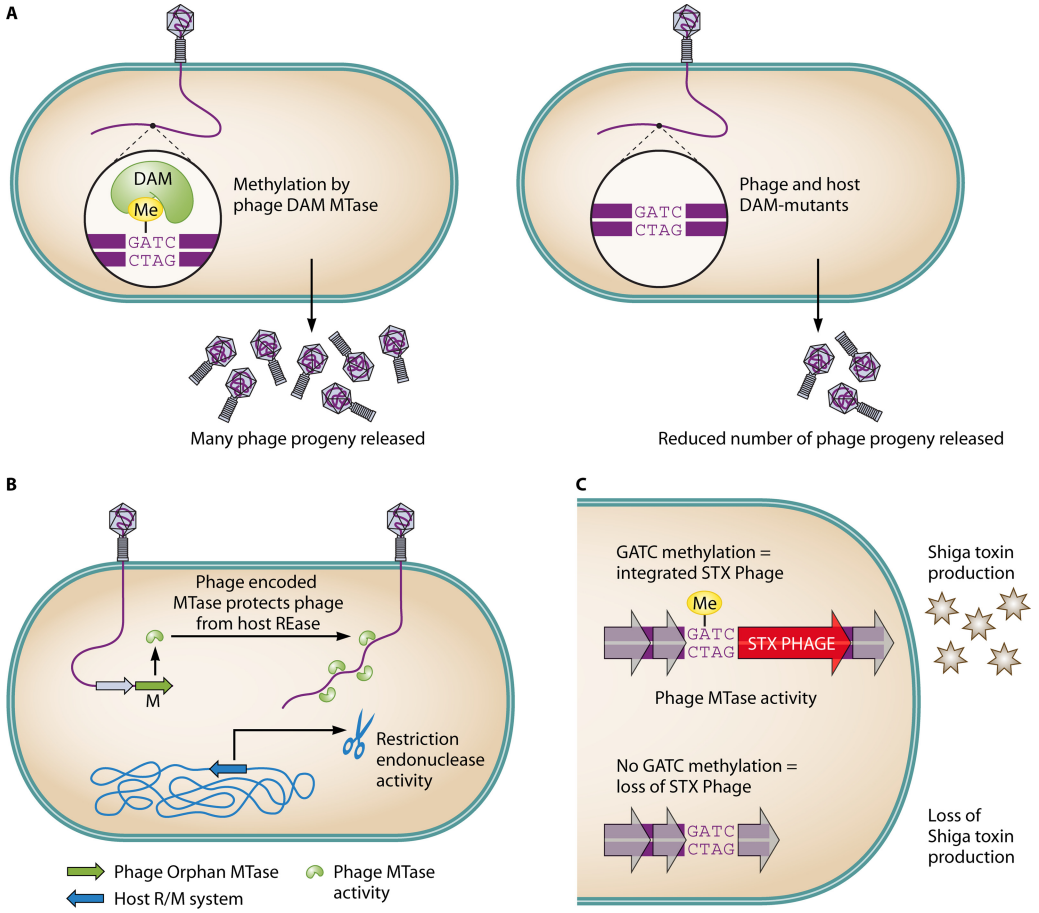

FIG 1 Potential advantageous effects of phage-encoded orphan MTases (see text for further details). (A) Methylation of the GATC sites within the *pac* region of the phage P1 genome by a self-encoded DAM MTase facilitates the efficient release of progeny phage. Generation of both *dam* mutant hosts and *dmt*$^-$ phage (phage DAM knockout) prevents methylation of the phage DNA during packaging, leading to a decreased level of phage progeny released. (B) Protection of phage genomes from host-encoded restriction endonucleases through the protection afforded by the phage-encoded orphan MTase. (C) DAM methylation is essential for lysogeny of the Shiga toxin-encoding phage 933W and release of Shiga toxin by EHEC. Loss of GATC methylation results in release of integrated phage and loss of Shiga toxin production by EHEC. Me, methyl group.

ignated Dmt) of 754 amino acids (48, 49), which has been observed to be active only during the lytic stage of the phage cycle (50), and packaging of phage P1 DNA is dependent on methylated GATC sequences within the 162-bp *pac* site (51). Furthermore, in the presence of a *dam* mutant host and *dmt*$^-$ (phage DAM knockout) phage mutant, a significant reduction in phage progeny was observed in comparison to phage propagations produced in the presence of either the host or phage MTase (51) (Fig. 1 A). Additionally, the promoters for the site-specific recombinase-encoding gene, *cre*, were found to be regulated by DAM methylation (52), and it is suggested that several other areas of the P1 genome are under transcriptional control by DAM methylation (48).

A number of orphan MTases have also been identified in *Bacillus* phages. Interestingly, these phages carry genes encoding MTases that can recognize more than one target sequence due to the presence of multiple TRDs (53). Such multispecific MTases have been identified in the temperate *Bacillus subtilis* phages ø3T, p11B, and SPβ (54), which each recognize two target sequences, while phage SPR carries a gene that encodes a type II MTase specific for three different recognition sites (55). Under conditions where the *Bacillus* prophage SPβ is induced, an orphan MTase becomes active during the lytic phage cycle and provides the phage with protection against digestion by various restriction endonucleases (56, 57) (Fig. 1B). *B. amyloliquefaciens* phage H2 carries a gene that encodes a multispecific m5c methyltransferase that was found to share high sequence homology with its counterparts on the genomes of ø3T, SPR, and $p11_s$ (58). Furthermore, the ability of these MTases to recognize more than a single target sequence may provide a particular phage with immunity to several R-M systems. DAM methylation plays a significant role in bacterial pathogenicity (59), and DAM-mediated methylation is also important for the maintenance of lysogeny of the 933W phage with a gene encoding Shiga toxin (60). As a result, the phage is kept in a temperate state allowing enterohemorrhagic *E. coli* (EHEC) to actively produce the virulent toxin (Fig. 1C). Although many of these proteins contain the conserved domains common among these MTases (61), the presence of such motifs is not essential for a functional enzyme. For example, the lactococcal phage ø50 was found to be resistant to the plasmid gene-encoded resistance systems (pTR2030) present in the parent strain, one being an R-M system (62). It was subsequently shown that both pTR2030 and ø50 shared sequence homology within a type II MTase-encoding

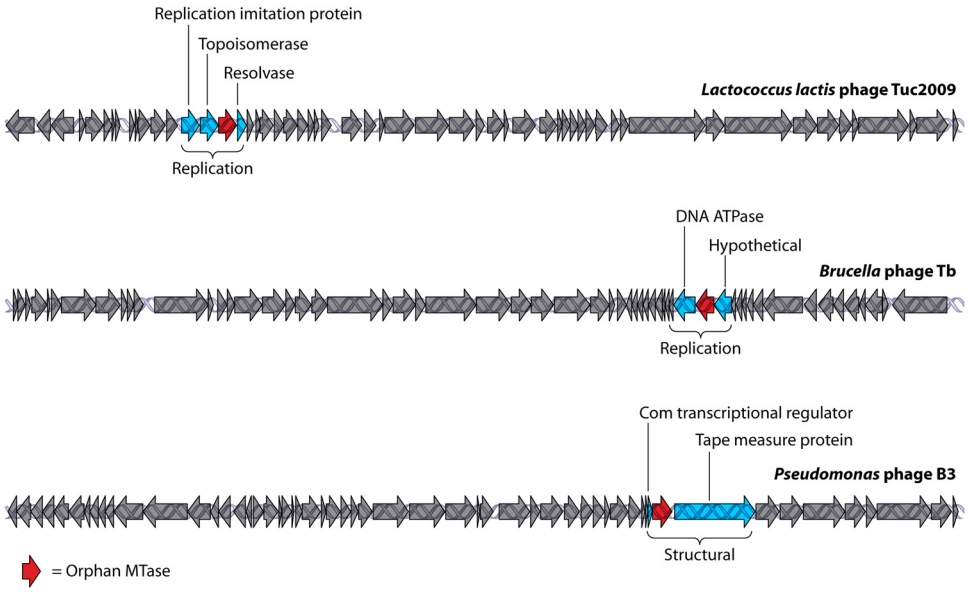

FIG 2 Genomic organization of lactococcal phage Tuc2009, *Brucella* phage Tb, and *Pseudomonas* phage B3 highlighting examples of orphan MTases and flanking genes to show that orphan MTases can be found in different locations in a given phage genome (regions encoding predicted structural and replication proteins).

gene, and furthermore, it was demonstrated that a single motif was sufficient to confer the phage with protection against the R gene of the R-M system (62). The *Myxococcus xanthus* temperate phage Mx8 was also found to contain an MTase-encoding gene, designated *mox*. This gene was found to be nonessential for lysogeny and presented no effect on the lytic phage cycle or superinfection immunity (lysogenic phage blocking subsequent infection of superinfecting phages) (63). Phage genomes are significantly smaller than their bacterial counterparts, and while the functions of many proteins in phage genomes are unknown, it is assumed that genes are retained only if they confer some benefit for continued survival (64–66). Therefore, the *mox* gene may play a role in phage genome protection. The locations of these MTase-encoding genes within phage genomes may provide an insight into their function. For example, the genome of lactococcal phage Tuc2009 harbors a putative MTase-encoding gene flanked by genes specifying a DNA replication protein, a putative topoisomerase, and a resolvase (67) (Fig. 2). A similar genetic arrangement is observed for *Brucella* phage Tb (68). The MTase encoded by the Tuc2009 phage gene may actively methylate DNA during replication to prevent digestion and protect progeny, but the MTase may also play a role in regulating DNA replication in terms of the timing of replication or potentially functioning in mismatch repair, which as mentioned above, is a trait also observed for certain bacterial MTases. *Pseudomonas* phage B3 possesses an orphan DAM-encoding gene within the structural module of the phage genome flanked by genes specifying a transcriptional regulator and the tape measure protein (TMP). It is imaginable that DAM methylation acts in a regulatory capacity to control the production of phage proteins such as the TMP, among others (69) (Fig. 2). Additionally, it was hypothesized that an adenine-specific MTase plays a role in regulating the cell cycle interactions of *Drosophila* and the intracellular symbiont *Wolbachia pipientis*, as prophages have been found only in strains that are in a symbiotic relationship (70), further highlighting that phage orphan MTases have roles similar to the roles of MTases found in bacterial cells.

The ability of phages to integrate the genes encoding orphan MTases into their genome may further contribute to the emergence of phages with broad host ranges, as the acquisition of an MTase-specifying gene would allow progeny phages to infect additional hosts by overcoming R-M systems. Such a notion is consistent with observations for *Salmonella* phage PVP-SE1 (71) and *Yersinia* phage PY100 (72), both of which possess an MTase and display a broad host range. While this minireview is primarily focused on orphan phage MTases, it is interesting that the *Staphylococcus aureus* quadruple-converting phage π42 was shown to harbor an R-M system, Sau42I, that confers resistance to the host against lysis by exogenous phages (73) and in doing so provides a selective advantage to the lysogenic phage. *Burkholderia cepacia* phage KL3 carries a gene that encodes an MTase that appears to be part of a functional defense module. Gene 47 encodes an EcoRII MTase associated with not only a cognate endonuclease but also a Vsr endonuclease (74). It has been proposed that the MTase functions in phage protection and that the endonuclease functions in bacterial DNA digestion, while the Vsr protein is thought to be responsible for postmethylation mismatch repair, the latter of which has been shown to be controlled by DAM-mediated methylation in bacterial cells as mentioned above (74). These studies indicate that phages have developed the ability to circumvent the R-M mechanisms of their hosts by the integration of orphan MTase-encoding genes and whole R-M systems, highlighting the ever-adapting nature of phages to become more advanced to outwit their bacterial hosts. While a number of studies on bacteriophage MTases have been performed, the abundance of these enzymes in phage genomes is relatively unknown.

To estimate the number of genes encoding putative orphan MTases among bacteriophage genomes, a manual search of the PubMed protein database (http://www.ncbi.nlm.nih.gov/pubmed/) was performed using the term "phage methyltransferase" which yielded 819 putative orphan phage MTases associated with a wide variety of bacterial species. Using the REBASE website (http://rebase.neb.com/rebase/rebase.html), a database was constructed to blast

these 819 retrieved proteins to determine whether these putative MTases belong to one of the three subtypes of type II MTases (m6a, m4c, and m5c) (for examples from each species, see Table S1 in the supplemental material). It should be noted that some of these MTases may not possess all the conserved motifs found within their bacterial counterparts; however, as mentioned above, it has been shown that a single motif can be enough to provide phage protection against R-M systems (62). Orphan MTases found in the currently available, fully or partially annotated phage genomes are associated with a diverse range of hosts and environments. These hosts include members of the family *Enterobacteriaceae*, the pulmonary pathogens *Burkholderia* and *Mycobacterium*, and the marine bacterium *Synechococcus* (Table S1). Additionally, phages infecting industrial strains such as the dairy starter culture *Lactococcus lactis* were also found to carry genes that encode MTases. Acquisition of (apparently) orphan MTases by bacteriophages seems to occur in diverse phage-host ecosystems with high cell densities, causing a competitive, high-number propagation race between phages and bacteria: bacterial counts in the gastrointestinal tract range from 10^4 CFU ml^{-1} in the stomach to 10^{12} CFU ml^{-1} in the colon (75), and *Synechococcus* on occasions can form blooms with 10^6 CFU ml^{-1} (76). Likewise, the relatively closed environment of a fermentation facility creates very high cell densities, mostly represented by a small number of strains/species. It was found that despite being present in such a large variety of phages, with the exception of some outliers, methyltransferases that were predicted to belong to a particular methylation type (m5c, m4c, or m6a), for the most part, were shown to group together (see Fig. S1 in the supplemental material). Several of the m6a and m4c methyltransferases were shown to form a phylogenetic group (Fig. S1), which is consistent with their classification.

SOURCE OF BACTERIOPHAGE MTases

Understanding the role of these orphan MTases also involves trying to find the origins of the MTase-encoding genes. Bacterial hosts containing genes that encode an R-M system and are infected by phages may, at low frequency, produce progeny phages that have become methylated and thus are resistant to such an R-M system (77). The resulting "modified" phages are thus due to methylation of the phage genome by the host-encoded R-M system and did not arise through the acquisition of genomic material. From the data and studies presented in this minireview, it is apparent that phages have gained the advantageous ability to permanently overcome such an R-M hurdle via the integration of a cognate orphan MTase. Exchange of genetic material between bacteria occurs naturally via HGT processes such as transformation, transduction, and conjugation (78). Bacteriophages play an important role in the lateral exchange of genetic material between bacterial hosts, and while this occurs in lytic phage often by recognition of pseudo-*pac* sites on host chromosomes, it is more common among the lysogenic phages, as during lytic phage infection, the host DNA can be substantially degraded (79, 80). Temperate phages can pick up bacterial genes that flank the integration sites as a result of an excision error and transfer these genes to a new host, known as specialized transduction. Specialized transduction has been demonstrated in several studies including *Brachyspira intermedia* (81) and *Bacillus* phages (82). Temperate phages likely attained their orphan MTase-encoding genes in this way, as no homology was found between *Bacillus* temperate phages and their hosts (57). It has been demonstrated that *E. coli* phage P2 is capable of transferring the EcoT38I R-M system into chromosomal DNA by means of HGT (83): approximately 30% of the genome of phage P2 and sequences homologous to the phage P2 attachment site were found flanking the EcoT38I R-M system genes. Evidence of the uptake and transfer of the EcoO109I R-M by HGT was also shown for the *E. coli*-infecting bacteriophage P4 (21). This provides strong evidence that temperate phages transfer genetic information encoding DNA modification enzymes, and therefore, we hypothesize that in certain cases temperate phages may retain the MTase gene due to a conferred advantage such as the ability to overcome host R-M systems or improved genome replication and/or regulation.

Errors in the DNA packaging process during lytic phage replication may also lead to HGT whereby DNA from the bacterial host (genomic and/or plasmid) can become incorporated into the phage capsid and subsequently introduced into a bacterium, a process referred to as generalized transduction (84). Furthermore, nonhomologous recombination is known to play a role in the mosaic nature of many phage genomes, allowing the exchange of genes as well as complete functional modules (85), and may be behind the emergence of these orphan phage MTases (86). Previous studies have shown that phages can exchange genetic material with host bacteria through homologous recombination events, for example lactococcal phage ul36 was shown to be subject to two genetic exchanges with prophage-like DNA located within the host chromosome (87). Orphan MTases may thus be acquired by temperate phages which consequently become incorporated in the bacterial genome through lysogeny. Subsequent to infection by a lytic phage, the prophage-encoded MTase may be transferred to the lytic phage during the phage cycle, giving rise to R-M-resistant progeny. This may be the case for phage 4268 where comparative genomic analysis of its lactococcal host revealed high sequence similarity to the chromosomally encoded MTase (88). As mentioned above, the functional MTase, LlaI, was shown to be acquired by a phage genome from a conjugative plasmid. While the exact mechanism of transfer is unknown, it is likely that the acquisition of the gene by the phage was due to HGT, e.g., utilizing nonhomologous recombination (62). This highlights the apparent regular occurrence of such exchanges during phage-host interactions and the role HGT plays in the spread of orphan MTases. While bacteria continue to fight back against phage infection, it is clear that phages are driven to evolve mechanisms that will eventually allow them to overcome these resistance systems, thus reaching a stable coexistence with their hosts. The acquisition of orphan MTase-encoding genes is one such mechanism.

FUTURE PERSPECTIVES

Exploitation of phage MTases for use in phage therapy. With the emergence of highly antibiotic-resistant bacteria, in particular among pathogens that are easily transmitted (*Enterococcus*, *Streptococcus*, and *Mycobacterium tuberculosis*) (89–91), renewed interest in phage therapy has emerged. A number of key elements have to be known before a phage can be applied as a therapeutic agent. These include using a well-characterized lytic phage with a broad host range, while avoiding the use of temperate phages. The determination of complete genome sequences of the phages is necessary to reveal the presence of any genes that may encode toxic or allergic compounds. Several recent reviews highlight the methods and challenges that accompany the use of phages as antimicrobial agents (92, 93). The success of phage therapy may be limited by the

inherent or acquired phage defense mechanisms of a targeted bacterial species. Studies have demonstrated that phage cocktails can be effective in phage therapy to compensate for phage resistance, although eventually bacteria and phages achieve environmental coexistence, thus rendering the phage therapy ineffective (94). An approach to aid therapeutic phages in circumventing bacterial resistance mechanisms may be to include phage isolates harboring orphan MTases. By including such phages with orphan MTases to the selection criteria for phage therapy, it could aid in the rapid removal of infectious bacteria by delaying the ability of host R-M systems to attack the incoming phage, thus prolonging the treatment's effectiveness and improving the overall benefits of phage therapy.

SMRT DNA sequencing and functional analysis of phage MTases. Characterization or identification/cloning of MTases using traditional molecular biology approaches can involve PCR amplification, restriction digestion, and overnight ligation of vector and target DNA, followed by transformation and selection via antibiotic plates. Furthermore, subsequent colonies have to be screened by PCR, plasmid profiling, and in most cases sequencing of the vector's multiple cloning site region to determine and verify the presence and sequence integrity of an insert (95). This approach, while successful, is rather laborious and time-consuming. A particular type of new generation sequencing technology has provided an alternative, fast, and attractive approach. Pacific Biosciences SR DNA sequencing technology is a recent high-throughput sequencing platform that can generate average read lengths of over 2,500 bp of whole phage genomes (96). Despite initial high error rates, on-going improvements of the technology and software have greatly increased read accuracy (97, 98). Each sequencing single-molecule real-time (SMRT) cell contains 150,000 zero-mode waveguides (ZMVs) (99), nanophotonic compartments containing a single DNA polymerase and a single strand of template DNA (Pacific Biosciences, Menlo Park, CA). The ZMVs have a tiny aperture that allows light to penetrate, creating a chamber for visualizing the activity of the DNA polymerase. Each nucleotide is linked to a unique fluorophore label attached to the phosphate of the base. As a nucleotide is incorporated, the fluorescent molecule is released and detected, thus allowing nucleotide sequence determination in real time. This sequencing technology can be applied to *de novo* sequencing and to base modification analysis (Pacific Biosciences, Menlo Park, CA). Recent studies have utilized this SMRT technology to characterize DNA methylation patterns by monitoring the time taken to incorporate each nucleotide by the DNA polymerase, the kinetic variation (KV) (100). Measuring the KV allows direct detection of modified nucleotides in the DNA template, including N-6-methyladenine and C-5-methylcytosine, as each has a unique kinetic signature (101). SMRT sequencing has enabled researchers to determine the identity and position of methylated bases, and from this information, the target sequence of the MTases encoded by genes on the genome can be derived (102). This technology can be easily applied to phage genomes, as they are relatively small, and a large quantity of data can be generated using a 5- to 10-kb insert library, ultimately to determine whether phage gene-encoded orphan MTases are functionally active and also to potentially establish the role they play.

CONCLUSION

The occurrence of genes that specify orphan MTases is relatively high at approximately 20% of the currently annotated phage genomes. While a number of studies have been carried out on MTases encoded by genes on temperate phages, little is known about their recognition sequences, source, or the precise role these genes play in enhancing the infectivity and survival of phage populations. It is apparent from Table S1 in the supplemental material that phages isolated from a diverse range of ecosystems possess integrated orphan MTase-encoding genes. However, it is unclear whether this is driven by the vast array of phage-phage and/or phage-host interactions or the necessity for survival or if certain phage species are better equipped for such genome modifications. More than likely, in the majority of cases, the incorporation of MTase-encoding genes into phage genomes may provide protection against host R-M systems. The continued isolation and whole-genome sequencing of phages as well as the use of next-generation sequencing may provide greater insight into the source of these MTases, their target specificities, and mechanisms of genome incorporation.

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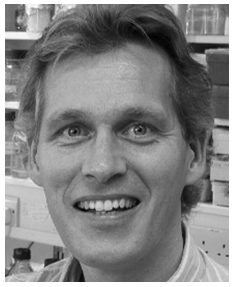

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