REVIEW ARTICLE

# Biofilm and Chronic Typhoid Carriers with Special Reference to Bacteriophage Therapy

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## Abstract

*Salmonella enterica* serovar Typhi is a human-restricted pathogen and the primary etiologic agent of typhoid fever with an incidence of 21 million cases each year, resulting in 200,000 deaths annually. About 3–5% of the individuals with an acute clinical or subclinical infection ultimately develop a chronic asymptomatic carrier state. These new chronic carriers are being added to the existing pool every year. This chronic carriage state not only serves as a reservoir for further spread of the disease *via* bacterial shedding in feces but is also being reported to be associated with malignant transformations in the biliary system. The acute and chronic carrier states are also becoming challenging to resolve with antibiotics due to the emergence of multiple drug-resistant strains. Moreover, biofilm formation is another hindrance in eliminating the infection. It is crucial to understand the development of each of these states to design and test targeted approaches to resolve the more recalcitrant chronic carriage. Bacteriophage therapy is emerging as one of the potential alternatives to deal with acute and chronic infection associated with biofilm formation. In this review, we have discussed the natural process of biofilm formation along with the intelligent role of bacteriophages to resolve such complicated infections, particularly in relation to typhoid.

**Keywords:** Antibiotics, Bacteriophage therapy, Biofilm, Chronic carrier, Typhoid fever.

*Journal of Gastrointestinal Infections* (2021): 10.5005/jp-journals-10068-3053

## Introduction

Bacteria are simple entities when one compares them with eukaryotic organisms. However, they have better adaptive capabilities to survive in various environmental conditions. The gene expression is modulated according to the availability of nutrients.[1] Where most eukaryotes already possess a multicellular system, bacteria can also shift from a free-floating single-cell (planktonic) state to a biofilm-like community against a harsh environment for millions of years.[2] Biofilm consists of a three-dimensional (3D) microbial structure (either aggregation of mixed or single species) enclosed within a self-produced extracellular matrix.[3] It appears that biofilms are a beneficial trait in pathogenesis, as it exhibits distinct metabolism and gene expression than their planktonic forms. The altered phenotype has increased tolerance to host immune response and exogenously administered antiseptics and antibiotics.[4]

Biofilms have evolved on earth for 3.4 billion years. They perform several biochemical cycling processes. Biofilms may be present in a free-floating form or can form on various biotic and abiotic surfaces.[5] The bacteria have acclimatized to live at 37°C; they find the human body a perfect biotic microenvironment for bacterial colonization and biofilm formation. The human body surfaces have a reserve of nutrients, humidity, pH apart from appropriate temperature. Interestingly in 1985, Costerton introduced biofilm in medical microbiology.[6] It has been stated that approximately 65% of microbial infections have a biofilm-related etiology. The microbial infections based on biofilm can be classified as (i) intrinsic to host tissue and (ii) associated with indwelling medical devices.[7] The intrinsic biofilm to host tissues leads to chronic infections such as cystic fibrosis, osteomyelitis, conjunctivitis, vaginitis, urethritis, nonhealing wound, bacterial endocarditis, dental caries, sinusitis, otitis media, periodontitis, etc.[8] The other type of biofilm-associated infections is usually associated with medical devices, e.g., catheters, pacemakers, heart valves, breast implants, contact lenses, endotracheal tubes, and orthopedic implants.[9]

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**How to cite this article:** Yadav VB, Nath G, Singh SK. Biofilm and Chronic Typhoid Carriers with Special Reference to Bacteriophage Therapy. J Gastrointest Infect 2021;11:36–41.

**Source of support:** Council of Science and Technology, Uttar Pradesh, and Department of Biotechnology (DBT), Government of India.

**Conflict of interest:** None

The evolution of biofilm on any surface primarily results in diseases of chronic nature. The inherent high resistance to antimicrobial substances and antibiotics is the added economic burden for the global healthcare system. Chronic typhoid carrier state is primarily a biofilm etiology in the gallbladder. These carriers are the sole source of typhoid and paratyphoid infection as the bacteria causing these conditions are human restricted. Further, it has been very strongly proposed that chronic bacterial infections often culminate into carcinogenesis.[10] The association between chronic typhoid carrier state and gallbladder cancer has been already reported with evidence.[11] In this review, we have highlighted the process of biofilm formation, mechanism of chronicity, and different modalities to tackle the issues of treating biofilm-associated diseases with special reference to chronic typhoid carriers.

## Basis of Biofilm Development

Understanding the fundamental concept of biofilm development will enable us to develop effective antimicrobial modalities for their eradication. Microbes colonizing and surviving in the human

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body face several stresses, including cellular and humoral immune mechanisms. In addition, variations in physical conditions (pH, oxygen concentration, nutrients, other competing microbes, osmolarity) occur at different body sites.

Both prokaryotic (bacteria) and eukaryotic (fungi) cells tend to form a biofilm,[12] but the composition may vary. Intriguingly, irrespective of the type of microbes involved and physical conditions, the complete series of events during the biofilm development are almost identical (Fig. 1). However, the actual processes under native conditions are pretty complex, varied, and dynamic.

## Composition of Biofilm

Production of an extracellular polymeric matrix is a biofilm's hallmark; however, the biofilms formed by most organisms commonly comprise of DNA, lipids, exopolysaccharides (EPS) and extracellular proteins. Furthermore, many of these proteins exhibit amyloid-like properties.[13] Thus, the biofilm matrix's production is primarily the critical key point for the success of biofilm communities in terms of propagation and survival of the cells.

Antibiotics penetrance for biofilm slows down by a factor of 2–3 due to the excretion of highly charged membrane-bound glycocalyx, which also plays a vital role in cohesion and adhesion with solid surfaces.[14] Another factor responsible for antibiotic resistance is the altered microenvironment and slow growth of the bacteria. It is known that within the biofilm, microgradients occur in the concentration of critical nutrients and oxygen, which results in heterogeneity in growth states extending from rapidly growing to metabolically inactive. As a result, the dormant bacteria in a biofilm can survive the antibacterial challenges.[15]

There is persistence in the biofilms, which evade killing by antibiotics and become resistant to chemical disinfectants. Although the proportion of this persistence is tiny, they evolve as a spore-like state. These mechanisms altogether increase the resistance of resident bacteria against conventional antibiotics by around 1000 folds.[16] In addition, the biofilms protect the resident bacteria against the immune system of host-mediated by impaired phagocytosis and complement system.[17]

## Strategies to Combat Biofilm Formation and Biofilms

The biofilm formation on abiotic and biotic surfaces can be minimized by removing the indwelling devices and coating the abiotic surfaces with antibiofilm substances.[18] Antifungal or antimicrobial surfaces have also been proposed to prevent biofilm formation.[19] Impregnation of antibiotics or disinfectants such as polyurethane polymers, loaded with the safest antibiotics, and photodynamic therapy (to kill photosensitized microbes) can also be used.[20]

The EPS protect the microorganisms from various antimicrobial agents. So, the substances with EPS degrading ability would expose the biofilm cells to antibiotic agents. Here, it is worth mentioning that bacteriophages encode a unique enzyme class called endolysins (peptidoglycan hydrolases).[21] Endolysins are primarily species-specific. Moreover, it is vital to know the bacteria present in the biofilm for bacteriophage-derived endolysin. Specific extracellular proteases (sarA, Sigma B, ESP) have also been reported for biofilm disassembly.[22] The addition of extraneous DNase and restriction enzymes for certain species has been reported to disrupt the biofilm matrix as eDNA is a significant cementing matter for EPS.[22] Neutralization of lipopolysaccharides may also result in disassembling of the biofilm. Change in membrane permeability due to alteration in membrane potential may also disrupt the biofilm. This alteration may be pursued by harnessing bacteriocins as antimicrobials alone or existing antimicrobials to target biofilms. Lantibiotics have already been reported to be effective at permeating biofilms.[23]

Any molecule damaging the plasma membrane will stop the cell division and affects the microorganism's viability. Some antimicrobial peptides (AMPs) (e.g., pyrrhocoricin, apidaecin, drosocin) use the exact mechanism to inhibit the cell division and act as antibiofilm. In addition, certain AMPs are known to inhibit adhesion as they stop the synthesis of adhesion molecules.[24] While EPS is an essential component of biofilm, few EPS have been found to inhibit the synthesis of polysaccharides in biofilm. Instead, they have been reported to induce dispersion of the performed biofilm.[25] Inhibition of the cyclic di-GMP signaling system leads to

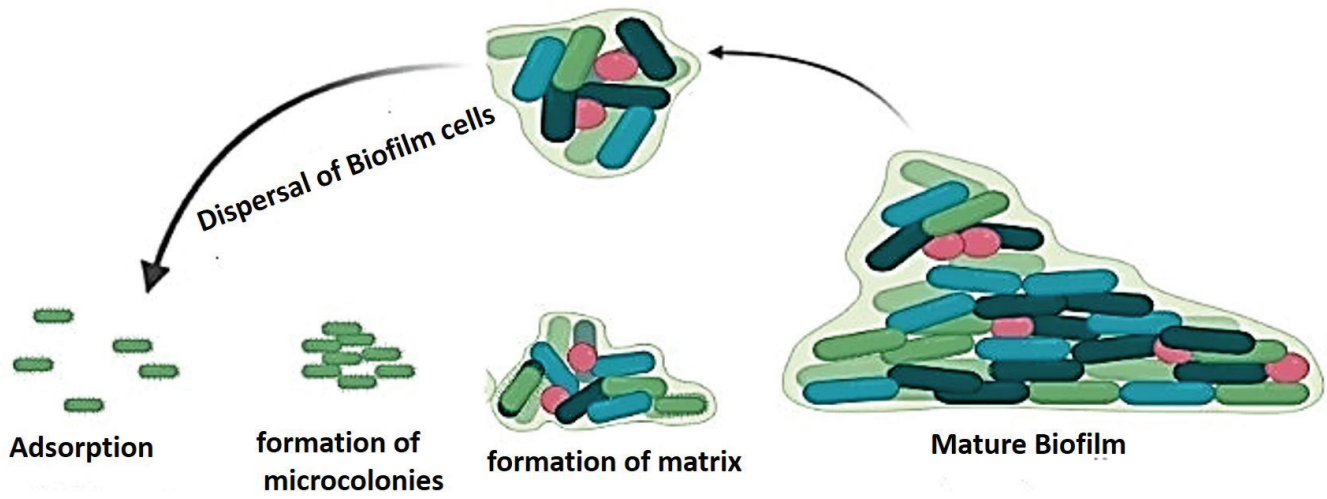



**Fig. 1:** Stages of biofilm formation

biofilm dispersal, and this phenomenon occurs under stress such as starvation, nitrosative conditions, etc.

Some of the secondary metabolites such as fisetin and esculetin affect biofilm maturation. As a result, they reduce the thickness of biofilm. Other agents, namely bispyridinamineoctenidine hydrochloride, have also been found to have antibiofilm activity, but the exact mode of action is unknown.[26]

## Chronic Carriage of *Salmonella* Infection

It is now established that asymptomatic *Salmonella* Typhi carriage may develop following symptomatic typhoid fever or even subclinical infection. Intriguingly, very famous German microbiologist, Robert Koch, very well predicted in 1902 that the main reservoir of *S. Typhi* is humans who are usually symptom-free but excreting the pathogen. At about the same time, two persons known as Typhoid Mary in the USA and Mr N in the United Kingdom were traced as the source of infection. The studies carried out later on, demonstrated that about 1–4% of individuals infected with *S. Typhi* become chronic carriers and keep shedding $10^4$–$10^5$ CFU/g of stool beyond 12 months of initial infection.[27] It is important to note that about a quarter of the chronic carriers never had a symptomatic infection due to *S. Typhi*, and possibly these individuals have a subclinical infection.

Since typhoid- and paratyphoid-carrying serotypes of *Salmonella* are human restricted, the persistence is of particular concern as a source of infection. The other serious concern is the recent report of a strong association between chronic typhoid carriage and cancer gallbladder.[11,28] Therefore, finding out the modalities to eradicate the bacterium from the entire human population seems significant.

As we have seen the grave public health concern of both acute infection and chronic carriage of typhoid-causing bacteria, it is imperative to understand the evolution of both states to design the strategies to combat the recalcitrant chronic carriage.

The key feature of chronic carriage of *S. Typhi* is successful colonization of the biliary system, especially biofilm formation on the surface of the gallbladder and gallstones.[29] As mentioned earlier regarding the development, maturation and dispersal of biofilm in general, the same phenomenon also occurs with this serotype of *Salmonella*. The biofilm protects the bacteria from the gallbladder's harsh environment, e.g., bile, host immune responses, and antibiotics.[30] However, in chronic carriage, the immune response of TH1 shifts from acute infection to TH2 when chronic carriage develops. Therefore, there is apprehension that chronic carriage causing *S. Typhi* may be genotypically different from acute infection. However, this speculation has been nullified by Ong et al.[31] report where complete genome sequencing of an isolate from chronic carrier revealed no difference from an isolate of acute typhoid fever.

## Strategies to Combat Persistent *Salmonella* Infection

Most of the time, the administration of antibiotics has been observed to be infective against persistent typhoidal infection. The gallbladder removal has been reported with some success but not in absolute terms as persisters have been reported in other body parts, e.g., liver, lymph nodes, bone marrow[32] Devraj et al.[33] have demonstrated that despite being genetically indistinct, two isolates from chronic carriers tended to form thicker biofilms with a higher level of eDNA and DNABII proteins than those formed by acute infection isolate. In addition, the authors have demonstrated that antibodies against DNABII proteins disrupted biofilm *in vitro.* The extracellular DNABII proteins consist of integration host factor and histone-like protein. These proteins are critical to the structural integrity of bacterial biofilms.

## Phages for Biofilm Removal

Biofilm formation is a form of cooperative group behaviors and probably the initial evolutionary structure of multicellular organisms. Different phages and bacteria evolve together in antagonistic, coevolutionary cycles, enhancing the speed of evolution of several traits, e.g., virulence and biofilm formation. Interestingly, biofilm gives shelter and protection to the bacteriophages either in the EPS or inside as prophage form. However, bacteria have several mechanisms to limit the access of phages.[34] As our understanding of the underlying mechanisms of coevolutionary interactions between biofilm and phages is getting bettered, biofilm's phage-based treatment can be designed.[35] Phage-based treatment modalities may include phages combined with antibiotics using a single phage or phage cocktail or phage-derived molecules such as enzymes and genetically modified phages.[36]

### Phage Therapy

The lytic phages are the key point to disrupt biofilms. The phages must penetrate, diffuse, and propagate through the biofilm. The phages harboring EPS-degrading enzymes can degrade the EPS matrix components and thus facilitate the penetration.[37]

It has also been reported that bacteria infected with phage undergo stress, leading to EPS-degrading enzymes release.[38] Often the bacterial biofilm may be multispecies. The cocktail of phages takes care of such conditions and the emergence of phage resistance in bacteria.[39]

#### *Genetically Modified Phages*

Transduction is the major issue raised by opponents of phage therapy. Further, many phages may not have the mandatory genes/gene products for penetration and degradation of biofilms. Therefore, the phage may be genetically modified to express EPS-degrading enzymes extracellular and hydrolases intracellular and without virulence and antibiotic resistance genes.[40] Specific temperate phages with phenotypic characteristics disrupting the biofilm may also be genetically modified to lytic phages.[41] The phage can be made with broad spectrum activity, programmable DNA nucleases associated with CRISPR in temperate phages can be used to reverse the antibiotic resistance.[42] Further combination of gold nanorods which, after infrared light, induces photothermal lysis of both target cells and phages will take care of transduction-mediated problem of gene transfer.

#### *Phages in Combination with Antibiotics*

The phenomenon of phage-antibiotic synergy can be utilized for better results in biofilm disposal. Often bacteriophages revert the antibiotic-resistant phenotypes.[43] However, phage–antibiotic combination has certain drawbacks also, i.e., the emergence of resistant bacteria may promote antibiotic resistance if resistant variants escape killing and decreased metabolic activity of cells may lead to decreased replication of the virus.[44]

## Phage-derived Enzymes

The enzymes encoded by phages called enzybiotics may help treat bacterial infection and biofilms. The lysins and depolymerases are the two powerful enzymes performing biofilm disposal and killing the bacteria.

- **Lysins:** Lysins are of two types; one is present in the phage tail as phage-associated lysins, acting on receptors after identification to degrade the cell was locally to facilitate the injection of the viral genome inside the host cell.[45] The other lysins are found inside the phage, which lyses the bacterial cell wall after the replication cycle. The unique feature of lysins as a therapeutic agent is that the lytic activity depends on the bacterial metabolic state. It means the phage lysins may also lyse the persisters.[46]
- **Depolymerases:** The polymerases produced by the phages are capable of degrading the extracellular substances of the bacteria, e.g., capsular polysaccharides, EPS, O-polysaccharides, and peptidoglycan. As these substances are abundantly present in the biofilm, they help the phages enter the biofilm's EPS structure.[47] Like lysins, depolymerase is also found in the bound form attached to the tail of phages.

Depolymerases may be divided into different groups. They are hydrolases, lyases, and triglycerol lipases. These enzymes are host-specific and highly diversified because phages and bacteria are pretty diverse and have intense horizontal gene transfer.[45,47] Depolymerases can be used to treat human and animal infections due to biofilms. These enzymes can enhance the penetration of the immune system by degrading the EPS matrix.[48] Interestingly, lysin and depolymerases have shown synergistic behavior in reducing the viable cells in the biofilm.[49]

## Problematic Biofilm in Humans

All the mucosal surfaces with commensal flora of the human body are prone to bacterial infections. Most such infections (>65%) are associated with bacterial biofilms. The oro-gastro-intestinal tract, periodontitis, gingivitis, dental caries, peptic ulcer, cholecystitis, ulcerative colitis, etc., are associated with biofilm. Pyelonephritis, chronic prostate cystitis, urethritis, etc., are also usually associated with biofilm formation. Ironically, bacteriophage therapy has not been tried on most of the infections mentioned earlier.[50] Therefore, it will be advisable to shift to bacteriophage therapy when conventional antibiotic therapy fails after 6 weeks of duration.

One such infection is cholecystitis, and cholelithiasis affects the gallbladder for the long term. If *S. Typhi* causes it, it may be an inducing factor for the biliary tract cancer apart from being a constant source of infection. The aforementioned mechanisms of bacteriophages dealing with biofilm suggest that they may be deployed to treat acute typhoid fever and eradicate its chronic carriage when all the available therapeutic drugs are ineffective.

Many case reports mention that the treatment of typhoid fever using bacteriophage therapy during the pre-antibiotic era was done in many parts of the world. However, due to the incidences of the severe reaction after therapy, most likely due to constituent endotoxins in the bacteriophage preparations, this modality could not continue. Further, the decline in the application of phage therapy could be observed because of the introduction of the then magic drug, antibiotics. However, we may go for bacteriophage therapy in the present scenario of antimicrobial resistance and antibiotics often failing to eradicate the chronic carrier state. Many types of research have been carried out in recent times about bacteriophage biology, including the therapeutic aspects. The endotoxin in the phage composition and a release after lysis of the infecting bacteria has been worked out. In animal models, safe doses in different clinical conditions have already been determined with septicaemia.[51] It will facilitate clinical trials on humans. The question of killing intracellular bacteria by bacteriophages has been addressed by Broxmeyer et al.[52] They have demonstrated the killing of *Mycobacterium avium* and *M. tuberculosis* by a mycobacteriophage delivered by nonvirulent mycobacterium. We have carried out the killing of intracellular *S. Typhi* using a bacteriophage cocktail (Unpublished data).

Further, an acute and chronic model of mimicking typhoid may be created in a susceptible mouse model using *S. Typhimurium* as a surrogate model. The efficacy of bacteriophages may be evaluated. Moreover, phage therapy may also be assessed by putting the mice on a chalcolithic diet with *S. Typhimurium* infection leading to biofilm formation. If encouraging results are seen, we may proceed with human cases as well, not only to treat the chronic typhoid carriers but also the acute infections. This therapy may ultimately result in the complete eradication of *S. Typhi* from the human population.

## Limitation of Phage Therapy and Preferable Approach

Although isolating bacteriophages is not a tough job for common target bacteria,[53] identifying therapeutic grade phages is complicated. Before starting phage therapy, knowledge of phage specificity toward other nontarget bacteria is also equally important. Importantly, bacteriophage genome sequencing is needed before their therapeutic application to confirm the absence of integrase genes (found in lysogenic type), antibiotic-resistant genes,[54] and genes for phage-encoded toxins. Furthermore, the formulation and stabilization of phages for use is bacteriophage dependent and optimized for each phage separately. This optimization is time-consuming and costly affairs of phage therapy clinical trials, which discourages the research and production of phage preparations.

The rapid development of resistance in host bacteria against phages in bacteria has been reported.[55] However, using bacteriophage cocktails to target different bacterial receptors and bacteriophage–antibiotics combined treatment prevents resistant development.[56] Furthermore, bacteriophages host range can also be expanded by genetic modification in phage tail ligand proteins.

Another approach of synthetic biology techniques generates various chimeric phages belonging to family T2, T4, and T7, targeting different bacterial receptors for synergistic therapeutic effects and delayed phage resistance development.[57]

Phage stability in the bloodstream is another obstacle in the path of phage therapy. Viruses used in therapy lose their potency soon due to the effect of humoral- and cell-mediated immunity. However, phage stability can be improved in circulation by altering the viral capsid proteins or through PEGlation (conjugation of PEG onto bacteriophages).[58]

Although bacteriophages are safe for humans,[59] phage purity is another severe concern before its therapeutic use. Phage lysates used in therapy may contain several harmful components, especially endotoxins (in the case of gram-negative bacteria) and protein toxins (produced by many pathogenic bacterial species). Due to their highly immunogenic properties, the endotoxins produced after the lysis of the bacteria aggravate the septic shock

*via* cytokine storm. The maximum permitted value of endotoxin in therapy is 5.0 Endotoxin Units /kg/h for intravenous injection. Therefore, removing these endotoxins is necessary before therapy, and today, many techniques are available to remove these harmful components. Some are PEG precipitation, membrane dialysis, ultracentrifugation, ion exchange chromatography, and extraction with 1-Octanol. Bacteriophage production in a cell-free system (synthetic bacteriophages) is another advanced synthetic approach to overcome these endotoxin-mediated side effects.[60]

## Conclusion and Future Perspective

Asymptomatic, chronic typhoid carriers have been recognized for over a century. Unfortunately, despite our increased understanding regarding the persistence of *S. Typhi* in the gallbladder, we still do not have an effective method to cure it. As we are convinced now that antibiotics cannot do the miracle in any case of biofilm formation if given alone, bacteriophage therapy is now (*) being seen as one of the potential modalities to deal with such infections. However, acquiring several genes by bacteriophages coding for several enzymes during the evolutionary process might help deal with persistent infection and long-term carriage of typhoidal and nontyphoidal *Salmonellae*.

## Acknowledgments

This work was supported by Council of Science and Technology, Uttar Pradesh, and by the Department of Biotechnology (DBT), Government of India. SKS is grateful to the Institute of Eminence, Banaras Hindu University, Varanasi, India, for providing the Malaviya Postdoctoral Fellowship.

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