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Title: Phage life cycles behind bacterial biodiversity

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Abstract: Bacteriophages (phages or bacterial viruses) are the most abundant biological entities in our planet; their influence reaches far beyond the microorganisms they parasitize. Phages are present in every environment and shape up every bacterial population in both active and passive ways. They participate in the circulation of organic matter and drive the evolution of microorganisms by horizontal gene transfer at unprecedented scales. The mass flow of genetic information in the microbial world influences the biosphere and poses challenges for science and medicine. The genetic flow, however, depends on the fate of the viral DNA injected into the bacterial cell. The archetypal notion of phages only engaging in predator-prey relationships is slowly fading. Because of their varied development cycles, environmental conditions, and the diversity of microorganisms they parasitize, phages form a dense and highly complex web of dependencies, which has important consequences for life on Earth. The sophisticated phage-bacteria interplay includes both aggressive action (bacterial lysis) and "diplomatic negotiations (prophage domestication). Here, we review the most important mechanisms of interactions between phages and bacteria and their evolutionary consequences influencing their biodiversity.

Keywords: bacteriophages, microbial evolution, horizontal gene transfer, biodiversity

1. INTRODUCTION

Although discovered in the late nineteenth century, phages still are one of the greatest mysteries of modern biology. These obligate intracellular parasites of prokaryotes undeniably make the most abundant group of biological entities in the world. The total number of bacterial cells on Earth is about 10^{30} , while the number of the phage particles is likely ten times higher [1]. Phages are commonly found in the biosphere, and the only factor limiting their occurrence is the presence of microbial hosts. Phages are almost everywhere and affect bacterial populations in all types of environments (marine, freshwater, soil, microbiome of multicellular organisms) [2].

The dynamics of the microorganism-phage interaction is perplexing; phage infections reach up to 10^{25} per second. Therefore, Earth functions as a giant bioreactor where bacterial and viral genetic materials are constantly undergoing random modifications driving the co-evolution of both groups [3]. Phage diversity is remarkable; for example, over 500 different phages assigned to over 30 different clusters have been isolated from just a single bacterial species like *Mycobacterium smegmatis* [4]. In practice, the isolation of a unique phage from the environment is possible only once. This is because phages acquire genes from the pool of phage and bacterial genomes, making the phage genomes a mosaic of exchangeable genetic modules. Recombination events cause changes in specific phage proteins or modify a large group of interdependent proteins [3,5].

Nevertheless, despite their broad genetic variability, phages have a surprisingly limited scope of lytic activity. Although phages infecting closely related bacterial species exist (e.g. within *Enterobacteriaceae* family) [6], the vast majority have specificity towards just one species, and some of them are specific to only a particular serotype or strain.

2. PHAGE LIFE CYCLES

There are four common phage life cycles: lytic, lysogenic, pseudolysogenic and chronic infection (Fig. 1). This review focuses on tailed phages, so chronic infection (typical for filamentous phages) will not be discussed and the reader can consult a recent review [7]. Each of these phage cycles involve at least five stages: adsorption, nucleic acid injection, assembly of virions, virion release, and further transmission [8].

Adsorption, one of the key steps in phage life cycles, requires the specific recognition of host surface proteins, lipopolysaccharides or other molecules (teichoic acids, fimbriae, flagella) on the bacterial cell envelope [9]. Successful recognition of bacterial surface receptors leads to permanent phage adhesion and allows penetration of the bacterial cell envelope and injection of the viral genetic material. Cell envelope penetration involves phage-encoded enzymes, such as the virion-associated peptidoglycan lysozyme, located on the phage tail, which hydrolyzes 1,4- β -linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues of the cell wall peptidoglycan backbone [10]. Depending on the phage type and physiology condition of bacterial cell, the phage genome remains free standing as a plasmid-like form (episome, in pseudolysogeny; Fig. 1C & 2E) or can be incorporated into the genome of the host (prophage, in lysogeny; Fig. 1D).

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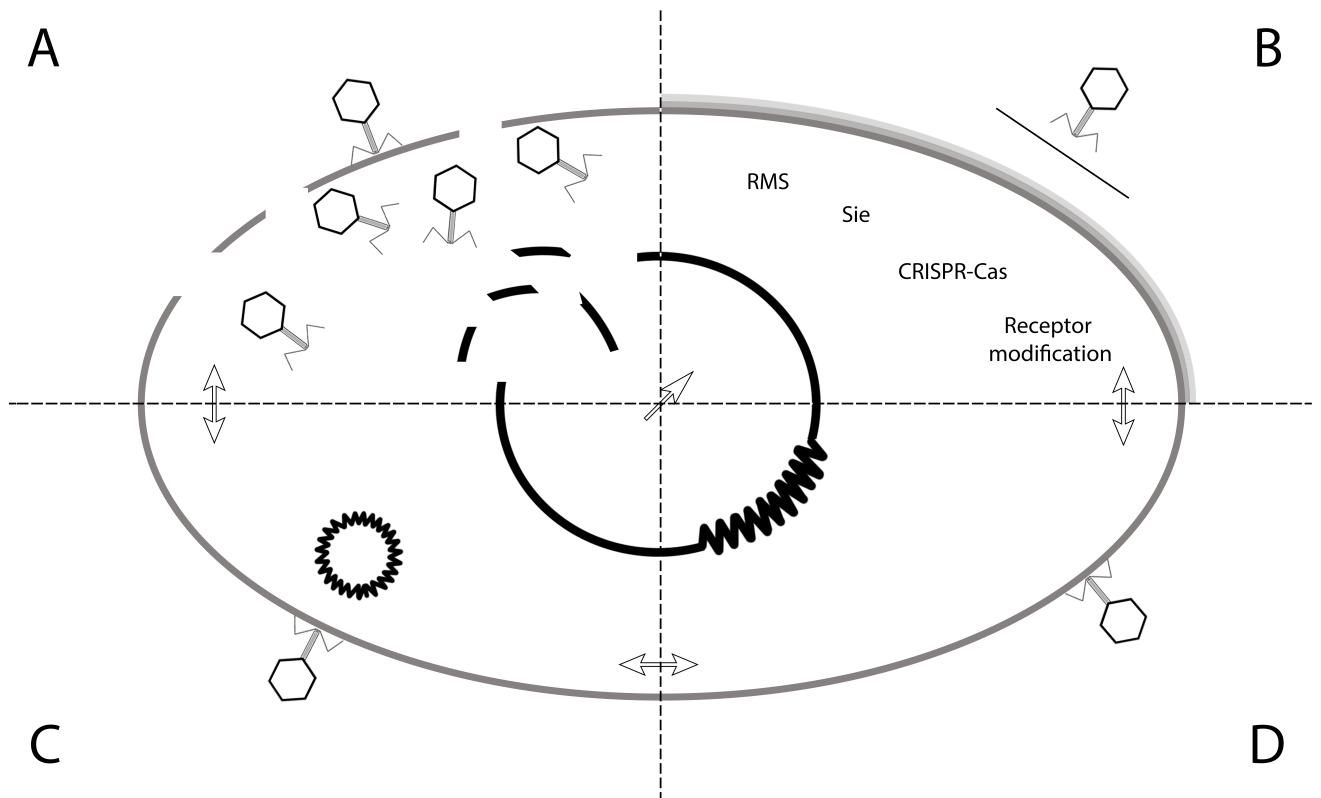


Fig. 1. Potential effects of phage infection. A – cell disruption during lytic cycle, B – phage development blocked by bacterial resistance mechanisms, C – phage genetic material forming episome (pseudolysogeny), D – phage genetic material integrating with bacterial genome (lysogeny).

Pseudolysogeny is related to starvation stress of the bacterial host and occurs in both lytic and temperate phages. After entering the host cell, the phage genetic material remains inactive in the form of a circular episome, and the development cycle is halted until environmental conditions improve. While the development cycle of lytic phages is simply stopped, infection by temperate phages may lead to two subpopulations of bacteria - lysogens and phage-carrying cells. In other words, a bacterial population exposed to temperate phages contains both lysogens and a stable fraction of productive phage-carrying cells. Unlike lysogens, the phage genetic material in phage-carrying cells segregates asymmetrically during bacterial cell division, resulting in infected and non-infected cell lineages [11,12] (Fig. 2D).

While being integrated into the host chromosome, a temperate phage can stay dormant as a prophage replicating along with the host genome. Occasionally, and mainly under stress conditions, the temperate phage proceeds to its lytic cycle (Fig. 1A). The lytic part of the phage cycle is identical for both lytic and temperate phages. Upon activation of the lytic cycle, phage infection induces the reprogramming of host genetic functions resulting in rapid replication of the viral genome and expression of structural and functional phage proteins. In subsequent steps, phage virion particles are assembled, loaded with the viral nucleic acid and released to extracellular environment upon lysis of the host cell. Bacterial membrane and cell wall disruption depends on specific lysis proteins such as endolysins (cell wall peptidoglycan hydrolases), holins (plasma membrane

disruption), and spanins (destabilization of the Gram-negative bacterial outer membrane) [13–16].

Recent reports of the Sorek research group [17] show that the initiation of lytic or lysogenic cycles in temperate phages depends on specific phage-encoded signal peptides. This unique discovery demonstrates the existence of a molecular communication system between phages (reminiscent of bacterial quorum sensing). Studies on the *Bacillus subtilis* phage phi3T showed that in the early phase of infection (soon after viral DNA introduction) one of the first expressed phage genes are *aimP*, *aimR*, and *aimX*. The AimP protein is enzymatically trimmed to a 6-residue peptide (termed arbitrium) and exported outside the bacterial cell. Arbitrium uptake by the neighboring cells occurs via bacterial oligopeptide permease transporter (OPP). The main function of arbitrium is inhibition of the intracellular phage protein AimR, which activates the transcription of the *aimX* gene (associated with the implementation of the lytic cycle). Consequently, in the initial phase of infection (at low arbitrium concentrations) phages propagate intensively by the lytic cycle. With an increasing number of infected bacterial cells arbitrium concentration increases, leading to a development cycle switch from lytic to lysogenic [17].

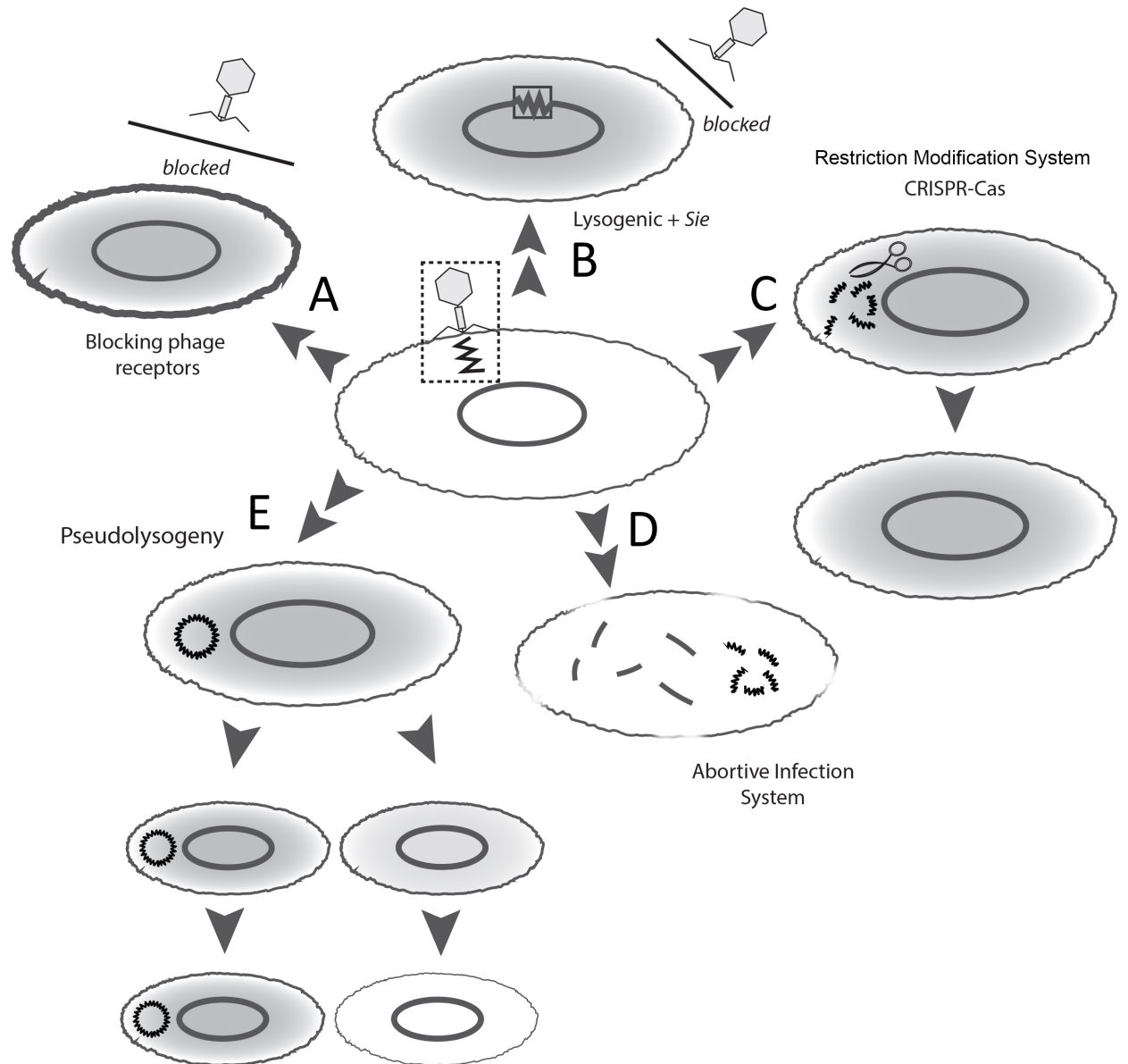


Fig. 2. Phage resistance mechanisms. A – phage receptor blocking, B – superinfection exclusion (Sie), C – phage genetic material cleavage (restriction-modification and CRISPR-Cas systems), D – abortive infection system (Abi), E – phage genetic material loss during asymmetrical cell division (pseudolysogeny).

3. PHAGES AS DRIVERS OF MICROBIAL EVOLUTION

The contribution of phages in horizontal gene transfer among bacteria is enormous. Transduction, the phage-mediated transfer of foreign DNA (phage or bacterial) into bacterial cells, can occur as a generalized or specialized event. In generalized transduction, random fragments of the bacterial DNA are included into phage virions instead of viral DNA. These pseudoviral particles can still adsorb to cells and eject the packaged DNA into the new host. Depending on the type of donor DNA (plasmid or chromosomal), the transduced molecule can be either integrated into the chromosome of the new host by

recombination or remain free in the cytoplasm replicating as a plasmid. Generalized transduction may occur during lytic step of both lytic and lysogenic life cycles [18]. In contrast, specialized transduction is typical of temperate phages and involves the prophage DNA excision from a specific integration site along with an adjacent part of the host genome. After packing into virions and infection of a new host cell, site-specific recombination (mediated by phage integrases) or homologous recombination (by bacterial recombinases) result in integration of the phage DNA into the host genome, usually at specific locations [19].

The appearance of phenotypic changes as a result of the integration of prophage into the host genome is called the

lysogenic conversion [20]. Usually, lysogenic conversion enhances bacterial fitness by increasing their pathogenicity [21]. Coexistence of phages and bacteria in the environment along with the horizontal gene transfer, allows the bacterial host for gene pool diversification, which facilitates the adaptation to new environmental conditions [22]. Thus, phage-mediated gene transfer allows bacteria to colonize novel ecological niches, which influences subsequent gene transfer with local populations deepening bacterial diversity [13].

The lysogenic cycle occurs when phage DNA integrates into bacterial genome and propagates jointly with the host DNA. Lysogenic bacteria also become immune to secondary infections by homologous phages and display increased bacterial fitness. Together, these properties influence community composition and survivability [21]. Further, prophages in bacteria infecting eukaryotic cells may provide critical functions for intracellular invasion, propagation and dissemination (Table 1). Upon lysogenic conversion, prophages spread bacterial virulence factors such as adhesins, toxins, enzymes and other proteins enabling invasion or providing resistance to host clearance mechanisms, antibiotics (Table 2) and even other phages (when the prophage genome comprises whole CRISPR array) [23]. Temperate phages also improve the pathogen's ability to form biofilms and influence biofilm dispersal [24]. Phage particles may transfer interspecific chromosomal islands and genetic information related to other phages (e.g. pyocins or specific protein secretion systems) [21].

Table 1. Bacteria pathogenic features, encoded by genes acquired via prophages integration [18,25,26].

Gene name	Phage*	Encoded feature	Host
<i>stx</i>	933, H19B	Shiga toxin	<i>E. coli</i> O157:H7
<i>ctxAB</i>	CTX ϕ	Cholera toxin	<i>Vibrio cholerae</i>
<i>C1</i>	Phage C1	Botulinum neurotoxin	<i>Clostridium botulinum</i>
<i>speA</i>	T12	Erythrotoxic toxin	<i>Streptococcus pyogenes</i>
<i>tox</i>	β -phage	Diphtheria toxin	<i>Corynebacterium diphtheriae</i>
<i>oac</i>	Sf6	O-antigen acetylase	<i>Shigella flexneri</i>
<i>entA, sak</i>	ϕ 13	Enterotoxin A, Staphylokinase	<i>Staphylococcus aureus</i>
<i>nanH</i>	Gifsy-2	Neuraminidase	<i>Salmonella enterica</i>

Two types of lysogenic conversion influencing bacterial pathogenesis can be distinguished. In one type, the expression of bacterial virulence factors takes place from the prophage during lysogeny, and in the other phage-encoded virulence factors are expressed during the induction of the lytic cycle mode [24,30]. Negative lysogenic conversion arises when phage DNA integration occurs in crucial regions of the genome for specific functions. In this case, the phage genetic material simply disrupts the integrity of particular bacterial genes [24].

Table 2. Antibiotic resistance encoded by genes acquired via prophages integration. The table focuses mostly on β -lactamases since β -lactam antibiotics are widely used due to their clinical efficacy and low toxicity [27–29].

Gene name	Encoded feature	Host
<i>mecA</i>	Penicillin-binding protein	<i>Staphylococcus aureus</i>
<i>bla_{TEM}</i>	β -lactamase	<i>Escherichia coli</i>
<i>bla_{PSE-1}</i>	β -lactamase	<i>Campylobacter jejuni</i>
<i>bla_{OXA-2}</i>	β -lactamase	<i>Pseudomonas aeruginosa</i>
<i>bla_{PSE}</i>	β -lactamase	<i>Salmonella enterica</i>

Even if the phage genome does not encode any virulence factors, prophage can affect bacterial toxin production and secretion by gene expression regulation. This kind of phage regulatory switch is also called active lysogeny [30]. Gene disruption may lead to gene inactivation and therefore creation of new phenotypes (e.g. lipase and β -toxin negative *Staphylococcus aureus* phenotype caused by integration of two relatively small prophages into genome – L54a and phi13) [31].

4. PROPHAGE DOMESTICATION BY THE HOST CELL

The viral genetic material can be modified inside bacterial host, which may lead to damage by nucleotide non-synonymous substitution or deletion of genes essential for the phage developmental cycle. This process may depend on random mutations or poorly understood bacterial mechanisms leading to a gradual degradation of the viral genome retaining only beneficial genes for the bacterium. Domesticated prophages are subjected to further changes resulting from spontaneous mutations or recombination with other (also unrelated) phages, plasmids and host DNA [32,33].

Phage genetic material in the form of prophages may comprise up to 20% of the bacterial genome. The presence of several phage-like sequences within the genome of almost every bacterial species underscores the generality of this phenomenon. Most of these prophages are no longer inducible and therefore referred to as “cryptic” prophages). Non-inducible prophages may be present in the host cell in four forms depending on the degree of genome degradation associated with the integration of viral and bacterial genetic material: defective prophages, satellite phages, bacteriocins and other prophage-related entities such as “gene transfer agents”. Simplifying the viral genome is a continuous process, but the selection mechanism works differently in particular bacterial species and also depends on environmental conditions. Therefore, some bacterial species retain a large part of the phage genome, while only the residual genes (e.g. bacteriocins) are preserved in others. Conceptually, the process of assimilation of prophage DNA by the host genome can be divided into several stages. The first stage of results in a defective prophage unable to perform the lytic cycle usually due to loss of functions (by

mutation or gene deletion) required for DNA packaging into the phage capsid [34]. The next stage results in a “satellite” phage (e.g. *Escherichia coli* phage P4) lacking the genes encoding the capsid structural proteins and requiring a specific helper phage to propagate [35]. Additional stages involve the progressive reduction of the phage genome to only residual genes encoding specific proteins (e.g. phage-tail-like bacteriocins) [34] or the formation of gene transfer agents. These are phage-like particles that transfer random elements of the bacterial genome to other bacteria. In this case, the mechanism of gene transfer resembles generalized transduction but in contrast to the phages, gene transfer agents do not carry the genes encoding the phage structure. Moreover, because of the small capacity of the head, these particles are unable to carry large amount of DNA necessary to generate functional phage-like particles [36].

5. BACTERIA AND PHAGE INTERPLAY – COADAPTATION

The interplay between bacteria and phages leads to co-adaptation. Thus, phage genes contribute to bacterial fitness under certain environmental pressures, and also the presence of the phage alters how bacteria interact with other phages. Indeed, not only the bacterial genome is the subject of phage-induced changes, but also the pattern of bacterial gene expression can be modified by phages to preclude superinfection by other phages. This can even include situations where two distinct integrated phages can lead to co-resistance [37]. The Red Queen hypothesis, introduced by Leigh Van Valen in 1973, assumes that species must constantly evolve and adapt not only to better reproduce, but also to effectively compete with other organisms in the ever-changing environment. This concept explains the two phenomena: co-evolution of competing species driving the extinction of slower-adapting ones and advantage of sexual reproduction over the asexual one (in case of the diploid organisms). The adaptive changes of one species within a particular niche usually causes selection pressure on other species what resembles an endless arms race [38]. The Red Queen hypothesis works in the relations of competition, predator prey and parasite-host as well. According to the Red Queen hypothesis, competition between phage and bacteria (predator and prey) leads to an endless “arm race” [39], since constant modification and selection of new features takes place leading to bacterial response to phage appearance and then rapid reaction of phage to resistance mechanism developed by the prey [40,41]. The classic lytic cycle leading to the lysis of almost the entire population of a particular bacterial species exemplifies the predator-prey relationship and co-evolution of phage and its host. The lytic cycle is the classical example of predator-prey relationship, where phages exploit particular bacterial population to propagate more effectively. In this case, phage predation results in the lysis of the vast majority of susceptible bacterial cells within the population [40]. However, the complete eradication of the microorganisms never occurs, because persisters (dormant, highly tolerant to antimicrobials variants of regular cells) arise in every bacterial community [42] as well as phage-resistant clones induced under the selective pressure caused by phage invasion. Thus, two types of cells are responsible for the

microbial community revival. Bacterial resistance to phages may be established through several possible mechanisms. Phage infection induces the activation of bacterial defense systems, such as restriction-modification, the CRISPR-Cas, and abortive infection, or leads to the survival of variants possessing modified phage receptor. No matter which of the abovementioned mechanisms occur, the emergence of phage-resistant clones causes a selective pressure on the phage [40].

Phage infection induces activation of bacterial defense systems, such as restriction-modification, the CRISPR-Cas (see below, paragraph 6.), and abortive infection [40]. CRISPR-Cas provides adaptive immunity to phages or plasmids, allowing bacteria to recognize and degrade foreign DNA entering the cell [43]. Conversely, phages develop mechanisms preventing abortive infection (e.g. through the production of bacterial-like antitoxins), disarm the CRISPR-Cas system (e.g. by mutations in protospacer-adjacent motifs or preventing formation of the CRISPR-Cas complexes), and inactivate restriction-modification systems (e.g. by methylation of phage DNA or reduction in the frequency of restriction sites by mutation) [41]. The situation becomes more complicated when the lytic phage infects a bacterium in unfavorable environmental conditions becoming pseudolysogenic (Fig. 2E). In this case, the host remains viable until the environmental conditions become more favorable for bacterial growth, thus providing a larger number of host bacterial cells for phage multiplication [11]. Pseudolysogeny was observed for the first time by Frederick Twort in 1915 [44], but for many years, researchers were unable to clearly define this phenomenon. Ripp & Miller described pseudolysogeny as an unstable state in the phage development cycle where the viral genetic material remains dormant (so called preprophage) despite injection into a sensitive host cell. There is neither integration into the genome nor lytic cycle initiation [45]. The direct cause of this halt in the phage development cycle remains unknown. However, pseudolysogeny typically is related to the starvation stress of the host, when the bacterium cannot replicate the phage genetic material or produce phage proteins efficiently. This state is a kind of adaptation of the phage, enabling to survive in the nutrient-deficient environment. Despite the preprophage cannot replicate the half-life of the phage particle increases significantly because it can be passed to host daughter cells. The preprophage resumes the regular development cycle once the nutrient conditions become permissive for the growth of the host population [30,46]. Generally, bacteria evolve by expanding their resistance while phages adapt by broadening the host range [47]. The relation between bacteria and phages is by definition antagonistic so their co-evolution is rather a reciprocal process. The selection pressure associated with the phage predation leads to the acquisition of new mechanisms of resistance. Conversely, the phages evolve towards extending the host range to capture emerging phage-resistant clones. Some phages possess more than one type of tail fibers/spikes recognizing host receptor. *E. coli* phages K1-5, and *Salmonella* phage SP6 produce two tail protein with endosialidase and lyase activity enabling the infection of different host serotypes [48]. Phage phi92 and PVP-SE1 from myoviruses have five/six different receptor binding

proteins being tail spikes or tail fibers [49,50]. The existence of phages possessing more than one sets of enzymatically active tail spikes, specific to different host receptors is an example of broadening the host range by acquisition of new enzymes using horizontal gene transfer. The interactions between phages and their hosts are not limited to recognition and adhesion. In fact, phage development cycle is adapted to the host biology at every single stage. The end result is that bacteria cannot develop resistance to all phages and phages cannot attack all bacteria cells [47]. This brings a trade-off, which is associated with the phenomenon of antagonistic pleiotropy and limited genetic capacity (too many genes is a considerable energy expenditure) [51].

Bacterial DNA sequences responsible for phage resistance are highly changeable, and some of them may even develop different cellular functions. This process is called “exaptation” (shift of function), which refers to two ecological situations: when the feature adapts to a new function by natural selection or when the origin of feature is not connected to natural selection but further is co-opted to a new function [52]. Neofunctionalization is preceded by modification of gene of interest like domain shuffling, modified regulation or duplication. The new feature appeared as a result of duplication, changing gene regulatory network, but the final step on the way of neofunctionalization is refinement of primitive feature [53]. Another example of exaptation is the change of a restriction-modification system, originally directed against foreign DNA molecules/phages, into a system participating in epigenetic modifications and regulating bacterial pathogenicity by turning on and off genes responsible for colonization, immune system avoidance or environmental adaptation [40]. That occurred by loss of REase activity with maintaining of MTase activity, which started to function as epigenetic regulator [54]. This may regulate the foreign DNA uptake and “bacterial suicide” by autolytic DNA degradation [40]. It can also methylate bacterial genome, leading to switching on and off different genes, what influence bacterial ability to adapt to new milieu, colonization and immune avoidance [55,56]. Other example of exaptation is a use of Toxin-Antitoxin module to cut mRNA, aborting reversible the translation event, as a response to stressing factors, like phage infection [57,58]. Further, lytic phages can provide selective pressure enabling the propagation of less virulent bacterial strains through modification or loss of surface structures that act as virulence factors and are also used as phage receptors [59].

Phages can mutate in bacterial monoculture, while higher diversity arising from independently acquired fragments of phage DNA (e.g. *via* the CRISPR-Cas system) makes phage adaptation more difficult [60]. CRISPR-Cas immune bacteria readily dominate among CRISPR-Cas deficient ones, since high variety of acquired phage DNA fragments makes phage adaptation (by point mutation) less likely [60]. Similarly, inhibition of coevolution occurs in phage tail fiber - bacterial receptor coadaptation. Bacteria can introduce unrestricted changes in the receptor while phage must adapt to a specific one [47]. However, every structural change of bacterial receptor is a trade-off; bacteria gain phage resistance, but often lose a virulence factor [47]. Since hypervariable genomic regions encode phage

receptors, receptor alternation becomes even more frequent by the acquisition of new genes from a foreign pool [61]. The structures used by phages as adhesion receptors may be crucial for bacterial pathogenicity and physiology, meaning every permanent change in the receptor composition and structure can be undesirable for bacteria, therefore some of them use so called phase variation, where the receptor expression can be reversibly inhibited [62]. Phage adhesion receptors usually are important virulence factors or they are required for key physiological processes. Examples of these receptors are smooth type of LPS, capsule, type-IV pili, which serve for pathogen attachment, protection against immune system activity (phagocytosis, complement activity) or biofilm formation. Thus, changes in the conformation or structure of bacterial surface components can reduce bacterial virulence. Some bacteria can eliminate the risk of negative consequences of receptor structural changes by phase variation. This mechanism temporarily inhibits the expression of a phage receptor [62]. But even though, the phages develop specific strategies to oppose that kind of changes, such as reverse transcriptase-mediated tropism used by *Bordetella* phage [63,64].

6. PHAGE – RESISTANCE MECHANISMS

Due to the multi-stage character of phage infection, bacteria may develop various methods of prevention of the invasion, which protect individual cells or the entire population (Fig. 2). Four main resistance mechanisms may occur: blocking the access of phages to bacterial receptors, preventing entry of phage DNA, digestion of nucleic acids and abortive infection systems. Phage adsorption is one of the most complex steps of the phage infection cycle, as phage particles must localize the proper receptor among thousands of cell envelope components. Some bacteria prevent absorption of the phages (Fig. 2A) by masking receptors with various components, removing or modifying them, or by forming an extracellular matrix. For example, different masking phage receptors strategies, such as production of masking lipoproteins (Llp) or cell-wall anchored proteins (protein A), have been described among strains of *E. coli* or *S. aureus*, respectively, which provide a physical barrier preventing phage attachment [65,66]. The receptor can be removed, as in *Campylobacter jejuni*, by inhibiting flagellin production [67,68]. Bacteria can also modify outer membrane structures, such as changes in the *E. coli* TolC and OmpA proteins [69–71]. Extracellular structures such as exopolysaccharide or lipopolysaccharide can also mask bacteria receptors [72,73]. Lipopolysaccharide is also a common receptor for the phage adhesion, and a common way to block phage infection involves changing the composition of the O-antigen lipopolysaccharide or shortening its structure [74]. The next group of resistance mechanisms involves preventing the entry of phage DNA (Fig. 2B). They are based on superinfection exclusion (Sie) proteins, which are encoded by either lytic or lysogenic phages. Sie proteins are anchored on the cell membrane or associated with other membrane components and prevent DNA from reaching cytoplasm of the host [75,76]. Prophages may encode more than one Sie system [77].

Another way to counteract phages is by cleaving the phage's DNA, typically using a restriction-modification or CRISPR-Cas systems (Fig. 2C). Both systems are directed against newly acquired foreign DNA and may work in synergy. Most bacteria have restriction-modification proteins classified into four groups (type I – IV) [78–80], which recognize and digest cytoplasmic DNA that is not methylated at appropriate sites. In some cases, the acquired phage DNA is already modified by phage methylases and protected from the restriction event.

The CRISPR-Cas system, mentioned before, consists of three main parts: regions of clustered, palindromic repeats, spacers and Cas proteins. The system comprises two classes, which incorporates five types. Types II and V belong to CRISPR-Cas class 2, while types I, III and IV are fitted to CRISPR class 1 [81]. Regardless of class the operation mode can be divided into adaptation to new nucleic acid, expression and interference. Adaptation begins with the recognition of invading DNA, prior fragmented with synergistic R-M system [82]. The fragment of the DNA is afterwards equipped with leader-end repeats and integrated into the system by endonuclease-acting Cas1 and Cas2. During the expression phase, the palindromic repeats region as well *cas* genes are transcribed to the pre-CRISPRs or pre-crRNAs, which are template for crRNA possessing newly acquired invading DNA fragment. This process differs between classes of CRISPR-Cas systems, nonetheless it leads to creation of protein and ribonucleoprotein complexes, which can recognize hostile DNA. When the expression is complete, the complexes locate and degrade the invading DNA [83]. CRISPR-Cas system has been identified in almost 40% of bacteria and 90% of *Archaea* representatives [84,85].

Another method of defense against phages is abortive infection (Fig. 2D), which involves stopping phage multiplication by inducing death of the infected bacterial cell death. The elimination of a low quantity of single cells enables the larger bacterial population to survive, since progeny virions are incomplete and cannot be released into the environment. Abortive infection systems may work at different stages of the phage lifecycle (replication, transcription, translation, or DNA packing) [71,86–88].

The best studied abortive infection systems for Gram-negative bacteria are described in *E. coli*. The two-component RexAB system causes the membrane potential disruption as the consequence of activation by protein-DNA complex formed during phage DNA injection. The second, working on the translation level, is the Lit system cleaving the elongation factor EF-Tu what leads to the inhibition of protein synthesis. Another is the anticodon nuclease Prr system or PifA based on membrane protein. An important mechanism of abortive infection systems involves toxin-antitoxins controlling bacterial the cell death via protein-protein, protein-RNA or RNA-RNA interactions (MazEF and hok-sok in *E. coli*). The most detailed abortive infection systems studied in Gram-positive occur in lactic bacteria, which have more than twenty different mechanisms (abortive infection systems A-Z) recognizing and stopping phage infection at various steps of the life cycle. [71,86–88].

7. PHAGES AS GLOBAL ECOSYSTEM REGULATORS

Due to their abundance and diversity, phages have a major influence on local and global ecosystems. By controlling the size of bacterial populations or communities, phages contribute to the redistribution of nutrients in the environment. In the marine environment alone, phage lytic activity releases about 10^8 tons of organic carbon daily [89]. Phages in the marine ecosystem are associated with the phenomenon of "microbial loop", which results in the recirculation of more than half of organic matter in the oceans. This revival of the food chain through the enrichment of the local environment in organic components available for microorganisms also transmit nutrients up to the food chain or alternatively, store them in the form of sea bottom sediments [20,90]. Apart from obvious effects on the carbon cycle, phages also participate in the redistribution of nitrogen and phosphorus, which directly affects the growth of phytoplankton [91,92]. The microbial loop operates not only on a global scale. Phages also affect the circulation of matter locally, such in the process of bioremediation of mine water or in the development of the intestinal flora [93,94].

8. BIOLOGICAL INTERACTIONS BETWEEN PHAGES AND MICROORGANISMS

The influence of phages on the environment extends beyond the regulation of microbial populations and the circulation of matter in nature. Sophisticated interactions between phages and their hosts go far beyond the typical predator-prey relationships (Fig. 3). Indeed, lysogeny shapes an ecosystem's metagenomics and influences the evolution of both microorganisms and phages. Temperate phages are involved in horizontal gene transfer, which often contributes to beneficial changes in the metabolism of the host. Lysogenic conversion under appropriate conditions results in the acquisition of novel virulence factors (e.g. exotoxins or the ability of biofilm formation), resistance mechanisms (e.g. for antibiotics or phages), and upregulation of the metabolism in general. All these benefits have a dynamic impact on the local environment by giving an advantage over other microorganisms and remodeling ecological niches [95]. The interactions between phages and microorganisms do not fall within the classical paradigm of parasitism. Indeed, depending on the type of the replication cycle, these relationships can be mutualistic or antagonistic, such as predator-prey, parasite-host and allelopathy (production of the substances that can have positive or negative influence on other organisms) [13,96]. In specific niches, phages condition bacterial abundance. Various phage-bacteria interactions can be distinguished. Although neutralism – lack of interactions between bacteria and phage (Fig. 3A), should be theoretically the most common state, it is difficult to prove since many of the other interactions can influence bacteria and phages indirectly. For example, "neutral" bacteria may benefit from the release of biomass after a lysis event, in which case this indirect interaction could be considered as commensalism (Fig. 3B).

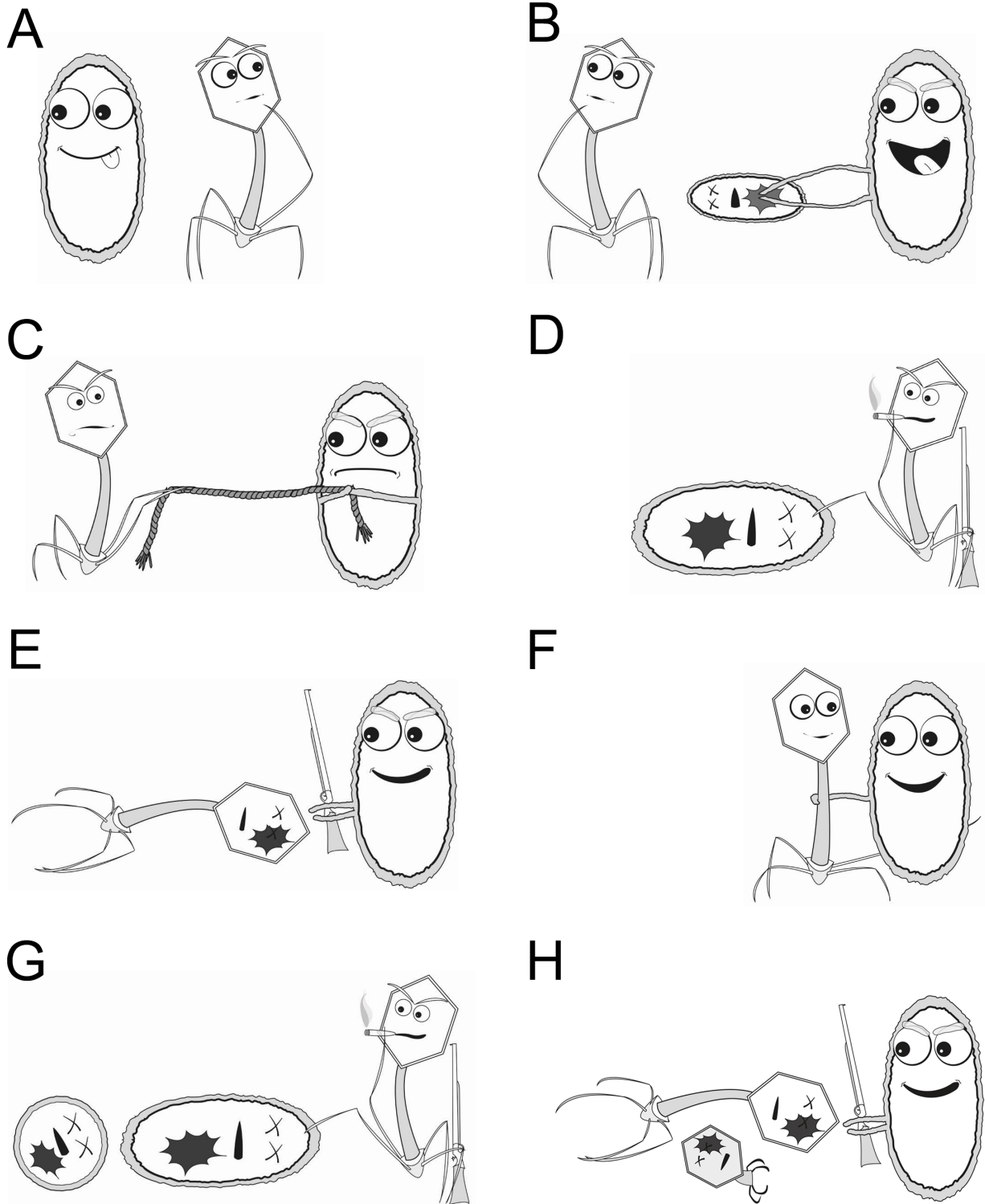


Fig. 3. Interactions between phages and bacteria. A – neutralism; B – commensalism, C – competition; D, E – predator-prey; F – mutualism; G, H – amensalism.

The most common interaction between phages and bacteria is competition (Fig. 3C). Phages compete for the host with other predators such as predatory bacteria (e.g. *Bdellovibrio*) or other phages [13]. Competition is connected to another antagonistic interaction – predator-prey, which can be observed for both phage and bacteria (Fig. 3D). Predation is one of the ways for phages to control

dominant bacterial populations, diversifying prokaryotes by allowing the non-dominants to co-exist [97,98]. Conversely, the predator-prey interaction may be adverse for phages (Fig. 3E) leaving them as a prey to the bacteria, which may use them as nutrient source [13]. Another type of interaction between phage and bacteria is mutualism (Fig. 3F), such as lysogenic conversion, which often increases the fitness of

the host and protects the prophage from unfavorable environmental conditions [99,100].

Amensalism implies an adverse effect on phages while the effect on bacteria is neutral, or *vice versa* (Fig. 3G). The example of negative interaction for phage and neutral for bacteria (Fig. 3H) can be the destruction of the non-antagonistic phage by the ecto-enzymes secreted by bacteria [13].

Lysogenic cycle, typical of temperate phages, can be considered in three ways. First, the genetic material of the phage is replicated and continuously propagated by the bacteria. Second, the integrated prophage often provides new virulence and resistance mechanisms that enhance the lysogen's competitive advantage and protect it from secondary phage infection (superinfection exclusion). This type of relationship can be described as mutualism [95]. Third, temperate phages can become allelopathic factors if the progeny phages are released in a heterogeneous bacterial community (infected and uninfected by phage). Population of lysogens in this case, wins the competition with non-infected strains by limiting their proliferation [101,102]. Thus, for genetically identical strains differing only in the presence of the prophage, this particular phage can be simultaneously allelopathic (for non-infected cells), and mutualistic (for cells carrying a prophage) [103].

9. PHAGE – BACTERIUM – ENVIRONMENTAL INTERPLAY

Phage ecology should be investigated as a three-sided interplay since the surrounding environment also influences phage-host interactions [23]. Even if the phage can kill most of the bacterial population *in vitro*, multiple factors (e.g. some chemical compounds, immune system, digestive enzymes or even transfer by circulatory system) can inhibit phage predation *in vivo*. Bacterial starvation can be seen as phage pseudolysogeny inducing factor, since this kind of phage lifecycle may support phage survival [45]. Traditional predator-prey interaction patterns can be found between bacteria and phages co-existing in simple systems where the phage mode of action is mainly lytic. Phages specific for saprophytic bacteria frequently carry out a lysogenic cycle (60-70% of gut bacteria are lysogenic) [23,24].

Abiotic factors (e.g. nutrient concentration) in environments occupied by particular phages and bacteria affect the generation of progeny phages better suited to the bacteria living in the same niche [104]. Similarly, the adaptation to higher temperature makes bacteria more resistant to phages, while phages become more infectious [105]. Propagation of animal and human pathogens at 37°C increases their motility, what helps to colonize new niches, but alters virulence factors expression. On the other hand, under these conditions there is a cost for phages in terms of developing phage resistance (Fig. 2). Further, motile bacterial clones (generated as a result of temperature selection) encounter phages more frequently, leading to faster co-evolution of bacteria and their predators [106]. In variable environments (with frequent and severe conditions fluctuations) and in nutritionally poor environments the

temperate phages are generally more successful than lytic or dormant ones [107].

10. BIOFILM COMPLICATES PHAGE – BACTERIA INTERACTIONS

Interactions between phages and microorganisms are much simpler to model in the context of free-living bacteria. However, this situation is rare in nature since bacterial populations usually reside in multicellular biofilms. Biofilms have a dense extracellular matrix containing exopolysaccharides, extracellular DNA, proteins, amyloid fibers, fatty acids, phages and, of course, bacterial cells of varied phenotypes and metabolic activities [108]. The structure of biofilms constantly evolves under the influence of environmental factors including phages. Temperate phages are actively involved in both the creation and dispersion of biofilms. For example, strains of *Pseudomonas aeruginosa* lacking of Pf4 prophage form less stable biofilm communities than wild type. Biofilm dispersion and release of the bacterial cells involved in new niche colonization are also limited in this case, since the absence of prophage prevents the degradation of the surface layers of the biofilm [109,110]. The thick biofilm matrix forms a physical barrier against external lytic phage invasion and antimicrobials. Unlike antibiotics, some phages are equipped with enzymes such as exopolysaccharide depolymerases enabling virions to penetrate into the biofilm matrix [111]. However, the phages have established such mechanisms during co-evolution for successful infection, and for the release of progeny from biofilm structure. Therefore, the local damage of the biofilm structure may not provide an enormous improvement in therapy when applied as supportive for standard antibiotics. Moreover, the possible bacteria-phage interaction scenarios become more complicated in the case of biofilm-forming bacteria due to the large phenotypic variation of microcolonies within biofilms [112].

11. PHAGE ABUNDANCE VERSUS BACTERIAL ABUNDANCE

According to Odum (1953), the complexity of the environment correlates with its productivity. However, different components of a particular environment are correlated with its productivity in different ways. More specifically, the diversity of bacteria is inversely proportional to the productivity of the environment and the diversity of phages [113]. Two models of bacterial population dynamics in natural environments can be considered [114]. A constant-diversity dynamics model, which dominates in environments where free-living cells co-exist and compete while constantly being exposed to phage predation, and a periodic-selection model more suitable for environments where phage pressure is limited (e.g. biofilms, host intracellular environment).

In the environments of limited nutrient availability, with no phage pressure, the susceptible strains can simply eliminate the phage-resistant ones because of fast rate of propagation. If phage predation occurs, both types of bacteria (phage-sensitive and phage-resistant) can co-exist,

depending on resistance costs and the actual state of environment [115]. Phage predation does not lead to bacterial elimination, but rather to the establishment of steady state where one phage can defeat another just by using the same host. More aggressive phages win the competition because of higher burst size, better adhesion rates or shorter latent periods. This competition underscores that the diversity of bacteria is always higher than the diversity of phages, which gives bacteria an edge in the "arms race" [116]

Under favorable conditions, the higher growth rate is beneficial for both bacteria and lytic phages [116]. According to "killing the winner" hypothesis, the dominance of susceptible bacteria population in limited niches implies accessibility of high numbers of the host cells for rapid phage propagation. As a result, the niche becomes colonized by other (phage-resistant) strains or species because of dominant population decimation [117]. Obviously, this is detrimental to the strain, which loses dominance. However, the lytic phage action against competitive strains is advantageous to the phage-resistant bacteria and switches the relation status from host-parasite to mutual companions [22]. The case of single lytic phage pressure described above is of course uncommon. In fact, in every niche there are various phages what increase the probability of cross-infections leading to considerable genetic and phenotypic diversity of bacterial strains [21]. This matter is far more complex due to the existence of the temperate phages, which may convert seemingly homogenous bacterial population into mixture of phenotypically different clones. Moreover, a superinfection exclusion (Sie) mechanism protects the bacterial host from secondary infection by closely related phages, being another factor driving phage evolution. Differences in phage-resistance, induced by temperate phages, lead to higher diversification among virulent lytic phages [116,118,119].

Knowles et al. (2016) propose the "piggyback the winner" model in which highly dense populations suffer greater selection pressure to initiate lysogenic cycles, which maintains both high density of bacteria and the phage population. In such situation, the prophage causes bacterial resistance to superinfection, [120]. Establishing phage resistance by mutation, which typically involves loss of gene function may lead to reduced growth rate (compared to phage susceptible bacteria) or susceptibility to phagocytosis [23].

12. CONCLUSIONS

The importance of phages for the environment and evolution of microorganisms cannot be appreciated without realizing that all processes and interactions occurring in the world of microorganisms have molecular underpinnings. Evolution promotes specific phenotypes in specific environments, as every ecological niche has its own set of biotic and abiotic factors, influencing the phage and bacterial populations and affecting the dynamics of their interactions. Ongoing arms race between bacteria and phages led to the emergence of phage-resistance mechanisms (RM, Abi, Sie, CRISPR-Cas, receptor modifications), and at the same time enabled the spread of

viral genetic material in various ways (lytic cycle, lysogeny, pseudolysogeny). The widespread phenomenon of horizontal gene transfer causes the constant mixing of phage and bacterial genomes and their almost unlimited transfer between strains and species. The natural mechanism of selection pressure, depending on environmental conditions, preserves or removes acquired genes contributing to the increased the versatility of both microorganisms and phages. Acquisition of new resistance and virulence factors by bacteria increased their pathogenicity, but the preservation of the acquired features depends mainly on the local environment. The introduction of a specific phage to the environment most often generates distinct bacterial clones with different phenotypes. Phages, by many considered as remedies against increasing antibiotic resistance, may also be Trojan horses of antibacterial therapy.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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