Combining Bacteriophages with Other Antibacterial Agents to Combat Bacteria

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1 Introduction

Antimicrobial resistance in the postantibiotic era revived interest in various alternative antibacterial agents that effectively control bacterial growth. In the focus of the academic community from that aspect are plant products, such as components of essential oils and plant extracts, animal antimicrobial peptides, as well as bacteripohages and their lytic enzymes.

2 Discovery of Bacteriophages and Antibiotics

Bacteriophages are viruses that infect bacteria and were independently discovered a century ago by a French-Canadian microbiologist Félix d'Hérelle (1917) and British physician Frederic Twort (1915). On September 15, 1917, d'Herelle presented to the Académie des Sciences by Dr. Emile Roux the invisible microbial antagonist of dysentery bacillus and introduced it as "bacteriophage" into the scientific community (d'Hérelle 1917). He also proposed to use these entities as antibacterial agents and successfully applied an oral phage preparation to treat bacterial dysentery (d'Hérelle 1919). Following this example, during the 1920s and 1930s several institutes in various locations preformed similar experiments and were controlled by d'Herelle in order to ensure the produced phage quality. In the Eastern Europe, Georgian scientist Georgi Eliava with help of Felix d'Herelle established Tbilisi Institute of Bacteriophages, Microbiology and Virology (Dublanchet and Bourne 2007). Besides this institute, several commercial laboratories and companies in France, Germany, and the United States produced phage preparations (Gratia 1922; Pockels

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1927; Straub and Appelbaum 1932; Straub and Rakieten 1932). However, the bacteriophage approach has undergone the mixed success at the time because of poor basic understanding of phage biology, lack of data from clinical trials, and general overexpectations.

The situation was dramatically changed when the first antibiotic penicillin from fungi of genus *Penicillium* was discovered by Scottish scientist Alexander Fleming in 1928 and purified by Florey and Chain in 1942 (Tan and Tatsumura 2015). Waxman (1947) introduced the term "antibiotic" in order to denote natural products of microorganisms that inhibit other microorganisms. Many antibiotics have been further chemically modified to expand their activity spectrum and to make molecules more stable under certain conditions. Rolinson (1979) denoted them as semi-synthetic antibiotics (e.g., amoxicillin). Finally, many other conventionally used antibacterial agents are called antibiotics, although they are neither natural microbial products nor modified natural compounds, but are exclusively obtained by chemical synthesis. These agents are called chemotherapeutics. Ehrlich (1910) discovered the first one: it was Salvarsan used to combat *Treponema palidum*. Thus, the term "chemotherapeutic" was originally introduced to describe a synthetic compound intended for bacterial growth control. Some agents today are called "antibiotics" since they are applied as antibacterial therapeutics, although according to the definition they are chemotherapeutics, such as fluoroquinolones. The confusion started when term "chemotherapy" has been widely used for anticancer therapy. Moreover, some natural products are called chemotherapeutics because of their application in anticancer treatment; for example, mitomycin C is obtained from bacterium *Streptomyces caespitosus* (Tomasz 1995). To avoid confusion, the term "chemical agent" will be used as common name for these agents, while terms "antibiotic," "semisynthetic antibiotic," and "chemotherapeutic" will be used as originally introduced by Waxman, Rolinson, and Ehrlich, respectively.

Following broad-range antibiotics appearance after the World War II, in Western Europe started a new era, so-called the golden age of antibiotics. Until the 1980s antibiotics era has experienced an extraordinary expansion in the treatment of diseases for which bacteria are etiological agents (Dublanchet and Bourne 2007), causing the phage therapy in the Western countries to be forgotten. Meanwhile, during 1960s and 1970s when antibiotics were at their peak in the West, phages remained a standard part of the healthcare systems in the USSR despite the wide acceptance of other antibacterial agents. One of the best-known centers for bacteriophage study and production of therapeutic phage preparations is the institute in Tbilisi, which from its founding till 1990s provided the entire Soviet Union with phage preparations against various infections. Since 1990s and the collapse of the USSR, the institute operates under the name of its founder, Eliava (Kutateladze and Adamia 2010). Another famous center with extended phage therapy experience in European Union is Ludwik Hirszfeld Institute of Immunology and Experimental Therapy of the Polish Academy of Sciences (Górski et al. 2009).

Nowadays, with emergence of antibiotic-resistant bacteria, phage therapy once again finds its part as a potential complementary or alternative way to treat or prevent various infective diseases. The main problem is a lack of evidence-based studies using modern standards as well as the lack of an adapted regulatory framework

(Verbeken et al. 2007; De Vos and Pirnay 2015). Besides great interest in the potential for phage therapy, there are some unsolved issues regarding its safety, raising the concerns of its actual utility (Loc-Carrillo and Abedon 2011; Lu and Koeris 2011). However, novel antibacterial agents do not represent the only solution for preventing rapid increase of drug-resistant bacteria. An effective solution could be novel approaches, i.e., strategies that include combinations of different antibacterial agents. Besides possible combinations of two or more conventional antimicrobial agents, conventional antimicrobials with other non-antibiotic drugs (e.g., some antipsychotic and anti-inflammatory drugs improve the antibiotic efficacy in vitro) and with plant bioactive compounds or bacteriophages should be (re) considered. Besides the more efficient bacterial killing with combine agents, the approach should prolong the emergence of antibiotic-resistant strains, although do not offer a permanent solution for problem of spreading bacterial resistance to chemical antimicrobial agents. Furthermore, many currently used antibiotics are toxic, are teratogenic, or cause reactions of hypersensitivity in therapeutic concentrations, and combined therapy with phages can decrease effective antibiotic concentrations. Finally, such therapeutic approach could contribute to easier commercialization of phage-based therapeutics.

In the light of re-born bacteriophage application, possible efficient combinations of different phages and chemical antibacterial agents are considered in this chapter.

3 Bacteriophages vs. Chemical Antibacterial Agents

There are several crucial differences between chemical agents and bacteriophages, when they are consider as antibacterial agents, summarized in Table 1.

As mentioned previously, chemical antibacterial agents are natural, semisynthetic, or synthetic compounds with determined chemical formulas. On the contrary, bacteriophages are microorganisms that consist of proteins and nucleic acids, originating exclusively from nature, where they are very abundant (approx. 10^{30} on the Earth) (Suttle 2005). In this regard, detection or synthesis of new chemical antibacterial agents is a time-consuming process and may take several years to accomplish, while new phage strains or even species can be easily isolated by standard procedures. Similarly, production of chemical antimicrobials is usually expensive and complex, while phage production is relatively cheap and simple. In the light of phage and chemical agent different nature, Kutateladze and Adamia (2010) suggested that phages as therapeutic remedies should not be regulated in accordance with the standards applied to antibiotics or phytopharmaceuticals but to regulate the phage preparations as "biological preparations" rather than "pharmaceuticals." Just to mention, the specific nature of phages, i.e., the fact that they are viruses along with the prejudice that they are "Stalin's cure" probably affected their current official approval and commercialization as antibacterials.

Chemical antimicrobials and phages differ in mode of action and activity range. Most conventional antibacterial agents show bacteriostatic, bactericidal, or bacteriolytic activity, depending on chemical class and applied concentration, while

Table 1 Comparison of chemical antibacterial agents and obligatory lytic bacteriophages

Characteristic	Chemical antibacterial agents	Obligatory lytic bacteriophages
Nature	• Chemically defined molecules	• Biological agents, i.e., bacterial viruses containing proteins and nucleic acid
Origin	• Natural, semisynthetic, and synthetic agents; ecologically unacceptable	• Natural agents (extremely common in the environment); ecologically acceptable
Mode of action	• Bactericidal, bacteriostatic, or bacteriolytic effect	• Bacteriolytic effect
Activity spectra	• All conventional chemical agents target more than one bacterial species and many have very broad activity spectra • Non-selective action affects the patient's microbiota • Determination of bacterial etiological agent is unnecessary for broad-spectrum antibiotics but an antibiogram is preferred)	• Narrow lytic spectra, usually specific for several strains of one bacterial species • The high specificity for pathogen; do not affect microbiota • Determination of bacterial etiological agent is necessary and determination of phage lytic efficacy is preferred
Side effects	• Multiple side effects (allergies, secondary infections, etc.)	• Humans are constantly exposed to phages without known consequences
Resistance emergence	• De novo resistance emerges less frequently • Resistance to antibiotics is not limited to targeted bacteria and spread by horizontal gene transfer	• Resistance is frequent • Phage-resistant bacteria remain susceptible to other phages having a similar host range • Rapid bacteriophage evolution, along with bacterial host cell
Pharmacokinetics	• Do not necessarily concentrate at the site of infection and then metabolized and eliminated from the body	• Multiplication at the site of the infection until there are no more bacteria and then they are eliminated by mononuclear phagocytic system ("intelligent" drug)
Mode of application	• Repeated doses are needed to eradicate bacteria; intravenous, intramuscular, topical, per os application	• Initial dose increases exponentially at the site of infection, so in many cases only one dose is needed; topical or per os application
Production	• Production is expensive and complex	• Production is low-cost and relatively simple
Discovery	• Development of a new chemical antimicrobial agent is a time-consuming process and may take several years to accomplish	• Phage isolation from the environment is relatively easy and fast process
Public attitude	• Widely accepted and applied	• Sporadically applied; there are many prejudices

obligatory lytic bacteriophages, used in phage therapy, exclusively act as bacteriolytic agents. Bacteriophages have usually narrow activity range, mainly being active against some strains of one species (Ross et al. 2016), while chemical agents have broad host range, frequently having effect against both Gram-positive and Gram-negative bacteria. The narrow host range seems to be a shortfall of bacteriophages, but it is also a desirable characteristic when stability of normal microbial communities of human body is considered, particularly gut and vaginal. Resistant cells to phages appear more frequently than to chemical agents, which is one of the main shortfalls of bacteriophage application. However, there are some hypermutable strains that can obtain resistance to chemical agents with rate similar as for bacteriophages (Auerbach et al. 2015). Although phage-resistant bacteria relatively rapidly evolve (Bohannan and Lenski 2000; Lenski and Levin 1985), the bacterial resistance mechanisms to chemical antimicrobials and phages differ substantially (Labrie et al. 2010) and developed resistance to phages is not spread by horizontal gene transfer, in comparison with chemical antimicrobial resistance. Finally, bacteriophages as viruses can also mutate along with phage-resistant bacteria, adopting their lytic spectra to new hosts (Kysela and Turner 2007).

Phages and chemical antibacterial agents have different pharmacology, i.e., pharmacodynamics—drugs impact on a body/microorganisms and pharmacokinetics—body's impact on a drug (Abedon and Thomas-Abedon 2010; Nilsson 2014). The phages are self-amplifying agents usually efficient in one dose and kill target bacterium, while chemical agents should be applied repeatedly, based on time or concentration-dependent antibacterial activity and kill bacteria indiscriminately. Their pharmacokinetics is also different: bacteriophages concentrate at the site of infection and are eliminated naturally from organism by mononuclear phagocytic system, urine, and feces, after disappearing of the targeted bacterial host cells from the site of infection. On the other hand, chemical agents do not concentrate at the infection site and metabolized prior to elimination.

It is clear that both phages and antibiotics possess certain advantages as antibacterial agents; thus one of the approaches to combat multidrug-resistant bacteria is to combine chemical and biological antibacterial agents.

4 Historical Examination of Interaction Among Chemical Antibacterial Agents and Phages

At the beginning of the twentieth century, when Felix d'Herelle has discovered phages, their application as antibacterial agents was a very attractive research field. Although discovery of penicillin dramatically decreased interest in phage therapy, some researchers still conducted these experiments even during 1940s and later.

In the context of antibiotic and phage interaction, the first experiments were conducted to examine antiviral effects of some antibiotics. For instance, it has been proven that tyrothricin from *Aneurinibacillus migulanus* (formerly *B. brevis*) and actinomycin A from soil *Streptomyces antibioticus* inactivate some phages (Neter 1942). Penatin from *Penicillium* sp. is proven to inactivate phages, decrease

plaque number and virion adsorption to cells, but phages still were able to cause lysis of bacterial host cells (Anderson 1943). Similarly, bacteriostatic concentrations of streptomycin delays phage lysis of staphylococci (Edlinger and Faguet 1950) and subinhibitory concentration of terramycin (oxytetracycline) inhibits cell lysis by phages (Edlinger and Faguet 1951). The same was confirmed for aureomycin (chlortetracycline) with *E. coli* and T3 phage (Altenbern 1953), while subinhibitory concentrations of chloromycetin (chloramphenicol) against T1 phage infecting *E. coli* allow lysis but reduce virion yield (Bozeman et al. 1954); the similar effect of chloromycetin was observed for *S. aureus* and its phages (Edlinger 1951); etc. Jones (1945) examined effect of phage combination with clavacin (mycotoxin patulin), streptothricin (antibiotic from *Streptomyces fradiae*), streptomycin, actinomycin, and penicillin against *E. coli* and *S. aureus*. Interestingly, the author found that streptothricin, streptomycin, and clavacin cause inactivation of various phages in bacteria-free filtrates (streptomycin irreversibly), whereas penicillin and actinomycin were without effect. All these findings rather indicate an adverse effect of antibiotics on phage multiplication, than on possible synergy.

The first observation of antibiotic-phage combination decrease of bacterial growth was reported by Neter and Clark (1944). They showed that exposure of bacteriophage to 2000 U of penicillin for 18 h did not affect the lytic activity of the bacteriophage and additive effect was observed when combination of bacteriophage and 0.2 U of penicillin was used against *S. aureus*.

The first set of detailed experiments clearly describing phage-antibiotic synergism was carried out by Himmelweit (1945), who after simultaneous application of phage and penicillin against *S. aureus*, drew the following conclusions: (1) penicillin does not affect the multiplication of *Staphylococcus* phage K, acting on staphylococcus S3K, nor does it interfere with the lethal and lytic action of this phage; (2) *Staphylococcus* phage K and penicillin together produce more rapid killing and lysis of staphylococcus S3K than either alone; and (3) the acceleration in the rate of lysis by bacteriophage is particularly with low concentrations of penicillin. Similar was observed by Nicolle and Faguet (1947), as well as Rountree (1947). Later, Yamagami and Endo (1969) observed that UV irradiation and subinhibitory concentrations of mitomycin C enlarge T4 plaque size and related it with bacterial cell filamentation and shortening of the phage latent period. However, the significance of these findings remained unrecognized until beginning of the twenty-first century.

Interestingly, during 1970s and 1980s there are several reports on interference of phages and antibiotics, used to elucidate phage biology and phage replication cycle characteristic. Using phage-antimicrobial combination it was proved that functional *E. coli* DNA gyrase is required for multiplication of some phages. For instance, replication of T5 was inhibited by novobiocin, coumermycin A, and nalidixic acid that interfere with bacterial DNA gyrase (Constantinou et al. 1986). Similarly, Bacillus phage SPO1 and PBS2 were inhibited by novobiocin and nalidixic acid that affect the enzyme functionality (Price and Fogt 1973; Alonso et al. 1981). Besides the DNA gyrase activity, novobiocin shuts off synthesis of early and late RNAs, diminishing transcription and thus phage production (Sarachu et al. 1980).

Bacterial transcriptional inhibitors, such as rifampin from *Streptomyces mediterranei*, streptolydigin from *S. lydigus*, and actinomycin D from *S. parvulus* also impair gene transcription of some *Bacillus*-specific phages (Dosmar et al. 1977; Osburne and Sonenshein 1980).

5 Reviving Idea of Phage Synergistic Combination with Other Antibacterial Agents

In the twenty-first century, first experiment on chemotherapeutic (enrofloxacin) and bacteriophages against *E. coli* was performed in vivo, using chickens as models (Huff et al. 2004). Inoculation of 10^4 CFU/mL in chicken air sac caused mortality in 68% of animals (Fig. 1). When enrofloxacin was added in water (50 ppm, 7 days), mortality was decreased to 3%, while mortality after one intramuscular injection of phage (unknown family, 10^9 PFU/mL) was 15%. However, when both agents were combined simultaneously, mortality was not recorded. Independently, or inspired by these results, many authors have examined the synergy among conventional antimicrobials and bacteriophages.

6 Phage Interaction with Chemical Antibacterial Agents

Among the first experiments conducted in order to examine phage and chemical agents interaction was conducted by Comeau et al. in 2007. They observed that sublethal concentrations of certain antibiotics can substantially stimulate the host

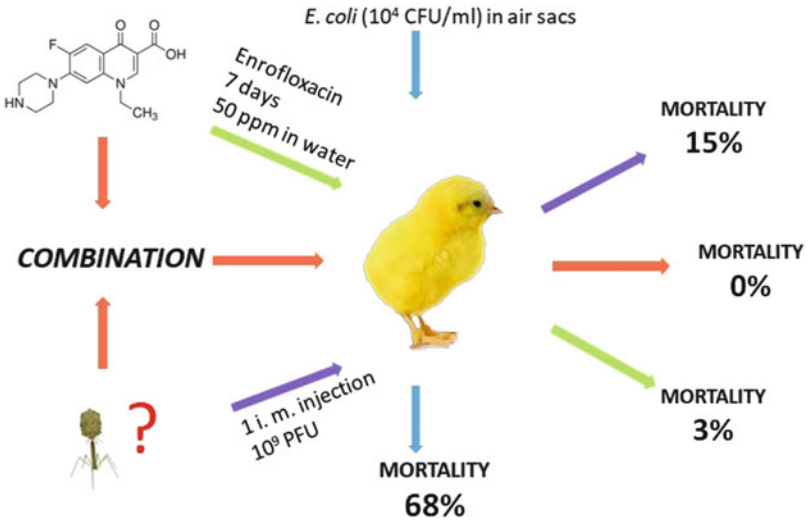

Fig. 1 The first in vivo experiment indicating chemical agent-phage synergy (Huff et al. 2004)

bacterial cell's production of some virulent phage and the combination was later applied to more efficiently decrease cell densities than either treatment alone. The authors defined the phenomenon of the synergy as "phage-antibiotic synergy" or PAS. However, in the experiments, for instance, ciprofloxacin was used, which is a synthetic agent. Thus, it seems that definition of the phenomenon as PAS is confusing: respecting the definitions of antibiotic, semisynthetic antibiotic, and chemotherapeutic (see Sect. 2) and avoiding confusion, here will be used term "chemical agent-phage synergy" or CAPS.

The CAPS is usually examined against planktonic cells but also against cells embedded in extracellular matrix, i.e., against biofilms (Table 2). It is well documented that bacterial biofilm mode of growth enhances resistance to antibacterial agents, making bacterial cells even 100–1000 times more resistant to antibiotics (Mah and O'Toole 2001) and 150–3000 times more resistant to disinfectants (Patel 2005). Biofilm occurrence during infection represents a limiting factor for therapeutic success, because the bacterial cells within biofilm are protected with matrix from physical, chemical, and biological stress (Hassan et al. 2011). Bacteriophages' anti-biofilm activity essentially has been shown to decrease the biomass and not to eradicate biofilm (e.g., Knezevic and Petrovic 2008; Knezevic et al. 2011; Fong et al. 2017, etc.), which raised a concern. Consequently, some authors have investigated the role of bacteriophages in combination with chemical agents to restore antibacterial activity. Thus, particular attention in this chapter will be paid to CAPS against bacterial biofilms.

6.1 Phage Interaction with Cell Wall Synthesis Inhibitors

More than half a century after Himmelweit observation of penicillin-phage K synergy (Himmelweit 1945), other beta-lactams have been examined from this aspect. Semisynthetic antibiotics also act synergistically with phages, for instance, sub-MIC of ampicillin (8 μg/mL) and a siphovirus TH (MOI = 1) reduced a number of planktonic cells of *P. mirabilis* for 5 logs (Yazdi et al. 2018). *P. mirabilis* biofilm removal for approx. 93% after 24 h was obtained with a high dose of ampicillin and larger number of phages (246 μg/mL and MOI = 100, respectively); ampicillin alone reduced biofilm for 44% and phages for 70%. The similar phenomenon has been observed for amoxicillin with a phage against *K. pneumoniae* planktonic cells and biofilms, where biofilm biomass was reduced for 3–4 log with ampicillin-phage combination (256 μg/mL and MOI = 0.01, respectively) as compared to ~2 log reduction when biofilm was exposed to bacteriophage alone (Bedi et al. 2009). Carbenicillin, a carboxypenicillin, exhibited synergy in combination with *P. aeruginosa* podovirus LKD16 (Torres-Barcelo et al. 2016).

Synergy was observed for first-generation cephalosporin cefazolin and phage SATA-8505 combination that reduced *S. aureus* biofilm for 3 logs, but only when treatment with phage preceded the antibiotic (Kumaran et al. 2018). While phage combination with second-generation cephalosporins has not been examined, the third-generation cephalosporins have been examined in the greatest extent. It was

Table 2 Combined effect of phages or their endolysins with various antibacterial agents

Bacterial species (strain)	Bacteriophage (family) or endolysin	Agent in combination	Effect	Reference
Staphylococcus aureus (S3K)	K (*Myoviridae*)	Penicillin	Synergy	Himmelweit (1945)
Escherichia coli (serotype 02)	SPR02 and DAF6 (unknown)	Enrofloxacin	Synergy	Huff et al. (2004)
Pseudomonas aeruginosa strains: (PAO1) (PAK)	Filamentous phages (*Inoviridae*): Pf3 Pf1	Tetracycline Gentamicin	Synergy Synergy	Hagens et al. (2006)
E. coli (MFP)	ΦMFP (*Siphoviridae*)	Cephalosporins (cefotaxime, ceftriaxone, ceftazidime, cefixime) Aztreonam Gentamicin, tetracycline Quinolones, mitomycin C	Synergy Synergy No synergy Synergy	Comeau et al. (2007)
S. aureus (Sa9)	Φ35 and Φ88 (unknown)	Nisin	Synergy followed by cross-resistance	Martinez et al. (2008)
Klebsiella pneumoniae (B5055)	Specific bacteriophage	Amoxicillin	Synergy	Bedi et al. (2009)
K. pneumoniae (B5055)	KPO1K2 (*Podoviridae*)	Ciprofloxacin	SYNERGY	Verma et al. (2010)
S. aureus (D43-a)	SAP-26 (*Siphoviridae*)	Rifampicin Azithromycin Vancomycin	30% biofilm cells alive 40% biofilm cells alive 60% biofilm cells alive	Rahman et al. (2011)
S. aureus (ATCC27700)	SA5 from Eliava preparation (*Myoviridae*)	Gentamicin	Synergy	Kirby (2012)
E. coli (ATCC11303)	T4 (*Myoviridae*)	Cefotaxime	Synergy in biofilm control	Ryan et al. (2012)
Pseudomonas fluorescens (SBW25 and a hypermutator strain mutS^{-})	SBW25ϕ2 (unknown)	Rifampicin	WT rif-resistant strains reverted to a rif-sensitive phenotype in the presence of phages	Escobar-Páramo et al. (2012)

(continued)

Table 2 (continued)

Bacterial species (strain)	Bacteriophage (family) or endolysin	Agent in combination	Effect	Reference
P. aeruginosa strains: (PA-4 U) (M2) (ATCC9027)	Pseudomonas phages: Delta (*Podoviridae)* 001A (*Siphoviridae)* Sigma-1 (*Siphoviridae)*	Gentamicin, Ciprofloxacin, polymyxin Ceftriaxone Ceftriaxone Ceftriaxone	No synergy No synergy No synergy Synergy with Sigma-1	Knezevic et al. (2013)
P. aeruginosa (PAO1)	LUZ7 (*Podoviridae)*	Streptomycin	Synergy	Torres-Barcelo et al. (2014)
P. aeruginosa (PAO1) *E. coli* B (ATCC11303)	BP-1 (unknown) T4 (*Myoviridae*)	Tobramycin Tobramycin	No synergy in biofilm reduction Synergy in biofilm reduction	Coulter et al. (2014)
Burkholderia cepacia	KS12 and KS14 (My*oviridae*)	Ciprofloxacin Tetracycline Minocycline Levofloxacin Ceftazidime Meropenem	Synergy; confirmed in vivo on *G. mellonella* model Synergy; confirmed in vivo on *G. mellonella* model No synergy No synergy No synergy Synergy; confirmed in vivo on *G. mellonella* model	Kamal and Dennis (2014)
S. aureus MDR (SA4 and SA7)	Φ (unknown)	Gentamicin Vancomycin Tetracycline	Synergy Synergy Synergy	Ali et al., (2015)
S. aureus (KACC 13236)	SA11 *(Siphoviridae)*	Ciprofloxacin	Synergy	Jo et al. (2016)
P. aeruginosa (PAO1)	Pyobacteriophage (phage cocktail)	Imipenem	Synergy	Papukashvili et al. (2016)
P. aeruginosa (various strains)	3 phages	Amikacin Meropenem	Synergy in 87% stains; for biofilm in 50% Synergy in 73% strains; for biofilm in 14%	Nouraldin et al. (2016)
E. coli (MG1655)	P1 (*Myoviridae*)	Ciprofloxacin	Lysogenization by bacteriophage P1 renders *E. coli* more sensitive to ciprofloxacin	Ronayne et al. (2016)

(continued)

Table 2 (continued)

Bacterial species (strain)	Bacteriophage (family) or endolysin	Agent in combination	Effect	Reference
P. aeruginosa (PAO1)	LKD16 (*Podoviridae*)	Carbenicillin Gentamicin Trimethoprim	Synergy Synergy Synergy	Torres-Barcelo et al. (2016)
P. aeruginosa (PA14)	NP1 (*Siphoviridae*) and NP3 (*Myoviridae*)	Ceftazidime Ciprofloxacin Colistin Gentamicin Tobramycin	Synergy against biofilm Synergy or facilitation against biofilm No synergy against biofilm No synergy against biofilm Facilitation against biofilm	Chaudhry et al. (2017)
P. aeruginosa (CHA)	phage cocktail PP1131	Ciprofloxacin Meropenem	Synergy in vitro and in vivo (endocarditis model) Synergy in vitro and in vivo (endocarditis model)	Oechslin et al. (2017)
Proteus mirabilis from UTI	TH (*Siphoviridae*)	Ampicillin	Synergy against planktonic cells and biofilm	Yazdi et al. (2018)
S.aureus (ATCC 35556)	SATA-8505 *(Myoviridae)*	Cefazolin Vancomycin Dicloxacillin Tetracycline Linezolid	Synergy Synergy Additive Additive Additive	Kumaran et al. (2018)
Acinetobacter baumannii MDR	KARL-1 *(Myoviridae)*	Meropenem Ciprofloxacin Colistin	Synergy No synergy Additive	Jansen et al. (2018)
P. aeruginosa (FADD1-PA001, JIP865, 20844n/m(s))	PEV20 (*Podoviridae*)	Ciprofloxacin Amikcin Colistin Aztreonam	Synergy, also after nebulization (except strain 20844n/m(s)) Synergy (except strain 20844n/m(s)) Synergy (except strain 20844n/m(s)) No synergy	Lin et al. (2018)

(continued)

Table 2 (continued)

Bacterial species (strain)	Bacteriophage (family) or endolysin	Agent in combination	Effect	Reference
S.aureus (Newman)	A PYO phage from Georgian PYO cocktail (*Myoviridae*)	Gentamicin Oxacillin Vancomycin Tetracycline ciprofloxacin Daptomycin Erythromycin Linezolid Rifampicin	10xMIC decrease phage density Modest phage growth at 10xMIC Antagonism at 10xMIC Synergy at 2xMIC; antagonism at 10xMIC Synergy at 2xMIC; 10xMIC prevented phage growth Modest phage growth at 10xMIC 10xMIC decrease phage density 10xMIC decrease phage density No synergy	Dickey and Perrot (2019)
P. aeruginosa (ATCC 39018)	RNA phage (unkonown)	Chlorine (sodium hypochlorite)	Synergy	Zhang and Hu (2013)
K. pneumoniae (B5055; O1: K2)	KPO1K2 (*Podoviridae)*	Iron antagonizing molecule $CoSO_4$	Synergy	Chhibber et al. (2013)
K. pneumoniae (B5055) and *P. aeruginosa* (PAO1); mixed-species biofilm	Klebsiella phage KPO1K2 and Pseudomonas phage Pa29 (*Podoviridae)*	Xylitol	Synergy	Chhibber et al. (2015)
E. coli (CECT 434)	CEB_EC3a (S*iphoviridae)*	Two Portuguese honeys	Synergy or additive	Oliveira et al. (2017)
P. aeruginosa (E2005-A)	ΦE2005-A (unknown)	*E. coli* HU2117 (beneficial strain; preformed biofilm on urinary catheters)	Synergy in decrease of *P. aeruginosa* adhesion	Liao et al. (2012)

(continued)

Table 2 (continued)

Bacterial species (strain)	Bacteriophage (family) or endolysin	Agent in combination	Effect	Reference
S. aureus (ATCC 13301 and CCARM 3080)	SA11 (*Siphoviridae*)	Probiotic strain *Lactobacillus rhamnosus* GG	Synergy	Woo and Ahn (2014)
Streptococcus pneumoniae (DCC1490, DCC1476, DCC1420, 8249)	Endolysin Cpl-1	Penicillin Gentamicin Levofloxacin Azithromycin	Synergy (depending on strain) Synergy (depending on strain) No synergy No synergy	Djurkovic et al. (2005)
S. aureus, clinical strains	Chimeolysin P16–17	Gentamicin	Augmentation	Manoharadas et al. (2009)
S. aureus (MRSA)	Chimeolysin ClyS	Oxacillin	Synergy	Daniel et al. (2010)
S. aureus (various MRSA strains)	Endolysin CF-301	Vancomycin Daptomycin	Synergy	Schuch et al. (2014)
S. pneumoniae	Endolysins Pal and Cpl-1	Endolysins Pal and Cpl-1	Synergy	Loeffler and Fischetti (2003)
S. pneumoniae (susceptible and MDR strains)	Endolysin Cpl-1 and Pal	Major pneumococcal autolysin LytA	Synergy	Rodríguez-Cerrato et al. (2007)
S. aureus (MRSA)	Endolysin LysK	Lysostaphin, *Staphylococcus simulans* bacteriocin	Synergy	Becker et al. (2008)
S. aureus (Sa9)	Endolysin LysH5	Nisin	Synergy	García et al. (2010)
A. baumannii (various strains)	Atilysin Arrt-175	Ciprofloxacin Tobramycin	No synergy No synergy	Defraine et al. (2016)
S. aureus (ATCC B1707, LAC, Newman, ATCC 29213)	Endolysin SAL200	Nafcillin, cefazolin Vancomycin	Synergy or indifferent effect Synergy or indifferent effect	Kim et al. (2018a)
S. pneumoniae (MDR)	Endolysin Cpl-711	Amoxicillin Cefotaxime Levofloxacin Vancomycin	Synergy Synergy No synergy No synergy	Letrado et al. (2018)

proven that cefotaxime, ceftazidime, cefixime, or ceftriaxone combined with phage ΦMFP act synergistically against *E. coli*, observed as plaque enlargement and phage titer increase (Comeau et al. 2007). Other studies confirmed these findings: cefotaxim-myovirus T4 combination showed synergy against *E. coli* biofilm (Ryan et al. 2012), ceftriaxone-siphovirus Sigma-1 against *P. aeruginosa* planktonic cells, as well as ceftazidime in combination with myovirus NP3 or siphovirus NP1 against *P. aeruginosa* biofilms (Chaudhry et al. 2017). Only two reports indicated lack of phage-beta lactam synergy: in the first dicloxacillin was combined with phage SATA-8505 against *S. aureus*, showing additive effect (Kumaran et al. 2018), and in the second report lack of synergy was observed in combination of third-generation cephalosporin ceftazidime and myoviruses KS12 and KS14 against *Burkholderia cepacia* (Kamal and Dennis 2014).

Carbapenems also were successfully combined with bacteriophages: imipenem synergistically caused dispersal of *P. aeruginosa* biofilm when combined with a commercial Pyobacteriophage preparation (Tbilisi, Georgia) (Papukashvili et al. 2016), while meropenem showed synergy against this bacterium when combined with a phage cocktail PP1131 in in vitro fibrin clots (Oechslin et al. 2017). The same was confirmed for meropenem and selected specific phages against *P. aeruginosa* both planktonic cells and biofilms (Nouraldin et al. 2016). Meropenem (>128 μg/mL) in combination with a myovirus KARL-1 (MOI = 10^{-1}) showed synergy with a complete clearance of *A. baumannii* liquid culture (Jansen et al. 2018). Interestingly, while combination ceftazidime-myoviruses KS12 and KS14 was not promising against *B. cepacia*, the same phages with meropenem showed synergy even in vivo using *Galleria mellonella* larvae model (Kamal and Dennis 2014).

Interaction of monobactam aztreonam and siphovirus ΦMFP against *E. coli* also was characterized as synergistic (Comeau et al. 2007). Thus, almost all current data show that phage-beta-lactam combinations have a great therapeutic potential.

Vancomycin is a glycopeptide antibiotic produced by *Amycolatopsis orientalis* that inhibits cell wall synthesis of susceptible bacteria (Watanakunakorn 1984). When it was combined with specific phages against *S. aureus* the synergy was recorded (Ali et al. 2015; Kumaran et al. 2018). High doses of vancomycin can antagonize phage multiplication, as shown for 10xMIC and a myovirus against *S. aureus* (Dickey and Perrot 2019).

This indicates that not only beta-lactams but also glycopeptide antibiotics are promising from the aspect of application, indicating generally a good combining potential of antimicrobials that inhibit cell wall synthesis with bacteriophages.

6.2 Phage Interaction with Antimicrobials that Disrupt Bacterial Cell Membranes

Phage combination with agents that affect bacterial cell membrane integrity has not been widely examined. Colistin and polymyxin B, cationic cyclic polypeptide antibiotics produced by *Bacillus* sp., disrupt cell membranes and are active against both Gram-positive and Gram-negative bacteria (Gupta et al. 2009). When they are

combined with bacteriophages, a lack of synergy was observed, and interactions were additive or indifferent. It was proven for combination of polymyxin B and siphovirus Sigma-1 or podoviruses Delta and 001A against *P. aeruginosa* planktonic cells (Knezevic et al. 2013); colistin and a siphovirus NP1 or myovirus NP3 against *P. aeruginosa* biofilm (Chaudhry et al. 2017); and colistin combined with a myovirus KARL-1 against *Acinetobacter baumannii* (Jansen et al. 2018). Similar was confirmed for non-therapeutic surfactants, such as sodium-dodecyl sulfate and bile salts that reduce phages' detrimental effect on bacterial growth (Scanlan et al. 2017). However, Lin et al. (2018) determined synergy between podovirus PEV20 and colistin against *P. aeruginosa* strains FADD1-PA001 and JIP865, but not against 20844n/m(s).

Another polypeptide antibiotic nisin from *Lactococcus lactis* is active against Gram-positive bacteria, generating pores in cell membrane but also interrupting cell wall biosynthesis through specific lipid II interaction (Prince et al. 2016). Leverentz et al. (2003) conducted an interesting research with nisin and phages against *L. monocytogenes* on honeydew melon and apple slices. On honeydew melons, bacterial populations treated with phages LM-103 and LMP-102 (1×10^7 PFU) and nisin at 200 and 400 IU were reduced for 1.5 and 2.5 logs, respectively, and on apple 0.6 and 0.3 logs, respectively, in comparison with bacterial number when only nisin was applied. A similar experiment was performed against *L. monocytogenes* on ready-to-eat pork ham slices—nisin (50 μg/L) and phage P100 (1.5×10^7 UFP/mL) in combination had a small anti-listeria effect at the beginning of the experiment, indicating antagonism between these agents. However, at 72 h, almost 3 log cycles of reduction were observed in the number of viable bacterial cells (Figueiredo and Almeida 2017). Nisin combined with phages Φ35 and Φ88 acts better against *S. aureus* in pasteurized milk (Martinez et al. 2008). Although decrease of bacterial CFU in comparison to more active agent (phages) was less than 2 logs, the interaction was described as synergistic (but it was rather additive). The experiments showed that nisin-adapted cells seriously compromised bacteriophage activity, changing adsorption and plaquing, while phage-resistant mutants were still sensitive to nisin. This finding suggests that cross-resistance between phages and this polypeptide antibiotic can be developed during time.

The available results indicate that phage combination with agents active against cell membrane integrity, except nisin, is not a good option from the aspect of potential application.

6.3 Phage Interaction with Antimicrobials that Inhibit DNA Replication

Antimicrobials that specifically bind to DNA gyrase and inhibit DNA synthesis were frequently included in contemporary studies of interactions with phages. However, ciprofloxacin was almost exclusively examined from this aspect, and in many studies a synergy with phages has been confirmed: with a siphovirus ΦMFP against *E. coli* (Comeau et al. 2007); with a podovirus KPO1K2 against *K. pneumoniae* biofilm

(Verma et al. 2010); with two myoviruses KS12 and KS14 against *B. cepacia* (Kamal and Dennis 2014); with a phage SA11 against *S. aureus* (Jo et al. 2016); NP1 siphovirus and NP3 miovirus against *P. aeruginosa* biofilm (Chaudhry et al. 2017); with myovirus P1 against *E. coli* (Ronayne et al. 2016); and with a phage cocktail PP1131 (Oechslin et al. 2017) or a nebulized phage PEV20 against *P. aeruginosa* (Lin et al. 2018). Although the results seem consistent, there are several reports indicating lack of synergy. For instance, ciprofloxacin was not successfully combined with siphoviruses and podoviruses against *P. aeruginosa* (Knezevic et al. 2013), as neither a myovirus KARL-1 against *A. baumannii* (Jansen et al. 2018). In addition, one more fluoroquinolone—levofloxacin—was unsuccessfully combined with *B. cepacia* myoviruses (Kamal and Dennis 2014).

Interestingly, Lu and Collins (2009) engineered a bacteriophage to overexpress proteins and to attack gene networks that are not directly targeted by antibiotics, showing that suppression of the SOS network in *E. coli* using engineered bacteriophage in vitro enhances killing with quinolones by several orders of magnitude and in vivo significantly increases survival of infected mice. They demonstrated that engineered bacteriophage enhanced the killing of antibiotic-resistant bacteria, persister cells, and biofilm cells; reduced the number of antibiotic-resistant bacteria that arise from an antibiotic-treated population; acted as a strong adjuvant for other bactericidal antibiotics (e.g., aminoglycosides and β-lactams); and furthermore reported that engineering bacteriophage to target non-SOS gene networks and to overexpress multiple factors also can produce effective antibiotic adjuvants.

Mitomycin C, used for therapy of some types of cancers and frequently applied to induce lytic cycle of temperate phages (Raya and H'bert 2009), kills bacteria through a growth-independent mechanism in contrast to most other antibiotics. It is passively transported and bioreductively activated, leading to spontaneous cross-linking of DNA (Kwan et al. 2015). Even this agent with a specific mode of action gives CAPS when combined with a siphovirus against *E. coli* (Comeau et al. 2007).

Considering available data, it is obvious that DNA replication inhibitors along with beta-lactams have a great potential in CAPS-based therapy.

6.4 Phage Interaction with Antimicrobials that Inhibit Transcription

Among antibiotics that inhibit RNA synthesis, only rifampicin (or rifampin) was examined in combination with bacteriophages. This antibiotic obtained from *Amycolatopsis rifamycinica* specifically binds to bacterial RNA polymerase, preventing transcription and consequently protein expression (Wehrli 1983). The augmentation of biofilm removal (with approx. 30% of survived cells) was confirmed for combination rifampicin-siphovirus SAP-26 against *S. aureus* (Rahman et al. 2011). Escobar-Páramo et al. (2012) examined resistance appearance of wild type and hypermutator strain of *P. fluorescens* in presence of phage SBW25ϕ2 and rifampicin. They noticed that the evolutionary response of populations under different treatments varied depending on the order in which the antimicrobials were added

and whether the bacterium was a hypermutator. In the study, wild-type rifampicin-resistant populations involved in biofilm formation often reverted to rifampicin sensitive when stresses were added sequentially. In contrast, when the mortality agents were applied simultaneously, phage populations frequently went extinct and the bacteria evolved antibiotic resistance. These authors concluded that evolutionary response to the combined effects of antibiotic and phages is difficult to predict, depending to some extent on the order in which the antimicrobial agents are added, and on bacterial mutation rates, where these two factors determine the way genetic diversity is created and maintained in bacterial populations during the process of adaptation. Similar study was carried out using *S. aureus* and a myovirus—regardless to mode of application (simultaneous or phage first and rifampicin second), antibiotic efficacy was not increased, but the phage completely prevented the ascent of rifampicin-resistant bacteria (Dickey and Perrot 2019).

The results of transcription inhibitor combination with phages are limited and although are not very promising, the further studies are needed.

6.5 Phage Interaction with Antimicrobials that Inhibit Translation

Phages have been combined so far with antibacterial agents that inhibit protein synthesis, but most studies were conducted using tetracyclines and aminoglycosides, while other groups were examined scarcely or neglected (e.g., amphenicols, linezolid, macolides).

6.5.1 Phage Interaction with Tetracyclines

The tetracycline is an antibiotic first isolated from *Streptomyces aureofaciens* that expresses its antibacterial activity binding to 30S ribosomal subunit. The tetracycline gives synergy with phage Φ against *S. aureus* (Ali et al. 2015) or with myoviruses KS12 and KS14 against *B. cepacia* (Kamal and Dennis 2014). Interestingly, the same *B. cepacia* phages did not act synergistically with a semisynthetic tetracycline, minocycline. The synergy was confirmed against *S. aureus* with a myovirus at 2xMIC, while 10xMIC was antagonistic (Dickey and Perrot 2019). However, a lack of interaction was observed by Comeau et al. (2007) in combination with siphovirus ΦMFP against *E. coli*, while in combination with SATA-8505 against *S. aureus* only additive effect was observed (Kumaran et al. 2018). The tetracycline derivatives glycylcyclines and fluorocyclines have not yet been examined from this aspect.

6.5.2 Phage Interaction with Aminoglycosides

Aminoglycosides are a large group of antibiotics obtained from *Streptomyces* sp. or *Micromonospora* sp. that binds to 30S ribosomal subunit, and the most frequently gentamicin is combined in in vitro studies with phages. This antibiotic was combined with *S. aureus* (Ali et al. 2015) or *P. aeruginosa* (Torres-Barcelo et al. 2016) specific phages and showed synergistic interactions.

Due to increased complexity of the pharmacodynamics in phage-antibiotic combinations, Kirby (2012) used the continuous culture system for *Staphylococcus aureus* to simulate the pharmacokinetics of periodic antibiotic dosing alone and in combination with lytic phage, while the further evaluation of the conditions governing the observed pharmacodynamics was analyzed using computer model representation of the system. This study proves that treatment with gentamicin induced a population of cells with a strong aggregation phenotype, and consequently an increased ability to form biofilm, but also induced increase of susceptibility to the phage action. This dual treatment with gentamicin and phage resulted in lower final cell densities than either treatment alone, and unlike in the phage-only treatment, phage-resistant isolates were not detected in the dual treatment. According to the results of this experimental approach, dual therapy can be more efficient than single therapy, particularly if there is an overlap in the physiological pathways targeted by the individual agents.

However, some studies showed lack of gentamicin-phage synergy: with phage ΦMFP against *E. coli* (Comeau et al. 2007) or sipho- and myoviruses of *P. aeruginosa* (Chaudhry et al. 2017).

Other aminoglycosides have been examined sporadically: streptomycin showed synergy with podovirus LUZ24 against *P. aeruginosa* (Torres-Barcelo et al. 2014), while amikacin gave synergy with podovirus PEV20 against some *P. aeruginosa* strains (Lin et al. 2018). Nouraldin et al. (2016) reported that an amikacin-phage combination eradicated biofilm in 50% of the *P. aeruginosa* isolates, while the meropenem-phage combination eradicated biofilm in only 14% of the strains. In the contrary to these results, Sillankorva et al. (2012) showed that the amikacin-phage combination for control *P. aeruginosa* biofilms was characterized as antagonistic. Similarly, tobramycin and T4 myovirus were successfully combined against *E. coli,* while significant enhancement activity of tobramycin-PB-1 phage combination against *P. aeruginosa* biofilm was not observed (Coulter et al. 2014). However, authors recorded significant reduction of the emergence of antibiotic- and phage-resistant cells in both *E. coli* and *P. aeruginosa* biofilms. For some tobramycin-phage combination the effect was only facilitative, such as in combination with phages NP1 (*Siphoviridae*) and NP3 (*Myoviridae*) against *P. aeruginosa*.

6.5.3 Phage Interaction with Other Translation Inhibitors

The linezolid is a synthetic agent from oxazolidinone group of antimicrobials that binds to the 50S subunit of the prokaryotic ribosomes, preventing formation of the initiation complex for protein synthesis (Livermore 2003). When combined with SATA-8505 phage against *S. aureus*, it showed additive effect (Kumaran et al. 2018). A macrolide azithromycin efficiently was combined with siphovius SAP-26 against *S. aureus* biofilm and after the treatment, only 40% of cells were alive (Rahman et al. 2011).

The results on therapeutic application of phages with translation inhibitors seem inconsistent and probably depend on host-antibiotic-phage system and mode of agents' application. Thus, further research is needed to elucidate CAPS with protein synthesis inhibitors.

6.6 Phage Interaction with Other Antibacterial Agents

Bacteriophages have been successfully combined so far with some inorganic agents. For instance, chlorine (210 mg/L) and RNA phages (3×10^7 PFU/mL) reduced biofilm growth for 94% and removed 88% of already formed *P. aeruginosa* biofilm (Zhang and Hu 2013). Similarly, cobalt (II) sulfate ($CoSO_4$) that antagonizes iron, in combination with a depolymerase-producing podovirus KPO1K2 synergistically inhibit formation of *K. pneumoniae* biofilms (Chhibber et al. 2013). Xylitol, a 5-carbon polyol sugar alcohol commonly used as a non-carcinogenic sweetener with anti-biofilm properties (Nayak et al. 2014), can be successfully combined with podoviruses KPO1K2 and a non-depolymerase-producing Pa-29 against multi-species biofilm developed by *P. aeruginosa* and *K. pneumoniae* (Chhibber et al. 2015). Interestingly, *E. coli* biofilm was successfully controlled by combining two Portuguese honeys with depolymerase-producing podovirus EC3a with recorded synergistic and additive effects (Oliveira et al. 2017).

Particularly interesting are studies that examined phage combination with other biological agents. For instance, the combined effect of probiotic *Lactobacillus rhamnosus* and siphovirus SA11 against *S. aureus* under the simulated intestinal conditions seems promising (Woo and Ahn 2014). The bacteriophage SA11 combined with the probiotic effectively reduced *S. aureus* number for 4 logs with relative decrease of virulence factor expression (adhesion- and efflux-related genes), supporting the application of bacteriophage to control the ingested antibiotic-resistant foodborne pathogens. Potential application of biological agents in biofilm control can be reflected not only in the treatment of the already established biofilms, but also in prevention of its formation on biotic and abiotic surfaces, especially in hospitals. Liao et al. (2012) showed that the combination of phages with a pre-established biofilm of *E. coli* HU2117 (a benign and potentially protective strain without P-fimbriae) was synergistic in preventing urinary catheter colonization by *P. aeruginosa.* Such pre-treatment of catheters decreased *Pseudomonas aeruginosa* adherence for approx. 4 logs, and neither *E. coli* nor phage alone generated significant decreases.

7 Combination of Bacteriophage Enzymes and Chemical Antimicrobials

A novel class of antibacterial agents derived from bacteriophages are (endo)lysins. These lytic enzymes are produced by progeny bacteriophages at the end of their replication cycle to degrade bacterial cell wall and liberate new virions. A typical endolysin contains C-terminal catalytic domain (CD) responsible for cell wall enzymatic degradation and N-terminal cell wall binding domain (CBD) responsible for substrate recognition (Yang et al. 2014). According to Fischetti (2008), lysins are defined as hydrolytic enzymes affecting bacterial cell wall (i.e., peptidoglycan) that selectively and rapidly kill ($\geq$3 log CFU in 30 min) specific Gram-positive bacteria, providing a targeted therapeutic approach with minimal impact on unrelated

commensal microbiota. The antibacterial activity of exogenously applied lysin is typically limited to Gram-positive bacteria, i.e., organisms without an outer membrane or surface lipids and waxes. Although endolysins have broader activity spectra than phages, their properties can be further improved by combining CD and CBD from various phages. These engineered chimeric lysins, named chimeolysins, usually possess extended bacteriolytic activity against various Gram-positive bacteria (Dong et al. 2015). Finally, the activity of endolysins has been extended to Gram-negative bacteria, by fusing endolysins with signal peptides that allow penetration through outer membrane of Gram-negative bacteria (i.e., artilysins) (Lai et al. 2011; Lukacik et al. 2012; Briers et al. 2011).

Although phage lysins and their products are very active against bacteria, some studies showed that they can be more active in combination with antibiotics. Application of antibiotics that inhibit cell wall synthesis theoretically could influence the effectiveness of lysins, as they can allow the unhindered passage of lysins to peptidoglycan, especially having in mind that the lysins are proteins ($\geq$25 kDa in size) with larger size than antibiotics (0.3–1.6 kDa in size) (Fischetti et al. 2006). The first such synergy was observed by Djurkovic et al. (2005)—they reported a synergistic effect of bacteriophage lytic enzyme Cpl-1 and penicillin or gentamicin against *Streptococcus pneumoniae*, while such effect was recorded neither with levofloxacin nor azithromycin, suggesting that observed synergistic effect is antibiotic-specific. These authors presumed that the increased access of the enzymes to their respective cleavage sites or the enhanced destructive effect of a two-dimensional digestion in the three-dimensional peptidoglycan was responsible for the observed synergy. Recently, in another study, combinations of CF-301 lysin with vancomycin or daptomycin synergized in vitro and significantly increased survival of mice in staphylococcal-induced bacteremia compared to treatment with antibiotics alone (Schuch et al. 2014). These authors confirmed the superiority of CF-301 combinations with antibiotics in 26 independent bacteremia studies, proving that combinations of CF-301 and antibiotics represent an attractive alternative to antibiotic monotherapies currently used to treat *S. aureus* bacteremia. Similar was confirmed against *S. aureus* when lysine LysH5 was combined with nisin (García et al. 2010), Sal200 with nafcillin and vancomycin (Kim et al. 2018a), as well as when LysK was combined with a bacteriocin lysostaphin from *Staphylococcus simulans* (Becker et al. 2008). Lysins also showed synergistic action when combined mutually (e.g., Pal and Cpl-1 against *Streptococcus pneumoniae*) (Loeffler and Fischetti 2003) or with *S. pneumoniae* autolysin LytA (Rodríguez-Cerrato et al. 2007). Interestingly, Letrado et al. (2018) examined combinations of endolysin Cpl-711 with various antibacterial chemical agents, and confirmed synergy with beta-lactams amoxicillin and cefotaxime, but not with fluoroquinolone levofloxacin and glycopeptide vancomycin (additive effect), indicating that synergy between endolysins and chemical agents, even when they inhibit cell wall synthesis, is not an universal phenomenon. Chimeolysins also show synergistic interaction with antibiotics—e.g., combination of beta-lactam oxacillin with ClyS, obtained by fusion of Staphylococcus phage Twort lysin CD with CBD from phiNM3 phage lysin, synergistically kill MRSA (Daniel et al. 2010). The activity of a chimeolysin P16–17, composed of N-terminal domain of the Staphylococcus phage P16 endolysin and

C-terminal domain of P17 minor coat protein, is augmented by gentamicin (Manoharadas et al. 2009). Besides combinations of lysins with conventional antimicrobials, other outer membrane-permeabilizing agents also were tested. In their study, Briers et al. (2011) showed that ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA) was the most suitable component to combine with endolysin EL188, as *P. aeruginosa* growth has been reduced up to 4 logs for 30 min.

The combined therapy of artilisyn Art-175 and ciprofloxacin or tobramycin against *A. baumannii* resulted in an improved antibacterial effect for both mixtures over that obtained with conventional antibiotic monotherapy, but no significant improvement compared to results obtained with Art-175 alone (Defraine et al. 2016). Since Art-175 kills the bacterial cells very efficiently, the authors explained lack of synergy by the fact that neither ciprofloxacin nor tobramycin can exert its action.

These in vitro studies demonstrate that bacteriophage-encoded endolysin combination with certain antibacterial agents is promising and that co-administration approach can overcome some disadvantages of phage therapy, such as phage narrow activity spectra.

Another interesting group of phage-encoded enzymes, which are part of virions, are exopolysaccharide depolymerases. Using the combinations of phages and antimicrobials (amoxicillin and ciprofloxacin) the eradication of *K. pneumoniae* biofilms was successful, and it was explained by presence of phage-encoded depolymerase with polysaccharide-degrading activity, which allowed the passage for the antibiotic to the biofilm cells (Bedi et al. 2009; Verma et al. 2010). Thus, bacteriophage virions can possess enzymes that dissolve the biofilm matrix or induce corresponding enzyme production by the host bacterium. This was reported for clinical mucoid *P. aeruginosa* strains from CF sputum, where bacteriophage-derived enzyme was able to depolymerize the alginic acid of *P. aeruginosa* (Hanlon 2007; Glonti et al. 2010). The enzyme alginase produced by bacteriophages could have the potential to improve the condition of CF patients by facilitating the expectoration of sputum, accelerating phagocytic uptake of bacteria, and perturbing bacterial growth in biofilms. The ability of bacteriophages to weaken and/or even destroy the biofilm matrix can also enable or improve the penetration of other antibacterial agents, which consequently can augment interactions of bacteriophages and conventional antimicrobials. Having in mind that in the most cases bacterium *P. aeruginosa* is important for producing the biofilm matrix within multispecies biofilm, the CAPS effect could also be extended to the other present bacterial species. Although those species might not be targeted by the specific bacteriophage/enzyme, they could become more susceptible to antibiotics, due to matrix destruction. However, to our knowledge, combinations of EPS depolymerases and antibiotics have not yet been examined.

8 Methods for Testing Effectiveness of Phage-Chemical Antimicrobial Combinations In Vitro

The methods used for phage and chemical agent interaction are not defined and thus methodology used in present studies varies significantly. The standardization of methods used for establishing the in vitro synergistic interactions among chemical

agents and phages could save time and resources, contribute to more precise interpretation of data and reproducibility, and finally may influence the greater interest of pharmaceutical companies to invest in order to obtain adequate preparations for the treatment infections caused by drug-resistant bacteria.

To determine synergistic interactions, the broth microdilution limited series method, agar dilution method, or disk diffusion method can be applied (Eliopoulus and Moellering 1996; Doern 2014). The synergy testing by disk agar diffusion methods provides qualitative screening of interactions between two antibacterial agents, but the results are best for studying antagonism. The zone diameters for individual antimicrobial agents of interest are determined using standard techniques and for the estimation of agent combinations use another Mueller-Hinton agar plate and place disks separated by distance equal to sum of the zone radii for each disk when tested alone. After incubation the interface of the zones of inhibition are observed. According to the Eliopoulus and Moellering (1996) synergism shows an enhanced zone, indifference show no change, and antagonism shows an abbreviated zone. However, when phage interaction with chemical antimicrobials is examined by disk diffusion method, phages are usually inoculated with host bacterium and a disc with chemical antimicrobial is placed on the surface of agarized medium. The increase of phage plaque diameter around disc with antimicrobial indicates synergistic activity (Comeau et al. 2007). The disc agar diffusion method provides only qualitative information; therefore, it could be used prior time-kill assay for qualitative screening of interactions. The synergy can be applied to increase plaque size and number for easier phage isolation or plaque enumeration (Los et al. 2008; Santos et al. 2009). The subinhibitory concentration of ampicillin (2.5–3.5 μg/ml) in bottom agar can enhance visibility of extremely small plaques on *E. coli* O157:H^- lawns. The subinhibitory concentrations of penicillin G, ampicillin, cefotaxime, or tetracycline with 5% glycerol in top agar enlarge plaques of a siphovirus PVP-SE1 specific for *Salmonella enterica* serovar Enteritidis (Santos et al. 2009). The best results were obtained with 5% glycerol and 0.5 μg/mL of ampicillin, 0.06 μg/mL of cefotaxime, or 1.5 μg/mL of tetracycline. Some antibiotics, e.g., kanamycin and rifampicin, did not cause this phenomenon.

In general, the most commonly used methods for synergy testing among various chemical agents are time-kill method and checkerboard method, and today there are different experimental designs based on these two methods. These methods for estimation of interactions among antimicrobial agents are in consideration when the predictability of synergism is unknown, as with a new antimicrobial agent, or when the predictability is unreliable because of the development of bacterial resistance or treatment failures.

One of the commonly used methods for determining interactions among antibacterial agents is broth microdilution checkerboard method. This is a two-dimensional, two-agent broth microdilution assay for evaluation of antimicrobial agents' combination against drug-resistant organisms (Verma 2007; Wagner and Ulrich-Merzenich 2009; Doern 2014; Aleksic et al. 2014; Knezevic et al. 2016). This method is based on broth microdilution susceptibility method for evaluation of the bacteriostatic and/or bactericidal activity of antibacterial agents' specific

concentrations in combination at a fixed time. Prerequisite for this assay is estimation of MIC values for each agent, in order to establish the range tested concentrations for antibacterial agents (e.g., usually from 1/32 × MIC to 4 × MIC). The concentrations of one agent in microtiter plate are decreasing horizontally and for another vertically, enabling to test wide range of concentration for two agents in combination. Detected in vitro interactions are calculated and interpreted as synergistic, additive, indifferent, or antagonistic depending on whether the antibacterial activity of agents in the combinations is greater than, equivalent to, or less than the activities of the agents when applied alone. The fractional inhibitory concentration (FIC) of each agent is calculated for each combination of tested agents as follows:

FIC of agent A = (MIC of agent A in combination) / (MIC of agent A alone).

FIC of agent B = (MIC of agent B in combination) / (MIC of agent B alone).

The type of interaction among tested antibacterial agents is determined by summation of FIC values to obtain fractional inhibitory concentration index (FICI):

FICI = FIC of agent A + FIC of agent B.

The summation of two agents' antibacterial activity can be interpreted as synergistic interaction if FICI $\leq$ 0.5, additive interaction if 0.5 $<$ FICI $\leq$ 1, indifferent interaction if 1 $<$ FICI $\leq$ 4, and antagonistic interaction if FICI $>$ 4. These definitions of interaction types differ among investigators, and these here represented are the most commonly used (EUCAST 2000; Schelz et al. 2006; Mulyaningsih et al. 2010; Bassole et al. 2011). Interactions among antibacterial agents also can be represented geometrically with isobolograms. When phage lytic enzymes are tested, this method is very convenient, as enzyme concentration can be expressed in the same units as antibiotics. In the light of bacteriophage combination, checkerboard testing is impossible to perform, as concentration of the phages cannot be expressed in the same units for calculating the FIC index. However, phage MOI can be expressed as protein concentration, which can be useful for PACS examination by checkerboard method.

The time-kill method is used for examining the rate at which concentrations of an antimicrobial agent kill bacteria (Verma 2007; Wagner and Ulrich-Merzenich 2009; Aleksic et al. 2014; Doern 2014; Knezevic et al. 2016). This method can be used to study both the time-dependent and concentration-dependent antibacterial activity of tested antimicrobial agents. It can be used for evaluation of new antibacterial agents and its combinations with other agents, and possibly offer an answer for treatment failure in clinical trials, where bacterial count during time may be crucial for therapy outcome. This method enables varying the time point in which the specific agent will be added (e.g., agents could be added in two or more different time points—simultaneously with phage or after appropriate delay), also the agents' dosage can be varied, and more than two agents could be tested simultaneously. Before performing the time-kill experiment, some parameters must be defined: (1) the MICs for the agents which will be used, (2) other concentrations of antimicrobial agent to test (e.g., usually two and four times MIC), and (3) sample time points, based on agents' mechanism of action and used organism growth rate (usually 0, 4, 8, 24, and 48 h after inoculation, and for agents with fast bactericidal activity, e.g., 1, 2, 4, 6, and 24 h). When bacteriophages are applied as one of the antibacterial agents, multiplicity

of infection (MOI) used in the experiment should be determined according to previously established bacteriolytic efficiency. The general experiment conditions (temperature, incubation period etc.) depend on the used test organism and experimental design. After the establishing the main parameters, the assay is conducted by adding standardized inoculum (5.5×10^5–1×10^6 CFU/mL) into broth media containing different concentrations of an antimicrobial agents, different combination of agents, and also into the broth without agent as a control of bacterial growth. At the previously determined sample time points the number of viable CFU/mL from each test tube are determined by performing serial dilutions and by plating onto nutritive media (preferably Mueller-Hinton agar). The plates are incubated and the results for each treatment and control are plotted versus time, to obtain time-kill curves. The results obtained for two chemical antimicrobials can be applied also for phage-chemical antimicrobial combination: if bacterial count (CFU/ml) decreased by $\geq$2 log for the phage-antibiotic combination compared to the more active single agent, as well as to the initial inoculum titer, the interaction is considered as synergy (Knezevic et al. 2013). Chaudhry et al. (2017) used another approach to estimate CAPS. For calculation, they used the cell density obtained in the control (C; no treatment), and the surviving cell density after treatment with agent A, agent B, and the combination of A and B (S_A, S_B, and S_{AB}). The fraction of cells surviving A is S_A/C, of cells surviving B is S_B/C, etc. For facilitation, the following inequality should be fulfilled: $\log(S_A) - \log(C) > \log(S_{AB})$—logC, i.e., $\log(S_{AB}) - \log(S_A) < 0$ and $\log(S_{AB}) - \log(S_B) < 0$. Thus, no facilitation (hence no synergy either) is when the effect of combined treatment is no better than the effect of the best single treatment. Synergy requires $S_A/C \times S_B/C > S_{AB}/C$, i.e., log(C) – log(SA) – log (SB) + log(SAB) < 0. The authors conservatively applied a 0.05 criterion to both tests for rejection.

The time-kill method is known as labor-intensive, due to determining the CFU number in multiple sampling times for various treatments. At each step in the protocol, plaque assays could be performed using the soft agar overlay method to enumerate phage titers (Kutter and Sulakvelidze 2005). According to the Pillai et al. (2005) the time-kill curves can be considered as a clinically relevant model if the applied concentrations of used agents represent those which can be achieved at the site of an infection. To make results comparable and the most accurate, a time-kill curve method should be standardized and used as a gold standard for further studies of CAPS.

Due to facts that the main challenge for the expansion of phage application is a necessity to perform large-scale clinical trials in accordance with US FDA or European guidelines, which are usually very expensive and take several years, above-described in vitro time-kill method represent a valuable first step in evaluation of CAPS for in vivo studies and further clinical trials.

9 Possible Mechanisms of Chemical Agent-Phage Synergy

The mechanisms that generally lead to pharmacological synergy of conventional antimicrobials and alternative agents imply (1) multi-target effect in which agents target different sites in/on the bacterial cell, (2) pharmacokinetics or

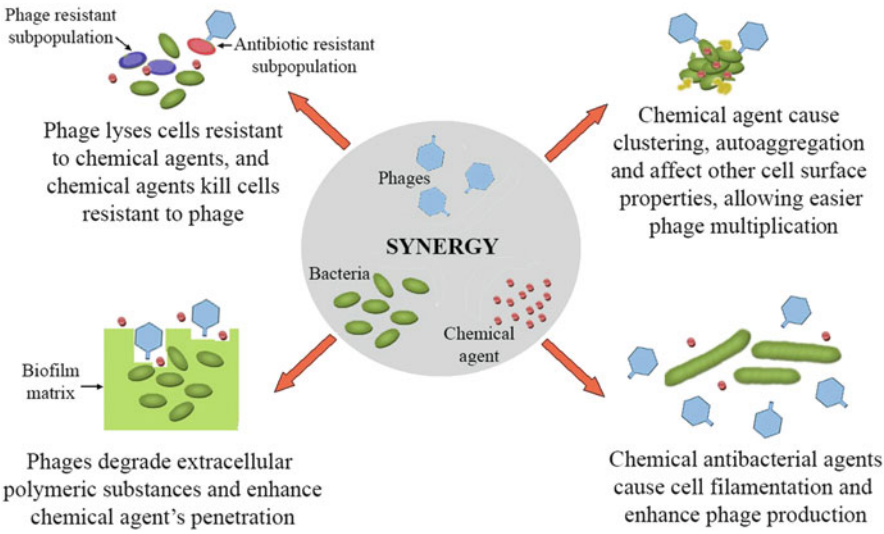

Fig. 2 Possible mechanisms of chemical agent-phage synergy (CAPS)

physicochemical effects (e.g., improvement of solubility or bioavailability), or (3) targeting a specific resistance mechanism of bacteria (Hemaiswarya et al. 2008; Wagner and Ulrich-Merzenich 2009). However, mechanisms of PACS seems to be different and some of them are following: (1) cell elongation/filamentation by antibiotic and subsequent phage multiplication; (2) prevention of phage- and/or antibiotic-resistant mutant occurrence; (3) phage change of cell permeability that allows antibiotic penetration; and (4) antibiotic effect on cell autoagreggation and other cell surface properties (Fig. 2).

Comeau et al. (2007) postulated that cell filamentation is responsible for the phenomenon, as beta-lactams and quinolones caused both filamentation and CAPS in *E. coli* and *Y. enterocolitica,* while gentamicin and tetracycline caused neither filamentation nor CAPS. Since antibiotics trigger SOS response, involvement of this reparative mechanism was examined in the CAPS onset, but it was shown that CAPS is independent from SOS response. The cell filamentation makes larger or altered pools of phage precursor molecules and removes inhibitors of phage assembly, facilitating cell lysis. The antibiotics also may accelerate cell lysis by, for instance, interrupting peptidoglycan synthesis and allowing holins/edolysins to further act in cell destruction. Furthermore, this was supported by an increase of plaque size when phages and antibiotics were combined. Not only the plaque size but also T4 burst size increased from 8 to 80 and 163 PFU mL^{-1} in combination with 1.86×10^{-4} and 7.43×10^{-3} μg mL^{-1} of cefotaxime, respectively (Ryan et al. 2012). Linezolid, tetracycline, and macrolides (telithromycin and clarithromycin) enhance a phage MR5 plaque diameter on MRSA lawns from 1.2 to 3.5 times, with reductions in the time of phage adsorption and the latent period (Kaur et al. 2012). The role of cell filamentation was further proved by Knezevic et al. (2013), indicating that a cell

elongation/filamentation appears to be a necessary but insufficient reason for phage-antibiotic synergy. Namely, both ceftriaxone and ciprofloxacin caused cell enlargement, but only ceftriaxone with a siphovirus gave a synergy, and not with a podovirus. It was also pointed out that some antibiotics can impede phage multiplication, by inhibition of DNA gyrase activity or protein synthesis, disruption of cell membrane integrity, etc., and that synergy may depend on specific phage-host and phage-antibiotic combinations. Importance of phage-host system was further confirmed, as combination of a myovius and ciprofloxacin resulted in a synergy during biofilm formation control (Sagar et al. 2016). Kim et al. (2018b) further contribute to the phenomenon elucidation, as observed that the phage adsorption efficiency was not changed after filamentation; although phage DNA and mRNA production was increased, a limited increase in protein production was noticed. The authors concluded that synergy is a result of a prolonged period of viral particle assembly due to delayed lysis, caused by the increase in the cell surface area and thus shortage of intracellular holins for aggregating and forming holes in the host membrane. Reactive oxygen species (ROS) stress by hydrogen peroxide also led to an increased production of phages, but heat stress does not. According to the current data, the antibiotics that cause cell elongation/filamentation play an important role in CAPS, but it is not the only explanation of synergy appearance, since antibiotics, which do not cause cell filamentation, also give synergy with phages.

The CAPS can be explained by the reduction of number of bacterial mutants resistant to phages and/or antibiotics. Simplifying this model, it can be stated that cells resistant to antibiotic are sensitive to phage and vice versa. Zhang and Buckling (2012), using a kanamycin-phage combination against *P. fluorescens,* observed a dramatical decline in bacterial population survival compared with single agent application, and ascribed this to prevention of bacterial resistance evolution. It is interesting that cells resistant to both agents suffered very large fitness costs. The mutant occurrence prevention during combined treatment can be an explanation for, e.g., a phage and tobramycin synergy (Coulter et al. 2014), since tobramycin does not cause cell filamentation. Using *E. coli* as a model, it was proven that the combined treatment decreased tobramycin-resistant cells for >99.99% and phage-resistant cells for 39%. On contrary, PB1 phage decreased tobramycin-resistant *P. aeruginosa* cells for 60%, while phage resistance was decreased 99%. Oechslin et al. (2017) confirmed that 2.5xMIC of meropenem and ciprofloxacin completely inhibit appearance of phage-resistant mutants. Significant impact of ciprofloxacin-phage (Jo et al. 2016) and linezolid-phage (Kaur et al. 2016) combination against resistance occurrence of *S. aureus* also have been documented. Similarly, when streptomycin and phage are applied, the best synergy was obtained when streptomycin was added 12 h after the bacteriophage treatment. It is interesting to mention that when *P. aeruginosa* was used as a model in CAPS, appearance of phage-resistant bacteria was not only short-term prevented but also long-term, regardless to antibiotics applied. In addition, CAPS had no impact on *P. aeruginosa* virulence, which is promising from the aspect of clinical application (Torres-Barcelo et al. 2016). In accordance with these findings, combined application of phages and rifampicin reduced appearance of antibiotic-resistant mutants, although synergy

was not recorded (Dickey and Perrot 2019). However, the combined treatment does not always lead to mutant occurrence prevention. In a microcosm system with *P. aeruginosa* PAO1 treated in a spatially structured environment with myovirus 14/1 and gentamicin combination, it was observed that phage–antibiotic rapidly lost the efficacy against both planktonic and biofilm populations due to rapid resistance evolution (Moulton-Brown and Friman 2018). Namely, phage selection correlated positively with increase in antibiotic resistance and biofilm growth was favored most in the combination treatment, with a relatively small cost of resistance. The authors concluded that spatial heterogeneity can promote rapid evolution of generalized resistance mechanisms without corresponding increase in phage infectivity, leading to failure of phage–antibiotic treatments in the evolutionary timescale. Still, these results support resistance prevention mechanism, considering that both resistant mutants and lack of synergy was observed in the experimental system.

It is evident that not only the type of antibiotic and phage, but also their order of application influence the final success of combined treatment, supporting the theory of decreased number of mutants. It was shown that when phages are added first, cephazolin and vancomycin more efficiently affect biofilm of *S. aureus* (Kumaran et al. 2018). For eight examined chemical antimicrobials, treatments with phage first, then antibiotic at either 2x or 10xMIC effectively reduced bacterial density (Dickey and Perrot 2019). The chemical antimicrobials at 2xMIC were ineffective against biofilm but effectively reduced the bacterial density of cultures treated previously with phage for 24 h. For some antimicrobials (ciprofloxacin, daptomycin, and linezolid), simultaneous treatment was as effective as sequential treatment, while for gentamicin, oxacillin, vancomycin, tetracycline, and erythromycin, sequential treatment with lower concentrations of antimicrobials (2xMIC instead of 10xMIC) was more effective. Interestingly, there was generally very little change in phage density when antibiotics were added at 2xMIC or 10xMIC, with an exception of gentamicin at 10xMIC that decreased phage number. The authors concluded that when it had a significant effect, phage pre-treatment improved the efficacy of low concentrations of antibiotics, but it decreased the efficacy of high concentrations of antibiotics. The influence of antimicrobials' order of application has also been observed for combination of ciprofloxacin and myovirus ELY-1 against *E. coli* (Lopes et al. 2018). The efficacy of the combined treatment varied with the antibiotic concentration and the time of antibiotic addition, being the best when MIC of ciprofloxacin was added 6 h after phage addition. Such treatment reduced the bacterial density and prevented the emergence of resistant variants for 1–2 logs. It was previously mentioned that CAPS varied depending on the order in which the rifampicin and phages are added and that sequential addition of agents in a biofilm system caused reversion of rifampicin-resistant mutants to sensitive (Escobar-Páramo et al. 2012).

Application of *S. aureus* as a model suggests that combined therapy can be more efficacious if there is an overlap in the physiological pathways targeted by the individual agents (Kirby 2012). For instance, bacteria treated with gentamicin show a strong aggregation phenotype and increased ability to form biofilm, but the aggregators are also more susceptible to phages than the parental cells. Thus, dual

treatment with gentamicin and the phage resulted in lower final cell densities than either treatment alone, and phage-resistant isolates could not be detected in the combined treatment.

According to some data, phages can improve antibiotic penetration into bacterial cells. For instance, besides the cell filamentation and autoaggregation, phage polysaccharide depolymerases can also contribute to the CAPS phenomenon (Sagar et al. 2016). As indicated, some phages possess the depolymerizing enzymes as a part of virions and can break down capsules and slime layers (e.g., *P. aeruginosa* alginate) around bacterial cells or in biofilm matrix (Hughes et al. 1998; Harper et al. 2014). This enzymatic degradation further potentiates antibiotic diffusion and cell penetration (Yan et al. 2013). Besides *Caudovirales*, *Inoviridae* (Pf1 and Pf3) also show CAPS in combination with gentamicin, tetracycline, chloramphenicol, or carbenicillin against PAK *P. aeruginosa* strain, whose cell wall is a barrier for antibiotic penetration (Hagens et al. 2006). The synergy is considered unique, since *Inoviridae* releases from a cell by extrusion, without lysis. It is believed that pores for extrusion allow antibiotic entry into a cell and consequently increase bacterial sensitivity to an antibiotic, leading to the synergism.

In a report of CAPS synergy in biofilm eradication, it was noted that the effectiveness of combined treatment of phages and amikacin on *P. aeruginosa* biofilm can be explained by altered surface charges of phage-resistant phenotypes and disruption of the biofilm matrix induced by some of the phages which can enhance the antibiotic penetration (Nouraldin et al. 2016).

Finally, other possible mechanisms should not be neglected. It has been proven that many prophages exist in sequenced bacterial genomes, as well as (pro)phage genetic elements in various strains (e.g., Knezevic et al. 2015). In this context, Fothergill et al. (2011) reported that the choice of antibiotic could dramatically affect the levels of free *Pseudomonas* phages, where ciprofloxacin and norfloxacin caused a level of phage induction higher than that observed with other CF-relevant antibiotics (tobramycin, colistin, ceftazidime, meropenem) against *P. aeruginosa* Liverpool epidemic strains (LES). Another well-known example is application of mitomycin C for prophage induction (Raya and H'bert 2009). Accordingly, antibiotic application can cause prophage induction from bacterial strains that contain complete prophages, by affecting lysis-lysogeny decision and thus can convert a lysogenic infection into a productive infection, which further contribute to enhanced bacteria lysis.

Probably all mentioned mechanisms play a role in synergy, but potential involvement of other still unrecognized factors or processes in the phenomenon onset should be further defined.

10 Shortfalls of Present Studies

There are several shortfalls of current studies: (1) negligence of bacteriophage biology, (2) existing prophages in bacterial hosts used as models, (3) variation in methodology, and (4) avoidance of CAPS mechanism elucidation.

In many studies, it is not clear which phage is used, as their phenotypic and genotypic characteristics were not examined. Thus, it is not clear whether the used phages are temperate or obligatory lytic. Temperate phages are not appropriate from the aspect of therapy, since they can carry various virulence factors, being able for both specialized and generalized transduction, as well as lysogenic conversion of bacteria, contributing to their virulence (Howard-Varona et al. 2017). For instance, Rahman et al. (2011) used a temperate phage induced from *S. aureus* to control biofilm, combining it with antibiotics. Even some obligatory lytic phages, whose procapsids are filled with DNA by head-full mechanism (e.g., T4), should be avoided in phage therapy, since they are more frequently involved in generalized transduction (Schneider 2017). Fortunately, current progress in the genome sequencing will diminish shortfalls of future studies.

As indicated, many bacteria carry prophages in their genomes, and their destiny during antibiotic action or infection with another bacteriophage is unclear. For example, more than 60% of *P. aeruginosa* strains contain at least one genetic element of Pf1-related phages (fam. *Inoviridae*), including strain K or PAO1 (Knezevic et al. 2015), used in the study of Hagens et al. (2006). The wild strain K is already infected with Pf1 phage, so application of this phage for CAPS in the experiments caused super-infection; similarly, PAO1 is infected with Pf4, so super-infection by Pf3 used by these authors can affect Pf4 phage production and thus cell survival. Furthermore, it was shown that lysogenization by myovirus P1 renders *E. coli* more sensitive to the DNA-damaging antibiotic ciprofloxacin (Ronayne et al. 2016). This implies that further attention should be paid on indigenous prophage destiny when CAPS is considered.

Variation in CAPS determination methodology is significant, so the results are rarely comparable, even if the same model strains, phages, or antibiotics were used. As mentioned above, a time-kill curve method should be a gold standard for CAPS estimation.

Finally, in the presented literature, CAPS mechanisms are not widely discussed, and there is a lack on studies focused on mechanism elucidation. These data can significantly contribute to understanding of CAPS and its better exploitation.

11 Advantages of CAPS Application

The advantages of chemical antimicrobial-phage combinations are numerous. First, it is proven that co-administration of phages and antibiotics could increase the phage efficacy by stimulating increased phage production, as seen in *Salmonella enterica*, *Escherichia coli,* and *Burkholderia cepacia* (Comeau et al. 2007; Kamal and Dennis 2014). Similar to multidrug therapy, combined phage-antibiotic therapy is less likely to fail due to bacterial resistance, because bacteria resistant to one agent still can be sensitive to the second agent and vice versa (Lu and Koeris 2011; Burrowes et al. 2011). Third, it is unlikely for phage- and drug-resistance to be acquired simultaneously, which is noted for some strains that carry multiple drug resistance determinants on mobile genetic elements (Partridge 2011). Fourth, the resistant

bacteria arising from combined phage-antibiotic treatment are usually less virulent comparing to resistant bacteria arising from drug treatment alone (Verma et al. 2009). The CAPS decreases effective antibiotic concentration, so reduces adverse effects of chemical antimicrobial agents, occurring after administration of high doses (Read et al. 2011). The CAPS can re-sensitize multidrug-resistant and pan-drug-resistant strains and thus present a possible treatment of potentially lethal infections. Finally, the synergy appears relatively fast, so in vivo bacterial numbers can be reduced for only several hours to the levels that the immune system can successfully cope with. Despite all these advantages, the clinical studies on phage-antibiotic application have not yet been conducted.

12 Concluding Remarks

According to the current studies, several consistencies related to the chemical agent-phage synergism can be observed: (1) CAPS is a strain-dependent phenomenon, being related to a strain's phage and chemical agent susceptibility; (2) it depends on mechanisms of chemical agent activity (that should not impair phage replication) and order of agents' addition/activity is sometimes crucial; (3) subinhibitory doses of various chemical antimicrobial agents can give synergy with phages, but most frequently the phenomenon is observed with beta-lactams and (fluoro)quinolones; (4) CAPS can be effective against planktonic cells, but also against bacterial biofilms; (5) there are several proposed mechanism of CAPS that probably play a role in the phenomenon; and (6) CAPS offers various advantages over single agent application. Accordingly, future studies should be focused on mechanism elucidation and clinical trials, to apply CAPS as new antibacterial armament.

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