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DNA MODIFICATIONS IN BACTERIOPHAGES: APPLICATIONS IN CYSTIC FIBROSIS AND COMBATTING MDR PATHOGENS

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Abstract. Bacteria and bacteriophages are engaged in a continuous evolutionary arms race, in which both sides rely on DNA-based defense and counter-defense strategies. Understanding these phage DNA modifications is essential for advancing engineered phage therapy, particularly against multidrug-resistant (MDR) pathogens. This review synthesizes current knowledge on chemically modified phage genomes and highlights how engineered DNA modifications such as those mediated by DpdA and related pathways enable phages to evade bacterial restriction modification systems and CRISPR-Cas immunity. Current study further examine bacterial countermeasures, including methyltransferase activity and antibiotic resistance determinants, with a focus on *Achromobacter* species, an emerging MDR pathogen associated with chronic lung infections in cystic fibrosis patients. By integrating evidence from LC-MS/MS, nanopore sequencing and bioinformatic approaches, we outline how these technologies reveal previously uncharacterized phage modifications. This review provides novel insights into how strategically engineered phage DNA can enhance therapeutic stability, broaden host range and overcome bacterial defenses, offering a promising framework for next-generation phage therapy design.

Keywords: *Phage DNA modifications, CRISPR-Cas, Achromobacter, bioinformatic, LC/MS/MS, efflux pump, MDR.*

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1. Introduction

Antibiotic resistance has escalated into one of the most urgent global health threats of the 21st century, undermining the effectiveness of current antimicrobial agents and limiting treatment options for multidrug-resistant (MDR) infections. The World Health Organization has repeatedly emphasized that MDR pathogens particularly Gram-negative organisms are responsible for rising morbidity, mortality and healthcare burden worldwide. As conventional antibiotics continue to lose efficacy, the need for innovative therapeutic strategies has never been more pressing. This urgent context has renewed scientific and clinical interest in bacteriophages (phages), viruses that infect and lyse bacteria, as a promising complementary or alternative approach to combatting antibiotic-resistant infections.

Bacteria, however, have evolved an extensive arsenal of defense mechanisms to protect themselves from phage predation. These include restriction-modification (R-M) systems, CRISPR-Cas adaptive immunity, abortive infection pathways, toxin-antitoxin systems and diverse nucleic acid recognition modules. Collectively, these defenses create

formidable barriers that limit the effectiveness of natural phages and pose significant challenges to the long-term success of phage therapy. Overcoming these bacterial immune defenses is therefore essential for optimizing phage-based therapeutics.

Phages, in turn, have co-evolved sophisticated counter-defense strategies to bypass bacterial immunity. Among these, chemical DNA modifications represent one of the most powerful and versatile mechanisms. Phages deploy a diverse set of nucleobase modifications such as 7-deazaguanine derivatives mediated by enzymes like DpdA to shield their genomes from host nucleases, prevent cleavage by R-M systems and evade CRISPR-Cas targeting. These DNA modifications not only enhance phage survival during infection but also provide a foundation for engineering next-generation therapeutic phages with improved stability, immune evasion and host range expansion. As a result, DNA-modified and DNA-engineered phages are emerging as a leading strategy to overcome the long-standing challenge of bacterial defense systems in phage therapy.

The clinical relevance of these innovations is particularly evident in chronic respiratory infections. Cystic fibrosis (CF), a genetic disorder affecting over 30,000 individuals in the United States, is characterized by impaired mucociliary clearance and persistent bacterial colonization. Among the pathogens associated with CF, species of *Achromobacter* have gained increasing attention due to their intrinsic and acquired resistance to multiple antibiotics. *Achromobacter* infections especially *A. xylosoxidans* are associated with lung function decline, treatment failure and poor clinical outcomes. Their ability to harbor multiple resistance determinants and evade antibiotic treatment underscores the need for alternative therapeutic modalities. Understanding how engineered phages can bypass bacterial defenses is therefore essential for developing precision phage therapies targeted toward MDR CF pathogens such as *Achromobacter*.

Recent advances in analytical and sequencing technologies have significantly expanded our ability to detect and characterize DNA modifications in phages (Wu *et al*., 2025). Techniques such as liquid chromatography-tandem mass spectrometry (LC-MS/MS), nanopore sequencing and computational bioinformatics have enabled the identification of previously unrecognized DNA modifications, their biosynthetic pathways and their roles in phage-host interactions. These tools provide essential insights into how phages adapt to bacterial defense systems and offer a framework for rational engineering of phages with enhanced therapeutic capabilities.

This review integrates current knowledge on phage DNA modification systems, with emphasis on DpdA-mediated pathways and newly emerging modification families. We examine how these chemical alterations enable phages to evade bacterial immune defenses, highlight their relevance for treating MDR pathogens such as *Achromobacter* in CF and evaluate recent technological advances that facilitate their detection. By linking molecular mechanisms to translational applications - such as therapeutic phage design, microbiome modulation, fermentation and food safety - we strive to offer a detailed insight into the role of engineered DNA-modified phages in countering bacterial defense systems and driving advancements in next-generation phage therapy (Chen *et al*., 2021; Crippen *et al*., 2020; Chanishvili, 2012; Clokie & Kropinski, 2009; Clokie *et al*., 2011; Hutinet *et al*., 2019; Gedara *et al*., 2019).

Restriction Modification system

During tests in which various strains of the same bacterial species were infected with bacterial viruses (bacteriophages or phages for short), the phenomena of restriction-modification (R-M) was found in the 1950s. It was found that the efficiency of plating on

permissive, non-restricting strains was nearly one, whereas the efficiency of plating on non-permissive, restricting strains was approximately five orders of magnitude lower. Efficiency of plating is defined as the proportion of phage particles capable of productively infecting the host bacterium and ultimately leading to plaques, i.e., observable foci of infection on host bacterium lawns. On the other hand, phage progeny that survived infrequent productive infections of restricting hosts were equally proficient at plating on strains that were restricting as well as strains that weren't. Additionally, following a single passage on the non-limiting strain, the offspring of the "modified" phages were no longer able to successfully infect the restricting strain. Phages that have been recovered from infections causing restricting strains are considered to have been "modified" by the restricting host because they lack a heritable alteration. Luria and Human found that the efficiency of plating or the percentage of phage particles that could successfully infect the host, was significantly lower on non-permissive, restricting strains of bacteria when bacteriophages infected them than on permissive, non-restricting strains (Loenen *et al*., 2014). Nevertheless, the phage progeny that succeeded in infecting the restricting hosts were thereafter highly effective in infecting strains that were neither restricting nor non-restricting.

The most common type of phage defense mechanism found in bacterial genomes, restriction-modification (RM) systems are frequently mentioned in discussions of bacterial defense against phage predation in both bacteria and archaea. Furthermore, RM systems have developed "moonlighting roles" in metabolism, gene control and recombination. Two enzymatic processes that both identify and react to a certain DNA sequence make up an RM system. If the sequence is not methylated, one of the enzymes cleaves it; if it is, the other enzyme adds methyl groups to the sequence to prevent cleavage. As a result, any foreign, non-methylated DNA that enters the cell including phage DNA that is intruding will be preferentially cleaved. To a certain extent, depending on the phage and the specific RM system, this offers immunity to infection (Bickle & Krüger, 1993; Kessler & Manta, 1990; Korona & Levin, 1993; Makarova *et al*., 2013; Mazur-Marzec *et al*., 2024; Vasu & Nagaraja, 2013).

Different types of modifications

Understanding how bacteria protect themselves against foreign DNA, particularly which of bacteriophages, was greatly aided by this discovery. It prompted additional investigation that revealed the molecular foundation of R-M systems, which are made up of two primary parts:

1) An enzyme known as methyltransferase (MTase) methylates the host's DNA to prevent cleavage.

2) An unmethylated foreign DNA-cleaving restriction endonuclease (REase) (Mazur-Marzec *et al*., 2024).

Based on their subunit composition, sequence recognition, cleavage position and cofactor need, R-M systems are divided into four primary types:

Hetero-oligomeric protein complexes with restriction and modification activities are classified as type I and the most prevalent type 1 enzyme, distinct REase and MTase are classified as type II and also type III and type IV are vary in terms of the makeup of their subunits and their recognition sequences (Vasu & Nagaraja, 2013). Phages have created a number of methods to get around R-M systems, including obtaining methylation patterns unique to each host. Coding for proteins that protect unmodified restriction sites or impede restriction endonucleases (Eriksen *et al*., 2022; Gao & Feng, 2023).

Life Cycle Structure and Genomics of Phages

A bacteriophage consists of several key parts, including the head (capsid), tail, tail fibers and baseplate. The head (capsid) is made of a protein and is typically polyhedral or icosahedral in shape and houses the phage's genetic material, which can be either DNA or RNA. Attached below the head is the neck or collar region. The tail, composed of protein, functions as a hollow tube that acts as a passageway for injecting the genetic material from the head into the host bacteria (Tavares, 2018). Because the tail fiber protein is highly variable and often consists of several subunits, it is possible to create a variety of tail fiber architectures by combining these subunits in different ways (Veesler & Cambillau, 2011). The baseplate often includes specialized proteins such as tail fibers and receptor binding proteins that are crucial for host recognition and attachment. The tail fibers, also made of protein, are located at the end of the tail and help the bacteriophage attach to the host bacteria. These tail fibers can be classified into long tail fibers (LTFs) and short tail fibers (STFs), each playing a role in the attachment process (Shkoporov & Hill, 2019; Yap & Rossmann, 2014).

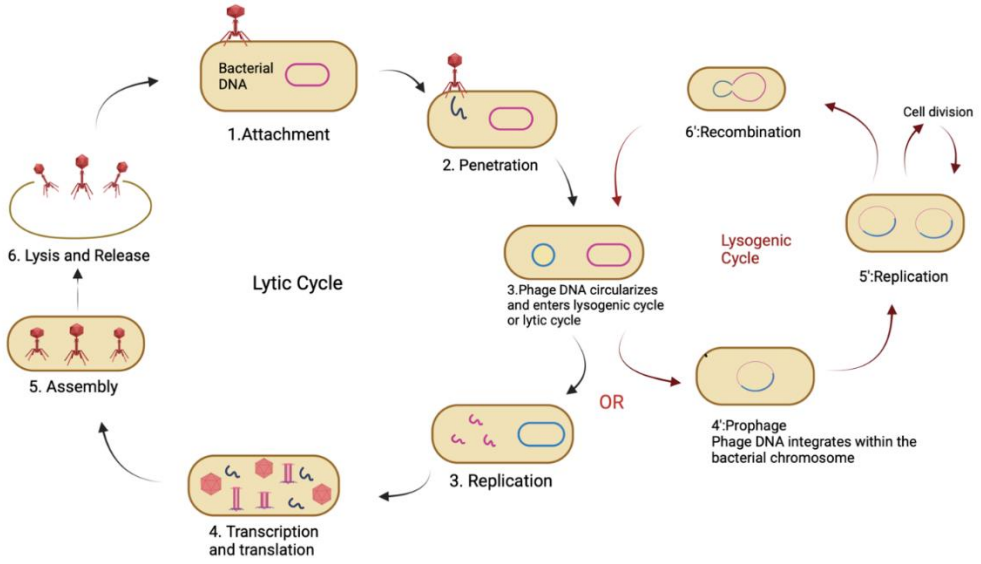

Figure 1. The life cycle of a phage can be lytic or lysogenic. The lytic cycle has 6 steps to infect a bacterium: (1) the attachment of phage to the receptors on the cell membrane of host bacteria, (2) penetration of its genome, (3) replication in the host cell by using their molecular machinery system, (4) transcription and translation, (5) assembly and (6) the release of the phage progeny. Lysogenic phages enter the (4') stage, are integrated into the bacterial chromosome and then start to replicate with the host cell **Source:** Tu et al. (2023)

Phage growth is influenced by a variety of parameters, including the phage bacterial adsorption rate constant, explosion size, latent period, bacterial growth rate, phage and bacterial elimination rates and the impact of controlled phage release (Leung & Weitz, 2017; Malik *et al*., 2017) the phage parasitic life cycle starts with the phage binding to specific host receptors, adapted from (Figure 1). Subsequently, the phage genome is replicated using the host machinery. The lytic cycle concludes with cell lysis, which releases the offspring into the surrounding environment. Phage attachment and infection mechanisms are facilitated by the production of protein structures known as tail fibers. Tail fibers exhibit significant variation between various phages and are highly selective

for bacterial surface structures. Infection and attachment can be divided into three stages: (1) adsorption, the initial contact with the bacterial surface; (2) “random walking” and reversible attachment along the surface of the bacterial cell and (3) irreversible binding to a target receptor (Moldovan *et al*., 2007; Tu *et al*., 2023).

The benefiting of Restriction and Modification System

The benefit of RM systems is helpful when the host first invades a region where new phage is present, as it gives the population time to grow and consequently, increases the likelihood of immune mutant emergence, the formation of a biofilm or other density-dependent mechanisms that improve phage-bacteria coexistence (Heilmann *et al*., 2012). Epigenetic DNA methylation plays a crucial role in bacteria by influencing gene expression and allowing the distinction between self-DNA and foreign elements such as phages and plasmids. The well-studied Type II RM systems consist of two distinct enzymes: DNA methyltransferase (MTase) and restriction endonuclease (REase).Although their functions are different, both proteins can identify the same shor particular DNA sequence (Pleška *et al*., 2016). By altering a particular sequence motif using a methyltransferase (MTase), restriction-modification (RM) systems shield host DNA from breakage by a corresponding restriction endonuclease (REase) while leaving invasive DNA susceptible. Other REases cleave methylated DNA and exist alone (Birkholz *et al*., 2022). Mammalian development depends on DNA methyltransferases (DNMTs), which move a methyl group from S-adenosyl-L-methionine (SAM), the universal methyl donor, to the 5-position of cytosine residues in DNA1. The DNMT family consists of four members: DNMT1, DNMT3A, DNMT3B and DNMT3L. DNMT3L lacks intrinsic enzymatic activity, in contrast to the other DNMTs2. On DNA, the remaining three family members are active (Jin & Robertson, 2013). Among prokaryotes, restriction-modification (R-M) systems are quite common (Makarova *et al*., 2013; Oliveira *et al*., 2014; Roberts *et al*., 2015). Because REase has strong endonucleolytic activity, the R-M systems could likewise constitute dangerous cargo for their hosts. A balanced expression of REase and MTase indicates that host death is most likely to occur (Pleška *et al*., 2016).

DNA Methyltransferase Mechanism:

DNA methyltransferases produce three different types of modified bases: N4-methylcytosine (m4C), N6-methyladenine (m6A) and 5-methylcytosine (m5C). They methylate specific bases in recognition sequences. Due to the fact that it is transmitted through maintenance methylation following DNA replication, this change is classified as epigenetic (Ishikawa *et al*., 2010). DNA methyltransferases (DNMTs) catalyze the transfer of a methyl group to DNA, primarily at the C5 position of cytosine in CpG dinucleotides. The mechanism of DNA methylation by DNMTs involves several key steps:

Recognition and binding, Base flipping and Methyl transfer

DNMTs recognize and bind to specific DNA sequences, often targeting hemimethylated DNA (DNMT1) or unmethylated DNA (DNMT3A and DNMT3B) (Jin & Robertson, 2013) (Figure 2). DNA methyltransferases (DNMTs) are a class of enzymes that catalyze DNA methylation (Eukaryotic Cytosine Methyltransferases, n.d.). The four members of the DNMT family in mammals are DNMT1, DNMT3A, DNMT3B and DNMT3L (DNA Methyltransferases and Their Roles in Tumorigenesis, n.d.). The

preservation of methylation throughout the genome depends on DNMT1 (Jeltsch, 2006). De novo methyltransferases are the names given to DNMT3A and DNMT3B. The catalytic activity of DNMT3A and DNMT3B is stimulated by DNMT3L. The maintenance methyltransferase DNMT1 reliably maintains the methylation pattern created by the de novo DNA methyltransferases DNMT3A and DNMT3B in conjunction with DNMT3L throughout cell division (Schübeler, 2015). The target cytosine is flipped out of the DNA double helix into the enzyme's active site (Zhang & Xu, 2017). Base flipping is a highly conserved molecular maneuver used across many functional contexts: not just in eukaryotic methyltransferases, but also in prokaryotic base modification readers, diverse DNA glycosylases and proteins involved in epigenetic signaling and DNA demodification. This widespread use emphasizes its essential role in precise and targeted DNA processing events (Hong & Cheng, 2016). S-adenosyl-L-methionine (SAM) serves as the methyl group donor (Kudo *et al*., 2024). DNMT mediated DNA methylation is essential for genomic imprinting, gene control and genome integrity. Developing targeted epigenetic therapeutics and understanding the intricate interactions between DNA methylation and other epigenetic alterations require an understanding of the mechanism of DNMTs (Adam *et al*., 2020; Regmi *et al*., 2023).

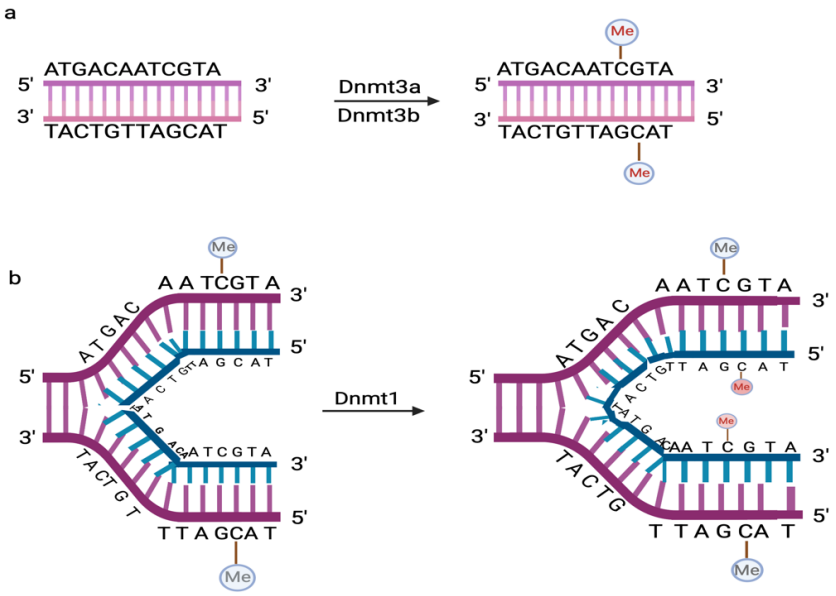

Figure 2. Mechanisms for DNA methylation. 5-methylcytosine (5mC) is created when a methyl group is transferred from S-adenyl methionine (SAM) to the fifth carbon of the cytosine residue by a family of DNA methyltransferases (Dnmts). (a) The de novo Dnmts, Dnmt3a and Dnmt3b, add methyl groups (red) to bare DNA. (b) During replication, the maintenance Dnmt, Dnmt1, keeps the DNA methylation pattern stable. The original DNA methylation pattern (gray) is retained in the parental DNA stand during semiconservative replication. Dnmt1 attaches itself to the replication foci and adds methyl groups (red) to the newly produced daughter strand (blue) in order to perfectly mimic the original DNA methylation pattern
Source: Moore et al. (2013)

DNA methyltransferases are bi-bi enzymes that use DNA and SAM as substrates to produce methylated DNA and S-adenosyl-L-homocysteine. The target base is flipped out of the DNA helix and bound in the enzyme's active site, enabling in situ methylation that is essential for processes like gene regulation and genomic imprinting. SAM is the methyl

group donor for DNA methylation, targeting the exocyclic amines of cytosine (N4) and adenine (N6) as well as the C5 atom of cytosine, which occurs most frequently in eukaryotes (Weigele & Raleigh, 2016).

CRISPR/Cas System

The CRISPR/Cas system is an innovative genome-editing tool, which acts as an adaptive immune system in archaea and bacteria protecting them from phages, plasmids and other mobile genetic elements (Allemailem, 2024). The intrinsic resistome of pathogens like Staphylococcus aureus includes many genes beyond those previously linked to drug resistance, showing how broad and varied innate bacterial defense strategies can be (Vestergaard *et al*., 2016). Intrinsic resistance describes a bacterium's ability to withstand antimicrobial drugs due to built-in, essential characteristics at the molecular level that are encoded in its genome and not acquired in response to antibiotics. Resistance can develop independently of antibiotic exposure, with bacteria occasionally undergoing natural genomic changes in resistance-related genes without the influence of antibiotic selection pressure (Poulton & Rock, 2022). Resistance can develop independently of antibiotic exposure (Figure 3), with bacteria occasionally undergoing natural genomic changes in resistance-related genes without the influence of antibiotic selection pressure (Poulton & Rock, 2022). Researchers have identified six main types (I-VI), categorized into two classes. Class 1 (Types I, III, IV) utilizes multi-protein effector complexes, while Class 2 (Types II, V, VI) employs single-protein effectors such as Cas9, Cas12 and Cas13.

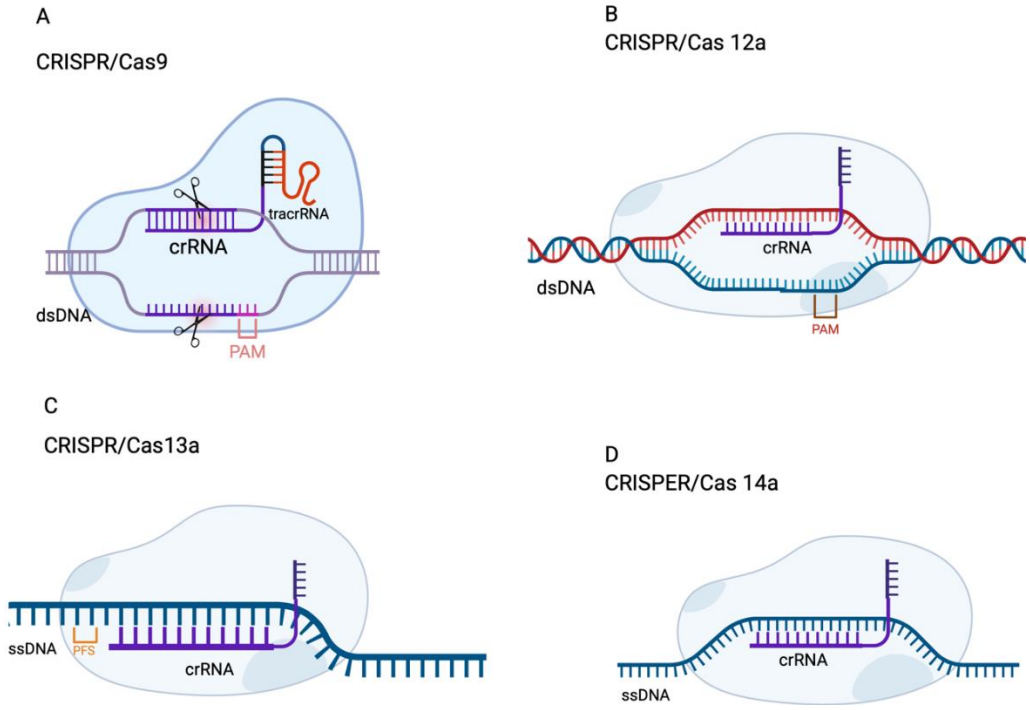

Figure 3. Gene editing schematic of four commonly used CRISPR/Cas systems (Cas9, Cas12a, Cas13a and Cas14)
Source: Bikard and Marraffini (2013)

The problem for R-M and CRISPR related systems is one of fine tuning the regulatory control over hazardous genes in a manner that produces high anti-phage immunity while limiting self-destruction (DNA Methyltransferases and Their Roles in

Tumorigenesis, n.d.; Eukaryotic Cytosine Methyltransferases, n.d.; Jeltsch, 2006; Schübeler, 2015). At the point of transfer to a new host cell, strict control over REase is particularly crucial. Since MTase has not yet provided protection, the genome is vulnerable to deterioration. If R-M systems are to facilitate the acquisition of the newly introduced gene by the new host, they must incorporate a time-dependent control mechanism that initially favors the expression and activity of the MTase (Adam *et al*., 2020; Kudo *et al*., 2024; Regmi *et al*., 2023).

In order to protect these sites from the action of the cognate REase, the MTase add s a methyl group whereas the REase cleaves at the recognition sequence (Moore *et al*., 2013; Weigele & Raleigh, 2016). Cas9's capacity to inhibit gene expression may serve as an additional defense mechanism against phage infections caused by mutants that have evaded detection. CRISPR-Cas immunity relies on a variety of RNA and DNA cutting enzymes throughout its various functional stages to bolster this nucleic acid-dependent immune mechanism (Bikard & Marraffini, 2013; Goldberg & Marraffini, 2015; Jeltsch, 2003; Leon *et al*., 2018; Morozova *et al*., 2016; Mruk *et al*., 2007; Mruk & Blumenthal, 2008; Oliveira *et al*., 2016; Pleška *et al*., 2016; Werbowy & Kaczorowski, 2016). Bi-bi enzymes known as DNA methyltransferases catalyze the transfer of a methyl group from S-adenosyl-l-methionine (SAM) to either the C5 of cytosine in situ within the DNA polymer or to the exocyclic amine of cytosine (N4) and adenine (N6). The target base is flipped out of the DNA helix and binds in the enzyme's active site during the methyl transfer reaction that is catalyzed by the enzyme. R-M systems play an important role in the coevolution of prokaryotes and their viruses because of their defense mechanisms (Rusinov *et al*., 2018).

The Biology of Antibiotic Resistance

Antibacterial resistance or MDR (Multidrug-Resistant), is the capacity of bacteria to endure in an environment where antibiotics are supposed to kill them. Even if the MDR is growing daily, technological advancements and a growing understanding of biological insights are also emerging concurrently. In order to survive under various antibiotic circumstances, bacteria can employ an intrinsic mechanism or an acquired resistance strategy (Blair *et al*., 2015). The expression of resistance genes against various targets is one of the resistance mechanisms and it can vary significantly depending on the growth conditions (Whittle *et al*., 2021). The number of infections brought on by MDR bacteria indicates that in recent years, the bacteria's insensitivity to antibiotics has increased significantly. Antimicrobial resistance remains a significant challenge to human health in Europe, as highlighted by various studies (Llor & Bjerrum, 2014). It is estimated that tackling the mortality caused by multidrug-resistant bacterial infections would require an annual investment of €1.5 billion from Europe's economy (Yang *et al*., 2021).

However, in the United States, MDR bacterial infections affect over two million individuals each year and are responsible for over 23,000 deaths. Likewise, these illnesses account for an additional $55 billion in healthcare and social costs each year in the United States (Prestinaci *et al*., 2015). The situation of MDR bacteria has gotten even worse due to the lack of new antibacterial medicines (Li & Webster, 2018). Furthermore, treating MDR bacteria with inadequate antibiotics encourages the growth of bacterial tolerance. Methicillin resistance, for instance, is present in 40-60% of Staphylococcus aureus strains obtained from various US hospitals and in some situations, vancomycin and carbapenem resistance as well (Ventola, 2015).

Intrinsic Resistance

At the molecular level, intrinsic resistance is based on essential or innate characteristics of bacteria that have evolved to withstand antimicrobial drugs. Due to the absence of antibiotic-based selective stress, microorganisms' inherent resistance trait occasionally undergoes natural genomic alternations (Cox & Wright, 2013). Even in the absence of antibiotic exposure, intrinsic resistance genes can undergo mutations, recombination or loss/gain of genetic elements as part of natural bacterial evolution. These changes can affect the expression or function of resistance mechanisms, sometimes increasing or decreasing susceptibility to antibiotics (Vestergaard *et al*., 2016).

Efflux pump

For an antimicrobial agent to have a long-lasting effect, it must remain in a microbial environment for a longer amount of time and in high concentrations. Nevertheless, certain bacteria have very efficient drug efflux pumps that remove the medication, leaving insufficient amounts for the drug to work as intended. While MDR pumps extrude a range of medications with distinct structures and functions, some pumps even force out particular antibiotics, including tetracyclines, streptogramins, lincosamides and macrolides (Zhang *et al*., 2024).

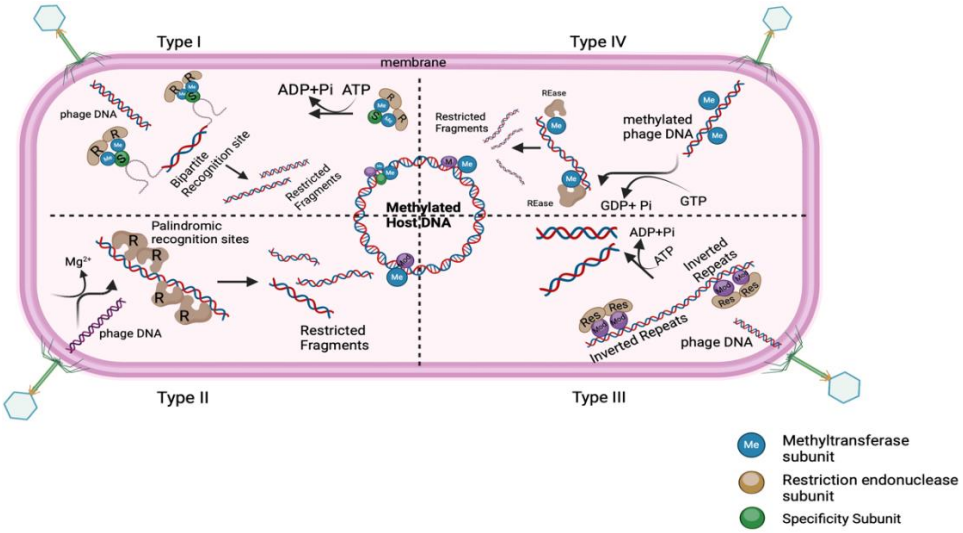

Figure 4: A methyl group is transferred from a donor molecule, usually S-adenosylmethionine (SAM), to a substrate by the enzyme methyltransferase. Within the enzyme complex, restriction endonuclease subunits carry out tasks that aid in DNA recognition, cleavage and modification. The coordination of the intricate tasks of DNA identification, cleavage and modification carried out by restriction endonucleases is made possible by the subunit organization

Figure 4 summarizes several molecular processes underlying MDR in bacteria. Antibiotic inflow is reduced when transmembrane proteins are downregulated or undergo structural alterations. Transmembrane efflux pumps become more numerous or active, expelling the antibiotics from the cell to reduce their intracellular concentration. By hydrolyzing or transferring certain chemical groups, some enzymes alter or break down antibiotics, making them useless. Certain proteins' target sites can be altered by binding site modifications or mutations in the genes producing the protein target. The target bypass is when an antibiotic binds to a novel protein without preventing it from doing its

job. Antibiotic-mediated inhibition is alleviated when target protection proteins physically associate with antibiotic target proteins (Allemailem *et al*., 2024).

Recent developments in various technologies have uncovered the finer points of many resistance mechanisms. These include the functioning of intricate efflux systems that indicate likely pathways for the development of inhibitors (Cox & Wright, 2013; Ray *et al*., 2017).

Different Approaches to Overcome MDR

Various strategies to stop the MDR include the use of vaccinations, combinatorial therapy, medication repurposing (Liu *et al*., 2021), anti-virulence compounds (Ménard *et al*., 2014) and drug loaded nanoparticles (NPs) (Allemailem, 2024). Silver nanoparticles are recognized as a "multifunctional nanoweapon" with significant potential in combating multidrug-resistant (MDR) bacteria. Their extensive antimicrobial capabilities and distinctive modes of action render them less prone to the development of resistance when compared to conventional antibiotics (Rai *et al*., 2012).

Cystic Fibrosis (CF)

About 30,000 Americans, mostly of European ancestry and 3,000 Canadians suffer from cystic fibrosis (CF), a genetically inherited illness. In CF patients, almost 1,800 distinct CFTR gene mutations have been found. The mutation in the CFTR gene prevents the CFTR protein from functioning correctly or at all. An ion channel is a specific class of protein that includes the CFTR protein. The CFTR ion channel transports carbonates and chloride ions from inside to outside lung cells. The CFTR protein forms a tube through which the chloride ions pass to exit the cell. Water is no longer drawn to the area outside the cell when a mutation in the CFTR protein causes the chloride ions to become stuck inside the cell. The mucus in the airways gets dry and thickens when there is less water outside the cells, which flattens the cilia. When the cilia are burdened with thick, sticky mucus, they cannot sweep effectively. Various organ systems, including the gastrointestinal and respiratory tract, are impacted by impaired CFTR function. Patients with cystic fibrosis (CF) create unusually thick mucus, which clogs small channels in organs. A century ago, gastrointestinal tract issues frequently resulted in infant death. However, with advancements in nutrition, lifestyle and medication, life expectancy has grown and patients with cystic fibrosis (CF) are now frequently alive into adulthood. Numerous treatments seek to lessen intestinal tract obstructions, release mucus from afflicted organs and reduce infections (Chen *et al*., 2021). However, the respiratory system continues to be a major worry for CF patients, even though many difficulties have been treated; in certain situations, lung transplants are necessary. In people with cystic fibrosis (CF), thick mucus discharges impair lung function and increase the risk of recurring viral, bacterial and fungal lung infections. The changed lung environment hinders the patient's immune system and makes them more vulnerable to bacterial, fungal and viral infections. Pseudomonas species are given much attention and since they are among the most common bacterial infections in people with cystic fibrosis. However, CF patients' lungs are frequently infected with other less well-studied bacteria.

Achromobacter

About 10% of lung infections in CF patients and other non-CF illnesses are caused by the Gram-negative bacteria Achromobacter sp. (Davies & Rubin, 2007). Additionally, pan-drug resistant infections have been reported and Achromobacter sp. naturally harbors

many antibioticresistance genes. Interest in bacteriophage therapy has recently increased due to the dearth of effective antibiotic therapies, particularly when compassionate use is warranted. Bacteriophages or lytic viruses, cause damage to the bacteria they infect. As a result, bacteria have developed defense mechanisms against phages. Restriction-modification systems and CRISPR-Cas are two examples of innate immune-like systems used in some defense mechanisms to break down phage DNA. These forces have led to discovering methods by which some phages express hypermodified DNA. While some phages change nucleotides after replication, others generate and integrate modified nucleotides during replication. A modified base may be used to wholly or partially replace a canonical base in either of the two changes to DNA processes (Crippen *et al*., 2020). In the never-ending battle between bacteria and phages, both entities continually improve their defenses and counterattack techniques. Phages have developed several strategies to get around these obstacles and one of their most prevalent methods is to change their DNA (Kot *et al*., 2020). Viruses specific to bacteria, known as bacteriophages or phages, are administered as part of phage therapy. Phages live parasitic lifestyles, infecting host bacteria and reproducing through their molecular machinery before lysing the host and releasing progeny. Unlike antibiotics, which are often effective against a broad range of bacteria, many phages are incredibly selective and can only infect and multiply in a limited range of hosts. The design of phage therapy techniques is time-consuming because of phage specificity. Furthermore, complete infection clearance is not guaranteed. No well characterized reference strains of Achromobacter are available, unlike those for Pseudomonas, Mycobacterium and many other pathogens. Therefore, before phages are used in a treatment, it must be isolated and characterized based on its ability to infect and reproduce in specific patient strains of bacteria. Single phage therapy is unlikely to eradicate an infection because of the likelihood of resistance mutations existing in bacterial 3 populations, the challenge of accessing every area of the diseased lungs and the likelihood of physiologically refractory bacteria. The goal of polyphage therapy, sometimes known as “Phage cocktails”, is to treat each bacterial infection with at least two genetically different phages to reduce these problems, particularly those involving resistant mutations. To decrease the likelihood that a single bacterial mutation may impart resistance to all phages in the treatment, it would be ideal for each phage to target distinct receptors and other host components and respond differently to host immunity. Nevertheless, phage-host dynamics in Achromobacter sp. are still poorly understood and a deeper comprehension of the molecular pathways necessary for phage infection could greatly enhance phage therapy.

Restriction enzyme digestion and LC/MS/MS (liquid chromatography coupled with mass spectrometry in a liquid phase) were used to detect phage DNA modifications. By making high titer lysates in the patient strain, we can tell that the specific phage can replicate in the specific strain in the patient. Restriction Digestion acts as the host’s defense system, so some phage DNA modifications could be identified. Some DNA modifications were identified by comparing the expected fragments from the Restriction Digestion of the gels and observed fragments from NEBCutter. It was predicted some modifications in these phages by obtaining data from the LC/MS/MS (the possible modifications that the phages may have). This approach enhances the efficiency and effectiveness of phage therapies. The recognition sequence of EcoD methyltransferase was determined using DNA with predefined sequences, which had been methylated by EcoD methyltransferase using tritiated AdoMet as the methyl donor. To identify the methylated sites within the recognition sequence, the methylated DNA fragments were

digested with Type II restriction enzymes. Subsequently, gel electrophoresis and fluorography were utilized to detect DNA methylation. The identified sites were then analyzed through a computer program to locate the consensus sequence shared across all labeled regions (Gulati *et al*., 2024).

Although it has not been very successful, the Oxford Nanopore Technology (ONT) platform has also been used to find restriction enzyme recognition sequences (Simpson *et al*., 2017). A thorough investigation of the full genome methylomes of several bacterial and archaeal species was carried out in 2016 using SMRT sequencing, which identified a large number of motifs methylated by methyltransferases and orphan methylases with corresponding restriction endonuclease systems (Blow *et al*., 2016). The development of new sequencing methods, such Pacific Biosciences' single-molecule real-time (SMRT) sequencing, has made it easier to conveniently characterize methylated patterns during large-scale genome mapping. Fluorescently labeled nucleotides are used in SMRT sequencing to identify methylated bases (Ardui *et al*., 2018). It involves a SMRT cell that immobilizes DNA templates through microscopic pores known as ZMWs (Zero-Mode Waveguide). Every nucleotide has a different fluorescent tag attached to it, which, when incorporated, creates a different signal. Methylated adenine (3-5 times) and methylated cytosine (5-7 times) demonstrate significantly longer fluorescence pulse durations compared to their unmethylated counterparts. The IPD (Interpulse Duration) ratio, which shows how long it takes to integrate a base at a certain location in comparison to an unmodified base, is examined by the RS Modification and Motif analysis tool (Rhoads & Au, 2015).

Figure 5 shows Pumps that are embedded in the cell wall or membrane can be produced by bacteria. These so-called efflux pumps are widely distributed in bacteria and can carry a wide range of substances, including nutrients and signal molecules. To reduce the concentration of antibiotics within the bacterial cell, several of these pumps can also move antibiotics out of the bacterium. Sometimes bacterial DNA mutations cause the bacteria to generate more of a particular pump, which raises resistance.

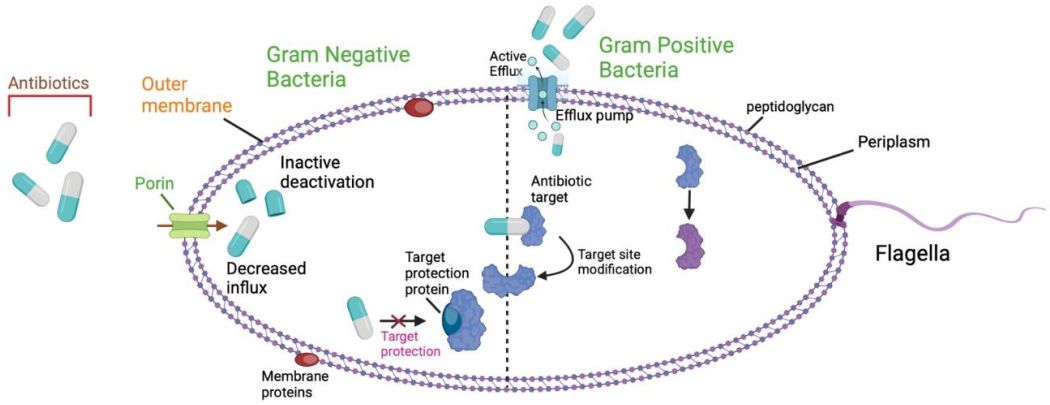

Figure 5. The antibiotic may no longer interact with the target if the bacterium's target undergoes structural or compositional changes as a result of bacterial DNA mutations. As an alternative, the bacteria can protect the target structure from the antibiotic by adding various chemical groups to it

Different kinds of modification

5-Methylcytosine (5mC): The most common and well-studied DNA modification, involves the addition of a methyl group to cytosine at CpG sites. Plays a crucial role in

epigenetic regulation and gene expression.5-Hydroxymethylcytosine (5hmC) formed by oxidation of 5mC by ten-eleven translocation (TET) enzymes. It is recognized as a significant epigenetic factor influencing stem cell renewal, the progression of cancer and the onset of neurological disorders.

Further oxidation product of 5hmC. 5-Formylcytosine (5fC) is an oxidized derivative of cytosine in DNA, recognized as both a key epigenetic marker and a crucial intermediate in active DNA demethylation. It is generated when TET enzymes further oxidize 5-hydroxymethylcytosine (5hmC), playing an important role in dynamic DNA modification and gene regulation processes (Raiber *et al*., 2015). Final oxidation product in the demethylation pathway, 5-Carboxylcytosine (5caC) is an oxidized derivative of 5-methylcytosine (5mC) in DNA, formed through iterative action of TET (ten-eleven translocation) enzymes as part of the active DNA demethylation pathway. This modification represents one of the final steps in the oxidation of 5mC, following the formation of 5-hydroxymethylcytosine (5hmC) and 5-formylcytosine (5fC) (Hashimoto *et al*., 2015). N6-Methyladenine (6mA) is recently discovered in eukaryotic genomes. Potentially serves as an additional epigenetic mark with regulatory functions. N6-Methyladenine (6mA) is a DNA modification involving the addition of a methyl group to the nitrogen at the 6th position of adenine. While 6mA is well known and abundant as a regulatory and protective DNA mark in prokaryotes, it has only recently been recognized as a potentially important and widespread epigenetic mark in various eukaryotic lineages, including protozoans, fungi, plants and animals (Luo *et al*., 2015).

Non-CpG methylation is observed in undifferentiated human embryonic stem cells. Involves methylation of cytosine where guanine is replaced by adenine, cytosine or thymine. Oxidative damage products: Such as 8-oxoguanine, which can result from environmental factors or cellular processes.

Detection Methods

Several techniques are used to detect and analyze these DNA modifications: ***LC-MS/MS (Liquid Chromatography-Tandem Mass Spectrometry)****:*

This method is widely considered a highly dependable approach for accurately identifying and measuring different DNA modifications. Allows for sensitive and specific detection of multiple modifications simultaneously (Wang & Lai, 2017). Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) is widely regarded as a "golden" technique for the accurate identification and quantification of DNA modifications due to its high sensitivity, specificity and ability to detect multiple modifications simultaneously. It effectively combines the physical separation power of liquid chromatography (LC) with the molecular identification and quantitation capabilities of tandem mass spectrometry (MS/MS) (Burke *et al*., 2021).

Bisulfite sequencing

Used primarily for detecting cytosine methylation. Antibody-based enrichment coupled with next-generation sequencing (Wang & Lai, 2017). While bisulfite sequencing is the conventional method for detecting cytosine methylation at specific positions, it has limitations in accurately quantifying overall DNA modifications and differentiating between closely related marks such as 5mC and 5hmC. In contrast, LC-MS/MS serves as a valuable complementary technique that allows for precise, sensitive and simultaneous measurement of various DNA modifications across different species. This combined methodology offers a more comprehensive insight into epigenetic

regulation and uncovers unforeseen biological patterns that may not be evident through sequencing alone (Burke *et al*., 2021; Varma *et al*., 2022).

TGT

The enzyme tRNA-guanine transglycosylase (tgt) is essential for altering both DNA and RNA. This is how TGT aids in identifying DNA alterations (Dai *et al*., 2023; Kumar *et al*., 2018; Tota & Devaraj, 2023). The enzyme tRNA-guanine transglycosylase (TGT) is an RNA- and DNA-modifying enzyme that catalyzes the site-specific exchange of guanine bases in tRNA and DNA with modified bases, serving important roles in cellular regulation and potentially in identifying DNA alterations (Tota & Devaraj, 2023).

TGT catalyzes a Base Exchange reaction in which a modified base is used in place of a guanine base in nucleic acids: TGT has historically been known to alter tRNA by introducing altered bases at particular locations, such as preQ1 (7-aminomethyl-7-deazaguanine). According to recent studies, TGT can alter DNA substrates in specific circumstances (Tota & Devaraj, 2023).

Direct DNA Labeling; TGT can be used to insert modified bases directly into DNA

It can exchange a target guanine for chemically modified preQ1 substrates linked to reporters like biotin or fluorophores. This allows for site-specific labeling of DNA, which can be detected using various analytical techniques (Tota & Devaraj, 2023). tRNA-guanine transglycosylase (TGT) is a remarkable enzyme that can be harnessed to insert chemically modified bases directly into DNA through a site-specific base-exchange reaction. This expands its natural role from modifying tRNA to a versatile biochemical tool for DNA labeling and detection (Tota & Devaraj, 2023).

Substrate Specificity: TGT recognizes specific DNA sequences:

The enzyme shows preference for certain base combinations in the recognition element, such as TGdU or dUGdU. The minimal recognition element for TGT activity is often a structured 17-nucleotide hairpin with the target guanine positioned in the loop, flanked by these preferred sequence motifs, which facilitate optimal enzyme-substrate binding and catalysis (Tota & Devaraj, 2023).

Detection of Natural Modifications:

TGT-related enzymes are involved in creating natural DNA modifications:

The Dpd modification system in bacteria uses TGT-like enzymes to insert 7-deazaguanine derivatives into DNA. These modifications can be detected and studied using TGT-based labeling techniques (Gedara *et al*., 2023). TGT-related enzymes not only play an essential biochemical role in RNA modification but have been repurposed in bacteria to modify DNA, forming the basis of the Dpd system and enabling advanced molecular tools for probing DNA modifications in biological and technological contexts (Nonekowski *et al*., 2002).

DpdA

DpdA (DNA phosphorothioate-dependent DNA alkyltransferase) is a key enzyme involved in inserting 7-deazaguanine modifications into DNA. Here are the main points about DpdA and its role in DNA modifications: DpdA is essential for inserting 7-deazaguanine bases into DNA. It has homology to tRNA-guanine transglycosylase (TGT) enzymes, suggesting a similar transglycosylase activity. DpdA is a crucial enzyme

responsible for the insertion of 7-deazaguanine bases into DNA, a distinctive DNA modification involved primarily in bacterial restriction-modification (RM) systems and phage DNA protection. This enzyme shows significant homology to tRNA-guanine transglycosylase (TGT), indicating that it utilizes a similar enzymatic mechanism involving transglycosylation (Gedara *et al*., 2023).

DpdA likely catalyzes a Base Exchange reaction, replacing a guanine base in DNA with a modified 7-deazaguanine base, specifically 7-cyano-7-deazaguanine (preQ0), through a transglycosylation mechanism similar to tRNA-guanine transglycosylase enzymes. This process involves the cleavage of the glycosidic bond between guanine and the DNA sugar, followed by insertion of the modified base, effectively altering the genetic information at that site. DpdA works in conjunction with DpdB, an ATPase that facilitates the Base Exchange by possibly remodeling DNA or enzyme conformations, thereby enhancing the efficiency of preQ0 insertion. Subsequently, DpdC enzyme further modifies the inserted preQ0 base by converting it into 2′-deoxy-7-amido-7-deazaguanine (dADG), adding a functional amido group to diversify the DNA base's chemical structure. Together, these enzymes form a coordinated system that introduces novel 7-deazaguanine derivatives into DNA, contributing to bacterial defense against restriction enzymes and possibly other genome surveillance mechanisms while illustrating a significant evolutionary repurposing of RNA modification machinery for DNA modification. This system has broad implications for bacterial genome protection, phage DNA resistance and biotechnology applications due to its sophisticated enzymatic interplay and novel nucleic acid chemistry (Gedara *et al*., 2023; Thiaville *et al*., 2016; Yuan *et al*., 2018).

DpdA is involved in inserting modifications like 2′-deoxy-7-cyano-7-deazaguanosine (dPreQ0) and 2′-deoxy-7-amido-7-deazaguanosine (dADG) into DNA. In some phages, it's associated with the insertion of 2'-deoxy-7-deazaguanosine (dG+) (Hutinet *et al*., 2019).

Biological significance

These 7-deazaguanine modifications protect phage DNA from host restriction enzymes. In bacteria, DpdA is often part of a restriction-modification system that inserts these modifications into DNA (Gedara *et al*., 2023). This modification system is widespread among both bacterial and phage genomes, highlighting an evolutionary arms race where phages hijack these base modifications to evade bacterial immunity, while bacteria use similar strategies to regulate their own DNA protection. Studies have identified multiple subfamilies of DpdA in different phages and bacteria, each adapted to insert distinct 7-deazaguanine derivatives, suggesting a rich diversity and ongoing evolution of this molecular defense mechanism (Hutinet *et al*., 2019). In vitro studies have reconstituted the activity of DpdA and related enzymes from Salmonella enterica serovar Montevideo. Expression of DpdA alone in E. coli led to the insertion of dPreQ0 into plasmid DNA. When co-expressed with other enzymes like Gat-QueC, it led to the insertion of dG+ modifications (Hutinet *et al*., 2019). DpdA and related genes are found in diverse bacteria and some phages (Gedara *et al*., 2023). DpdA and related genes are omnipresent in many bacterial and phage genomes, reflecting a widely conserved mechanism to modify DNA with 7-deazaguanine derivatives for defense and survival, adaptable to various genomic and ecological contexts (Jung *et al*., 2025).

Bioinformatics

The possible effects of DNA alterations on chromatin structure, gene expression and other biological functions can be anticipated using bioinformatic methods. Integrating transcriptomic, proteomic and structural genomics datasets with modification data may be one way to do this. Researchers may effectively evaluate large-scale sequencing data, spot patterns of DNA alterations, link them to gene expression and genomic characteristics and develop theories regarding their biological importance by utilizing these bioinformatic techniques. This thorough examination is essential to expanding our knowledge of epigenetic control and how it affects a range of biological functions and illnesses.

Methylation-Expression Correlation

Tools like MethGET can correlate DNA methylation data with gene expression data to investigate the impact of modifications on gene regulation (Teng *et al*., 2020). Tools like MethGET (Methylation and Gene Expression Teller) are powerful bioinformatics platforms designed to correlate genome-wide DNA methylation data with gene expression profiles to investigate how DNA methylation impacts gene regulation. DNA methylation is a major epigenetic modification that adds methyl groups to cytosine residues, typically influencing gene activity without altering the DNA sequence itself. The relationship between methylation and gene expression varies depending on the genomic context, species and environmental conditions, making it essential to analyze these data together comprehensively (Teng *et al*., 2020).

Visualization Tools

Packages like DNA ModAnnot offer customized data visualization functions to represent modification patterns across the genome (Hardy *et al*., 2021). Packages like DNA ModAnnot provide specialized tools for the analysis and visualization of DNA modification patterns on a genome-wide scale, specifically designed to handle data from long-read sequencing technologies such as Pacific Biosciences (PacBio) and Oxford Nanopore Technologies (ONT). These technologies uniquely enable the direct detection of modified DNA bases with nucleotide-level resolution. DNA ModAnnot is an R package offering a comprehensive toolbox that integrates several key functionalities: rigorous filtering of DNA modification data to reduce false positives, annotation of modified bases concerning genomic features and a collection of tailored visualization tools. This modular design allows users to customize analysis pipelines for different types of DNA modifications, including 6-methyladenine (6mA) and 5-methylcytosine (5mC) (Hardy *et al*., 2021).

Nanopore Data Analysis

Tools like NanoMod can detect DNA modifications using raw electric signals from Nanopore sequencing. In Nanopore sequencing, DNA strands pass through a nanopore, causing characteristic disruptions in ionic current. Different DNA bases and modified bases generate distinct current signals influenced by the immediate sequence context around the base. These tools compare signal distributions between modified and unmodified samples to identify modifications. NanoMod provides a robust computational framework that leverages raw Nanopore signals and comparative statistics to map DNA modifications at single-base resolution, facilitating large-scale functional genomics studies involving both natural and synthetic DNA modifications (Liu *et al*., 2019).

Figure 6 provides a schematic overview of the process, illustrating how DNA modifications like 5mC, 6mA and 7-deazaguanine protect phage genomes from restriction enzymes. It also summarizes the main modification types and enzymes involved, including DpdA.

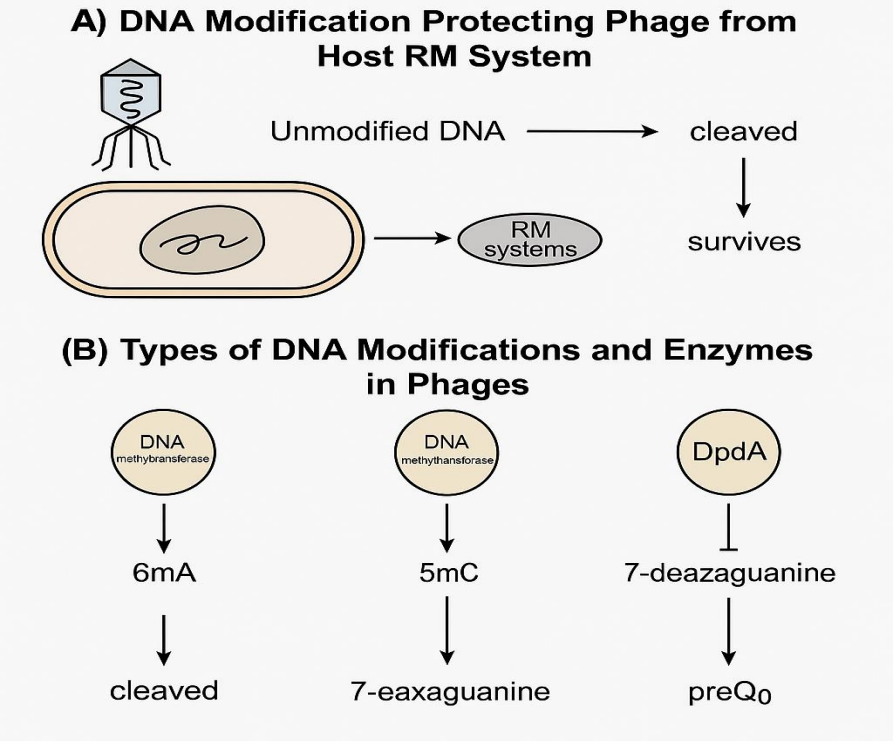

Figure 6. (A) Phage DNA modifications protect against bacterial restriction-modification (RM) systems. Unmodified DNA is cleaved, while modified DNA (e.g., via methylation or 7-deazaguanine substitution) survives. (B) Summary of common phage DNA modifications (6mA, 5mC, 7-deazaguanine) and their corresponding enzymes (DNA methyltransferase, DpdA), highlighting their protective pathways

Challenge

The ongoing arms race between phages and bacteria, including phage acquisition of DNA modification systems and bacterial countermeasures, requires continuous study. Understanding this dynamic evolution is necessary for practical applications such as phage therapy or biotechnology (Ryu, 2021). Many phage genomes remain poorly characterized, with approximately half of their genes encoding proteins of unknown function. This lack of annotation impedes understanding of the diversity and mechanisms of DNA modifications in phages, including the enzymes responsible and their biochemical pathways (Łobocka *et al*., 2021).

2. Conclusion

The rising prevalence of multidrug-resistant (MDR) bacteria highlights the pressing need for alternatives to traditional antibiotics. This review explores how phage DNA modifications, particularly mechanisms like DpdA-mediated 7-deazaguanine incorporation, enable phages to evade bacterial restriction-modification systems, CRISPR-Cas immunity and other defense mechanisms. Such findings shed light on the

molecular dynamics of phage-host interactions and serve as a basis for engineering phages with enhanced therapeutic resilience, expanded host range and greater resistance to bacterial immune systems. Beyond therapeutic use, phages engineered with tailored DNA modifications hold significant potential for various biotechnological applications, including food safety, stabilization of microbial fermentation processes and precise modulation of the microbiome. Advancements in tools such as LC-MS/MS, nanopore sequencing and bioinformatics are further enhancing our ability to detect, analyze and refine these modifications for both clinical and industrial purposes. Collectively, these advancements underscore the broad translational possibilities of DNA-modified phages, offering a robust platform for developing next-generation phage therapies to combat the escalating threat of antibiotic resistance.

Conflict of interest

The authors declare that they have no competing interests.

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