Combating intracellular pathogens using bacteriophage delivery

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ABSTRACT
Intracellular pathogens reside in specialised compartments within the host cells restricting the access of antibiotics. Insufficient intracellular delivery of antibiotics along with several other resistance mechanisms weaken the efficacy of current therapies. An alternative to antibiotic therapy could be bacteriophage (phage) therapy. Although phage therapy has been in practice for a century against various bacterial infections, the efficacy of phages against intracellular bacteria is still being explored. In this review, we will discuss the advancement and challenges in phage therapy, particularly against intracellular bacterial pathogens. Finally, we will highlight the uptake mechanisms and approaches to overcome the challenges to phage therapy against intracellular bacteria.

1. Introduction
Several bacterial pathogens have developed strategies to survive within a mammalian host cell and are known as intracellular bacteria. These pathogens modulate the intracellular environment, create a suitable niche, and bypass the harsh consequences of encountering the host immune system. Intracellular bacteria can be classified as obligate, which are unable to grow outside a host cell like *Chlamydia trachomatis* (*C. trachomatis*) and *Coxiella burnetii*, or facultative, which can grow outside and inside a host cell for e.g. *Salmonella enterica* (*S. enterica*), *Mycobacterium tuberculosis* (*M. tuberculosis*) and *Listeria monocytogenes* (*L. monocytogenes*) (Armstrong and Hart 1971; Gaillard et al. 1987; Malik-Kale et al. 2012; Bastidas et al. 2013). These pathogens account for high mortality in humans. About 1.4 million patients succumbed to tuberculosis in 2019 (World Health Organization 2019). In 2015, 90,300 deaths occurred from non-typhoidal salmonellosis and about 178,000 people died of typhoidal salmonellosis (Mortality 2016). Additionally, other pathogens like *Staphylococcus aureus* (*S. aureus*), *Escherichia coli* (*E. coli*), and *Pseudomonas aeruginosa* (*P. aeruginosa*) can also invade and survive within host cells (Sukumaran et al. 2003; Fraunholz and Sinha 2012; Garai et al. 2019).

A major challenge for treatment intracellular bacteria is the inability of many antibiotic agents to cross the mammalian cell membrane barrier (Kamaruzzaman et al. 2017). The rising concern over antibiotic resistance demands novel treatment approaches. Phages are viruses that infect and lyse specific host bacteria and their application as therapeutics is termed as phage therapy. There is a large reservoir of around $10^{31} - 10^{32}$ phages in the biosphere, which could be tapped in, to solve the challenge of antimicrobial resistance (Wittebole et al. 2014; Kortright et al. 2019). Multiple review articles on phage therapy have been published recently (Carvalho et al. 2017; Gordillo Altamirano and Barr 2019; Mohan Raj and Karunasagar 2019; Melo et al. 2020), however, to our knowledge, only a few review articles have exclusively focussed on developing new strategies to deliver phages for targeting intracellular pathogens (Nieth et al. 2015; Azimi et al. 2019). In this review, we have primarily discussed the advancement in phage therapy to control intracellular pathogens. We have also highlighted pertinent challenges and possible solutions for phage therapy to tackle intracellular bacterial infections.

2. Intracellular bacteria
Intracellular bacterial pathogens are classified in facultative if able to grow either inside or outside a host cell or obligate if unable to grow outside a host cell. Intracellular pathogens have the inherent ability to cross the mammalian cell membrane via multiple...
Table 1. List of intracellular bacteria, their mechanism of entry, target cell and phages available in literature.

| Sr. No | Intracellular bacteria          | Mechanism of entry | Target cell          | Phages available | Reference                  |
|--------|---------------------------------|--------------------|----------------------|------------------|---------------------------|
| 1      | *Mycobacterium abscessus*       | Receptor mediated  | Macrophages          | phiT46-1         | (Amarh et al. 2021)       |
| 2      | *Mycobacterium tuberculosis*    | Receptor mediated  | Macrophages          | D29, TM4, D56A   | (Ford et al. 1998; 1998; Mayer et al. 2016) |
| 3      | *Shigella flexneri*             | Trigger mode       | Epithelial cells     | Sfi-1, Sfi-1-Sf25| (Ahamed et al. 2019; Doore et al. 2018) |
| 4      | *Shigella dysenteriae*          | Trigger mode       | Epithelial cells     | SF-9             | (Faruque et al. 2003)     |
| 5      | *Listeria monocytogenes*        | Zipper Mode        | Epithelial cells     | AS11, P100, LMP1, LMP7| (Guenther et al. 2009; Lee et al. 2017; Ahmadi et al. 2020) |
| 6      | *Salmonella typhimurium*        | Trigger mode       | Epithelial cells/    | P22-B1, P22, PBST10, PBST13, PBST32, and PBST 35 | (Jung et al. 2017) |
| 7      | *Yersinia Pestis*               | Zipper Mode        | Macrophages          | PhiA1122, Yep-Phi| (Garcia et al. 2003; Zhao et al. 2011) |
| 8      | *Yersinia enterocolitica*       | Zipper Mode        | Epithelial cells/    | Yersinia Phage X1| (Xue et al. 2020)         |
| 9      | *Staphylococcus aureus*         | Zipper Mode        | Macrophages          | phiIPLA35 (phiIPLA35), phiIPLA88 (phiIPLA88) | (Garcia et al. 2009) |
| 10     | *Chlamydia*                     | Trigger mode       | Epithelial cells     | Chp2, Chp3, CPG1, CPGAR39 (CPG1) and Chp4 | (Silva-Dominiak et al. 2013) |
| 11     | *S. enterica*                   | Trigger mode       | Epithelial cells/    | ZCSE2            | (Mohamed et al. 2020)     |
| 12     | *E coli*                        | Trigger mode       | Macrophages          | K1F              | (Scholl and Merrill 2005) |
| 13     | *Mycobacterium leprae*          | Receptor mediated  | Epithelial cells     | No               |                           |
| 14     | *Coxiella burnetii*             | Trigger mode       | Epithelial cells     | No               |                           |

*Trigger mode- A Macropinocytosis-related Process involves type 3/4 secretion system.
**Zipper mode- A Clathrin- and Actin-mediated Internalisation Process.

mechanisms (Moulder 1985; McClarty 1994). These pathogens can penetrate and localise in both phagocytic (for instance, macrophages) and non-phagocytic (for instance, endothelial/epithelial cells) hosts (Galan 1994). Organisms like *Mycobacteria* utilise the natural phagocytic ability of the macrophages to invade via receptor mediated endocytosis and reside within them (Riley 1995; Ibarra and Steele-Mortimer 2009). On the other hand, pathogens like *C. trachomatis*, *S. aureus*, and *Shigella* have specialised mechanisms to induce cytoskeletal reorientation that generates phagocytic activity in non-phagocytic epithelial cells (Jensen et al. 1998; Sinha and Herrmann 2005; Kumar et al. 2006; Hybiske and Stephens 2007). Unlike these pathogens which can invade specific cell type, *Salmonella* has the ability to infect both the macrophages and the non-phagocytic epithelial cells. These pathogens utilise several mechanisms of cellular entry (Table 1). Some pathogens like *Listeria* and *Yersinia* have surface proteins that binds to specific host cell receptor and induces a signalling cascade event that facilitates clathrin mediated endocytosis (Zipper mode) (Van Nhieu and Isberg 1991; Pizarro-Cerdá et al. 2012). Alternatively, pathogens like *Salmonella* and *Shigella* utilises its type 3 secretion system to translocate effector molecules into the host cell that triggers actin rich membrane ruffles necessary for endocytosis (Trigger mode) (Tran Van Nhieu et al. 2000; Patel and Galán 2005). Both mechanisms are extremely crucial to infiltrate non-phagocytic epithelial cells.

Upon internalisation, these pathogens reside within specialised intracellular compartments known as phagosomes and/or endosomes (Figure 1). In phagocytes, these phagosomes are destined to lysosomal fusion, where the low pH and degradative enzymes destroy the pathogens. However, most of these intracellular pathogens have evolved strategies to block phago-lysosomal fusion and subvert the harsh bactericidal environment within the lysosomes. Interestingly, some pathogens can escape from the phagosomes and survive in the cytoplasm (Fraunholz and Sinha 2012; Peng et al. 2016) (Figure 1). For instance, *M. tuberculosis* arrests phago-lysosomal fusion and even survives in the cytoplasm and lysosomes, thus creating a diverse niche for its survival within macrophages (Armstrong and Hart 1971; Jamwal et al. 2016). *S. aureus* can survive and replicate within the phago-lysosomes in a low pH condition (Brouillette et al. 2003). These diverse mechanisms employed by the pathogens to reside within the
3. Drawbacks of antibiotics to curtail intracellular bacterial infection

The effectiveness of an antibacterial agent depends on its local concentration, which should be sustained at a therapeutic level to eliminate the pathogen. Exposure to a suboptimal dose of antibiotics leads to the selection of drug-resistant bacteria or the development of a tolerant phenotype (Odenholt et al. 2003). Some of the hurdles that encountered during antibiotic treatment while targeting intracellular pathogens are illustrated in Figure 2.

Drugs like macrolides and quinolones are readily taken up by phagocytic cells. Despite the intracellular accumulation of antibiotics, efflux pumps present on mammalian surface and endosomal membranes, like P-glycoproteins and multidrug resistance proteins (Kim et al. 1997), lower the intracellular drug concentration within a host cell (Poole 2005; Fu and Roufogalis 2007). This hampers the local intracellular concentration required for killing the pathogen. For example, reduced intracellular activity of several antibiotics like fluoroquinolones, macrolides, streptogramins, lincosamides and rifampicin was observed against L. monocytogenes in multidrug resistant protein 1 overexpressed carcinoma cell line (Nichterlein et al. 1998). P-glycoprotein inhibitors (verapamil, ciclosporin, and GF 120918) and multidrug resistance protein inhibitors (gemfibrozil or probenecid) were shown to be effective in increasing the intracellular accumulation of the antibiotics in mammalian cells; however, disparate effects of various inhibitors are observed and their efficacy is dependent on the antibiotics used (Seral et al. 2003; 2003; Michot et al. 2005).

Apart from intracellular drug concentration, subcellular localisation of bacteria also governs the efficacy of antibiotics (Ong et al. 2005; Seral et al. 2005; Greenwood et al. 2019). Some pathogens like S. aureus and M. tuberculosis have an inherent ability to survive within the phago-lysosomal compartment with a low pH condition of 4.5–5.0. Maintaining the activity of antibacterial agents at such a low pH is another challenge (Baudoux et al. 2007; Lemaire et al. 2011).

Stress induced by antibiotics and host oxidative response can cause the transformation of bacteria into a non-replicating metabolic state, known as persisters (Grant and Hung 2013; Fisher et al. 2017). Numerous bacteria like M. tuberculosis, P. aeruginosa, Salmonella and S. aureus can persist in a growth arrested state with reduced metabolism which results in high tolerance to antibiotics over a long period of time (Grant and Hung 2013). For example, internalisation of S. typhimurium within the macrophage vacuolar environment induces phenotypic heterogeneity that results in the formation of non-replicating persister cells (Helaine et al. 2014). Wayne and Sohaskey had extensively discussed the non-replicating nature of M. tuberculosis within the host that causes latent infections that are often resistant to conventional treatment (Wayne and Sohaskey 2001). Although there are alternative approaches like intermittent drug doses to target persisters, these have not been widely explored in clinics (Cogan 2006) (Figure 2).

Phagocytes produce reactive nitrogen and oxygen species as a response to invading pathogens (Fang 2011). The antimicrobial function of reactive oxygen species (ROS) has been well documented in killing intracellular pathogens and inducing signalling events to trigger an inflammatory response (Spooner and Yilmaz 2011). Interestingly, recent studies have reported that the consequence of respiratory burst leading to ROS production failed to kill intracellular pathogens like S. aureus and was shown to induce tolerance to multiple antibiotics (Rowe et al. 2020). Similarly, connection between ROS and antibiotic tolerance has been established in M. marinum where intracellular ROS resulted in increased expression of drug efflux pump (Adams et al. 2011; Ramón-García et al. 2009). Although not experimentally proven, another explanation could be DNA mutagenesis caused by ROS exposure leading to physical damage to the genetic material (Beckman and Ames 1997;
Bertram and Hass 2008). Error-prone polymerase activity in response to the stress induced by respiratory burst might lead to random mutations causing antibiotic resistance or tolerance (Tang et al. 2000; Hori et al. 2010). (Figure 3).

4. Advances in phage therapy to combat intracellular pathogens

Phage therapy is emerging as a last-resort treatment for patients with antibiotic-resistant infections. In a recent report, Dedrick et al. showed that phage therapy could be used for treating disseminated drug-resistant M. abscessus infection in a young cystic fibrosis patient (Dedrick et al. 2019). A cocktail of genetically engineered phage effectively cleared M. abscessus infection when delivered intravenously and led to wound closure and resolution of infected skin nodules (Dedrick et al. 2019).

For phages to be effective against intracellular infections, they need to reach the intracellular site where the bacteria reside. The studies on intracellular phage delivery can be grouped into two categories - free phage delivery and carrier-mediated delivery of phages.

4.1. Free phage delivery

Within their host, intracellular pathogens are shielded from numerous effector molecules and cells due to the protective semi-permeable plasma membrane. Phages are high molecular weight molecules (> 10 megadaltons) that are unable to passively diffuse across the plasma membrane. However, there are several mechanisms that can be utilised by phages to overcome this biological barrier. The possibility of phage interaction and transport through mammalian cells became evident in the early 1970s when high doses of phages against V. cholerae were administered orally for the treatment of diarrhoea in humans and about 10^2 pfu/mL phages were observed in the blood throughout treatment duration (Monsur et al. 1970). Similarly, phages have been found in different organs after intravenous delivery. Recently, a phage mixture was administered intravenously for the treatment of a patient with disseminated drug resistant M. abscessus, and a substantially high titre of phages was detected in sputum and faeces, in addition to serum (Dedrick et al. 2019). Phages have also been detected in the brain tissue after intranasal delivery (Dor-On and Solomon 2015) and even foetal tissue after systemic administration (Srivastava et al. 2004). Nguyen et al. demonstrated...
rapid transcytosis of phage T4 from apical to basolateral chambers in a variety of cells like Madin-Darby Canine Kidney (MDCK) cells, T84 cells (colon epithelial), Caco-2 cells (colon epithelial), A549 cells (lung epithelial), Huh7 cells (hepatocyte epithelial cell-like), and hBMec cells (brain endothelial) (Nguyen et al. 2017). It was suggested that phages traffic through the Golgi apparatus. Phage UAB_Phi20 was also shown to be transcytosed across Caco-2 cells and human colon tumorigenic (HT-29) cells (Otero et al. 2019). In addition to transcytosis, phages have been reported to utilise phagocytosis, endocytosis and pinocytosis to enter mammalian cells (Figure 3). Phagocytosis is carried out by professional phagocytes, usually immune cells such as monocytes, macrophages, and dendritic cells. These cells can engulf invading bacteria, particles, or cell debris and eventually degrade them in lysosomes. Endocytosis is a constitutive process carried out by all mammalian cells for the uptake of nutrients and small molecules. Clathrin and caveolae-mediated endocytosis are two major types of endocytosis, among others, observed in mammalian cells. Pinocytosis involves uptake of large amounts of extracellular fluid, which is also a constitutive process in mammalian cells. Phages can be engineered to enable penetration through the gut. The peptide YPRLLLTP (identified by in vivo bio-panning protocol) displayed on the capsid of M13 phages facilitated translocation across the intestinal lining (Duerr et al. 2004). Phage interaction with mammalian cells for therapeutic implications has been extensively reviewed (Żaczek et al. 2019; Górski et al. 2020). Tian et al. showed that in HeLa cells and MCF-7 breast cancer cells, M13 enters through clathrin-mediated endocytosis and micropinocytosis (Tian et al. 2015), while it uses caveolae-mediated endocytosis for human dermal microvascular endothelial cells. The internalisation efficiency of M13 phage was enhanced several log folds by genetically modifying the phages to display cell-penetrating domains 3D8 VL transbody or TAT peptide (Kim et al. 2012). These modifications resulted in different modes of phage internalisation and fate as they interact with distinct cell surface glycosaminoglycans. 3D8 VL-M13 utilised the caveolae-mediated endocytosis and remained stable after internalisation for more than 18 h in the cytosol. TAT modified phage was mainly internalised via clathrin and caveolae-mediated endocytosis and were found in multiple subcellular compartments and were degraded in lysosomes within 2 h of internalisation. Overall, this report focuses on the efficiency rates at which engineered M13 displaying various peptides can be delivered into the mammalian cells (Kim et al. 2012).
Phage opsonisation by serum proteins can trigger phagocytosis (Van Belleghem et al. 2018). Møller-Olsen et al. showed internalisation of fluorescently labelled phage K1F-GFP into human urinary bladder epithelial cells via phagocytosis (Møller-Olsen et al. 2018). These phage K1F-GFP into human urinary bladder epithelial cells via phagocytosis (Møller-Olsen et al. 2018). These phages were successful in killing both extracellular and intracellular E. coli EV36-RFP infection in T24 urinary epithelial cells. The authors used a SYTOX dead cell stain and estimated the co-localisation of SYTOX with E. coli RFP using confocal microscopy. Phage treatment showed 77% co-localisation compared to 29% in untreated samples (Møller-Olsen et al. 2018).

Zhang et al. showed endocytosis and accumulation of S. aureus phage vB_SauM_JS25 in non-phagocytic MAC-T bovine epithelial cells over time (Zhang et al. 2017). Microscopic evaluation showed that about 12% of cells were positive for phages. A time-dependent intracellular killing (1–1.5 log order reduction in 12 h) of S. aureus by phage vB_SauM_JS25 was observed in MAC-T bovine epithelial cells. The extracellular bacteria was eliminated by adding lysostaphin (20 μg/ml) 1 h after infection (Zhang et al. 2017).

Lehti et al. described that endocytic uptake of E. coli PK1A2 phage into live eukaryotic neuroblastoma cells is dependent on the presence of polysaccharide residues on the mammalian cell surface (Lehti et al. 2017). The phages show initial adsorption to the polysaccharide receptor which enabled uptake. Phage adsorption and internalisation were lower when there was less sialic acid on the cell surface or when sialic acid was added separately as a competitive binding site. Many internalised phages (~30%) were found to be active even after 24 h. The phages eventually localised to the lysosome and became undetectable in 48 h (Lehti et al. 2017).

Peng et al. showed that free mycobacteriophage D29, a lytic phage, significantly reduced the intracellular M. tuberculosis count in peritoneal macrophage cells (Peng et al. 2006). Mouse peritoneal macrophages were infected with M. tuberculosis H37Rv for 4 h followed by washing with three times with Hank’s buffered salt solution (HBSS) to remove all extracellular bacteria. Phages treatment were given for 24 h and 48 h post-infection. Compared to the control group (no phage treatment), a high dose of phages (2.0 × 10^7 pfu/well) effectively lowered the viable intracellular bacterial count by 76% after 24 h and 92% after 48 h (Peng et al. 2006). Additionally, Lapenkova et al. investigated the effect of D29 on the mouse macrophages infected with virulent mycobacterial strain H37Rv (Lapenkova et al. 2018). Phage D29 (10^8 pfu) was incubated with infected macrophages for 24 h, followed by washing and re-incubation with fresh D29 for another 24 h. Intracellular bacteria (H37Rv) were plated after disrupting the infected macrophage membrane by two freeze/thaw cycle and allowed to grow from 3 weeks. Results depicted a 10-fold reduction in CFU counts in phage treated samples compared to control samples (Lapenkova et al. 2018). Promising results were also observed against other opportunistic intracellular pathogens like E. coli and S. aureus. Capparelli and group documented that in vitro phage (M^5a) treatment of intracellular S. aureus infected peritoneal macrophages resulted in 70% reduction in CFU (Capparelli et al. 2007). To check for efficacy of M^5a against local infection, S. aureus was administered subcutaneously followed by M^5a treatment 4 days later. Phage treatment resulted in a 2-log fold reduction in bacterial CFUs compared to untreated group and led to 97% survival of mice infected with lethal doses of S. aureus A170 strain. Although in in vivo studies, there was no direct evidence about whether the phages were effective against extracellular or intracellular bacteria (Capparelli et al. 2007). The authors further showed a significant role of the phage in controlling Methicillin-resistant S. aureus (MRSA) infection (100% survival of mice treated with 10^9 pfu/mice compared to 20% survival in the untreated group) (Capparelli et al. 2007).

Phage therapy against facultative intracellular pathogen Burkholderia pseudomallei was successful both in vitro and in vivo (Guang-Han et al. 2016). When phage C34 was added prior to infection, the survival rate of B. pseudomallei infected lung epithelial cells (A549 cell line) increased by 2-fold in vitro. However, no significant effect was observed when the phage was delivered post-infection. The authors further showed that pre-treatment (24 h before infection) and post-treatment (2 h after infection) also protected (33% survival compared to no survival in untreated) the intranasally infected (B. pseudomallei) mice which highlights the prophylactic and therapeutic potential of phages. Nevertheless, no direct evidence of reducing intracellular bacterial load was documented in vivo (Guang-Han et al. 2016). Similar study in support of prophylactic ability of phage treatment has been reported for M. tuberculosis infection using D29 phage (Carrihy et al. 2019). C57 black mice were exposed to aerosolized phages (7.7 ± 0.3 log_{10} PFU/mouse) 30 min prior to 50–100 CFU of M. tuberculosis H37Rv. Compared to untreated mice, 70% reduction in CFU count was observed in phage treated animals after 24 h of infection (Carrihy et al. 2019). Kolenda et al. reported that in uninfected cells, only ~100 pfu/mL were recovered from the osteoblasts after 24 h of incubation with free phages (10^7–10^9 pfu/mL) while in S. aureus infected osteoblasts, 10^5–10^7 pfu/mL of phages were recovered.
indicating that phage entry into the osteoblasts was dependent on re-infecting bacteria and the phages were proliferating within the bacteria. This was supported by co-incubation with vancomycin and rifampin that reduced the extracellular re-infecting bacteria and resulted in only \( \approx 10^3 \) pfu/mL present intracellularly (Kolenda et al. 2019). Detailed investigation in this study revealed that phages could get internalised only after adsorbing to the re-infecting bacteria however they were ineffective in reducing intracellular CFU despite internalisation. This lack of efficacy could be either loss of the phage activity in the intracellular environment or induction of bacterial dormancy within osteoblasts which may have inhibited propagation of phages. However, no evidence was provided in the study to clarify these speculations. Studies which have discussed free phage delivery for intracellular pathogens have been summarised in Table 2.

To improve therapeutic outcomes for intracellular infections, it is essential to enhance phage uptake by infected cells and target the intracellular bacteria.

### 4.2. Carrier Mediated phage delivery

Although free phage therapy is effective in some instances, as mentioned above, there is a need to improve the delivery of the phages intracellularly. This can be achieved by a “Trojan horse” approach that involves encapsulation or loading of phages onto carriers. Studies on carrier mediated phage therapy have been summarised in Table 3. Broxmeyer et al. reported a novel strategy of phage delivery via non-pathogenic strain \( M. \) smegmatis infected with phage TM4 (Broxmeyer et al. 2002). Neither \( M. \) smegmatis alone nor phage TM4 treatment alone affected intracellular \( M. \) tuberculosis and \( M. \) avium count within RAW 264.7 cells. Interestingly, TM4 infected \( M. \) smegmatis was phagocytosed and colocalized with \( M. \) avium containing vacuole after internalisation. This resulted in an approximately 100-fold reduction in the bacterial load after 48 h of treatment \( \textit{in vitro} \) (Broxmeyer et al. 2002). The results were further validated \( \textit{in vivo} \) by Danelishvili et al. to treat disseminated \( M. \) avium infection using phage TM4 adsorbed on \( M. \) smegmatis that accounted for significant reduction in bacterial load in the spleen. Although significant, direct evidence of phage mediated intracellular bacterial killing was not presented in animal model (Danelishvili et al. 2006). The idea of vectorisation proposed by Broxmeyer and group (Broxmeyer et al. 2002) ensured the delivery of active phage to the target site after subcutaneous administration. While innovative, the administration of live bacteria in patients is risky for patients. The use of biomaterials such as liposomes and polymeric particles can provide a suitable alternative.

| Sr No. | Intracellular pathogen targeted | Phage used for the study | Experimental model/cell type | Reference |
|--------|---------------------------------|--------------------------|-----------------------------|-----------|
| 1      | \( \text{Chlamydia psittaci} \) | phiCPG1                  | HeLa cell                   | (Hsia et al. 2000) |
| 2      | \( \text{M. tuberculosis} \)    | Mycobacteriophage D29    | Primary cells: mouse peritoneal macrophages | (Peng et al. 2006) |
| 3      | \( \text{S. aureus} \)         | M^A                      | Peritoneal mouse macrophages/has in vivo results also | (Capparelli et al. 2007) |
| 4      | \( \text{M. ulcerans} \)       | D29                      | Murine footpad model        | (Trigo et al. 2013) |
| 5      | \( \text{S. aureus} \)         | MR-5                     | Peritoneal mouse macrophages | (Kaur et al. 2014) |
| 6      | \( \text{S. aureus} \)         | vB_SauM_J525             | Bovine Mammary Epithelial Cells (MAC-T) | (Zhang et al. 2017) |
| 7      | \( \text{B. pseudomallei} \)   | C34                      | BALB/c mice MAC-T           | (Guang-Han et al. 2016) |
| 8      | \( \text{E. coli PS-AmpR} \)   | Uncharacterized (from sewage) | in vitro effect on bacteria | (Porter et al. 2016) |
| 9      | \( \text{S. typhimurium} \)    | P22-B1,P22,PBST10        | Peritoneal mouse macrophages | (Jung et al. 2017) |
|        | \( \text{ATCC19585} \)         | PBST13,PBST32,PBST35     | (RAW 264.7)                 |           |
|        | \( \text{ATCC19585} \)         |                          | Urinary bladder epithelial cell line, T24 (HTB-4) | (Møller-Olsen et al. 2018) |
|        | \( \text{CCARM8009} \)         |                          | A 15-year-old patient       | (P22-B1)   |
| 10     | \( \text{H37Rv} \) (virulent strain of mycobacteria) | D29                      | Peritoneal mouse macrophages | (Lapenkova et al. 2018) |
| 11     | \( \text{E. coli} \)           | K1F                      | Human cerebral microvascular endothelial cells (hCMEC) | (Møller-Olsen et al. 2020) |
| 12     | \( \text{M. abscessus} \)      | Muddy, BPs, ZoeJ (genetically engineered) | in vitro effect on bacteria | (Kolenda et al. 2019) |
| 13     | \( \text{E. coli strain EV36} \) | K1F                      | MG63 osteoblastic Cells HepG2 cells | (Porter et al. 2016) |
| 14     | \( \text{S. aureus} \)         | PP1493, PP1815, and PP1957 |                          |           |
|        |                                |                          |                            |           |
| 15     | \( \text{Salmonella spp} \)    | SR sp1                   |                            |           |
for clinical translation as several such systems are already clinically approved (such as AmBisome and Doxil) (Campoccia et al. 2013; Fenton et al. 2018). A few approaches have been developed to encapsulate phages in liposomes and polymers (Puapermpoonsiri et al. 2009; Cinquerrui et al. 2018). For instance, 100 nm cationic liposome-encapsulated phages, KPO1K2, (average phage size of $\approx 50$ nm) formulated by conventional approach (lipid thin film hydration) ensured effective intracellular delivery inside Klebsiella pneumoniae ($K. pneumoniae$) infected macrophages (Singla et al. 2016). Gentamicin was added to the culture media to kill any extracellular bacteria and only focus on intracellular bacteria. The authors reported that cationic liposomes carrying phages caused 94.6% killing of intracellular $K. pneumoniae$ compared to free phages which accounted for 21% killing after 24 h (Singla et al. 2016). In addition, liposome-encapsulated phages were also shown to be protected against neutralising antibodies compared to free phages (Singla et al. 2016). Another group reported that intraperitoneal delivery of liposome-encapsulated phage cocktails was effective against $K. pneumoniae$ infected burn wounds in BALB/c mice model (Chadha et al. 2017). In this study, phage entrapped liposomes with an average diameter of 229 nm showed an encapsulation efficiency of $79.2 \pm 5.6\%$. After 72 h of treatment, liposome entrapped phage cocktail showed 1–2 log order reduction in bacterial counts compared to free phages in skin, blood, and liver. Although the phage activity on killing intracellular bacteria couldn’t be established from the in vivo experiment (Chadha et al. 2017).

Along with liposomes, nanocrystals and polymers can also serve as an efficient tool for phage encapsulation and delivery (Ma et al. 2008; Colom et al. 2017; Loh et al. 2020) (Figure 4). Fulgione et al. reported that biomimetic hydroxyapatite (HA) nanocrystals effectively delivered Salmonella phage (SR $\varphi$1) intracellularly (Fulgione et al. 2019). HepG2 cells were infected with $10^6$ CFU/mL of $S. enterica$ serovar Rissen followed by treatment with free SR $\varphi$1 ($10^7$ PFU/mL), only HA nanocrystals or equivalent phage-loaded HA nanocrystals for 24 h. Intracellular CFU count revealed reduced count of $10^5$ CFU/ml for HA-SR $\varphi$1 compared to $10^8$ CFU/ml in free phage and only HA group. Extracellular bacteria was eliminated by using gentamicin for 3 h post infection. The authors also confirmed enhanced stability of HA-SR $\varphi$1 at pH 4.0 compared to free phages suggesting a potential use of such mineral crystals at low pH conditions like phagolysosomes (Fulgione et al. 2019).

Overall, these studies suggest that the use of carrier-mediated delivery systems in the form of liposomes or polymers can be utilised for efficient intracellular phage delivery.

### 5. Challenges towards intracellular phage therapy

While phage therapy holds the potential to tackle intracellular bacterial infections, several challenges constrain their translation into clinics. Apart from the usual challenges for translation of phage therapy for extracellular bacteria such as bacterial defence mechanisms (CRISPR/CAS machinery, restriction-modification system) (Barrangou et al. 2007; Enikeeva et al. 2010; Dupuis et al. 2013; Watson et al. 2019), bacterial accessibility (Sousa and Rocha 2019) and phage stability in in-vivo conditions (Nobrega et al. 2016), there are several other challenges for intracellular phage therapy. Intracellular bacteria can develop resistance against phage infection by multiple mechanisms. When these pathogens establish infection intracellularly, they encounter multiple...

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**Table 3. Carrier mediated intracellular phage delivery.**

| Sr No. | Intracellular pathogen targeted | Phage used for the study | Experimental model/cell type | Reference |
|-------|--------------------------------|--------------------------|-----------------------------|-----------|
| 1     | $M. tuberculosis$ / $M. avium$ | Mycobacteriophage TM4 (Adsorbed on $M. smegmatis$) | Mouse peritoneal macrophage cell line, RAW 264.7 | (Broxmeyer et al. 2002) |
| 2     | $M. avium$                     | TM4 (Adsorbed on $M. smegmatis$) | Female C57BL/6 black mice | (Danelishvili et al. 2006) |
| 3     | $M. tuberculosis$              | Phage lissyfp + Mycobacteriophage TM4 (Liposomes) | Human macrophage cell line (THP-1) | (Nieth et al. 2015) |
| 4     | $K. pneumoniae$                | KPO1K2 (Liposomes)       | Peritoneal mouse macrophages | (Singla et al. 2016) |
| 5     | $K. pneumoniae$                | KØ1, KØ2, KØ3, KØ4 and KØ5 Isolated from sewage (Liposomes) | Male BALB/C mice | (Chadha et al. 2017) |
| 6     | $Methicillin Resistant Staphylococcus aureus$ | MR-5 (Liposomes) | Female BALB/C mice | (Chhibber et al. 2018) |

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environmental stresses such as nutrition deprivation, hypoxia, or changes in pH (Trastoy et al. 2018). Such conditions can induce a state of dormancy and render the bacteria metabolically inactive, thus preventing phage amplification and cell lysis (Fisher et al. 2017). Studies on phage infection on bacteria under dormant, acidic, and hypoxic growth conditions are limited. It was shown by Swift et al. that phage D29 was not effective in lysing the bacterial host *M. smegmatis* in a hypoxic environment (Swift et al. 2014). On the contrary, some studies have reported successful phage infection in stationary phase host bacteria (Piuri and Hatfull 2006; Bryan et al. 2016). A recent study has reported that a cocktail of phages was effective in inhibiting *M. smegmatis* growth under acidic, hypoxic and stationary phase growth conditions (Kalapala et al. 2020). Such investigations are critical for designing phage formulations that will be effective in lysing bacteria in their intracellular niche. In addition, stress conditions can trigger the production of outer membrane vesicles that carry phage receptors and can act as a decoy and bind phage while the host bacteria remains unaffected (Manning and Kuehn 2011; Reyes-Robles et al. 2018). Intracellular pathogens may also be sequestered in vacuoles and phages may not be able to target such infections.

It is evident that the occurrence of phage-resistance is inevitable and thus bacterial susceptibility testing is critical before initiation of therapy. Unfortunately, resistance can also emerge during treatment. The application of a phage cocktail is suggested to mitigate the problem. Unlike antibiotics, there are numerous lytic phages for most bacterial species that can be used to form phage mixtures and overcome resistance. Phage resistance can emerge if the bacteria modify its cell surface receptor and blocks attachment of phages. It is expected that similar to extracellular bacteria, use of phage cocktails would decrease the probability that bacteria would develop resistance against all the phages at once. Hence, rational designing of a phage cocktail, identification of bacterial receptors targeted by each phage becomes imperative to design phage cocktails that target diverse bacterial receptors. This would ensure that the evolving phage-resistant population will still be susceptible to other phages in the cocktail (Takeuchi et al. 2016; Gordillo Altamirano and Barr 2020; Yang et al. 2020). A phage cocktail was shown to reduce development of phage tolerance in *M. smegmatis* and *M. tuberculosis* compared to treatment with single phages (Kalapala et al. 2020). Broad-range lytic phages need to be identified or developed and tested against clinical strains and demographically predominant strains in addition to lab strains (Gu et al. 2012; Malik et al. 2017; Forti et al. 2018). Synergy between phages and antibiotics is a widely reported for extracellular bacteria (Comeau et al. 2007). The combinatorial effect of phages along with antibiotics has been investigated in multiple opportunistic pathogens like *E. coli*, *S. aureus*, *K. pneumoniae*, *Acinetobacter baumannii* and *Enterococcus faecalis* (extensively reviewed in (Gordillo Altamirano and Barr 2019; Tagliaferri et al. 2019)). However, there is need to study phage-antibiotic

**Figure 4.** Various phage delivery technologies. Multiple encapsulation and delivery techniques have been implemented to increase the stability and intracellular delivery of phages.
synergy in context of intracellular bacteria as it could pave way for combinatorial treatment to prevent rapid rise in resistance against phages and antibiotics.

The host defence mechanism can exert additional barrier to the circulating phages and influence the pharmacokinetics depending on the route of administration. Immune response can act on phages both by innate and adaptive response. Innate responses include complement mediated lysis or opsonisation of phages, and eventual phagocytosis and degradation by mononuclear phagocyte system (MPS) (Krut and Bekeredjian-Ding 2018). Phages are mostly found in liver or spleen after intravenous delivery which harbours most of the MPS cells (Hodyra-Stefaniak et al. 2015). Antibody production against phages has also been reported that can reduce efficacy of phage therapy (Hodyra-Stefaniak et al. 2015; Krut and Bekeredjian-Ding 2018). Kim et al. showed that attachment of hydrophilic polymers, such as polyethylene glycol, to phages increases the half-life while in circulation and decreases susceptibility to innate and adaptive immune response (Kim et al. 2008). Carrier mediated delivery of phages as discussed above could also modulate the adaptive immune response but needs to be further tested (Singla et al. 2016; Agarwal et al. 2018).

In addition, engineering approaches such as synthetic biology-based phage modification, development of chimeric phages that exhibit strong lytic activity (Lu and Collins 2007; Mahichi et al. 2009) and biomaterial-based delivery can significantly improve the performance of the phage formulation by enhancing uptake by infected cells and targeting the intracellular niche as discussed before (Wijagkanalan et al. 2008; Nitta and Numata 2013; Citorik et al. 2014).

Several phase 1 trials with phages have been conducted which validate the safety of the formulation in healthy volunteers. A list of clinical trials utilising phages for therapy has been tabulated (Table 4). It is expected that the results of these trials would pave the way for bringing phage therapy into regular clinical practice.

### Table 4. Recent phage therapy clinical trials.

| Sr No. | NCT number | Pathogen/Infection | Phage/ Cocktail | Phase of the study | Reference |
|--------|------------|--------------------|-----------------|--------------------|-----------|
| 1      | NCT02664740 | S. aureus and MRSA/ Diabetic Foot Ulcer mono-infections | Not mentioned | Phase 1,2 | (United States of America clinical trial database, n.d.) |
| 2      | NCT04287478 | E. coli and K. pneumoniae/ Urinary Tract Infections | Personalized cocktail | Phase 1,2 | (United States of America clinical trial database, n.d.) |
| 3      | NCT03325475 | S. aureus, P. aeruginosa, or K. pneumonia/ Second degree burn wounds | Phage Cocktail-SPK | Phase 1 | (United States of America clinical trial database, n.d.) |
| 4      | NCT03808103 | Adherent Invasive E. coli /Patients with Crohn’s disease | EcoActive | Phase 1,2 | (United States of America clinical trial database, n.d.) |
| 5      | NCT04191148 | E. coli / Lower urinary tract infections | LBP-Ec01 | Phase 1 | (United States of America clinical trial database, n.d.) |
| 6      | NCT0216010 | E. coli and P. aeruginosa/ Burn wound infections | Described in (Merabishvili et al. 2009) | Phase 1,2 | (Jault et al. 2019) |
| 7      | NCT03140085 | Uropathogens/ Urinary tract infections | PYO phage | Phase 2/3 | (Leitner et al. 2017) |
| 8      | ACTRN12616000002482 | S. aureus/ Chronic Rhinosinusitis | AB-SA01 | Phase 1 | (Ooi et al. 2019) |
| 9      | NCT02757755 | Healthy volunteers | AB-SA01 | Phase 1 | (United States of America Clinical Trial Database n.d.) |
| 10     | NCT00937274 | E. coli / Diarrhoea | T4 phage cocktail | Phase 1 | (Sarker et al. 2016) |

### 6. Future strategies for phage targeting to intracellular bacteria

Despite the advances in the application of phage as a potential therapeutic agent against bacterial pathogens, additional refinements are necessary to ensure sustainability. Some of the major questions that need to be addressed are in terms of the delivery of phages at the site of infection. Since intracellular pathogens are generally present inside specialised vesicles within a host cell, new strategies must be designed for intracellular trafficking of phages/particles to various subcellular sites like phagosomes, phagolysosomes, or some escaping from these vacuoles to the cytoplasm. Chemical conjugation of peptides or administration of small molecules along with phage carrying particles for enhanced uptake can be an innovative strategy to deliver them into the specific subcellular sites. Effective uptake of phage particles can be facilitated by cell-penetrating peptides (CPP). Apart from endocytosis, CPPs are directly translocated by toroidal pore and barrel stave pore formation (detailed uptake mechanism
reviewed elsewhere) (Madani et al. 2011; Gestin et al. 2017). These features of CPPs evade endosomal or vesicular trapping and ensure proper targeting of cargos to specific organelles. Hussain et al. conjugated vancomycin-carrying nanoparticles with cyclic 9-amino-acid peptide CARGGLKSC (CARG) that specifically accumulated in staphylococcal infected lung and skin but not in normal uninfected tissues (Hussain et al. 2018). The peptide targets bacterial surface components as was observed by in vitro labelling. Infected mice treated with intravenous injections of the CARG-conjugated vancomycin particles (one-day post-infection) showed 100% recovery and long-term survival (Hussain et al. 2018). Engineered nanoparticles with specific homing peptides ensured targeted delivery into the intracellular niche harbouring the pathogen. Additionally, genetically modified phages expressing certain peptide sequences on their outer surface can facilitate the uptake process and organellar targeting. By the virtue of protein-ligand interactions, particles can be engineered for targeted delivery using the specific receptors present on the cell surface. For instance, a 2.5-fold increase in cellular uptake was observed with liposomes comprised of 7.5% mannosylated cholesterol compared to bare liposomes in alveolar macrophages (Wijagkanalan et al. 2008). In another report, Yang et al. demonstrated that mannose-functionalised star-shaped antimicrobial polycarbonates were effective compared to control polymer in causing a 3-fold reduction in intracellular M. bovis BCG CFU/mL count after 72 h treatment in THP-1 monocytic cells (Yang et al. 2016). Use of such ligand functionalised polymer could increase the efficiency of intracellular delivery of phage and should be explored further.

Carrier mediated delivery was shown to be more effective in reducing intracellular pathogen as phage internalisation by infected cells was enhanced (Broxmeyer et al. 2002; Nieth et al. 2015; Singla et al. 2016). There are several methods of achieving high phage encapsulation within various carriers. The cost of the polymers and lipids is low compared to biomolecules and generally encapsulation and purification processes are rapid and can be done in a few hours. Cinquerrui et al. proposed a microfluidics-based nano-encapsulation of phages in sub-micron sized liposomes using phospholipid 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) and cholesterol (Cinquerrui et al. 2018). The size of liposomes could be modulated by varying the concentration of cholesterol and regulating the hydrodynamic conditions. Phage T3 with a diameter of around 65 nm and Phage K with a head of around 80 nm and a tail length of approximately 200 nm were used in the study. Interestingly, the compact tail-free head of phage T3 showed higher encapsulation yield compared to phage K (Cinquerrui et al. 2018). Considering the large size of the mycobacteriophages, Nieth et al. bypassed the conventional liposome formulation with alternative approaches by performing rehydration of lipid films by gel-assisted giant unilamellar vesicle formation and inverse emulsion (Nieth et al. 2015). In gel-assisted vesicle formation, lipid film was rehydrated on dried polyvinyl alcohol (PVA) gel using 10% sucrose solution. In inverse emulsion technique, a step-wise lipid bilayer formation occurs where the inner layer was created by water-in-oil emulsion and was placed on top of another oily lipid solution (outer layer) which was emulsified by titration with a blunt end syringe. In both these techniques, a moderate to high encapsulation efficiency was observed (approx. 50% phage positive vesicles in case of gel-assisted technique and almost 100% phage positive vesicles in case of inverse emulsion technique). Both the techniques resulted in large vesicles of approximately 15–20 μm. Conversely, conventional liposome formulation resulted in particle size of around 5 μm but had low encapsulation efficiency compared to the gel-assisted technique. This could be due to large size of phages and low phage concentration used at the time of synthesis. When cell uptake experiments were performed with 5 μm size liposomes, a 4-fold higher uptake was observed in THP-1 macrophage cells compared to the free phages. However, in vitro efficacy of the phages in reducing intracellular bacteria was not reported (Nieth et al. 2015).

Several studies have successfully shown the use of polymers to develop nanocarriers for intracellular antibiotic delivery which can be adapted towards delivery of phages (Toti et al. 2011; Pei et al. 2017; Fenaroli et al. 2020). For instance, polymers like poly (lactic-co-glycolic acid) (PLGA) encapsulated azithromycin enhanced the efficacy of the drug to reduce intracellular Chlamydia infection by decreasing the area of inclusion (proportional to infection load) by 50% in lung epithelial cells compared to the free drug (Toti et al. 2011). Similarly, particle engineering approaches for intracellular delivery of vancomycin showed specific release at lower pH and enhanced MRSA killing by 5-fold compared to the free drug in J774A.1 macrophages (Pei et al. 2017). Such mechanisms will ensure minimal loss of drug while in circulation, thus maintaining an optimal drug concentration only inside pathogen containing vesicles with low pH compared to free drug (Kamaly et al. 2016; Malik et al. 2017; Fenaroli et al. 2020). Phages can also be encapsulated within PLGA microparticles using a
water/oil/water double emulsion process (Puapermpoonsiri et al. 2009). Phages against *S. aureus* and *P. aeruginosa* were encapsulated with 18% and 27% efficiency respectively within 10 μm polymeric microparticles. Modifications were made to the standard double emulsion process to minimise the interaction of phages with the organic solvent which can cause denaturation. The resultant dry powder formulation of phages had good aerosol properties but a low shelf life (Puapermpoonsiri et al. 2009). Phage inactivation due to exposure to organic solvents like ethanol, acetonitrile, dimethylsulphoxide, and dimethylformamide hamper the efficacy of the phage-loaded microparticles (Matsubara et al. 2007). Alternative surfactants like polyvinyl alcohol can be employed to improve formulation stability (Malik et al. 2017). Additionally, phages can be adsorbed on the particle surface to alleviate concerns of denaturation during the fabrication process. Agarwal et al. showed that a mixture of three to five phages can be adsorbed on polymeric (PLGA) particles (Agarwal et al. 2018). The authors were able to load ~10^6 phages/mg of particles and phage-particles were effective against *Pseudomonas aeruginosa* cystic fibrosis mice. Phage adsorbed microparticles significantly reduced *P. aeruginosa* infection by 1.5 log fold in cystic fibrosis mice compared free phages (Agarwal et al. 2018). Although these studies have not focussed on intracellular phage therapy, they provide technologies that can be adapted and explored against intracellular bacteria.

Use of engineered phages to broaden the host range (Yehl et al. 2019), conversion from lysogenic phages to lytic forms (Moons et al. 2013; Zhang et al. 2013), expressing cell-penetrating peptides (Fulgione et al. 2019) and incorporate enzymatics such as endolysins (Haddad Kashani et al. 2017) and phage encoded cell wall degrading enzymes into their genomes have been shown to be effective *in vitro*, which have paved a way for future opportunities. For instance, Xu et al reported enhanced internalisation of T7 phage by mammalian cells when human immunodeficiency virus type 1 TAT peptide was present on its surface (Xu et al. 2018). The authors reported 2-log orders higher uptake of modified phage in mammalian cells (kidney epithelial cells) compared to T7 not modified with TAT peptide (Xu et al. 2018). In another instance, increased internalisation of engineered M13 was reported by HeLa cells (Bhattarai et al. 2012). M13 was genetically engineered to express an integrin binding peptide (RGD) on the major viral coat proteins. The engineered phages demonstrated a 4-fold increase in uptake by HeLa cells compared to wild-type phages (Bhattarai et al. 2012). Dedrick et al. engineered a lytic derivative of phage ZoeJ by precisely removing the repressor gene identified as gene 45 which can efficiently kill *M. abscessus* (GD01) (Dedrick et al. 2019). Using a cocktail of engineered phages (Muddy, BPs33ΔHT-HRM10, and ZoeJΔ45) they reported effective killing of infectious *M. abscessus* strain (Dedrick et al. 2019). Phages can also be genetically engineered to endure acidic environments encountered *in vivo* by displaying phospholipids on the phage capsid (Nobrega et al. 2016). pH-responsive biopolymers like Eudragit® S100 have been shown to protect phage activity from acid damage at pH as low as 2.0 compared to free phages (Vinner et al. 2019). Interestingly, phages were conditioned for maximum release (70%) in a simulated intestinal fluid with pH 7.0 compared to 40% at pH 5.0 (Vinner et al. 2019). The use of pH-sensitive biopolymers as a carrier for intracellular phage delivery may result in specific release of phages within specialised organelles and facilitate the killing of bacteria residing within these organelles. Endocytosis mediated uptake of particles sometimes results in endosomal compartmentalisation and renders it inaccessible to the pathogen residing in the cytoplasm. Cationic polymers containing several secondary and tertiary amines are known to induce osmotic stress by entrapment of protons in the endosome membrane, a phenomenon known as “proton sponge effect”. Trapped protons increase the membrane potential that causes an influx of chloride ions into the endosome. This raises the osmotic pressure which eventually ruptures the endosome and facilitates cytosolic delivery (Freeman et al. 2013; Bus et al. 2018). These mechanisms could be utilised for efficient delivery of phage-loaded particles directly into a specific intracellular site of infection.

### 7. Concluding remarks

Phages are natural predators of bacteria and serve as excellent therapeutic agents against various bacterial infections. Although phage therapy has been well practised against several bacterial infections, studies focusing on the therapeutic efficacy of phages against intracellular infection have remained largely unexplored. By the virtue of residing within a specific intracellular compartment, these intracellular pathogens are challenging to treat. With rising concern over antibiotic resistance, phage therapy could be an alternative approach to reduce such bacterial infections. In this review, we have focussed on discussing the efficacy of phage delivery against intracellular infection. An overview of studies that have used phages in free and
encapsulated form has been highlighted. Liposomes and biopolymers could serve as an efficient carrier to deliver phages into the intracellular milieu. Mechanisms to increase the uptake of phages by the infected mammalian cells are needed to enhance the therapeutic efficacy of phages against intracellular infection. Additionally, the challenges and future strategies pertaining to intracellular phage therapy has also been discussed. These challenges provide ample scope for research to develop phages as a therapeutic approach towards combating intracellular bacterial infection.

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