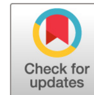

DOMAIN 12 BACTERIOPHAGE

An Overview of Diverse Strategies To Inactivate *Enterobacteriaceae*-Targeting Bacteriophages

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ABSTRACT Bacteriophages are viruses that infect bacteria and thus threaten industrial processes relying on the production executed by bacterial cells. Industries bear huge economic losses due to such recurring and resilient infections. Depending on the specificity of the process, there is a need for appropriate methods of bacteriophage inactivation, with an emphasis on being inexpensive and high efficiency. In this review, we summarize the reports on antiphagents, i.e., antibacteriophage agents on inactivation of bacteriophages. We focused on bacteriophages targeting the representatives of the *Enterobacteriaceae* family, as its representative, *Escherichia coli*, is most commonly used in the bio-industry. The review is divided into sections dealing with bacteriophage inactivation by physical factors, chemical factors, and nanotechnology-based solutions.

KEYWORDS bacteriophages, inactivation, antiphagents, *Enterobacteriaceae*, bionanotechnology

Bacteria-based processes are among the most important in biotechnology and dominate multiple branches of the food and agriculture industries. Many biotechnology companies exploit the natural metabolic properties of bacteria to produce drugs (1), vaccines and antibiotics (2, 3), insecticides (4, 5), dairy products (6, 7), enzymes, biofertilizers (8), organic acids (9), precursors of polymers (10), fuels (11, 12), and solvents (13). *Escherichia coli*, a member of the *Enterobacteriaceae* family, is the most commonly used species (14). The sales of drugs of microbial origin surpass 13 billion U.S. dollars annually (15), and the biotechnology industry is one of the fastest-developing industries globally (16).

The closures of bacteria-based factories result in substantial economic burden to companies, consumers, and the product market. One of the most common reasons for shutting down bacterial factories is infections caused by bacteriophages, i.e., viruses attacking and killing bacteria. Bacteriophages are highly specific in action. exclusively infecting bacterial cells (17), while causing no significant threat to animals and humans (18, 19). They are believed

to be the most widespread biological entities on Earth, totaling approximately 10^{31} bacteriophage particles (20). It is estimated that only 0.001% to 0.1% of the global bacteriophage metagenome is known, and many species of bacteriophages remain undiscovered (21). A single bacteriophage particle (virion) consists of the genome (dsDNA, ssDNA, (+)ssRNA, dsRNA) within a protein capsid (22). Most known bacteriophages belong to the order *Caudoviricetes* (tailed-phages), whose representatives are characterized by the dsDNA genome and an icosahedral capsid with fibers attached to the tail (23). A single family of enveloped bacteriophages, *Cystoviridae*, is also known (24, 25). The size of the virion is usually about 50 to 200 nm; some filamentous bacteriophages of the family *Inoviridae* (e.g., M13) may even reach a length of 400 nm (26). Additionally, larger bacteriophages have been isolated from marine environments with dimensions above 800 nm (27 to 29). However, there are smaller phages (e.g., MS2 phage, 26 nm diameter).

Bacteriophages have evolved precise biological functions that enable them to carry out host identification, subsequent metabolism requisition, and reproduction (30). A temperate bacteriophage integrates its genetic material into the host's chromosome, forming a prophage. The bacteriophage genome can be transmitted as a prophage sequence to daughter cells at each subsequent cell division, in a process called the lysogenic cycle. External factors, such as stress, can activate the prophage, leading to the lytic cycle. This allows for taking control over the host and forcing it to produce copies of the virion. Virulent bacteriophages exclusively propagate through the lytic cycle. The release of progeny virions usually results in the death of the host cell, with limited examples of bacteriophages causing chronic infections (31).

Phages are difficult to eliminate, especially in routine cleaning and disinfection (32). Therefore, bacteriophage infection often spreads rapidly, leading to resilient contaminations, followed by heavy product loss (33). This effect is amplified while operating in biofoundries on large scales (34). These contaminations are extremely damaging to academic laboratories and bacteria-based industries alike (35). Bacteriophage contamination was first reported by Whitehead and Cox in 1935 in a dairy culture, leading to the study of phages for inactivation (36). Today, 1% to 10% of batches of products in the dairy industry are lost to bacteriophages. Over 70% of biotechnological companies have routinely encountered problems with phage contamination (37), with several companies being forced to shut down entirely due to bacteriophage contamination (38). Companies are reluctant to admit to bacteriophage contaminations; therefore, the issue is still poorly addressed.

Due to the difficulty in deactivating bacteriophages in an operating bioreactor, resources are invested in reducing the probability of bacteriophage infection, both at the design level (by optimizing protocols for wastes and raw materials handling, biofoundry and process layout, or personnel training) and by employing microbiological strategies (periodic changes of bacterial strains, cocktails of bacterial strains, and development of bacteriophage-resistant mutants) (39, 40). Additionally, antiphagents (antibacteriophage agents) can provide a more direct solution by eradicating bacteriophage contaminations.

Other reviews on phage inactivation (41) have focused on particular inactivation methods (42) or environmental conditions (43). This review presents a systematic overview of the various approaches for bacteriophage inactivation. *Escherichia coli*, and some other representatives of the *Enterobacteriaceae* family, are the most frequently used models in research practice and in the industry; coliphages are the most commonly used models in research practice. In the industry, coliphages are preferred for phage inactivation or stabilization protocols. Therefore, we focused on this group of bacteriophages. To provide an extensive view of the current trends, we briefly discussed the inactivation methods of bacteriophages that infect commonly used bacteria in the industry. We grouped these methods based on their nature and character, briefly describing the mechanism of action of each group.

PHYSICAL FACTORS

Temperature. Physical bacteriophage inactivation treatments include thermal inactivation, hydrostatic pressure, radiations, electric field, osmotic shock, and variations in pH. Thermal treatment is commonly applied for bacteriophage inactivation at mild or moderate temperatures (i.e., 50 to 95°C) (44). Disinfectants are generally tested in synergy with elevated temperatures to increase efficacy (45). While low temperatures (i.e., below 40°C) are used against pathogenic bacteria, the same does not work against

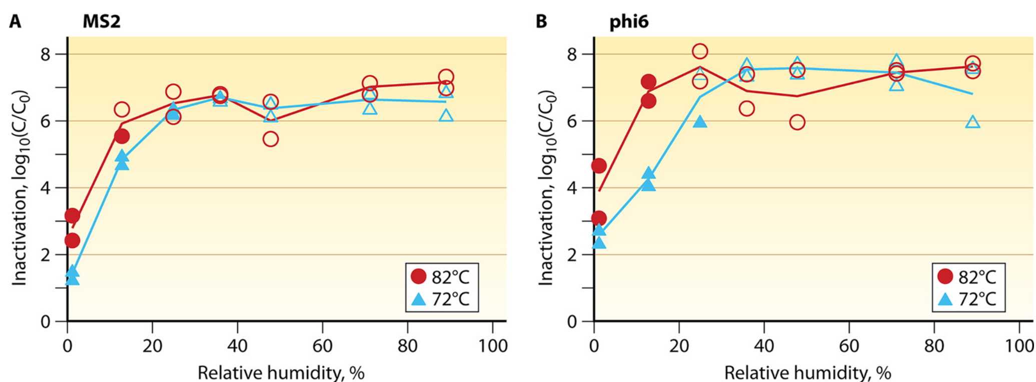

FIG 1 Inactivation of MS2 bacteriophage (A), and Phi6 bacteriophage (B), at 72°C and 82°C for various relative humidity (RH) when viruses were suspended in culture media. Open faded symbols indicate virus inactivation beyond assay detection limits. The image was adapted from Rockey et al. based on the CC BY 4.0 License (53).

viruses. The persistence of enteric viruses is higher at low temperatures (46).

Thermal treatments cause morphological changes in bacteriophage particles, such as the parting of the head and tail structures, aggregation of bacteriophage tails, and release of DNA (47). A group led by Evilevitch showed the immediate ejection of DNA through the vortex portal, a protein structure that is a "gate" to the capsid (part of the virus, where genetic material is stored) at high temperatures (65 to 70°C) (48, 49). They also found that the DNA of Lambda bacteriophages (targeting *E. coli*) exhibits temperature-induced transition resulting in structure, energy, and mobility variations. Below the transition temperature, DNA has restricted mobility. This phenomenon delays or completely prevents its release, even when the capsid is "opened" by a receptor molecule (50, 51). This result is the highest activity of the Lambda bacteriophages at temperatures just above the transition of DNA but not too high, which could trigger the "ejection through portal" mechanism.

The effect of elevated temperature on bacteriophages was also studied by Brié et al. (52), who studied MS2 bacteriophages. MS2 is a bacteriophage targeting *E. coli* F+ strains and is considered a good model for rhinoviruses. Above critical temperature (72°C), MS2 virions were destroyed, and the genome was released. At lower temperatures, the bacteriophages remained virulent: no "ejection" mechanism was reported (52). Rockey et al. studied the effect of various relative humidity (RH) and high temperatures (53). It was observed that RH of 20% was essential for effective heat inactivation of MS2 and Phi6 bacteriophages (Fig. 1).

For bacteriophages to be deactivated, the temperature must be high enough to exceed the energy barrier characteristic for a given bacteriophage and trigger at least one mechanism of deactivation (50, 54). However, it is important to note that some bacteriophages can resist extreme conditions, such as boiling in 90°C for 15 min (42). Bacteriophages can survive the processing environment, including pasteurization procedures (72 to 75°C, 15 to 30 s), due to their high thermal resistance (55). Higher temperatures, especially in dairy industries, also lead to a subsequent denaturation of whey proteins. Overall, thermal treatments are difficult to implement in biotechnological processes, limiting their potential as an effective antiviral treatment modality.

Pressure. To avoid the undesirable consequences of heating, novel nonthermal inactivating processes have been gaining popularity in the last few years. In this regard, high-pressure systems are a promising technique, because they ensure the retention of chemical and physicochemical properties of the final products (42). High hydrostatic pressure (HHP) and high-pressure homogenization (HPH) are two such pressure-based techniques that have been well studied (42). High-pressure processing (HPP), in particular, was a successfully implemented alternative to nonthermal processing technology in the food industry so far (56). DHP (dynamic high pressure), as the name suggests, is a dynamic process that allows the treatment of large quantities of the product (57).

It has been observed that bacteriophage inactivation is proportional to the pressure and the number of passes (42, 57, 58). It has also been reported that a pressure of 300 MPa at a

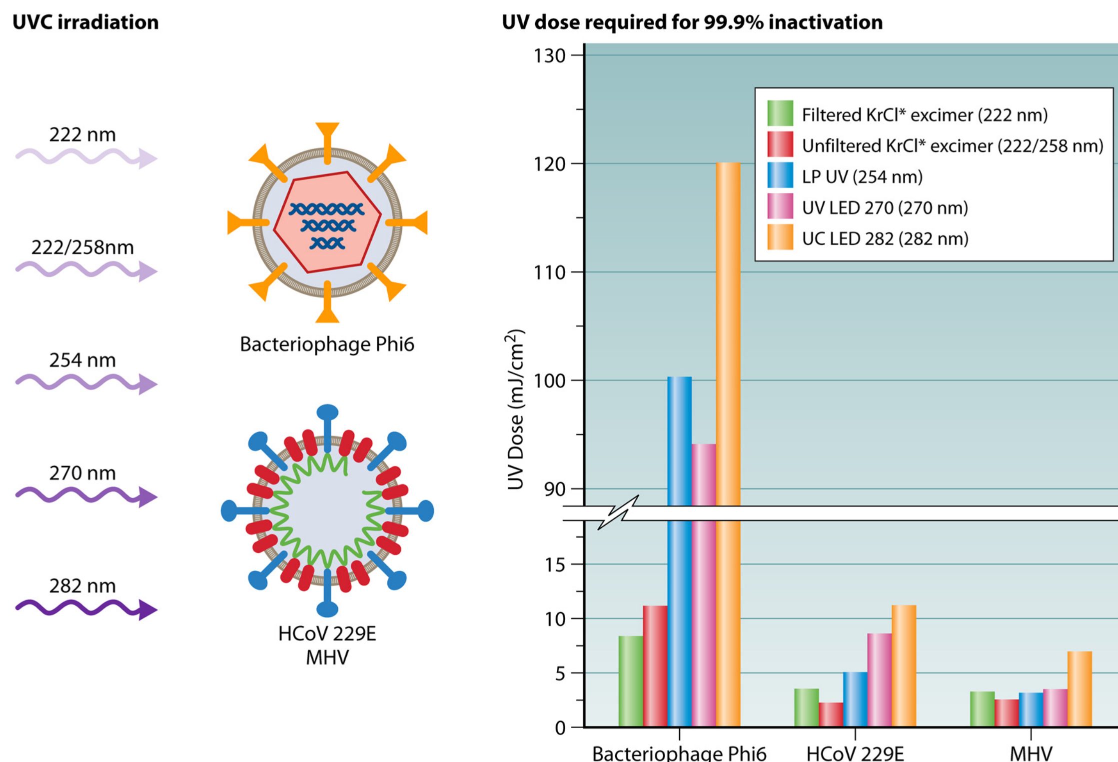

FIG 2 Inactivation of models of coronavirus by UV-C irradiation. In this figure, Phi6 bacteriophage and murine hepatitis virus (MHV) were exposed to various wavelengths of UV-C to cause 99.9% inactivation. As a control, the wavelengths used for krypton chloride (KrCl*) generation were used. Adapted from reference 96 with permission of the publisher (copyright 2021 American Chemical Society).

temperature range of 25 to 40°C can cause a 2log (99%) reduction in the bacteriophage titer (56). However, such studies are opposed by other experiments showing bacteriophages' resistance toward high pressure. For example, little or no inactivation of *E. coli* Qβ and c2 bacteriophages was observed in culture media after treatment at $\leq$400 MPa. A complete inactivation (8log) was achieved when this pressure was increased to 800 MPa (59).

It is hypothesized that the long form of the prolate head makes bacteriophages more sensitive to high-pressure treatments than other isometric structures that are more stable (57). Most bacteriophages, however, show resistance to high pressures. *Salmonella* bacteriophages, for example, are unaffected by HPP up to 250 MPa (56). This suggests that pressure might be effective, but is not a universal antiphagent.

Radiation and electric field. The inactivation of bacteriophages via radiation relies mostly on the generation of free radicals (60). These radicals affect the viral genome, causing damages in the genome (61). The first experiments using radiation for the inactivation of bacteriophages were performed over 40 years ago, using high-energy electrons (60, 62). The effect of X rays (63 to 70), along with the X-rays (67) and gamma rays (71 to 79), were investigated as bacteriophage-inactivating factors.

Nowadays, less harmful radiation and more precise applications are preferred. With the SARS-CoV-2 pandemic, virus-radiation research underwent a resurgence. UV radiation is a compromise among ionizing properties, ease of use, and limited risk. Its impact on bacteriophages is known in laboratory conditions, including experiments in water solutions (80 to 95). To enhance the virucidal effects of UV radiation, irradiation is sometimes combined with various photocatalysts, e.g., titanium dioxide (90, 92). Usually, UV radiation of a wavelength from the spectra of UV-A and UV-C is used (Fig. 2). However, near-UV radiation was also found effective (96 to 101).

The widespread usage of next-generation lasers allowed for bacteriophage inactivation in a more precise way. Laser-

based methods use different wavelengths (including UV spectrum), but as monochromatic laser light instead of dispersed light. All of these methods are characterized by short times of irradiation since relying on femtosecond (102 to 109), picosecond (110), or subpicosecond lasers (111) (femto- and picoseconds adequately).

Recently, photoinactivation-based methods have become increasingly popular. In principle, photoinactivation is inactivation by visible light combined with a light-sensitive indicator that releases reactive oxygen species (ROS) (112). Two types of photoinactivation may occur: (i) type I—excited photosensitizer returns to the ground state with oxygen, forming oxygen radicals; and (ii) type II—energy is transferred from triplet state photosensitizer to ground-state oxygen, forming singlet state oxygen (113). Microbial photodynamic inactivation (PDI), especially bacteriophage photoinactivation, was very effective in bacteriophage removal from wastewater (113 to 121).

Another bacteriophage-inactivating factor is the electric field. Since bacteriophages' capsids are composed of different proteins, each has a charge and isoelectric point (122) and is sensitive to electric field changes. A pulse electric field (PEF) is used most frequently. Even though this method is usually used for bacteria elimination, PEF seems to be also efficient for bacteriophage inactivation in a relatively short time—up to 15 min (123 to 125). The application of low-intensity and low-frequency electric fields as an antiphage factor is rare nowadays. Staczek et al. showed that low-frequency electromagnetic fields alter the replication cycle of MS2 bacteriophage (126). Richter et al. used electric field to orient virions for sensing applications and did not observe any adverse effects (127, 128). Grygorcewicz et al. showed a rotating electromagnetic field's positive effect on the infectivity of bacteriophages (129).

Rahaman et al. proved the complex application of electric field and electrochemical multiwalled carbon nanotubes (EC-MWNT) could be used for both MS2 bacteriophage inactivation and bacteriophage removal by filtration. Authors reported the reduction of bacteriophage titer by about 7log when the potential of 2 V was applied during the filtration through the MWNT filter at a constant permeate flux 140 L * m^{-2} h^{-1} (130). Vecitis et al. used a similar protocol for the simultaneous removal of *E. coli* and MS2 bacteriophages from water solutions. The decrease in bacteriophage titer was similar (7log), but the authors also reported the elimination of 7log *E. coli* within 30 s when the potential of 3 V was applied (131).

Another method for bacteriophage inactivation is the usage of discharge-generated ions, which are particularly effective against airborne viruses. In such protocols, the electric field is applied to the carbon-brush fibers, creating a nonuniform field that ionizes the air. Depending on the number of ions per bacteriophage particle, discharge-generated air ions inactivate up to 4log of coliphage MS2 (132) and against coliphage PhiX174 (133). The effectiveness of this protocol remains similar in water solutions (123). Kettleson et al. reported the usage of an electrostatic precipitator (ESP) for 2log reduction of coliphages T3 and MS2 (134). Also, Drees et al. described the electrochemical method for bacteria and bacteriophage inactivation. As model bacteria, *E. coli* ATCC 15597 and *P. aeruginosa* ATCC 15224 strains were used; the effect on bacteriophages was tested on coliphage, MS2, and *Salmonella*-phage PRD1. The exposure of *E. coli* to 5 mA direct current resulted in a decrease in bacterial titer by about 2log within 5 min, while the same current caused the reduction of MS2 bacteriophage titer by 2.5log within 20 min (135).

The usage of radiation for bacteriophage inactivation appears to be effective and quick. However, the use of radiation is nonspecific, making it dangerous for personnel and corrosive to labware. Due to the nature of irradiation, all radiation-based inactivation protocols are effective on flat surfaces, as the radiation cannot pass through certain materials. Apart from UV radiation, most protocols require expensive equipment and qualified personnel.

Osmotic shock and pH. Exposing bacteriophages to significant changes in the concentration of salts in the solution causes the destabilization of bacteriophage particles. Virions are destabilized by the osmotic shock and burst (136), causing their inactivation (137). Previous reports show that a 10-fold elevation in salt concentration inactivates up to 90% of the bacteriophage population (138). This process is also rapid, taking effect within a minute of concentration change (139). The effects of osmotic shock on bacteriophages were investigated mainly in the 1960s and 1970s (140 to 142), but were largely overlooked as a sterilization technique. Osmotic shock can also be used to prepare bacteriophage ghosts (143, 144), viral protein shells devoid of genetic information.

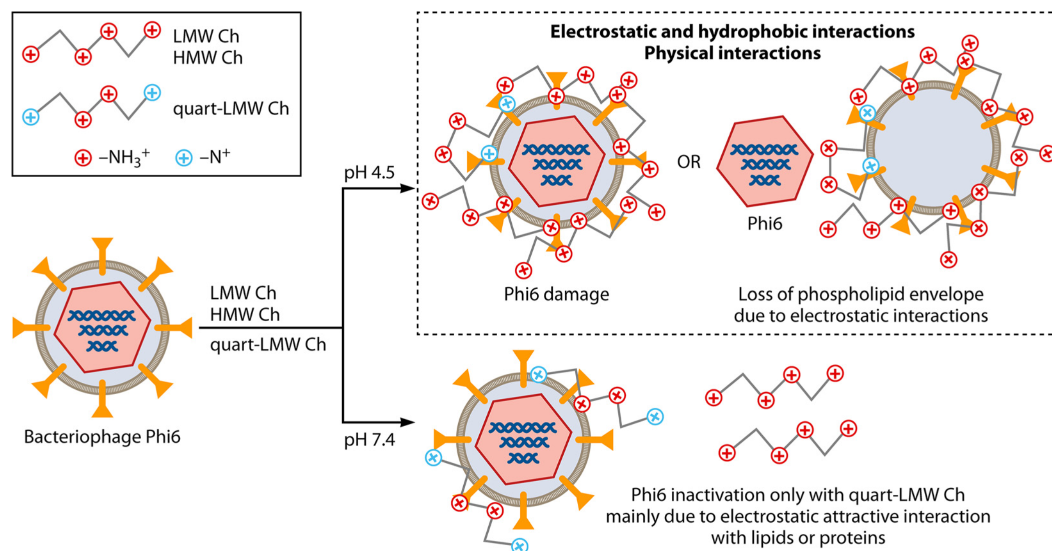

FIG 3 The inhibitory mechanism of chitosan antiviral agents on enveloped bacteriophage Phi6, using low molecular weight chitosan (LMW Ch), quaternary LMW Ch, and high molecular weight chitosan (HMW Ch). The image was adapted from Plohl et al. based on the CC BY 4.0 License (298).

Although pH change is not commonly used for bacteriophage inactivation, bacteriophages can undergo inactivation when stored in improper pH conditions. Studies have shown bacteriophage inactivation within 15 s when subjected to a very acidic pH (pH = 1) (145). Bacteriophages are much more susceptible to pH changes than other viral species; e.g., coliphages undergo up to a 5log titer decrease in viral particles at basic pH levels (pH 9 or 10), as opposed to a 1log titer decrease in SARS-CoV-2 viruses stored at the same pH (146). Some bacteriophages, such as coliphage PhiX174, show higher resistance toward pH change compared with other species such as MS2, which are completely inactivated in an acidic pH (147).

pH-resistant bacteriophages are desirable for use in bacteriophage therapy in humans through oral delivery methods; the use of genetically modified coliphages T7 has been describe in the literature. However, the phages are prone to attack by enzymes present in the gastrointestinal tract and must be adequately shielded (148).

CHEMICAL FACTORS

Polymers. The trend toward sustainable and low-toxicity approaches in phage inactivation methods led to research in bacteriophage-inactivating polymers. As a first choice, poly-amino acids appeared promising, especially poly-L-lysine. Some inactivating properties were found for L-lysine monomers, such as commercially available α-poly-L-lysine (100 monomers) (149, 150), and ε-poly-L-lysine (~30 monomers). The latter is produced by bacteria belonging to the *Streptomyces* species and was proved capable of effective inactivation of coliphages T4 and T5 (151). Additionally, the antiphage properties of another poly-amino acid, poly-DL-alanine, was also confirmed (152). Both polylysine and polyalanine are believed to bind to phosphate moieties of bacteriophage DNA, causing its precipitation and subsequent inactivation (151).

Polymeric substances can also be applied as micelles. Polymeric amphiphiles combine a stable structure with antiviral properties (153). The mechanism of action is the prevention of bacteriophage absorption by the host cells. For example, many viruses have lectin receptors. Since the PEG-polylactide copolymer surface modified with galactose can interact with lectins, these receptors can be blocked by the copolymer (154). These prohibit the virus from absorption and prevent its amplification.

Recent reports suggest certain polysaccharides and their derivatives, such as chitosan (a derivative of chitin) (155) (Fig. 3) or poly(*N*-2-hydroxyethyl acrylamide) (156), can exhibit antiviral properties. Chitosan appeared to be particularly effective in the inactivation of coliphages and bacteriophage 1-97A, causing the reduction of bacteriophage titer of about 5log (157). It is known that such polymers can inactivate mature bacteriophages and inhibit bacteriophage

replication, although the particular mechanism of action remains unknown (158).

Proteins. Another class of molecule capable of exhibiting antiphage activity is proteins, with the most popular being eukaryotic antibodies. Antibodies exhibit high specificity for their target antigen and are generated by the immune system in response to a viral infection (159, 160). Through a complex interplay between various cell types, the immune system recognizes bacteriophages as foreign, potentially hostile, T-dependent antigens and develops bacteriophage-specific antibodies to combat the infection (161). Additionally, blood plasma proteins can inactivate most bacteriophages (162).

Natural extracts. Natural extracts are bioagents against many microbial infections and are popularly being incorporated as disinfectants in industries due to being readily available and economically viable on an industrial scale.

Tea is one of the most popular beverages worldwide. In traditional Chinese medicine, it has been used for thousands of years due to its health benefits (163); antibacterial and antiviral properties of tea extract help bolster the immune system. Tea extracts have previously displayed inactivating properties against microbes (164).

Other extracts of herbs such as *Thymus vulgaris* (thyme) (165), *Scutellaria baicalensis* (166), and *Salvia rosmarinus* (rosemary) (167) are also employed for antiviral studies. Blueberry extracts have caused a complete inactivation of MS2 bacteriophages for 7 days (168).

Pomegranate juice inactivates human enteric viruses such as MNV-1; however, it is inefficient against bacteriophages (169). This is a vivid demonstration that the quest for a universal plant extract effective against a wide range of bacteriophages has not been successful and remains an unlikely possibility.

Bacteria are also known to produce compounds that provide resistance against bacteriophages, by intercalating with their DNA and inhibiting replication. Recently, a review paper discussing antiphage small molecules produced by bacteria summarized the reports on numerous compounds inactivating *E. coli* phages, including MS2, f2, fd, Lambda, and T-phages (170). There are 11 such compounds known so far, of which daunorubicin, doxorubicin, epirubicin, and idarubicin are most commonly studied and applied in various industries. These molecules were first found to be produced by *Streptomyces* spp. (171). Other natural defense molecules increase the permeability of bacterial cells; examples of such compounds are dequalinium chloride and di-benzimidazole (172). One of the main drawbacks of using natural extracts is that they are nonspecific in their targeting.

Commercially available disinfectants. Phage eradication includes routine cleaning processes and disinfectants (32). Basic methods include the appropriate choice of equipment, process design, and extensive cleaning and sterilization (38). Chemical treatments encompass several disinfectants such as benzalkonium chloride, chlorhexidine, hydrogen peroxide, triclosan, polyvinylpyrrolidone-iodine, alkaline detergent mixtures, potassium peroxymonosulfate, and quaternary ammonium compound-based sanitizers, to name a few (173 to 175). In laboratory practice, chemical agents such as Virkon S, Triclosan, and ethanol (75% concentrated) are often used to prevent bacteriophage contamination (176 to 178).

Several viral disinfectants are commercially available, with a range of active substances such as formaldehyde, caustic soda, potassium peroxymonosulfate, and acetic acid, which inactivate phages in a variety of methods (179). While alcohol disinfectants target protein denaturation, aldehyde agents disrupt proteins via alkylation (180). Some of the most successful compounds responsible for virus inactivation include quaternary ammonium, glutaraldehyde, and sodium hypochlorite (181). The mode of action of quaternary ammonium compounds is reliant on the disruption of the viral envelope with subsequent release of the nucleocapsid, or the prohibition of viral fusion and subsequent replication (182). The action of sodium hypochlorite relies on the release of chloride ions (181) and targeting amino acids on the surface protein (183).

Sanitizers also contain certain active substances against viruses; some include ethoxylated nonylphenol, potassium peroxymonosulfate, and Triclosan (5-chloro-2-(2,4-dichloro phenoxy) phenol). Potent food additives such as oxidizing agents, halogenated agents, and alcohols are also explored to be added to the substrates of food industries (184). Bacteriophages are generally resistant to common soaps, antibacterial liquids, and antiviral solutions (e.g., Virusolve, dish soap, and Line-Antibacterial 70) (185).

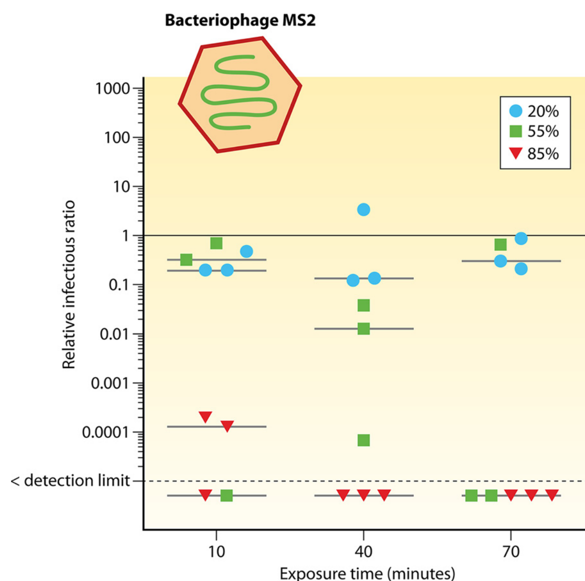

FIG 4 Ozone effect on bacteriophage MS2 infectivity at three levels of relative humidity and three exposure times. The solid line represents the reference value without ozone. The dotted line represents the detection limit. Twenty percent RH values are represented by circles (●), 55% RH by squares (■), and 85% RH by triangles (▼). The image was adapted from Dubuis et al. based on the CC BY 4.0 License (299).

Some experiments also suggest ozone as an antiphage agent as it can break the protein capsid and release the viral genome, thereby hampering adsorption to the host cell (186) (Fig. 4). The inactivating properties of gaseous ozone were observed against coliphages MS2, PhiX174, and T7, and *Pseudomonas* bacteriophage Phi6 on the gelatin-based medium surface (187). Aerosolized bacteriophages were effectively inactivated by ozone by about 3log, with the inactivation titer dependent on ozone concentration (188). Mik and Groot described the inactivation of coliphage PhiX174 by ozone and ozonized cyclohexane, observing the decreases of bacteriophage titer by 6log and 5log (189). For filamentous bacteriophage f2 (targeting *E. coli* K-12 strain), the exposure to 0.06 mg/L ozone for 600 s resulted in the complete inactivation of bacteriophages (9log decrease of bacteriophage titer) (186). For coliphage T1, the reduction was about 5log (190). Additionally, ozonation appears to be an efficient antiphagent in aqueous solutions. Komanapalli and Lau reported the inactivation of Lambda bacteriophage in SM buffer solution within 10 min by about 8log (191). However, another paper described that the presence of kaolin and activated sludge reduced the effectiveness of ozonation against coliphage T2. This may be due to absorption of bacteriophages on the surface of suspended solids (192). Recently, a water sterilization method for applying ozone nanobubbles (NB-O_3) was proposed and was shown to inactivate *Aeromonas hydrophila* bacteriophage pAh6 effectively. After 5 min of treatment, a decrease in bacteriophage titer of about 6log was observed (193).

One of the most prevalent chemical disinfection methods is chlorine, mostly in food-related industries. A clear drop in the PFU/mL (plaque formation unit per mL) of MS2 is observed using chlorine (194). However, some countries, such as Switzerland, Austria, and Germany, have banned large-scale chlorination as chlorine in drinking water may be carcinogenic in nature (195).

The harmful effects of ozone and chlorine on living organisms (including humans) due to their oxidizing properties have been known for more than 40 years (196). Additionally, the usage of chlorine and ozone is limited to specific conditions because of their tendency to corrode metals and alloys (197). On the other hand, using milder disinfectants, e.g., ethanol, is not effective against bacteriophages (41).

Reactive oxygen species (ROS). Reactive oxygen species (ROS) have been identified as an effective platform for developing antimicrobial agents (198). Hydroxyl radical, superoxide anion, singlet oxygen, alpha oxygen, and peroxides are the most common examples of reactive oxygen species generated by different techniques. Bacteriophages can be inactivated by producing ROS, resulting in DNA cleavage and virus inactivation. Such a mechanism was witnessed when the compound resveratrol forms a complex with Cu(II) and results in the redox cycling of copper. This results in the generation of ROS, which inactivates bacteriophages (199).

Decontamination can also be carried out using singlet oxygen ($^1O_2$) generated, for example, by fullerol suspensions (200) and cationic porphyrins (201). The antiviral properties of titanium oxide surfaces are due to ROS generation by photo-excitation (202). In other experiments, ROS-containing water nanodroplets are electro-sprayed to inactivate bacteriophages (198). Some nanoparticles can also inactivate bacteriophages by generating reactive oxygen species (ROS) (203, 204). In other experiments, surface discharge of ROS and reactive nitrogen species (RNS) were utilized to inactive bacteriophages (Fig. 5) (205). Plasma or plasma-activated water was added to bacteriophage suspensions, and the subsequent fall in infectivity was recorded. Plasma treatment of 80 s resulted in more than 99.99% bacteriophage loss, while an exposure of 100 s caused complete inactivation of T4 bacteriophages.

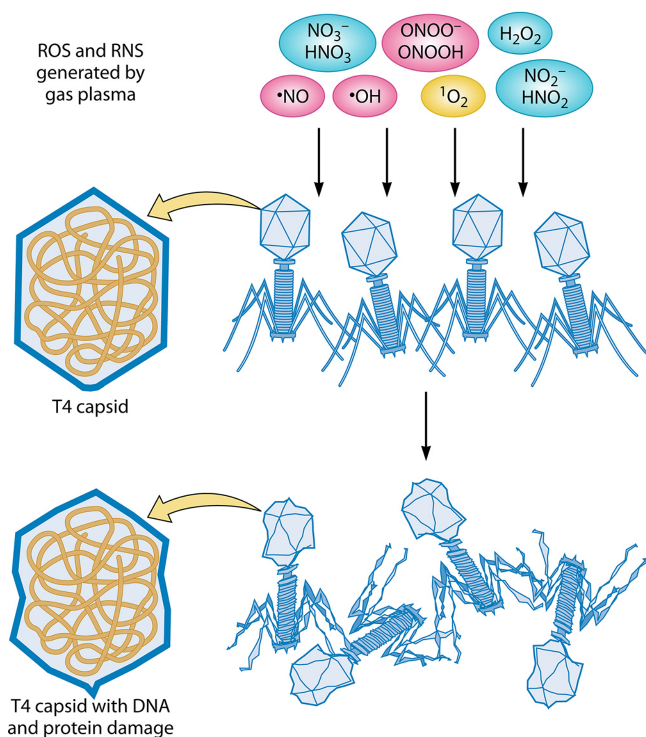

FIG 5 The inactivation of bacteriophage T4 by reactive oxygen species (ROS) and reactive nitrogen species (RNS) of plasma. The image was adapted from Guo et al. based on the CC BY 4.0 License (205).

Notably, not all studies found it efficient to inactivate bacteriophages by ROS (206). Some literature points out that bacteriophages can inhibit ROS formation, providing possibilities for bacteriophage-based cancer treatment (207, 208). This also makes this method not a universal antiphagent.

Salts. A critical factor affecting bacteriophage stability and activation is the presence of specific salts (ions) in bacteriophage suspension in water-based buffers. For instance, T-phages (targeting *E. coli* strains) require magnesium salts in the environment for proper amplification (209). A deficiency in these salts can lead to the inactivation of bacteriophages (210). Another example is the inactivation of calcium-dependent bacteriophages by the complexation of calcium cations (211). Calcium is required for the proper structure change of the bacteriophage baseplate, which is essential for bacteriophage DNA release during the infection (212).

Certain metal salts are usually used as antiphagents, with the most important heavy metal salts, including lead (210), mercury (210, 213, 214), copper (199, 215 to 224), and cadmium (225, 226) salts. It is generally accepted that heavy metal cations bind to bacteriophage proteins, causing changes in folding that lead to the loss of structure and function (227). This happens due to the binding of metal ions in the salt to specific amino acids sequences (228). There are also reports on the inactivation of bacteriophages with the ions of light metals, such as sodium, potassium, or calcium (210, 229, 230) on *Salmonella* bacteriophage PRD1.

Additionally, "precious metal salts" can be used for bacteriophage inactivation. Silver is the cheapest in this category, making it an economically viable option. Another advantage of silver compounds is their customizability, enabling the regulation of antimicrobial activity by changing the attached ligand (231). Although silver nanoparticles are often used for phage inactivation, silver ions have been used as well (232, 233). Other precious metal salts are platinum (234 to 238), gold, and palladium salts, although they are not as frequently used as silver salts (236).

Some metal salt combinations can provide reversible bacteriophage inactivation, such as potassium cyanide. Cyanide (CN−) ions bind to bacteriophage capsids causing temporary inactivation. When complexed with metal cations, e.g., gold (Au^{3+}), the inactivating effect is overcome, leading to phage reactivation (239). Additionally, high concentrations of sodium citrate have been described as bacteriophage-inactivating, causing the decomposition of viral capsids (240).

In additional to metal salts, metal oxides can also be used as influential bacteriophage-inactivating factors. The most frequently used are zinc oxide and titanium (II) oxide. Their mechanism of action relies mainly on the generation of ROS or photoinactivation, as described in previous chapters.

The major drawback of the usage of salts, especially salts containing metals, is their effects on living organisms and the environment. When released into the atmosphere, heavy metals tend to aggregate in soil (241), building up to toxic concentrations in soil bacteria and plants. Additionally, they are not specific against bacteriophages (242).

NANOPARTICLES

Nanotechnology is often applied to microbiology to tackle the growing concerns of contamination. The extent of toxicity of nanomaterials can be mediated by their size, charge, and composition (243). Nanoparticles may achieve viral disinfection

via the release of toxic ions (244 to 246), ROS generation (203, 204), or by blocking specific viral proteins (247).

New biomedical applications have unraveled with the growing advancements of nanotechnology linked with microbiology, especially with silver nanoparticles (AgNPs). More than 500 tons of nanoparticles per year are now supplied to meet different industries' demands, drawing attention to their biological activity, safety, and mechanism of action (248). AgNPs attack bacteriophages via three mechanisms: adsorption, the release of ions, and ROS generation (249). It was also shown that AgNPs could bind to the exposed C-terminal amino residues and cause up to 96% reduction in PFU/mL of bacteriophages, thereby assisting in decontaminating commercially viable products (246).

Experiments combining silver nanoparticles (AgNPs) with bacteriophages have resulted in a lower MIC of AgNPs (from 1.1% to 0.13%) and minimum bactericidal concentration (from 2.15% to 0.25%) compared to silver nanoparticles alone (250). Some experiments with colloidal silver nanoparticles have presented a complete inactivation of MS2 and T4 bacteriophages, with a starting concentration of 10^3 PFU/mL (251). Moreover, bacteriophage contaminations can also be detected by using silver nanoparticle-based inks (252). Other antiviral applications of silver include silver-nanoparticle-decorated silica hybrid composites for water disinfection (253), to coat air filters (254, 255), amine-functionalized glass substrate immobilized with silver nanoparticles (251), and silver-doped titanium dioxide nanoparticles (256) for drinking water treatment. Antiviral inactivation by silver nanoparticles is enhanced by impregnation with granular-activated carbon (GAC) (245). A coating of GAC modified with AgNPs on household filters resulted in 3log reductions in PFU/mL of T4 (257).

Richter et al. worked on negatively charged gold nanoparticles coated with various ratios of negative (11-mercapto 1-undecanesulfonic acid) and hydrophobic (1-octanethiol) ligands. The study aimed to establish ratios that could inactivate bacteriophages without damaging bacterial cells. Such nanoparticles have the potential to be directly used in applications that require selective removal of bacteriophages (247).

Iron nanoparticles also play an essential role in the decontamination of bacteriophage infections. Hematite nanoparticles can decontaminate up to 1.5log of MS2 bacteriophages within 45 min (258). Smaller sizes of iron nanoparticles result in a greater inactivation effect by providing a larger surface area for inactivation and allowing easy dispersion (259). Zero-valent iron nanoparticles (nZVI) are popularly used to inactivate bacteriophages along with some eukaryotic viruses (260). Bacteriophages react uniquely to nZVI, with some studies showing that M13 bacteriophages are most vulnerable against nZVI (7log inactivation), while T7 bacteriophages show maximum resistance. Moreover, the nZVI effects on UZ1 bacteriophage targeting *Klebsiella aerogenes* in an aqueous system have been explored (261).

Effects of nanoparticles of zinc, fullerene, titanium dioxide, gold oxide, and copper oxide on bacteriophage inactivation have also been explored (204, 262 to 264). Examples of inactivation by metallic nanoparticles include spray-dried alumina granules (265) and silver and zinc oxide nanoparticles (263). Metal oxide nanoparticles (ZnO, $ZnTiO_3$, MgO, and CuO) can also be combined with UV irradiation to inactivate bacteriophages (266).

The antiviral properties of nonmetallic nanoparticles are mostly limited to carbon nanomaterials (267). Carbon nanotubes (CNT), single-walled or multiwalled, seem to be the most promising bacteriophage-inactivating structures. By increasing surface area, the number of bacteriophage particles absorbed on that surface is much higher. This approach was effective in water solutions and bioaerosols (130, 131, 268 to 275). Other carbon nanostructures were seen as potentially promising antiphage factors, such as carbon dots (276, 277), fullerene (262, 278, 279), fullerol (200, 280, 281), graphite (282), graphene (283), and graphene oxide (GO) (284).

Silica nanoparticles, although less studied and used, operate with a similar mechanism of action. Enhanced bacteriophage absorption and attenuation on the surface of such nanoparticles were described (285, 286). No inactivation by a suspension of silica nanoparticles was noticed (286), although bacteriophages can be immobilized on silica particles, which can result in an apparent decrease in bacteriophage titer. Cademartiri et al. presented that silica particles modified with poly(ethylene) glycol can absorb bacteriophages targeting foodborne pathogens, including *E. coli* (VB-EcoM-AG2), *S. enterica* serovar *Enteridis* (VB_SenS-AG11), and *Shigella boydii* (VB_SboM_AG3). Depending on the bacteriophage, a decrease in titer up to 8log (10^8 PFU/mL) was observed after overnight incubation with these particles (287). Bone et al. reported the immobilization of T4 bacteriophage on 1 μm amino-functionalized silica particles. Flow cytometry proved that a single piece of

such particles could bind about 20 bacteriophages and that the number of absorbed bacteriophages is proportional to the particle's diameter (288).

Similar to metal salts and ions, nanocompounds can be harmful to the environment (289, 290). Moreover, there are already reports on the recognition of nanoparticle-resistant bacterial strains, which suggest that the overuse of nanocompounds for bacteriophage inactivation may cause widespread nanoparticle resistance (291, 292), showing similarity with antibiotic resistance in bacteria. Although promising, the nano-related approach requires proper waste management procedures and regulatory clearance before being used on an industrial scale.

Inactivation of other groups of bacteriophages. Despite *Enterobacteriaceae*-targeting bacteriophages being the target of this review, interesting methods for bacteriophage inactivation have been described for other species of phages.

For examining temperature inactivation, Lactococcal bacteriophages appear to be good models. This is due to their increased resistance to high temperatures and pressures compared to coliphages (57). Elevated temperatures generally inactivate cold-active bacteriophages (infectious at ≤4°C). Bacteriophage 9A, an example of a cold-active thermolabile bacteriophage, is rapidly inactivated over a temperature range of 25 to 55°C (293). Bacteriophages P001 and P008 experience a fall in titer by 7log and 2log orders, respectively, when the temperature is elevated to about 75°C, ranging from 16 s to 120 min, depending on the phage (47).

Unfortunately, 9log unit inactivation of the heat-resistant P1532 bacteriophage at 95°C leads to more than 95% whey protein denaturation (44). Other resistant bacteriophages, like bacteriophage P680, are detected even after a heat treatment at 95°C for 30 min (294). Phi6, a unique representative of enveloped bacteriophages, was also an object of examination of its pH tolerance (295). Moreover, the complex effects of iron, aluminum, nickel, chromium, and copper salts were investigated on this bacteriophage. The authors claimed the virucidal properties of metal salts rely on the change of pH due to salt hydrolysis (233). Nonmetal salts have very limited application; there are but several reports on the inactivation of dairy bacteriophages with ammonia (296) and its salts, namely, ammonium chloride (42) and ammonium sulfate (297).

Conclusion. Bacteriophage contamination is a growing concern for bacteria-based industries. Such infections tend to recur and are resilient to sterilization protocols. A variety of approaches have been explored to fight bacteriophages. Within the vast literature of phage inactivation, solutions for *Enterobacteriaceae*-targeting bacteriophages are the most crucial, as *E. coli* is a model bacterium in most laboratories and industries. Further research into phage inactivation is needed for the generation of novel antiphagents of higher efficiency and broader application. In this paper, we reviewed different techniques adopted to reduce bacteriophage titers.

Antiphagents can be primarily categorized as physical and chemical. Nanotechnology also contributes significantly to the inactivation of bacteriophages. While bacteriophages resist most physical factors, extreme conditions such as high temperature (95°C and above), high pressure, intense irradiation, and sudden changes in pH cause significant reduction in bacteriophage titers. Several chlorine-based disinfectants also inactivate bacteriophages, especially in industries and laboratories. The examples of usage of each method are summarized in Table 1.

Despite this plethora of antiphage approaches, industries consistently struggle with bacteriophage contaminations. A universal antiphagent, effective against all kinds of bacteriophages and applicable in every condition, is yet to be found and is unlikely to exist, given the large number of phage species and the variation in genotypes between them. Each method has drawbacks and limitations and are more suited for specific context-dependent applications. Bacteriophages are generally resistant to the effects of temperature and electric fields, and radiation usage is expensive and requires specialized equipment. Chemical disinfectants and ROS-related approaches are corrosive and nonspecific. The effectivity of pressure and osmotic shock protocols for bacteriophage inactivation is relatively poor. Nano-related strategies and metal compounds are harmful to live organisms and the environment. Polymers seem to be the most promising group of compounds for usage as antiphagents. However, the use of biodegradable polymers for bacteriophage inactivation requires further development. We conclude that there is a pressing need to design antiphagents that are active against bacteriophages while ineffective against bacterial cells, as well as being safe to humans, the environment, and equipment. Such agents are primarily required for biofoundries that endure heavy losses due to bacteriophage infections. Novel antiphagents have the potential to be active against

TABLE 1 Summarization of different methods for the inactivation of *Enterobacteriaceae*-targeting bacteriophages

Inactivation method	Conditions	Phage	Host	Reference
Physical factors				
Temp	72°C	MS2	*E. coli* Hfr K-12	52
	72°C, 84°C	MS2	*E. coli* ATCC 15597	53
Pressure	250 MPa	Nonspecified	*S. enterica*	56
	800 MPa	Qβ	*E. coli* ATCC 1288	59
Radiation	UV-A (62 mJ*cm^{-2})	MS2	*E. coli* 15597	90
	UV-A (10 mJ*cm^{-2})	PhiX174	*E. coli* 13706	90
	UV-A (30 mJ*cm^{-2})	PRD1	*S. typhimurium* LT2	90
	Near-UV	T4	*E. coli* B	96
	Near-UV	T7	*E. coli* B	97
	UV-C (35 mJ*cm^{-2} / 300 10 mJ*cm^{-2})	λNM1149	*E. coli* LE392	98
	Femtosecond laser (425 nm)	M13	*E. coli* TG-1	102
	Femtosecond laser (425 nm)	M13	*E. coli* JM103	109
	Femtosecond laser (425 nm)	MS2	*E. coli* C3000	109
	White light (40 W*m^{-2})	Qβ	*E. coli* 13706	113
	White light (40 W*m^{-2})	T4	*E. coli* 13706	113
Electric field	Streamer corona discharge	MS2	*E. coli* C3000	123
	Electromagnetic field (60 Hz)	MS2	*E. coli* ATCC 15597	126
	EC-MWNT	MS2	*E. coli* ATCC 15597	130
	Direct current (5 mA)	MS2	*E. coli* ATCC 15597	135
	Direct current (5 mA)	PRD1	*Salmonella choleraesuis* subsp. *Choleraeusi* ATCC 23564	135
	Discharge-generated ions by electric field	PhiX174	*E. coli* ATCC 13706	135
Osmotic Shock	Salt concn	T4B	*E. coli* B	138
	Salt concn	T4	*E. coli* B	140
	Low pH	T3	*E. coli* ATCC 11303	145
	High pH	Coliphages	*E. coli*	146
	Low pH	T7	*E. coli* BL21	148
Chemical factors				
Polymers	ε-poly-L-lysine	T4	*E. coli* B	149
	ε-poly-L-lysine	T5	*E. coli* B	149
	Chitosan	T2	*E. coli* B2	158
Proteins	Anti-phage antibody	T4	*E. coli*	161
	Anti-phage antibody	Coliphages	*E. coli*	162
Natural extracts	Blueberry extract	MS2	*E. coli* Hfr K-12	168
	Small molecules	MS2	*E. coli*	170
	Small molecules	f2	*E. coli*	170
	Small molecules	Lambda	*E. coli*	170
Commercially available disinfectants	Ozone (0.06 mg/L)	f2	*E. coli* ATCC 15766-B	186
	Ozone	MS2	*E. coli*	187

(Continued on next page)

TABLE 1 (Continued)

Inactivation method	Conditions	Phage	Host	Reference
	Ozone	PhiX174	*E. coli*	187
	Ozone	T7	*E. coli*	187
	Ozone	T1	*E. coli* IFO 13168	187
	Ozone	Lambda	*E. coli*	191
ROS	plasma exposure (100 s)	T4	*E. coli* JM109	205
	Fe2+	PhiX174	*E. coli* WR1	206
Salts	Cd+ ions	T-phages	*E. coli*	210
	Metal ions (Na^+, K^+, Ca^{2+})	PRD1	*S. typhimurium* LT-2	239
	CN- ions	T4	*E. coli*	243
Nanoparticles				
Metal nanoparticles	Silver nanoparticles	MS2	*E. coli*	243
	Silver nanoparticles	T4	*E. coli*	243
	Iron nanoparticles	MS2	*E. coli*	258
	Zero-valent nanoparticles	f2	*E. coli* 285	260
	Multisized nanoparticles	M13	*E. coli*	266
Nonmetal nanoparticles	Carbon-nanotube sponges	MS2	*E. coli* ATCC 15597	270
	Multiwalled carbon nanotubes	MS2	*E. coli* DSMZ 5695	272
	Carbon nanodots	T4	*E. coli*	277
	C60 fullerene	MS2	*E. coli* ATCC 15597	278
	Silica particles with polyethylene glycol	VB_SenS-AG11	*S. enteritidis* C417	287

eukaryotic viruses offering an effective tool to fight future pandemics more effectively.

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