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Bacteriophage endolysins and their role in eradication of bacterial biofilms

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Abstract

Biofilm is the protective coating that the bacteria use to thrive in their environment without being damaged by radiation or the effects of antibiotics. Nosocomial infections that are often caused by biofilms have been demonstrated to be very challenging to cure because of their complicated molecular structure and resistance to antibiotics. Biofilms of bacteria that form on the medical equipment pose a risk to patients and facilitate the transmission of infection. Progress has been made in eliminating bacterial biofilms by combining bacteriophage with antibiotics for a synergistic impact and employing the phage-lysin efficiently. The aim of this study was to explore the potential efficacy of phages and lysins alone and/ or in conjunction with antibiotics to combating biofilm conformation and eradication. This review article is broadly divided into two parts; the first section focused on molecular mechanism of biofilm formation and risk of bacterial biofilms in the hospital settings. The second part of the review is giving an insight on bacteriophage derived lytic protein-endolysin, which has emerged as a potential alternative to eliminate bacterial biofilms, and should be explored to combat infections caused by them.

Keywords: Lytic protein, Bacterial biofilm, Exo-polysaccharide, Nosocomial infections, Device related biofilm

1. Introduction

Bacteria are abundant and can be found almost anywhere. Based on its unique composition; each bacterium performs a specific role and possesses unique characteristics. In terms of anatomy, some bacteria have tails while others have pili all over their bodies. In a symbiotic relationship, a bacterium as a unicellular creature receives nutrients and physiological cues from a multicellular host in an exchange for helping to support the host. The symbiotic microorganisms include the bacterium *Escherichia coli*, which lives in the small and large intestine and breaks down sugars (Scheithauer *et al*., 2016). Mammals depend on their microbial flora for a few metabolic processes, including ATP and vitamin

syntheses, and for the innate anti-pathogen defence mechanisms (Rimondini *et al*., 2016). The bulk of the microbial flora is found in or on the skin; oral cavity, mucosa, gastrointestinal tract, and urogenital tract. Bacteria on Earth have always had two unique lifestyles; mainly planktonic (free-floating) and sessile (attached to a surface) (Bjarnsholt, 2013). Biofilm bacteria exhibit distinct metabolism and gene expression than their planktonic counterparts, and present an altered phenotype with increased tolerance to the host immune defence mechanisms and the exogenously administered antimicrobial substances. Bacteria undergo changes after colonizing an organism's biotic and abiotic surfaces; resulting in the formation of a cover that protects the bacteria from the host's innate immune system and exogenous antimicrobial substances (Guzmán-Soto *et al*., 2021). The exo-polysaccharide matrix or "slime" is another name for this barrier. In 1684, Antony van Leeuwenhoek was the first scientist to notice the germs in matter from his own teeth; but it wasn't until a paper was published revealing that the word "biofilm" was used and defined (Bjarnsholt, 2013). Bacteria produce biofilms, which aid in their adaptation and growth. It's a swarm of bacteria that have banded together and put themselves into a matrix they created. Bacteria must utilize a variety of survival tactics to breach the human immune system and cause a wide range of diseases and damages, because the biofilm matrix comprises proteins; polysaccharide, and eDNA (provided by the microorganisms in their environments) (Hall-Stoodley and Stoodley, 2009). Bacterial biofilms are essential to the microbes' existence, because they provide these microorganisms with additional advantages that allow them to thrive in a variety of habitats. Biofilms can grow on both biotic and abiotic surfaces; therefore they can be detected in both the natural environment and the medical field. Because of the existence of biofilms on the hospital surfaces and medical equipment; bacteria can persist as resources and can be easily transmit to the patients (Khatoon *et al*., 2018). Biofilms are preferred because they permit the microbes to persist inside the host despite of the innate immune defences (Vestby *et al*., 2020). As a survival strategy in the biofilm's low-oxygen environment, bacteria have been recorded to convert their metabolism; gene expression, and protein production in the direction of low metabolism and favoring the reduced cell division (Donlan and Costerton, 2002). These bacterial changes contribute to the antimicrobial resistance; by deactivating the antimicrobial targets or lowering the requirement for cellular functions. During a biofilm infection, both the innate and acquired host immune responses are triggered simultaneously; however these responses are unable to eradicate the biofilm pathogen and instead they hasten the development of a collateral tissue damage (Hall-Stoodley and Stoodley, 2009; Chen *et al*., 2013).

2. Bacterial biofilms related nosocomial infections

Nosocomial infections are a type of infectious illness that occurs in a healthcare setting. They are also known as hospital-acquired illnesses and/or healthcare-associated infections. Nosocomial infections are detected at least 48 hours after a patient's initial hospitalization; but not before. These infections are potentially fatal as they can cause sepsis and other complications. It has been documented that many medical procedures favor the growth of microorganisms that are resistant to multiple antimicrobials; leading to nosocomial infections. In fact, avoiding these infections may be easier if you follow the CDC's (Centre for Disease Control and Prevention) safety guidelines (Openda and Nyokong, 2023). *Enterococcus faecalis*; *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus viridans*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, and *Pseudomonas aeruginosa* are the most prevalent Gram-positive and Gram-negative bacteria that produce biofilms on the medical equipment. About 40-50 % of these infections are connected to prosthetic heart valves; 50-70 % of catheter biofilm infections and 87 % of bloodstream infections, which are to be caused by *Staphylococcus aureus* and *Staphylococcus epidermidis* (Darouiche, 2004). Bacterial biofilms are linked to over 65 % of all

bacterial illnesses (Jamal *et al*., 2018). Various devices have been reported to have different infection rates; with 2 infections per thousand bone implants, 2 infections per thousand of common prostheses, 4 infections per thousand of mechanical heart valves, 10 infections per thousand in case of ventricular shunts, 4 infections per thousand of defibrillators, and about 40 % of the infections are attributed to ventricular-supported devices (Vestby *et al*., 2020). Inflammation of the heart's vascular endothelium and pulmonic valves are known medically as "native valve endocarditis" (NVE). This typically occurs due to an infection with streptococci; staphylococci or enterococci (Elgharably *et al*., 2016). Under these circumstances, microbes can gain access to the circulatory system through the digestive tract; the urinary system, and the mouth and throat. Following removal of the invading bacteria by the immune system; a condition known as non-bacterial thrombotic endocarditis (NBTE) develops at the site of the damage, causing the platelets; red blood cells, and fibrin to clump together and form a thrombus (Kuipers *et al*., 2021).

3. Hospital-devices-related biofilm infections

The indwelling medical devices that regularly harbor biofilms include the central venous tubes; artificial heart valves, dialysis machines with peritoneal catheters, prosthetic joints, urine catheters, and oral prostheses. Based on the microorganisms involved; the two primary forms of biofilms that can be generated are single-species biofilms and multiple-species. This is obligatory based on the tools and equipment utilized and the amount of time required to become effective (Maki *et al*., 1991; Donlan, 2002). For example, there are two types of contact lenses available: mainly the soft and hard. Both the plastic and glass lenses can harbor microorganisms. The categorization they receive is based on several variables, such as the construction equipment; frequency of disposal, wear arrangement, and design. Using scanning electron microscopy, *P. aeruginosa* biofilm has been detected on the contact lenses of a keratitis patient. The contact lenses, which are frequently maintained in lens storage containers, are another potential biofilms target. As a result, the lens storage boxes have been recognized as a potential source of bacterial contamination (Waghmare and Jeria, 2022). Biofilm conforms on all the central venous catheters; but its location and extent vary with the long insertion period of the catheter. Biofilm formation, for instance; is greater on the exterior face of the short-term catheters (10 days) than it is in the catheter interior of long-term catheters (30 days). The state of the patient's discharged fluid throughout the central venous catheter can influence the proliferation of microorganisms. Gram-positive bacteria do not easily survive in the intravenous fluids (Jamal et al., 2018); however *P. aeruginosa*, *Enterobacter* spp., and *Klebsiella* spp. do. Prosthetic valve endocarditis occurs when the microorganisms colonize the artificial heart valves or devices that are used to replace the damaged tissues. The Gam-negative bacteria, including *Bacillus*; and *Enterococcus*, as well as the Gram-positive *Streptococcus* spp.; *Staphylococcus aureus*, and *Staphylococcus epidermidis*, are also blamed for this infective endocarditis. These microorganisms may have entered the body through the epidermis or through an indwelling device, such as the central venous catheter and/or a dental filling (Garrett *et al*., 2008). Damage can occur during surgical installation of the prosthetic heart valves; if the platelets and fibrin accumulate at the site of opening and on the apparatus. Microorganisms are better able to colonize these areas than the other cell types (Donlan *et al*., 1999). During a surgery; a urinary catheter (usually constructed of silicon or latex) is inserted into the patient's body to monitor the urine output. For the urinary system; catheters are placed in the urethra and lead to the bladder, which can have either an open or unrestricted system. A urinary tract infection (UTI) develops within a few days if urine is drained in an open collecting center; as opposed to a closed one. In an unrestricted catheter system, when urine is collected in a plastic bag; the likelihood of urinary tract infections is reduced. The most common pollutants and biofilm formers on this device include mainly Gram-negative bacteria, viz. *E.*

coli; *E. faecalis*, *P. aeruginosa*, *P. mirabilis*, and *K. pneumoniae*, as well as *Staphylococcus epidermis*; as an example of Gram-positive bacterium (Gunardi *et al*., 2021).

4. Blooming of a bacterial biofilm

There is a four-step procedure that bacteria go through to adhere and create a biofilm (Fig. 1). A specific form of signaling termed as a quorum sensing is seen for strong production of the biofilm. This process helps in the regulation of genes that leads to the strongest cell density; in order to control several functions such as virulence and bacteriocin synthesis. The major steps of biofilm formation are as follow:

4.1. Attachment to the surface: The bacteria adhere to surfaces via projections such as pili and flagella; as well as by physical forces, including van der Waal's and electrostatic contact. Initial attachment is reversible and mainly governed by hydrodynamic and electrostatic forces; however hydrophobic projections and abilities provide the bacteria with the strength they need to firmly cling to surfaces, since they lessen the repelling force between the bacteria and the surfaces. Bacteria prefer the non-polar and water-repellent materials such as Teflon, more than the polar and water-repellent materials such as the metals and glass (Tuson and Weibel, 2013; Vasudevan, 2014).

4.2. Construction of a micro-colony: Following bacterial cell attachment and stability; a process of microbial cell multiplication and division is initiated *via* chemical signaling within the extracellular matrix (EPS). Because of this; more micro-colonies will likely to arise. These communities are not made up of single bacterial cells but rather than many cells that exchange the substrates and metabolic products with one another. Several reports have shown that multi-species biofilms have better properties compared to the single-species biofilms; in terms of biofilm mass, community cell count, better metabolic activity, and enhanced tolerance to the antimicrobial agents (Sadiq *et al*., 2021; Zhao *et al*., 2023a). The syntrophic associations involve several metabolically distinct bacterial strains that depend on each other for the uptake of particular substrates for their energy needs, which tend to flourish in the biofilm's hospitable environment (Otto, 2013).

4.3. Maturation: At this point in biofilm formation; the microbes involved with one another use auto-inducer signals. They are able to stabilize; expand their population, and begin quorum sensing as a result of this connectivity. A large number of gene products are released during this process, which adds up to the EPS matrix. Soon, water channels develop within this aggregated EPS matrix, which act as a biofilm's circulatory system by transporting the nutrients and removing the wastes (Costa *et al*., 2018).

4.4. Detachment of a biofilm: Finally, microorganisms rapidly reproduce and disperse; undergoing a change in the state *i.e*. from stationary to a mobile state. This is completed by detachment of the bacterial cells from the small colonies; first, their transfer to the other substrates, and finally their attachment to a new surface (Shen *et al*., 2018). Some bacterial species do not favor synthesis of the extracellular polysaccharide; where the bacterial cells disperse directly into the environment, or shear force may play a role in the detachment process. The passive mechanical detachment and active detachment of bacterial cells from a biofilm can also occur. This phenomenon is referred to as seeding dispersion. During the detachment process; microorganisms in a biofilm begin to produce a variety of polysaccharide degrading enzymes, which aid in the microorganisms' ability to escape from the surface and colonize a new location (Flemming and Wingender, 2010). Several bacterial spp. such as *E. coli*, *P. aeruginosa*, and *P. fluorescens*, produce an enzyme called N-acetyl heparosan lyase, whereas the *Streptococcus* produce another enzyme termed hyaluronidase, which aid in the lysis of the external matrix and subsequent detachment of the EPS. To facilitate a shift from a location, the bacterial cells in this phase increase the production of proteins that are involved in flagella development. Meanwhile, separation of cells allows the microorganisms to roam and infect new areas.

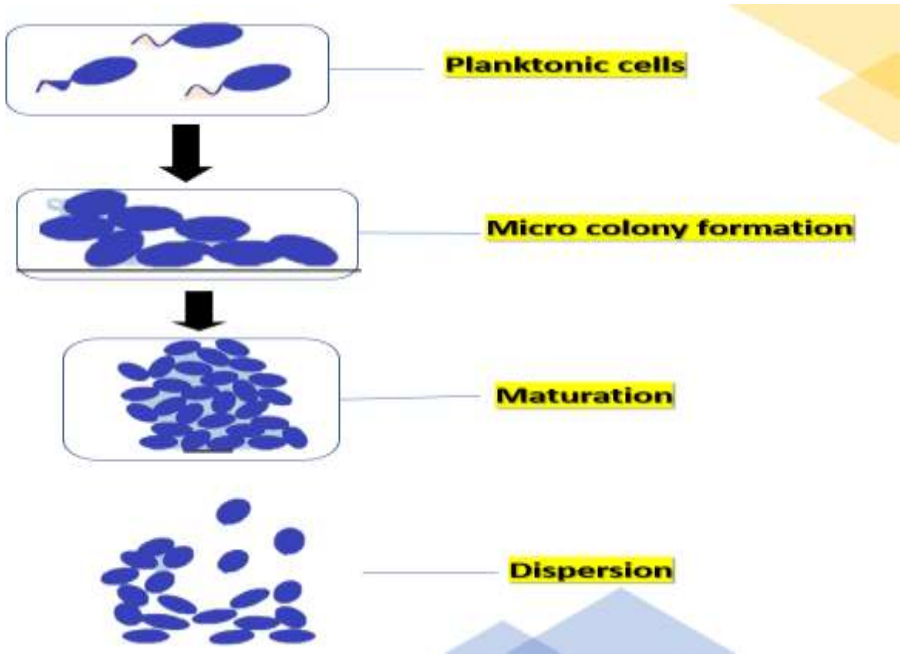

Fig. 1: The structural formation and dispersal of a bacterial biofilm

5. Structure and function of the bacterial biofilm

Microorganisms living together in an extracellular matrix are called biofilms. Because of the matrix's protective and favorable conditions; the resident microorganisms are able to survive better. Biofilms can increase the bacterial resistance to antibiotics by more than a factor of a thousand, compared to the free-living planktonic bacteria. The matrix in EPS can limit the diffusion of antimicrobial agents or even neutralize the effects of antibiotics. The proteins; lipids, polysaccharides, and nucleic acids are some of the biomolecules that make up the matrix (Jurcisek *et al*., 2017). Although the bacterial species differ in their elemental matrix pattern; the bacterial biofilms are highly structured with specific functions and properties. Different substances coordinate to complete the vital activities, for example the exo-polysaccharide maintains the structure, while the extracellular DNA (eDNA) protects the integrity of the bacterial biofilm, etc. (Okshevsky and Meyer, 2015). For the multiple bacterial species; in order to establish an inclusive interacting community, the protective extracellular matrix within a biofilm must be sufficiently compatible with all the biofilm-forming microorganisms (Yao *et al*., 2022). The two main components, including the extracellular DNA and the nucleoid associated protein, and their functions in bacterial biofilms are described below.

5.1. eDNA

Since Avery; Mcleod, and McCarty's demonstrations that eDNA was responsible for metamorphosis, it has been known that the bacteria release DNA. Several bacterial spp. can transform; that is, they can absorb eDNA and incorporate its genetic traits. To complete transformation, the bacteria often need to be in a designated state. While the role of transformation in evolution is a mystery, this process plays a significant role in the vertical gene transfer. The biofilm has benefited from this to a significant degree (Whitchurch *et al*., 2002). As a result, the

presence of released eDNA is more likely to be caused by evolution than by chance, and has since been observed to play a role in the release of chromosomal DNA in the competent non-typeable *Haemophilus influenzae*.

Nonetheless, initial studies indicated that eDNA is likely to continue production of structures in the biofilm matrix (Pontiggia *et al*., 1993). This production facility demonstrated that eDNA is ubiquitous in the bacterial biofilms. The study conducted by Whitchurch *et al*. (2002) introduced a validation that *P. aeruginosa* bacterial biofilms relied on eDNA during their development. Biofilm development is inhibited when the DNase is co-cultured with bacteria. Following that, samples from the diverse single-species biofilms are collected; and over time the biofilm disturbance becomes increasingly resistant to the DNase (Jurcisek *et al*., 2017). This led to an understanding that the formed eDNA is important only during biofilm formation and not afterwards. It has been also reported that the mammalian host itself is an important source of eDNA. The guardians of the innate immune system, *i.e*., the poly-morphonuclear leukocytes (PMNs), can undergo a transformation in which their chromatin is released; resulting in the production of a substance called a neutrophil extracellular trap (NET). The release of PMN eDNA in a net-like array can either kill the individual bacteria or prevents biofilm formation. First; using eDNA from NETs increases the concentration of the naturally occurring antimicrobial components, such as histones, which can kill any bacteria that have become trapped in the NETs. This helps in preventing the bacteria from leaving the biofilm and minimizing the pathogen proliferation (Goodman and Bakaletz, 2022). Second; researchers discovered that the bacterial eDNA has a lattice-like structure regardless of its origin (Bockelmann *et al*., 2006).

5.2. Nucleoid associated protein (NAP)

Although bacteria lack histones; they do have proteins known as NAPs, which influence the overall structure of the bacterial chromatin. While certain NAPs are exclusive to eubacteria; the DNABII family of small proteins that is generally less than 100 amino acids long, can be found in every known genome (Dey *et al*., 2017). The histone-like HU protein that is discovered in *E. coli* strain U93 and the homologous Integration Host Factor (IHF) has the same subunits, and can operate as a homo- or heterodimer (Goodman and Bakaletz, 2022). Although both of these NAPs have DNA binding domains; IHF is more selective in its exact binding sequence, whereas the HU proteins do not have any binding specificity. As the energy of binding for these proteins is greatly influenced by a bend conformation of DNA; the DNABII proteins bind to bend DNA with more affinity, or they can even cause the DNA to bend upon binding. Due to this reason, these proteins have been found to binding preferably at Holiday Junction (HJs); a recombinant intermediate that has a cruciform shaped DNA structure. Any two adjacent arms of HJs can mimic a bend DNA conformation; thus providing the DNABII with an opportunity to bind with higher affinity (Kamashev *et al*., 1999). The lattice structures of eDNA in a biofilm also mimic the presence of many contiguous HJs; thus the presence of higher amounts of DNABII proteins in the extracellular matrix has been reported. In addition, they are known to stabilize the extracellular DNA dependent matrix of the bacterial biofilm (Devaraj *et al*., 2021).

6. Supremacy of the bacterial biofilms

The extracellular matrix (EPS) acts as a framework for bacterial cellular adhesion to the outer membrane (Mitchell *et al*., 2016). It also functions as a mediator between the biofilm cells and their environment. Moreover, EPS shields the bacteria from the environmental hazards, as well as the antimicrobial compounds; chemicals, desiccation, radiation, and other threats. In addition, it provides the bacterial unit within the biofilm with a steady supply of nutrients; thus, preserving the biofilm's capacity to respond to the various changes in the surrounding environment. EPS is the key for physicochemical behavior of the microbial biofilms; however the pathogen type;

biofilm age, and environmental factors (*i.e.*, desiccation; pH, oxygen, nitrogen, temperature, and nutritional deficiency) all influence the EPS composition (Muhammad *et al*., 2020). The biopolymers in EPS make up super cement that helps explain the notorious nature of the biofilm communities. These biopolymers include polysaccharides; proteins, e-DNA, and phospholipids (Seviour *et al*., 2019). The type and abundance of the major constituents of EPS (*i.e.*, polysaccharides; proteins, lipids, DNA, and other polymeric substances) is dependent on the environmental conditions and the type of bacteria (Costa *et al*., 2018; Seviour *et al*., 2019). Like in Gram-negative bacterial biofilm; the polysaccharides are mainly neutral and poly-anionic; in contrast to that in Gram-positive, where it is mainly cationic (Donlan, 2002). The source of proteins is primarily the secretory proteins, such as enzymes and structural proteins of the pili and fimbriae. The other important constituents include eDNA and lipids, which help in bacterial attachment (Flemming *et al*., 2016).

Bacterial biofilms have been associated with a number of diseases; such infections have a higher tendency to become chronic and resistant to the conventional antibiotic treatments. Various reports have shown how biofilms contribute to the pathogenesis of many infectious diseases, which are related to cardio vascular; respiratory, digestive, auditory, urinary, and reproductive systems. The biofilms can be formed intra-cellularly and may even be a cause of cancer (Vestby *et al*., 2020). With respect to the tolerance against the various antibiotics; the planktonic bacteria when develop the capacity to form biofilms, they can spread the disease throughout the host body. The planktonic microorganisms may be eliminated by antibiotics and the host defences. Antibiotics destroy the majority of the biofilm bacteria; however, there is a tiny but considerable population of cells that can act as the starting point of an infection (Høiby, 2017). The bacterial unit exhibits structural and physiological changes, which are attributed to the differential regulation of the coding genes; in response to an increase in the hazardous substances and the toxic end-products inside the biofilm (Hall-Stoodley and Stoodley, 2009). When oxygen and nutrients are in short supply within a biofilm; the bacteria adapt to the stress conditions and undergo lower metabolic functions, which make them more resistant to the antimicrobial therapy; by either eliminating the drug targets or reducing the cellular functions (Vestby *et al*., 2020). The bacterial spp. affect the biofilm's development; host susceptibility, lipid biosynthesis, iron accumulation, gene regulation, toxin efflux, DNA shape, and other activities (Uruén *et al*., 2020; Singh *et al*., 2021). Today, antibiotic resistance is the main factor contributing to the difficulty of treating the bacterial diseases caused by biofilms. The slowing of bacterial growth; the acquisition of phenotypic variation by the bacteria forming biofilms, the inactivation of antibiotics by enzymes released by the bacteria, the difficulty of antibiotic penetration into the biofilm, and the electrostatic charge of the EPS that attracts the antibiotics with unequal charges, are the four main factors that contribute to the rise of antibiotic resistance among the bacteria (Khan *et al*., 2021; Zhao *et al*., 2023b). The antibiotic-resistant microorganisms that are found in biofilms can sustain their persistent shape. When EPS is present, the antibiotic effectiveness is decreased.

Several studies have supported that inhibiting the Quorum Sensing (QS) system is a crucial strategy for removing the biofilm, because the microorganisms use this system to coordinate their biofilms. Bacterial infections in the body can activate QS for the formation of biofilm and unwanted products. Because QS barriers prevent the bacteria from communicating with one another, they become more susceptible to the host's immune responses and the antibiotic reactions (Jiang *et al*., 2019). Thus, targeting the QS can turn out to be a potential therapy for the bacterial biofilm related diseases. Inhibition of QS system; reduction of the interaction amongst the bacterial cells, and decreasing the production of harmful substances, are all known as Quorum quenching (QQ) (Dong *et al*., 2001). There can be many ways to achieve QQ, such

as quorum-quenching enzymes that inhibit a QS signal or QS inhibitors, and the chemicals that disrupt the QS pathway. All such compounds are called quorum sensing inhibitors (QSI). Unlike the conventional chemotherapeutic agents; QSI will not affect the microbial growth in a biofilm; thereby excluding the chances of antimicrobial resistance development (Zhao *et al*., 2020). For example, the use of the QS system by the Gram-negative bacterial pathogens can be stopped through utilizing several strategies, including preventing the production of the AHL by the enzymes (Dong *et al*., 2001).

7. Bacteriophages and their organization

Bacteriophages; often known as phages, are a particular class of viruses that can only infect and spread throughout the bacteria. Since the bacteriophages may be found practically anywhere; they represent the most prevalent type of life on earth (Mushegian, 2020). Their size; shape, and genetic make-up vary greatly. The nucleic acid genome of every phage is encased in a capsid protein shell, which protects the DNA and makes it easier for the virus to infect the subsequent bacterial host cell. Using electron microscopy, the in-depth images of different phage types demonstrated that they have heads; legs, and tails (Boyd, 2012). The phages use Brownian movement rather than genuine mobility to go where they need to go (de Sordi *et al*., 2019). Bacteriophages exhibit striking host specificity; thus, they infect typically only a single species of bacteria and/or even strains within this species. Both the lytic and lysogenic replication mechanisms are used by the bacteriophages (Fig. 2). During lytic cycle, phage's genome is inserted into a vulnerable host bacterium. Several copies of the original phage assemble are synthesized quickly from the viral genome; whereas the capsid proteins are synthesized with the help of host cell. The new bacteriophage is released as the dead bacterial host cell is lysed. Lysogenic replication is similar to lytic replication in that the phage is primarily responsible for passing on its genetic material; however, this does not kill the host bacteria. The phage genome gets integrated into the chromosome of the bacterial cell or is kept as an episomal element; thus, allowing the phage to multiply and pass on to the next generation of bacterial cells. Prophages are those phages whose genomes are already integrated into the bacterial cells; while lysogens are the bacteria that host them. In response to the shifts in their environment, the prophages can kill their hosts by switching back to a lytic replication cycle; in contrast to the lysogenic phages that allow the bacteria to reproduce and pass the phage genome to the following generation without being killed. When there are environment changes; the prophages have the ability to destroy their hosts by returning to a lytic replication cycle (Pham *et al*., 2018). Bacteriophages depend on the microflora that already exists in a host in order to infect and reproduce within this host. This microflora, which can be further subdivided into pathogenic and non-pathogenic species are essential for promoting the gene transfer (Holmes, 2000; Fortier, 2017). Bacteriophages have the ability to modify the microflora to fit to their needs, in addition to their potential for horizontal gene transfer. Even though wild phages probably do have a positive impact on the wild bacterial populations; there are numerous challenges in the therapeutic use of lytic bacteriophages as antimicrobial therapies (Motlagh *et al*., 2016). One explanation is that there are many diffrent types of bacteria in the wild, which have evolved phage resistance. One well-known instance is the CRISPR-Cas9 system, which has been first created as a bacterial defence mechanism against the bacteriophage invasion (Doore *et al*., 2018). Nonetheless, it is increasingly being used as a laboratory tool for manipulating the heritable traits. Phages are far more immunogenic than the antibacterial medications, and they are also swiftly eliminated from the circulation through the reticulo-endothelial system. Because of their size compared to the antimicrobial medications; the phage's therapeutic potential will probably be limited to the topical methods unless effective ones are developed. A number of researchers have proposed that using phage enzymes, which can break the bacterial cell walls, may be a more effective method to removing the bacterial biofilm (Motlagh *et al*., 2016).

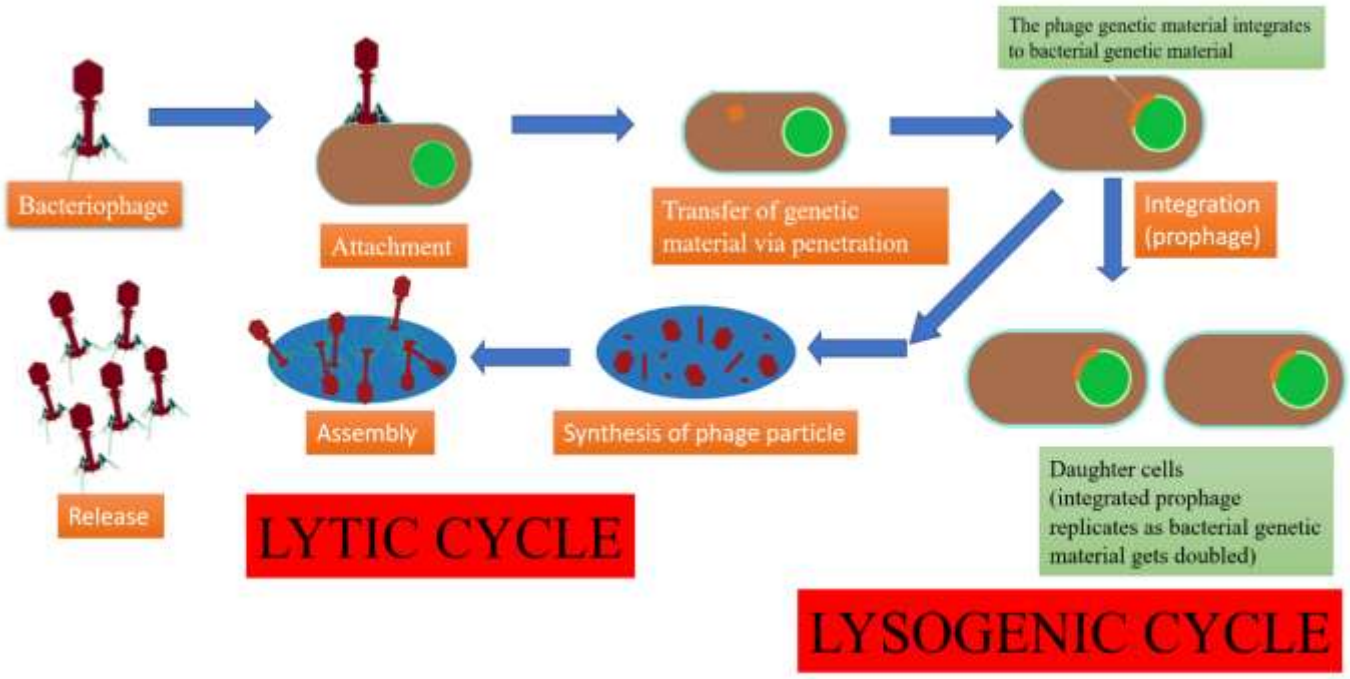

Fig. 2: The lytic and lysogenic lifecycles of the bacteriophage

8. Clinical importance of the bacteriophages

Phages are crucial to the medical industry for a number of reasons; one such is that many dangerous bacterial toxins, mostly exotoxins are encoded by bacteriophages. The cholera toxin produced by *Vibrio cholerae*; the diphtheria toxin produced by *Corynebacterium diphtheriae*, the botulinum neurotoxin produced by *Clostridium botulinum*, the double toxin produced by *Clostridium difficile*, and the Shiga toxin produced by *Shigella* spp., are few examples of these bacterial toxins (Wagner and Waldor, 2002). These bacteria will either not be dangerous at all or be substantially less toxic; if the toxin producing gene has not been transferred by the phage. The paralysis brought on by the botulinum toxin appears to have the opposite effect from that of the cholera toxin, which may have help the phage and its host to reach their next victim by inducing diarrhea (Santiago-Rodriguez and Hollister, 2019).

Many characteristics of bacteriophages are clinically significant. In the first place, they can act as vectors for vertical gene transfer, which includes the transfer of genes for antibiotic resistance (Brown-Jaque *et al*., 2018). They can also be used to insert genes into particular strains for clinical applications; however, this application is still in the testing stage. A third significant clinical use of bacteriophages is as biomarkers to identify the presence of their host in a challenging environmental sample (Łusiak-Szelachowska *et al*., 2020a). As the host bacterium is likely to be present if the phage is present; this is frequently used to evaluate the fecal contamination of water sources. Moreover, it is possible to create bacteriophages that produce a visual signal when they

infect their hosts; through inserting a gene or set of genes that are known as reporter genes, which produce easily identifiable end product in the form of a signal. An example of such reporter gene is the bacterial *lux* gene or firefly's *luc* gene, which can be inserted in the phages, and thus they will generate results in the form of bioluminescence only after infecting their hosts (Smartt and Ripp, 2011). Another significant use of the bacteriophages in epidemiology is through phage typing. Each strain of a certain bacterial species can be connected to a pattern of susceptibility and resistance to each phage type; by infecting this strain with a standardized panel of phages (Mohammed, 2017). Many of the fundamental discoveries in molecular biology have become possible through the use of bacteriophages.

9. Phage's association and interaction with the human body

In healthy people, several microorganisms make up the normal microbiota; predominated by bacteria. For example, bacteria such as *E. coli*, *Streptococcus* spp., and Bacteroidetes commonly reside in the small intestine, whereas Firmicutes and Bacteroidetes predominate in the large intestine; colon and feces (Zuo *et al*., 2019). Because of the ample presence of bacteria, the human gut represents also a home to ss-DNA as well as ds-DNA phages (families *Siphoviridae*, *Myoviridae*, and *Podoviridae*) (Manrique *et al*., 2017). As the majority of viral microbiome in human gut is bacteriophages, they have a key role in functions of the gastrointestinal microbiome. Though the full potential of these phages in the healthy gut microbiome is still not completely understood; however, many reports have shown a disturbance or dysbiosis of the gut microbiota under the disease conditions. As per an estimate, approximately 10^{15} phages reside in the human gut; however, their count may vary as per the health conditions of the body. The number of coliphages is more in the colorectal cancer than in the normal healthy control; thus it is strongly suspected that the phages may have an important role in the gut homeostasis (Łusiak-Szelachowska *et al*., 2020b). In contrast to the virulent phages; most of the phages found in various regions of the human body are temperate. Furuse *et al*., (1983) reported that about 56.5 % of the phages in fecal samples obtained from the healthy people are temperate and only 2.5 % are pathogenic. In contrast, higher frequency of phages has been reported in samples collected from people with leukaemia and intestinal or respiratory problems; recording 13.2 % and 20 % virulent phages, respectively (Furuse *et al*., 1983).

There are several factors that have been studied to govern the population and activity of the gut phages (Sutton and Hill, 2019), including: i) the physical separation *i.e*. phage multiplication requires the bacterial host not to be present only, but it must be metabolically active; thus, a dormant growth phase of the bacterial host will be transiently resistant to the phage infection; ii) phase variation; where a bacterium can develop a capsular polysaccharide, thus can't be detected by the phage; iii) the bacteria can have various defence mechanisms that target different steps of the phage multiplication cycle, such as modification of the membrane proteins; using restriction enzymes to degrade the injected phage genome, activating the CRISPR-Cas system, restricting the phage genome replication, and/or blocking the virion assembly. In a recent investigation, the gut viromes of nine out of ten healthy human volunteers; have not revealed an overrepresentation of the temperate phage marker genes (*i.e*., integrase and point-specific recombinase genes) (Santiago-Rodriguez and Hollister, 2019). These results led the scientists to speculate that phages survive in the human stomach through mechanisms other than the phage genome integration.

According to the current knowledge, phages are crucial in determining the makeup of the microbial community, because they promote the bacterial diversity and vertical gene transfer. The most dominant phage in the human gut has been identified as crAssphage of the Caudiviraeles family. In 2014, it had been reported for the first time that crAssphage was six times more frequent than the other phages, and had been identified to be present globally (Dutilh *et*

al., 2014). Crass family for *Bacteroides intestinalis* is identified and well-studied. It has been reported to provide an ecological advantage to its host, such as the phage infecting the cynaobacteria that possess photosynthetic genes as the auxiliary metabolic genes (Hurwitz *et al*., 2013). Another example of co-existence of a phage and its host is the phage-mediated transformation; as observed in the lysogenic phage carrying cholera and shiga toxin (Muniesa *et al*., 2012). Intestinal mucosa plays a crucial role in maintaining the human health. In contrast to persons with ulcerative colitis; healthy individuals have increased number and variety of Caudovirales phages in their gut mucosa, according to a research conducted by Zuo *et al*., (2019). Another study raises the idea that antibiotic resistance genes (ARGs) are present in the phage microbiome of healthy persons (Maszewska *et al*., 2018). Approximately, fifty feces samples from healthy individuals who denied using antibiotics in the three months preceding sample collection were analysed. The feces of 72.7 % of these healthy people have at least one ARG in the phage DNA (Fu *et al*., 2010).

Many recent studies have demonstrated that a variety of illnesses, including obesity; diabetes, metabolic disorders, diarrhea, inflammatory bowel disease (IBD), and malnutrition, may alter the gut microbiota (Maszewska *et al*., 2018). It may be challenging to determine if changes to the virome and microbiota are the cause of the disease condition, according to Garmaeva *et al*., (2019). Tetz *et al*., (2017) studied the effects of exposure to a bacteriophage cocktail against *Enterobacteriaceae*; *Staphylococcaceae*, *Streptococcaceae*, and *Pseudomonadaceae*, on the intestinal permeability and relative abundances of the bacterial flora in the overall gut microbial community. They concluded that the phage lysate increased the intestinal permeability in the experimental animals (*i.e*., a rat model); when administered orally for 10 days. The markers of impaired permeability, including the lactulose/ mannitol ratio; the plasma endotoxin concentrations, and the serum levels of the inflammation-related cytokines have increased.

The gut microbiome is a very dynamic and an interactive community; with lytic phages attacking and eliminating certain bacteria, which in turn modifies the gut microbiome. In addition, it has been shown that the phages can influence the production of metabolites by their bacterial hosts, which are known to affect the mammalian host, including neurotransmitters; amino acids, and bile salts (Hsu *et al*., 2019). The Caudovirales phages, which make up the majority of the human virome, may have a considerable impact on the intestinal physiology and composition of the gut bacterial microbiome. According to the study conducted by Norman *et al*., (2015), the Caudovirales phage diversity and certain bacterial community members have significant associations in people with inflammatory bowel disease (IBD). Similarly, cases of ulcerative colitis (UC) and Crohn's disease (CD) are both found to have an abnormal form of the enteric viriome. Studies based on patient samples and animal models; using fecal samples or intestinal biopsies for metagenomic studies, have shown that CD patients have higher population of the temperate phages, and those that are infecting the bacterial orders of Alteromonadales; Clostridiales, *Retroviridae* family, and *Clostridium acetobutylicum*. Furthermore, a decreased abundance of *Microviridae* phages; in addition to the ulcerated mucosa of the CD intestinal biopsies, has less VLPs than the non-ulcerated mucosa (Nishiyama *et al*., 2020). Similarly, for the UC patients; less phageome diversity and decreased mucosal smoothness has been reported, compared to the healthy individuals (Qv *et al*., 2021). The phages infecting *Escherichia*; *Lactobacillus*, *Bacteriodes*, and *Enterobacteria* have increased; whereas the Clostridiales phages decreased. However, it is observed that a person’s disease status is more accurately reflected by the bacterial community rather than the viral community; though the viral diversity has been used as viral biomarkers for UC, CD, and IBD (Qv *et al*., 2021).

10. Single or cocktail bacteriophages and phage lysins used in eradicating the bacterial biofilms

Due to their small size and ability to penetrate through the bacterial cell wall; the bacteriophages are proving to be potential candidates for eradicating the bacterial biofilms (Singh *et al*., 2022). The microbial infection may be treated with phages- in combinations or singly. Phages have been used to treat the bacterial biofilms on the medical devices. Their therapeutic ability is mainly focused on the lytic phages; as they kill their host bacterial cells; are host specific, have minimum toxicity, and have no side effects or antimicrobial resistance (Singh *et al*., 2022). All approaches of phage therapy have been tried and studied, *viz*. phage monotherapy; phage cocktail, phage-antibiotic combinations, phage-derived proteins, and lastly recombinant phage and/or phage-products. All approaches have been extensively studied with better understanding of their advantages and disadvantages. For example, the phage monotherapy may be slow for acute infections where a quick response is required. This problem can be tackled by using a mixture of phages; commonly called phage cocktail (Melo *et al*., 2016). Such phage mixtures with different activity will target the host bacteria *via* more than one mechanism of action; making it more detrimental for the bacterial pathogen than a single therapeutic challenge. Phages have also been used in synergistic combination with antibiotics, which has shown better results, as compared to using a drug alone (Motlagh *et al*., 2016). Lastly, in multiple studies; the potential of the phage derived enzymes is also being explored and has shown promising results (Singh *et al*., 2022). These enzymes help the virus in infecting or propagating in its host; either by causing lysis of the host cell wall for the release of virions (endolysins), or by creating pores in the cytoplasmic membrane (holins), and/ or by digesting the polysaccharide components of the bacterial cell wall (depolymerases) (Peng *et al*., 2017). Thus, the bacteriophages are one of the most promising alternate approaches to replace the antibiotic therapies and to minimize the antimicrobial resistance (Principi *et al*., 2019). Two successful examples of the phage based compounds include the Staphylococcal phage Sb_1 and a polyphage PYO, which have come in the markets as treatments for the biofilms of the methicillin resistant *Staphylococcus aureus* (MRSA) (Tkhilaishvili *et al*., 2020).

The urinary tract infections (UTIs) are one of the most common chronic infections. Phage combinations and single phages have been used *in vitro* to prevent *P. mirabilis* and *P. aeruginosa*, which are the bacteria responsible for catheter-associated UTIs; from forming biofilms on the catheters. It is important to assess the effectiveness of a single phage against the combination of phages for treating the biofilms. As reported by Fu *et al*., (2010), the effectiveness of a single phage against *P. aeruginosa* is only for 24-48 h; after which regrowth of the biofilm and appearance of resistant strains has been observed. However, when a five-phage cocktail is used in the same settings; a 99.9 % reduction in biofilm formation and equally significant delay in the emergence of resistant strains has been reported. A phage cocktail of two phages Isf-Pm1 and Isf-Pm2 has been studied against *P. mirabilis* biofilms; using cell adhesion assay in Vero cells and a phantom bladder model. The dual-phage mixture has shown a significant reduction (65 %) of the biofilm mass; along with down-regulation of the key genes involved in biofilm formation (Mirzaei *et al*., 2022). On a 24-hour-old biofilm of *P. mirabilis* that causes catheter-associated UTI; a three-phage combination removed the biofilm at a similar or slightly reduced concentration compared to a single phage (Maszewska *et al*., 2018).

The acquired antibiotic resistance genes and biofilm structure have also been associated to oral disorders, such as periodontal disease and peri-implant diseases (Szafrański *et al*., 2017). To ascertain how the phages affect the oral biofilm bacteria; several studies have been conducted on *A. actinomycetemcomitans*; *E. faecalis*, and *Streptococcus mutans* (Pinto *et al*., 2016). *A. actinomycetemcomitans* can cause abscesses; infectious endocarditis, and periodontitis. *E. faecalis* is included in having infections in the dental root canal

and getting dental implants. *Streptococcus mutans* can potentially lead to dental infections linked to caries. A reduction in the bacterial counts from 2-3 log to complete bacterial removal has occurred across all the trials; as a result of phage application alone. Even after the phage has eliminated 95 % of the *A. actinomycetemcomitans* bacterium; the biofilm matrix persisted. The abundance of *Streptococcus mutans* and *E. faecalis* bacteria in biofilms has been reduced *in vitro* by 5 logs after phage application (Szafrański *et al*., 2017). Intermittent root canals have been recorded where vancomycin-resistant enterococci infections are most common (VRE). *E. faecalis* phages may be used to treat dental root canal infections that have a biofilm. In a diseased root canal model; treatment with *E. faecalis* phage *in vivo* effectively decreased *E. faecalis* biofilm (Khalifa *et al*., 2016).

A promising new method for biofilm reduction involves using the phages in conjunction with antibiotics, and monitoring their behavior when they work synergistically. Certain antibiotics become more potent at lower concentrations when combined with phage. If phages are supplied before the antibiotics; some bacterial biofilms might be more easily eliminated. When combination of phages and the most potent antibiotics (1 MIC) is employed; a biofilm that has grown on the epithelial cell layers for 8 hours is reduced dramatically after 12 hours of therapy (Abedon, 2019). About 24 hours following the phage therapy along with gentamicin and tobramycin; statistically significant killing in 48-hour old *P. aeruginosa* biofilm on the plastic shells has been observed (Chaudhry *et al*., 2017). The substantial synergistic effect of phages and ciprofloxacin at sub-minimum inhibitory concentrations (MIC), which has been used for treating *P. aeruginosa* infections associated with biofilms of cystic fibrosis and wound infections, has been confirmed by Chang and co-workers as well (Chang *et al*., 2019).

It has been demonstrated that phage derived endolysins work well against the bacteria that are still resistant to the antibiotics. Schuch and co-workers have reported a bacteriophage lysin CF-301 to target the *Staphylococcus aureus* biofilms. The biofilms of coagulase-negative Staphylococci; *Streptococcus pyogenes*, and *Streptococcus agalactiae* are also sensitive to disruption by the CF-301 lysin; along with *Staphylococcus aureus* biofilms formed on the polystyrene; glass, surgical mesh, and catheters. In catheters, CF-301 removed all biofilms within 1 hour and has killed all the released bacteria by 6 h (Schuch *et al*., 2017). Similarly, P128 as an anti-staphylococcal protein is investigated alone and in combination with standard antibiotics to kill *Staphylococcus aureus* in different environments associated with clinical infections. The lysin P-128 showed potential anti-biofilm activity as detected by colony forming units (cfu) reduction. When lysin P-128 has been tested in combination with several antibiotics (*i.e*., vancomycin; gentamicin, ciprofloxacin, linezolid, and daptomycin), which are known to be poor inhibitors of *Staphylococcus aureus* biofilms; P128 displayed significant synergistic antibiofilm activity (Nair *et al*., 2016). In a recent study conducted by Nandi *et al*., (2022); phage genomes specific for *Acinetobacter baumannii* have been searched for specific antimicrobial peptides. They reported the presence of at least fourteen anti-microbial peptides in eight endolysins of *A. baumannii* specific phages (Nandi *et al*., 2022). Thus, the lysins from *Staphylococcus aureus* phages are found to be promising novel agents for clearing the staphylococcal biofilm infections *in vitro*.

Conclusion

As summarized in the current study, it is difficult to treat chronic infections originating due to biofilms, because of the ease of aggregation of the planktonic bacteria. Therefore, diagnosis of biofilm related infections is imperative and an effective therapy for such infections is required. In recent times, lot of focus has been given to phage therapy, and deeper insights are obtained on its mechanism of action. It is now understood with sufficient evidence that bacteriophages or phage-derived lysins may be an effective method to treat infectious diseases caused by the bacterial biofilms. According to some reports,

there is less biofilm suppression when only a single phage is utilized. In addition, it is also emphasized that using a phage combination rather than a single phage is preferable; since it maximizes the benefits of a phage and reduces the growth of the phage-resistant bacteria; especially in the biofilm-related nosocomial infections. Combination of phage and antibiotics is also demonstrated as a superior antibiofilm therapy. Some antibiotics perform better at lower concentrations when combined with a phage or its protein. It is necessary to conduct more future studies; especially by utilizing an *in vivo* biofilm model, to determine the processes by which the different antimicrobial agents can destroy the bacterial biofilms.

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Conflict of interest

The authors declare that they have no conflict of interests.

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