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Unveiling the Microbial Arms Race: Exploring Bacterial-Bacteriophage Coevolution and Its Impact on Phage Therapy

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Abstract

Bacteria are some of the first organisms to ever exist in the biosphere, being prokaryotic organisms, they have been present on Earth for the past 3 billion years. Soon afterward, however, certain retroviruses evolved to infect these organisms soon becoming the most populous entity in the biosphere. For decades, researchers have delved into the various interactions between bacteria and bacteriophages due to their coevolutionary dynamics. Through this research, scientists eventually were able to discover a plethora of various mechanisms that bacteria have utilized to prevent bacteriophage infections, including but not limited to, receptor inactivation, modification, clustering, and CRISPR-Cas systems. Bacteriophages have developed superb countermeasures to these defenses over billions of years of coevolution. Scientists are trying to understand these coevolutionary dynamics to develop better potential antibiotic treatments using bacteriophages, as the prevalence of antibiotic-resistant bacteria has become a critical issue in modern healthcare. The excessive utilization of antibiotics has accelerated the prevalence of resistant strains of bacteria, rendering conventional antibiotics ineffective against once-treatable infections. This phenomenon can dramatically reduce patient outcomes and pose a substantial challenge to public health systems. In response to this crisis, virologists are actively exploring alternative therapeutic strategies to combat antibiotic resistance. One such novel approach that is becoming increasingly more studied is bacteriophage therapy, which utilizes viruses that selectively target and kill specific pathogenic bacterial strains. By sparing beneficial bacteria and offering a safe solution, bacteriophage therapy presents a promising avenue for addressing the growing threat of antibiotic resistance.

Keywords: CRISPR-Cas systems, antibiotic resistant, bacteria

Unveiling the Microbial Arms Race: Exploring Bacterial-Bacteriophage Coevolution and Its Impact on Phage Therapy

Delving into the Battle Between Bacteria and Bacteriophages

The continual war between bacteria and bacteriophages may very well be one of the deadliest and oldest to ever exist on Earth (De Sordi, Lourenço & Debarbieux, 2019). Bacteria have continually been under siege by ssRNA retrovirus bacteriophages for billions of years, with nearly 70% of all marine bacteria being infected, and 40% of all marine bacteria dying each and every day (De Sordi et al., 2019). With bacteriophages being some of the most populous entities in the entirety of the biosphere - an estimated population of around 10^31 (Clokie et al., 2011) - bacteria cannot entirely prevent themselves from becoming infected. However, this does not imply that bacteria have simply allowed this onslaught to continue without evolving a series of ingenious defenses to survive against such infections, and have rather adapted over billions of years to gain resistance (Rostøl & Marraffini, 2019). These bacterial defenses include, but are not limited to receptor inactivation, modification, and clustering, CRISPR-Cas systems, as well as biofilm formation and gene transfer (Buckling & Brockhurst, 2012; Rostøl & Marraffini, 2019). Despite these intricate defense systems, bacteriophages have still managed to be successful in their pathogenesis, and as was discussed earlier, are able to outwit these systems through a series of ingenious strategies and mechanisms (Egido, Costa, Aparicio-Maldonado, Haas, & Brouns, 2022). It is truly awe-inspiring to understand the details regarding how both of these seemingly invisible entities present in our biosphere are continually in a never-ending arms race to attack (Golais, Hollý, & Vítkovská, 2013). However, upon closer inspection, we may even see a sort of symbiosis that exists between the two entities (Naureen et al., 2020). We will discover how both bacteria and bacteriophages - through extensive genetic mutations and natural selection - have managed to be successful in propagating themselves and maintaining their

population levels while keeping their invaders and hosts, respectively, at bay (Moura de Sousa, Pfeifer, Touchon, & Rocha, 2021; Shaer Tamar & Kishony, 2022).

Coevolution of Defense and Attack Strategies Between Bacteria and Bacteriophages; CRISPR-Cas System

The earliest defense that bacteria employed against bacteriophages that invaded them was an extremely simple but considerably effective strategy. As with nearly all viral particles, bacteriophages must bind to complementary receptors on the surface of the plasma membrane of bacterial cells in order to gain entry through receptor-mediated endocytosis (Naureen, et. al). The most simple method of preventing this receptor-mediated endocytosis is extremely simple - the removal of receptors that the bacteriophages could hijack in order to enter and infect the bacteria. As a result of this defense mechanism, bacteria that lacked such viral entry receptors were able to prevent bacteriophages from binding and injecting their genetic material, and therefore inhibiting bacteriophage infection from being plausible. However, this strategy against the prevention of viral infection comes at a steep cost for bacteria - they may lose important cellular receptors that are crucial for certain life sustaining metabolic functions (Koskella et al., 2014). Bacteriophages can subsequently capitalize on this weakness and can evolve to continually target receptors that serve essential and life-sustaining functions for the bacteria, therefore rendering a loss of the receptor a non viable option. This gave a large advantage for bacteriophages during the early stages of their coevolution with bacteria, since bacteria were left with no choice but to preserve these cellular receptors that were exploited by bacteriophages to bind and inject their genome. This advantage did not last for an extended duration of time, however, and bacteria soon gained a new capability - the ability to modify receptors rather than causing them to have a complete loss of function.

Bacteria are able to achieve this in a plethora of ways: the most notable involving the modification of surrounding structures to cause a greater physical hindrance. This would prevent bacteriophages from binding to both non essential and metabolically crucial receptors, and eliminate the need for bacteria to terminate the function of these receptors. There are several fascinating examples of this that have been discovered by scientists in recent years, including the modification of lipopolysaccharides (LPS) on gram-negative bacteria such as E. Coli and *Serratia marcescens* (Hasan et. al, 2022). Another notable example of such an evolved receptor modifications in the OmpC outer membrane protein are present in certain variants of a common species of nitrogen fixing bacteria found in the soil, *Pseudomonas fluorescens* (Hasan et. al, 2022). All of these bacteria have evolved over an extended period of time to either modify their surface glycocalyx receptors or the tertiary structure of their membrane proteins. They are the simplest defense in the arsenal of defenses bacteria employ against infectious phages and prevent the most common root cause of pathogenesis - adsorption to the surface of cell membranes. This strategy of receptor modification and clustering by bacteria proved to be extremely effective for hundreds of millions of years - that is, until bacteriophages developed a new structure; tail fibers. Tail fibers are protein appendages near the baseplate of bacteriophages below their protein capsid. These fibers provided bacteriophages with an immense evolutionary advantage, allowing them to inject their genomic information rather than relying on receptor-mediated endocytosis. This allows for them to simply use bacterial receptors as locations to adhere to, after which they propel their genome into the cytoplasm of the host bacteria. This process is summarized in the figure displayed below.

Figure 1

The following figure outlines the 5 main steps pertaining to the process of bacteriophage infection and replication.

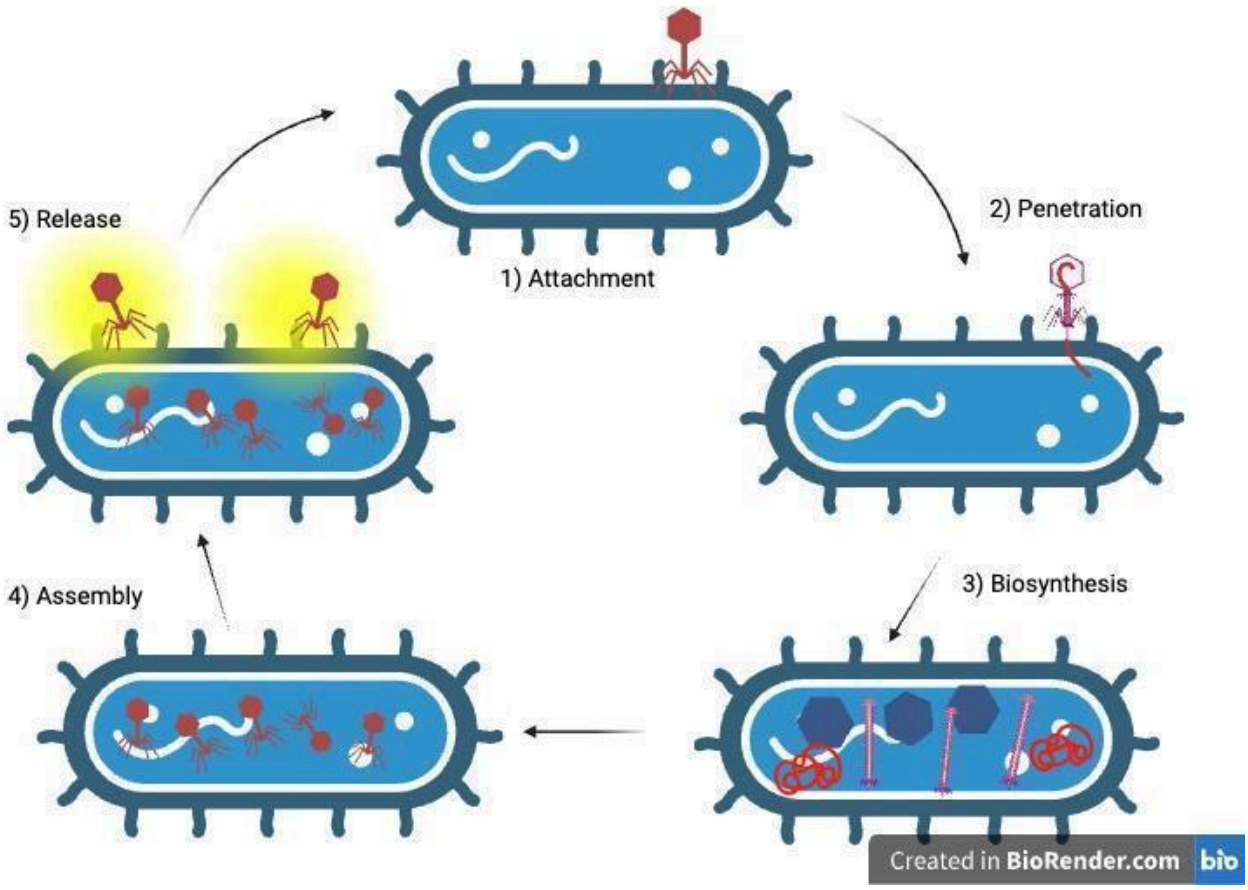

Figure Description:

The first step pertains the attachment of the bacteriophage to the bacterial cell. The bacteria must adsorb to the surface of the plasma membrane of the bacteria. Afterward, it will then inject its genomic RNA material, which is then transcribed into DNA through reverse transcription. Lastly, this DNA genome is implemented as a provirus into the bacterial cell which then replicates new bacteriophage viruses. These viruses are then released into the external environment through lysis of the bacterial cell.

Coevolution of Defense and Attack Strategies Between Bacteria and Bacteriophages; CRISPR-Cas System

As is often the case, the simplest defenses can prove to be immensely effective, and this was certainly true in the case of bacteria's defense against phage infections. After all, the strategy of inactivating, modifying, and clustering receptors was immensely effective in defending some bacteria from a great deal of bacteriophage infections. However, the ingenious attack strategy and the short replication cycle that allows for rapid evolution of bacteriophages enables them to occasionally bypass any modifications made to the functionality of cell membrane receptors. Once bacteriophages adsorb and endocytose into bacterial cells, they then can inject their provirus (genetic material, often in the form of DNA or RNA), which then can be integrated into the genome of the cell (Rostol et. al., 2020). However, bacteria have evolved an incredibly intricate and advanced defense system against this stage of infection as well. CRISPR, namely the CRISPR-Cas system, utilizes CRISPR RNA (crRNA) interference in order to prevent bacteriophage genomic material from becoming integrated into the bacteria's DNA plasmid. The CRISPR-Cas system, as the name implies, consists of two separate components - a CRISPR array component and a Cas enzymatic protein component. CRISPR, an abbreviation for Clustered Regularly Interspaced Short Palindromic Repeats, are a series of short repeating DNA segments present in the genome of bacteria that are separated by even shorter repeating DNA sequences known as spacers. When required, these short CRISPR DNA sequences can be transcribed into crRNA segments that are highly specific that bind to viral DNA, including the (+) stranded DNA provirus of bacteriophages synthesized through reverse transcription that are integrated into the genome, and degrade it (Rostol et. al., 2020). In order for this complement binding and degradation process to occur, however, crRNA sequences must associate with guiding RNA sequences (gRNA) and an enzyme known as Cas-9, an enzymatic protein part of the Cas family

of proteins. All proteins in the Cas family are responsible for degradation and splicing functions in both prokaryotic and eukaryotic cells, such as the Cas-3 enzyme being utilized in self-lysis processes during apoptosis (programmed cell death). As one may expect, the Cas-9 protein degrades the exogenous injected DNA genome of the bacteriophage upon complementary crRNA binding (Díaz-Muñoz et al., 2014). This strategy proves very effective against phages that have managed to inject their genomic information and is a nearly foolproof defense strategy. Bacteriophages' quick replication cycle means that they can evolve with incredible speed, and can thus alter their genomal sequence within a short period of time - however, the CRISPR-Cas system utilized by bacteria has a high level of specificity and adaptability in order to bind to unfamiliar exogenous DNA sequences (Rostol, et. al., 2020). Despite the effectiveness and adaptability of the CRISPR-Cas system, bacteriophages are not defenseless by any means. They have instead evolved to incorporate a plethora of anti-CRISPR proteins along with the DNA genome they inject into bacterial cells, the two most notable examples being AcrIIa4 as well as AcrIF1 (Rostol, et. al., 2020). AcrIIa4 is an inhibitor of the Cas 9 endonuclease and binds to a site on Cas 9 to alter its conformation through allosteric regulation. In other words, the binding of this protein to the Cas 9 enzyme changes its shape, preventing it from being able to bind to crRNAs (CRISPR RNAs) and forming the CRISPR-Cas complex necessary for the destruction of bacteriophage genome (Díaz-Muñoz et al., 2014). On the other hand, AcrIF1 interrupts the signaling cascade started by the CRISPR-Cas system (Yu et al., 2020). Although the knowledge regarding these mechanisms are limited, scientists theorize AcrIF1 cleaves gRNAs (guiding RNAs) in order to prevent them from directing the binding of the CRISPR-Cas complex to complementary bacteriophage DNA. Both of these anti-CRISPR proteins are effective in many instances in preventing the CRISPR-Cas system from destroying bacteriophage DNA, but bacteria are continually evolving along with phages, and the CRISPR-Cas system is continually

evolving to be resistant to these proteins. In addition, bacteriophages will inevitably develop new proteins to combat mutations that provide bacteria with resistance to older anti-CRISPR proteins. Through bolstering our understanding of the aforementioned evolutionary dynamics between bacteria and bacteriophages, scientists are researching and currently developing a promising new therapy for bacterial infections known as phage therapy. Phage therapy could be an excellent solution to combat the ever growing problem of antibiotic resistance (Lin, et al., 2017). As bacteria become more resistant to antibiotics due to us liberally utilizing them to not only treat diseases in humans but diseases in livestock that we consume as well, they may be able to outpace the rate at which new antibiotic medications can be developed. This is where the strengths of phage therapy can come into play - after all, scientists would not need to develop new phage therapies synthetically in a lab, but could rather identify bacteriophages that infect certain pathogenic bacteria in nature (Shkoporov et al., 2022). Since the coevolutionary process between bacteria and bacteriophages is continuous, new bacteriophages that could infect mutated strains of bacteria could always be extracted from the environment and replicated in culture. In addition, bacteriophages are highly specific parasites - they pose no known negative side effects to humans, and are completely harmless to our eukaryotic somatic cells (Lin, et al., 2017). Each bacteriophage species is only able to bind and adhere to a specific bacterial species and does not have receptors at its baseplate and tail fibers that could potentially bind to the glycolipid receptors of eukaryotic cells. Another promising aspect of phage therapy is that different bacteriophage variants only infect very specific pathogenic strains of bacteria (Lin, et al., 2017). This ensures that bacteriophages utilized in phage therapy do not infect and kill beneficial bacteria in our gut microbiome, which could pose devastating consequences.

In recent years, there have been several single patient investigational new drug (SPIND) cases that suggested that phage therapies could have an unprecedented efficacy in the treatment

of antibiotic-resistant infections. The first known single-patient clinical use of bacteriophage occurred in March of 2016 at the University of California, San Diego during which a patient suffering from a multi-antibiotic-resistant *Acinetobacter baumannii* infection was intravenously administered with bacteriophages (University of California, San Diego, 2017). Against the staggering odds of surviving the infection, the critically ill patient immediately responded to the treatment, regaining consciousness rapidly, and was fully discharged from the hospital in 3 months (University of California, San Diego, 2017). This clinical use ignited the interest of clinical researchers worldwide, and bacteriophage treatments began to be utilized experimentally on critically ill patients whose MDR infections were having no response to the antibiotics they were receiving (Hitchcock, 2023). In total, the Antibacterial Resistance Leadership Group (ARLG) recorded 63 different clinical SPIND cases, during which phage therapy was utilized after all other antibiotics and therapeutics failed (Hitchcock, 2023). Out of these 63 cases, an astonishing 51 of these cases lead to favorable outcomes (i.e. clinical improvement and complete recovery) (Hitchcock, 2023). Another recent retrospective analysis documented in Oxford University's academic journal displayed similar success of the improvement in patient outcomes (Green 2023). Out of the 12 patients who received extensive intravenously administered phage therapy, 8 of the patients eradicated the MDR bacteria present in their system, prevented its pathogenesis, and were on their way to a full recovery (Green, 2023).

The prospects of phage therapy are not limited to acute MDR bacterial infections, but can potentially be utilized to even treat life-threatening side effects of chronic conditions, notably cystic fibrosis. Individuals suffering from cystic fibrosis are particularly vulnerable to infections from certain strains of bacteria, including *Pseudomonas* aeruginosa. An estimated 17% of *Pseudomonas* aeruginosa strains are highly resistant to a multitude of antibiotic medications that often have high efficacy against other pathogenic bacteria targeting the lungs. One proposed

mechanism for this resistance are the biofilms that are produced by this bacteria as a defense from host immunological defenses. In two young CF patients suffering from an acute MDR *Pseudomonas* aeruginosa infection, phage therapy proved to be a roaring success. One patient, a 6-year-old girl who was suffering from a resistant *Pseudomonas* aeruginosa infection, was provided with inhalable phage therapy coupled with 3 mL phosphate-buffered saline [PBS] and 10 mM of magnesium sulfate (Hahn, 2023). Although her recovery from the infection was gradual, the inhalable phage therapy proved to be effective in reducing the concentration of MDR *Pseudomonas* aeruginosa in her lungs (Hahn, 2023). Unfortunately, she passed away 6 months after her recovery from phage therapy due to respiratory failure of the lungs due to an overproduction of mucus caused by her CF, and the inability to find a matching lung donor (Hahn, 2023). The second patient was a 26-year-old woman who was also facing an MDR *Pseudomonas* aeruginosa infection similar in pathogenesis to that of the first patient and was in the advanced stages of lung disease (Hahn, 2023). Similar to the first patient, she received inhalable phage therapy, but with higher plaque-forming units (PFUs) of bacteriophages in correlation with the greater PFUs of MDR *Pseudomonas* aeruginosa present in her lungs (Hahn, 2023). The second patient recovered immediately after her treatment and was fortunately able to receive a timely lung transplant after her phage therapy, after which she made a full recovery.

With no apparent negative side effects and only potential for upside when treating antibiotic-resistant bacterial infections, one may wonder as to why phage therapies haven't already been widely adopted as a mainstream treatment. This has to do with a few key downsides and limitations to phage therapy. For one, scientists have not clinically approved a method for utilizing bacteriophages for the treatment of systemic infections. So far, scientists have only been able to utilize bacteriophages in topical ointments and other treatments in which only primary bacterial infections are targeted (Hatfull et al., 2022). However, simply injecting

large concentrations of bacteriophages intravenously is not sufficient for the treatment of systemic infections. A saline solution providing the bacteriophages with a favorable environment is necessary, along with immunosuppressive drugs. This is due to the fact that our immune system would identify bacteriophages as foreign entities, subsequently sequestering and destroying them, even though they pose no jeopardy to our health. Another large obstacle that would need to be overcome by clinical researchers is isolating and culturing bacteriophages rather than infecting pathogenic bacteria (Hatfull, et al., 2022). This is easier said than done, as most bacteriophages exist in remote and hard-to-access areas, and finding an exact species of bacteriophages for each bacterial infection would be incredibly time-consuming and costly (Lin et al., 2017). Despite these potential downsides, however, as more funding and research is being directed into the development of phage therapies, these hurdles will likely be overcome in the next few decades.

Concluding Remarks

All in all, the coevolution between bacteriophages and bacteria is one of the oldest and deadliest battles to occur in the entirety of the biosphere. The mechanisms that both entities utilize are truly awe inspiring, and display the number of defenses and attack strategies that can arise from billions of years of evolution. It is remarkable to learn about the simplest (receptor inactivation) and most complex (CRISPR-Cas) systems that bacteria utilize to defend themselves against bacteriophage infections. However, it is arguably more fascinating to view the coevolutionary dynamics that allowed for bacteriophages to outwit these amazingly intricate defense systems. By understanding these mechanisms, we can potentially harness the power of bacteriophage therapies. Bacteriophages could be utilized in the near future as a therapy against bacterial infections, especially those that could not be treated with traditional antibiotics. The possibilities are limitless, and understanding this coevolutionary relationship is a foundational

stepping stone to save millions of lives around the globe each year from resistant bacterial infections.

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