Chapter 9

Bacteriophage Therapy: An Alternative for the Treatment of *Staphylococcus aureus* Infections in Animals and Animal Models

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Abstract

Staphylococcus aureus causes hospital-acquired (HA), community-acquired (CA) and companion animal and livestock-associated (LA) infections. Molecular epidemiology studies suggest that although host specificity may be associated with specific genetic lineages, recent human-to-animal and animal-to-human transmissions related to mobile genetic elements have been described. Gene transfers include virulence and antibiotic resistance genes, thus making it difficult to control multidrug resistance *S. aureus* infections. Bacteriophages (phages) and endolysins, the enzymes responsible for bacterial lysis by phages, are alternatives to the use of antibiotics for the control of *S. aureus* infections. In this work, we review current advances in the development of phage therapy and the study and design of recombinant endolysins to treat *S. aureus* infections. Preliminary results of bacteriophage isolation based on molecular epidemiology knowledge show that bacteriophages are specific of genetic lineages and that this strategy may be used as an approach to isolate and evaluate new bacteriophages for therapy.

Keywords: bacteriophage therapy, endolysins, enzybiotics, antibiotic resistance, molecular epidemiology

1. Introduction

1.1. *Staphylococcus aureus* as a zoonotic pathogen

Besides infecting human hosts in hospital-acquired (HA), in community-acquired (CA) infections as an opportunistic pathogen and in food poisoning by enterotoxic strains, *S. aureus* has also been isolated from animal hosts, both in livestock-associated (LA) and in companion animals' infections. Due to the raise of methicillin-resistant *S. aureus* (MRSA) strains, this feature was included as a phenotypic marker to identify *S. aureus*, and now they are described as MRSA or methicillin-sensitive *S. aureus* (MSSA). Molecular epidemiology approaches helped to the

Genetic lineage	Original described host	Further reports	Other features
ST1	Human	Cow, horse, chicken, pig[a]	–
CC5	Human	Chicken, turkey, dog[b,c]	ST5. Major HA clone; dog isolates in Japan and Spain
ST8	Human	Horse, cow, fish[d]	USA300. Major CA clone; fish isolates in Japan
ST9	Pig	Chicken	–
ST22	Human	Cat, dog[e,c]	EMRSA-15 global CA epidemic clone
CC97	Cow	Human, pig[f,g]	Loss and acquisition of virulence gene and pathogenicity islands lead to change in host specificity; recent transmission between cattle and pigs in Slovenia and Italy
ST121	Human	Rabbit	–
CC126	Cow	–	–
CC130	Cow	Sheep, deer[h]	In semiextensive red deer farm in Spain
CC133	Sheep	Goat, cow, cat[b], dog[c]	Cat isolates from Japan; dog isolates from Spain
ST239	Human	Cow	HA clone in Europe; isolates from bovine milk in Turkey[i]
CC705	Cow	–	–
CC385	Chicken	Wild birds	–
ST398	Pig	Human, cow, chicken, horse, dog[c]	Acquisition of genetic elements to evade immune response in new hosts. $mecA_{LGA251}$ (*mecC*); Spanish kennel dogs isolates
ST425	Cow	–	–
ST1464	Sheep	–	–

Modified from Refs. [5, 59, 60]; [a][61]; [b][62]; [c][63]; [d][64]; [e]ST deduced from homology between pet and human strains by PFGE and by *spa*-typing [65]; [f][7]; [g][8]; [h][66]; [i][67].

Table 1. Animal-associated genetic lineages of *S. aureus*.

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understanding of the genetic structure of the *S. aureus* genetic population dynamics and hence in making predictions on transmissions between humans and animals. Multilocus sequence typing (MLST) is one of those molecular approaches. MLST analyzes the allelic combination of seven-to-nine (in *S. aureus* and other bacterial species) housekeeping genes that are randomly distributed along the genome. Mutations in *S. aureus* genes (*arcC, aroE, glpF, gmk, pta, tpi* and *yqiL*) are registered in an open public database (http://saureus.mlst.net) hosted at the Imperial College of London and supported by the Wellcome Trust Foundation. Each allele for each gene is designated with a specific number, so the allelic profile of a strain is designated by the numbers of alleles designated for each gene in the order described previously. Each allelic profile is designated with a sequence type (ST) number. STs sharing six or less alleles are grouped in clonal complexes (CC) in which the STs with the highest frequencies and number of shared alleles are designated as founder or subfounder clones, giving the name to the CC or related subgroups [1, 2]. Genetic lineages represented by a particular ST or CC are associated with specific hosts and geographical distributions. Some of them were originally described as specific for human or animal hosts and further reports associated them with animal or human transmissions, respectively, thus suggesting the zoonotic potential of *S. aureus* lineages. **Table 1** shows the major genetic lineages of *S. aureus* associated with animal hosts.

It is important to establish that the original description of a genetic lineage associated with a particular host followed by posterior reports of association with other hosts may not represent the evolutionary story of that lineage; it may only represent the original interest for the host due to the anthropocentric reasons or by the importance of the animal host as a food source or its contact with the human owner.

ST398 is one of the most reviewed cases of a clone showing animal-to-human transmission. Due to the whole-genome sequencing of strains from human endocarditis and bovine mastitis, differences in genomic content suggested that ST398 may be originated in humans. By loss, acquisition and reacquisition of pathogenicity islands or a staphylococcal chromosomal cassette related to methicillin resistance (SCC*mec*), and particular virulence genes like those encoding Panton-Valentine leukocidin (PVL) or the tetracycline resistance gene tet^R, ST398 susceptible to methicillin was originally transmitted from humans to animals and then back to humans as a methicillin-resistant strain [3, 4]. Similar events may occur for bovine-specific clones from CC97. Staphylococcal protein A (*spa*) and clumping factor A (*clfA*), which are important in human pathogenesis, appear as nonfunctional mutants in bovine isolates, suggesting that they are not important for bovine colonization. Alleles of von Willebrand factor are specific for each host, and pathogenicity islands seem also specific for each host [5,6].

Reports of interspecies transmission of *S. aureus* infections are becoming more frequent. In a study of CA-MRSA distribution in Slovenia, ST398, an originally pig-associated genotype, was found in 9.9% of the cases [7]. CC97 was first described as associated with bovine mastitis cases and now has also been found in humans and pigs. Of particular interest is the case of a multidrug-resistant LA-MRSA genotype from Italy that has been transmitted to pigs as MSSA and spilled back after methicillin resistance acquisition [8]. Ovine-associated *S. aureus* isolates are represented by CC133. In a global survey in Western Europe and Mediterranean countries, CC700 and CC522 were also ovine-associated. This distribution differs from North and South

America and Australia, where CC133 is the major ovine clone. Isolates from CC97 (bovine-associated), CC5, CC8 and CC30 (human-associated) were also found in this report, indicating high interspecific transmission of these genotypes [9]. Among zoological park animals in Greece, human-associated lineages ST80, ST8 and ST15, some of them with human pulsotype by PFGE analysis, suggest human-to-animal transmission [10]. ST80 and ST15 genetic lineages were also found in companion animals with close human contact in a veterinary teaching hospital in Greece. Panton-Valentine leukocidin (PVL), a necrotic toxin involved in skin infection, was found in 68.2% of MSSA isolates and in 50% of MRSA isolates, reinforcing the probable human origin of those strains. Also ST398 MRSA isolates were found that belong to the human cluster [11]. *S. aureus* has also been associated with wildlife animals. Studies in Spain demonstrate the presence of ST398 (pig- and human-associated) and ST1 (human-associated) MRSA isolates harboring the novel *mecC* methicillin resistance gene (see below) in either red deer, Iberian ibex, wild boar or Eurasian griffon vulture, suggesting a probable human origin of these isolates [12–14]. All of these examples represent the high transmission capability of apparently species-specific *S. aureus* genetic lineages and urge to the implementation of both molecular epidemiology surveillance and novel infection controls.

Antibiotic resistance is also a major problem of *S. aureus* infections. There is a constant interchange of mobile genetic elements modifying the virulence arsenal of *S. aureus* genetic lineages. This suggests that genetic background may be considered for the design of modern strategies to control *S. aureus* infections.

After the discovery of penicillin by Alexander Fleming in 1928 and its application to treat *S. aureus* infections in 1940, the first penicillin-resistant *S. aureus* strains were reported by 1945. Later in 1959, methicillin appears as an alternative to the use of penicillin. By 1961, the first methicillin-resistant *S. aureus* (MRSA) strains were reported. A similar story occurred for vancomycin-resistant (VRSA) and vancomycin-intermediate (VISA) *S. aureus*. Methicillin resistance is encoded by a staphylococcal chromosome cassette named SCC*mec* containing the *mecA* or *mecC* ($mecA_{LGA251}$) genes conferring resistance in humans and animals, for which at least 11 variants have been described. Apparently, these cassettes originated from a macrococcal *mecB* gene, which originated *mecA* (SCC*mec* and chromosomal forms) and *mecC* in staphylococci [15]. *mecC* has been almost exclusively associated with SCC*mec* type XI and located in animal strains from different STs and CCs [16], suggesting an intense intergeneric mobilization of SCC*mec* cassettes. VRSA strains seem not to be a major problem since only a dozen of clinical strains has been reported in the last decade. Vancomycin resistance is mediated by a complex of four genes (*vanA, vanH, vanX, vanY*) carried in a transposon. These modify a D-alanyl residue to D-lactate rendering the peptidoglycan structure resistant to vancomycin binding. *vanA* plasmids have also been reported, one of them being efficiently transmissible. This may predict that in the future, VRSA will also become a public health problem. Spontaneous mutants giving raise to VISA clones within vancomycin-susceptible *S. aureus* (VSSA) populations are known as heterogeneous-VISA (hVISA). hVISA/VISA is difficult to detect because on a first screening isolates behave as VSSA. Under the presence of vancomycin, VISA individuals are selected, and on a second screening, they behave as VISA. hVISA/VISA phenotypes have been associated with mutations in around 20 different genes

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that divert metabolism to peptidoglycan synthesis. Peptidoglycan then entraps vancomycin. hVISA/VISA reports are becoming more frequent in the literature, and it is to date considered of more relevance than VRSA. Staphylococci also present multidrug resistance genes such as *erm* (conferring resistance to macrolides, lincosamides and streptogramin B—MLS_B-) and *vga* (conferring resistance to lincosamides, pleuromutilins and streptogramin A) genes. Some of these genes are located in plasmids or transposons that are highly mobile genetic elements [16]. All of these evidences suggest that antibiotic resistance is becoming a major public health problem for the control of *S. aureus* infections, so alternative biotechnological approaches different from classical antibiotic treatments must be used in the future to control *S. aureus* infections. Bacteriophage therapy is one of those approaches.

1.2. Bacteriophages

Bacteriophages are viruses that infect only bacteria. They coevolve with their hosts optimizing its spread and release mechanisms from the bacterial cell to the environment and cause (in the case of lytic bacteriophages) lysis of the bacteria. They are also a major driving force in *S. aureus* evolution as a pathogen since many virulence genes are mobilized between different strains by means of transduction [17]. Bacteriophages are the most abundant biological entities of nature, although they are present in all environments, it is in aquatic systems where they are in greater proportion [18, 19]. Early indications of the presence of viral particles were reported in 1896 when bacteriologist Ernest Hanking observed that from the waters of the river Jumma in India, they identify a "substance" with antimicrobial activity against *Vibrio cholerae* and this substance was also heat labile and capable of passing through the filters of porcelain used at that time [20]. Two years later in 1898 Gamaleya observed a similar phenomenon in *Bacillus subtilis*. In 1915 and 1917, Twort and D'Herelle, respectively, discovered the viral particles called bacteriophages [21]. Frederick Twort in 1915 reported antimicrobial activity against *Staphylococcus aureus* suggesting that it could be viral particles among other possibilities. As of D'Herelle, he coined the term bacteriophage in 1917; this discovery was due to their previous studies to develop a vaccine against dysentery where he observed lytic plaques later named as bacteriophages [22]. In 1923, the National Institute of Bacteriophages in Tbilisi Georgia was established. Since then, the search for lytic bacteriophages for the biological control of infectious diseases has been in the scene.

1.3. Generalities

Bacterial viruses (bacteriophages or phages) possess genetic material in the form of DNA or RNA; morphologically, they consist of a head and a tail both constituted of protein. The head is the core package of nucleic acid surrounded by a protein shell or capsid also called lipoprotein. The tail varies on complexity from one bacteriophage type to another [23]. According to their lytic activity, they can be divided into two groups: lytic and lysogenic bacteriophages. When bacteriophages infect their host, they reproduce and the process ends with lysis of the bacteria and release of viral progeny. This is known as the lytic cycle. When the bacteriophages are able to integrate its genetic material into the bacterial genome and thus reproduce for several generations together with their host's genome, they are called temperate phages and

they reproduce by a lysogenic cycle [24]. Bacteriophages which possess double-strand DNA express highly specific enzymes called viral-associated peptidoglycan hydrolyses (VAPGH) that bind to the bacterial cell surface and cause disruption of the cell wall to inject their DNA into the host cell [25]. The filamentous phage releases their viral progeny without causing the death of the bacteria [18], while nonfilamentous phages cause bacterial lysis by synthesizing endolysins (enzymes encoded by double-strand DNA phages) that hydrolyze peptidoglycan as part of an holin-endolysin system. The endolysins and holins are synthesized at late stages of phage infection. Endolysins accumulate in the cytoplasm until viral particles are assembled and holins form pores in the membrane allowing cytoplasmic translocation of endolysins through the membrane for peptidoglycan degradation [26]. Furthermore, single-stranded DNA or RNA bacteriophages synthesize "lysines" which interfere or inhibit the synthesis of the bacterial peptidoglycan [27]. The VAPGHs and endolysins are able to degrade the peptidoglycan when applied externally, which is why these enzymes represent an alternative to be used as enzybiotics in Gram-positive bacteria [28]. Bacteriophages and their endolysins are highly specific, infecting or hydrolyzing only a single species of bacteria attaching to specific receptors on the surface of host cell. The specificity of interaction between phage attachment structures and host cell surface receptors determinates host range. [29].

2. Bacteriophage reproduction

2.1. Lytic cycle

Phages replicate inside bacterial host and the process finalizes with lysis of the host and spreading of phage progeny. Phage replication includes the following steps [30]:

1. **Adsorption**. Phage attachment to a specific host cell in a process involving interaction with receptors on the surface of a susceptible host cell and an infecting virus. There are two major types of receptors: components of a bacterial cell like lipopolysaccharide, peptidoglycan, outer membrane proteins and teichoic acids, and fimbriae-type receptors like *pilli* or flagella.
2. **Nucleic acid injection**. Through the tail, phage injects its genetic material into the cell after peptidoglycan degradation behind pore formation (by VAPGH). The phage coat protein that includes capping head and tail structure remains attached to the bacterial surface.
3. **Replication**. After injection of its nucleic acid, phage expresses early genes that redirect host synthesis machinery to the reproduction of viral nucleic acid and proteins.
4. **Assembly and packing phage particles**. Once the viral components are synthesized, the genetic material is encapsulated in its protein coat, and complete virus particles are formed.
5. **Phage progeny release**. Phage late proteins like holins and endolysins or murein synthesis inhibitors are produced, and they are responsible for the lysis of the host cell and the release of viral particles to the environment.

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2.2. Lysogenic cycle

The lysogenic cycle comprises the same steps as lytic cycle, but after penetration of the genetic material, the phage nucleic acid is inserted into the chromosome of the bacteria and is replicated as a segment of the own bacterial genome for one or more generations without metabolic consequences for the bacterium. After this cycle, the genetic material of the phage can be excised from the bacterial chromosome and enter into a lytic cycle; usually, this occurs under physiological stress or damage of the genetic material.

3. Endolysins

The term endolysin was coined until 1958 to refer to the phage component responsible for the bacterial lysis. Lytic phages present a genetic cassette encoding a holin-endolysin system. At the end of the reproductive cycle, once mature viral particles have been assembled, holins are synthesized in critical concentrations and inserted into the cell membrane, creating pores for the translocation of endolysins, previously accumulated in the cytoplasm, to reach the peptidoglycan structure [19]. Endolysins are classified according to its enzymatic activity (**Figure 1**) in: (1) N-acetylmuramoyl-alanine amidases, which hydrolyze the amide bond

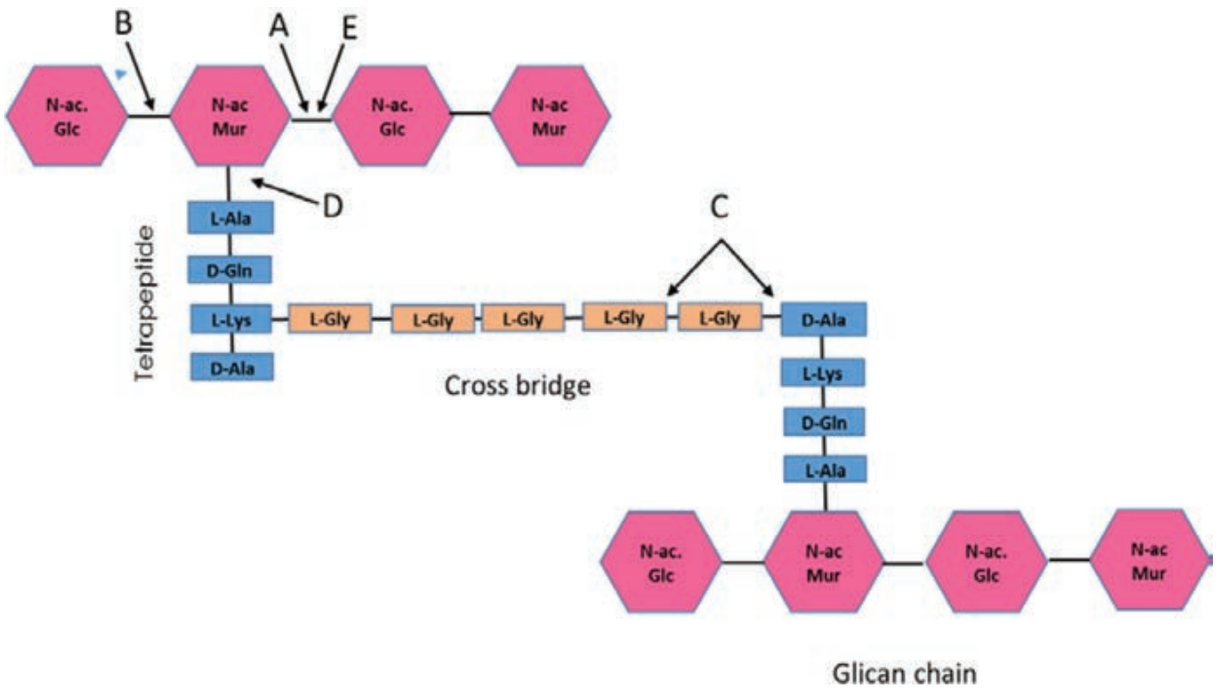

Figure 1. Enzymatic activities of endolysins. (A) N-acetyl-muramidase catalyzes the hydrolysis of N-acetylmuramoil-β-1,4-N-acetylglucosamine. (B) N-acetylglucosaminidase catalyzes the hydrolysis of N-acetylglucosaminil-β-1,4-N-acetylmuramine. (C) Endopeptidase hydrolyzes peptidic bonds on amino acids chains linked to the glycan moiety or in the pentapeptidic bridge. (D) N-acetylmuramoyl-L-alaninamidase hydrolyzes the amide bond that connects the glycan with the amino acids. (E) Transglycosylases attach the glycosidic β-1,4 bonds resulting in the formation of a 1,6 anhydrous ring in N-acetylmuramic acid (modified from Barrera-Rivas et al. [19]).

between the N-acetyl-muramic in the glycan chain and the L-alanil residues; (2) endo-β-N-acetylglucosaminidases, which hydrolyzes the N-acetylglucosamine β-1,4-N-acetylmuramine acid linkage; (3) N-acetyl-muramidases, which catalyze the hydrolysis of N-acetylmuramoil-β-1,4-N-acetilglucosamine bond; (4) transglycosylases, which disrupt β-1-4 glycosidic bonds by forming a 1–6 anhydride ring in the N-acetylmuramic residue; (5) endopeptidases, which may hydrolyze both the tetrapeptide linked to the glycosil moieties and the pentapeptide entrecrossing bridge [31, 32].

Endolysins encoded by double-stranded DNA bacteriophages have a molecular weight between 25 and 40 kDa [33]. Most of endolysins are composed of at least two functional domains: one containing the catalytic activity located generally in the N-terminal domain and one responsible for the recognition of a specific substrate associated with the C-terminal domain. In some cases, more than one catalytic domain or more than one recognition domain are present [19]. The recognition domain usually joins to specific molecules in the bacterial cell envelopes such as monosaccharides, coline or teichoic acids [34]. Endolysin activity is usually species specific, although there have been reports of endolysins with a wider substrate range. Besides, the cell wall recognition domain is not always essential for endolysin activity. The endolysin got a wider substrate range, but it conserved certain specificity, since it was no active against all bacteria. Studies of crystallography and mutation analysis with endolysin *PlyL* against *Bacillus anthracis* led to propose that the C-terminal domain of this endolysins inhibits the activity of the catalytic domain by particular intermolecular interactions. This inhibition is released when the C-terminal domain binds to its particular ligands in the target cell wall, thus acting as a regulatory domain [35]. Most of the reported endolysins from phages against *S. aureus* have two catalytic domains and a cell wall recognition domain being LysK one of the must studied endolysin models. LysK has a cysteine/histidine-dependent aminohydrolase/peptidase (CHAP) catalytic domain that hydrolyzes the peptidic bond between the D-alanine of the oligopeptide chain attached to the sugar backbone and the first glycine of the pentaglycine bridge that is typical of *S. aureus* peptidoglycan and confers resistance to lysozyme. CHAP presents the higher activity of both hydrolytic domains. LysK also has an N-acetylmuramoyl L-alanine amidase or amidase-2 (Ami-2) catalytic domain which catalyzes the hydrolysis of the N-glycosidic bond between the N-acetylmuramic residue and the L-alanine of the oligopeptide attached to the sugar backbone. A third domain called SH3b is responsible for the specific recognition of cell wall components, strain specificity and modulator of hydrolytic activities [36, 37]. Endolysin 2638A has similar triple domain structures: an amino-terminal domain with endopeptidase activity, a central Ami-2 domain (with the highest activity in this phage) and a SH3b cell wall recognition domain [38]. Modular structure of *S. aureus* endolysins has allowed the construction of chimeric endolysins by the combination of catalytic and/or recognition domains. An example is the endolysin Ply187AN-KSH3b, which is a translational fusion of the CHAP domain of phage Ply187 and the cell wall recognition domain SH3b from LysK endolysin. This endolysin was effective in a mouse model of endophthalmitis that also decreased inflammatory response and protected the retina from tisular damage [39].

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4. Evolution of phage therapy

Since the discovery of bacteriophages, it raised the idea of using them for treatment of bacterial infections. D'Herelle began testing the therapeutic effects of phages, using animal models such as chickens and cows first, which provided successful results. Subsequently, there was carried out human testing and the development of phage therapies became more extensive. In 1923, the development of phage-based therapy strengthened with the foundation of the Eliava Institute in Tbilisi, Georgia, in the former USSR. In 1940, they began to commercialize phage in the United States. During World War II, phage cocktails were used to treat diseases such as dysentery and gangrene in the soldiers of the former Soviet Union. Their application was topical, oral and intravenous, although the latter favors the immune response of the individual treated due to the protein content of the virus, resulting in the elimination of the phage from the body [40–42]. Until a few years ago, therapies were based solely on the administration of the complete bacteriophage, but it was until 2000 that the studies for the identification and purification of lytic enzymes to treat infections caused by bacteria begun. In addition to using bacteriophages and their enzymes as enzybiotics (enzymatic activities with antibiotic effect) in the treatment of infections in humans, animals and agriculture, they are also used in the food industry as preservatives and disinfectants [19]. After the discovery of penicillin, the development and commercialization of antibiotics in the 1940s and 1950s soon occupied the global antibacterial market. The lack of knowledge of the biology of phages, the lack of studies of epidemiology of diseases and also a lack of control during the preparation of therapeutic

Bacteriophages	**Antibiotics**
Advantages	• Doses are easy to determine
• More abundant entities in nature	• Broad spectrum of action for the treatment of several infections; immediately used without identifying the specific strain causing the infection.
• They are natural enemies of bacteria	
• Ecologically friendly	
• Don't affect normal microbiota	
• Bacteria don't develop resistance	
Disadvantages	• Production of synthetic or semisynthetic antibiotics can contaminate environment
• Just a small number of phages are effective as therapeutic agents	• Destroy all bacteria cells including normal microbiota
• It is necessary to identify the specific strain causing the infection to use the specific and active phage	• Bacteria develop resistance

Table 2. Advantages of bacteriophages over antibiotics.

stocks led to a temporary delay in the research and development of phage therapy. In early studies of phage preparations, successful results showed high antimicrobial activity in *in vitro* and *in vivo* assays; however, in subsequent trials, some phages had little or no ability to destroy bacteria or became lysogenic [43].

Because of the concern in the treatment of diseases caused by pathogens with multiple resistance to antibiotics, it has revived the interest in the development and use of the bacteriophage therapy and their enzymes to treat diseases in animals and humans. Phage therapy has been used in plants, animals and humans with varying degrees of effectiveness; in addition, bacteriophages have some potential advantages over antibiotics but also have some disadvantages [44] (**Table 2**). The specificity of phage-host interaction permits the use of some phages in therapy because they do not have influence on normal microbiota in humans, animals, plants, food or inert surfaces. On the contrary, the use of broad spectrum antimicrobials has an effect on the eradication of a wide range of infecting pathogens but also kills bacteria from the natural microbiota thus causing a disequilibrium in the host normal microbiota and promotes secondary bacterial or fungi infections or even physiological or endocrinological disorders.

5. Bacteriophages and its interaction with animals

There is a high diversity of phages in microbial communities living in symbiosis with animals, for example, in the pig digestive tract and in the cow rumen [45, 46]. In the animals gut microbiota, there is a complex ecosystem with approximately 500 species of microorganisms, which are interacting with mutual benefits [47]. When the abundance of one of those bacteria changes and alters the dynamic equilibrium, it results in some disorders or disease in the host. Phages play an important ecological role for the health regulating the relative amount of the different bacterial strains in microbiota. On the other hand, the presence of phages in animals could present some disadvantages for health. When phages insert into the bacterial genome genes that encode toxins like Panton-Valentine, Shiga and diphtheria toxins [48, 49] or some other virulence factors, further excision may be aberrant, leading the phage genome to carry those virulence genes by transduction. These aberrant phages may insert in new hosts and transfer virulence properties. In fact, some genetic elements related to virulence may be originated from aberrant prophages. Also, prophages confer its host resistance to the infection of other phages. In addition, phages can also impact in host immune response through modifications in bacteria's antigenicity. Density of host bacteria determines the ability of phages to infect and reproduce because phages encounter their host through random collision. There are four models in the literature explaining the behavior of phages and bacteria in the regulation of animal microbiota. (A) "**kill the winner**": phages are more abundant than bacteria but don't infect them because of the lower abundance of its host, when some strains overgrow, phages can depredate and kill them by lysis, and system comes back to an initial healthy equilibrium. (B) "**kill the relative**": some phages are reproduced from lysogenic strains so they don't need to be abundant; strains with prophages produce phages that kill their genetically related strains which aren't resistant to the phage. The result is an advantage in the

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abundance of lysogenic population in comparison with non-lysogenic strains. (C) "**community shuffling**": temperate phages act negatively on their host, temperate phages kill their host under some stress situation and this don't occur with non-lysogenic strains. Positive feedback could take place if massive lysis causes host reactions like inflammation on another immune response. This causes an imbalance in the microbiota and in some cases disorders or diseases related to the change in populations. (D) "**invade the relative**": prophage propagates itself by infecting new hosts without lysing them, but establishing lysogeny [47]. Other contribution of phages to bacteria strains in animal microbiota is when phages function as vectors of virulence, for example, changing the expression of antigens in outer membrane like O-antigens [50], giving to bacteria genetic adaptation; it results in new and more virulent strains for the animal host.

6. Phage therapy in animal infections caused by *S. aureus*

The use of bacteriophages or bacteriophage cocktails and the use of endolysins represent a potential alternative for the treatment of infections caused by *S. aureus*. Although several diseases caused by *S. aureus* in animals have been described in a previous section, most of the research in phage therapy has been done for bovine mastitis, so it will be the central point of this section.

7. Mastitis and *S. aureus*

Mastitis is characterized by the inflammation of the mammary gland in one or more quarters of the udder accompanied of leukocyte production, mainly monocytes and blood serum proteins such as cytokines, chemokines and interleukins [51]. It is caused mostly by contagious pathogens such as *S. aureus* and *Streptococcus* spp. and environmental pathogens such as *E. coli*. Also, in less proportion, mastitis can be caused by or promoted by injury, allergies and neoplasias [52]. Mastitis causes large economic losses in the milk and dairy products industry for about 2 billion of dollars each year in the USA [53]. Among the pathogens causing mastitis, *Staphylococcus aureus* is considered a causal agent of great concern because of the low cure rate of *S. aureus* infections by antibiotic treatment and its ability to persist in a herd in the form of undetected subclinical infections [54]. Vaccines for the treatment of mastitis have limited efficacy. Cure rates for antibiotic treatment are often lower than 15%. This is caused by the poor penetration of the gland by antibiotics allowing *S. aureus* to survive inside the epithelial or phagocytic cells. Antibiotic resistance in *S. aureus* is also a growing concern, with overall rates of antimicrobial resistance in bovine *S. aureus* isolates varying widely by region [55]. The continued emergence of MRSA strains in humans and animals points to the need to develop new antimicrobial agents or therapies treatment for this pathogen. The treatment of bacterial infections with bacteriophages and their derivatives is such an option. **Table 3** describes those approaches.

Experiment	Observations/treatment	Reference
Bacteriophages		
Use of phage K to treat cow with subclinical mastitis. Twenty-four lactating Holstein cows with pre-existing subclinical *S. aureus* mastitis were treated. Prior to experimentation with dairy cows, the phage preparations were screened in mice to determine acute toxic effects	Treatment consisted of 10 ml intramammary infusions of 1.25×10^{11} PFU of phage K and infusions with saline for control, administered once per day for 5 days. The cure rate was established by the assessment of four serial samples collected following treatment The cure rate was 3 of 18 quarters (16.7%) in the phage-treated group, whereas none of the 20 saline-treated quarters were cured which were already infected with *S. aureus*. Phage-infused healthy quarters continued to shed viable bacteriophage into the milk for up to 36 h postinfusion	[54]
Study of bacteriophage (M^{Sa}) active against *Staphylococcus aureus*, including methicillin-resistant staphylococcal strains	A lethal dose of *S. aureus* A170 was given to mice; phage M^{Sa} rescued 97% of mice and completely eradicated bacteria in vivo within 4 days of phage treatment; when applied to nonlethal (5×10^6 CFU/mouse) 10-day infection, the phage also fully cleared the bacteria The phage M^{Sa}, delivered inside macrophages by *S. aureus*, kills the intracellular staphylococci *in vivo* and *in vitro* Phage M^{Sa} was well tolerated by the animals, it drastically reduced inflammation, and it did not stimulate the production of neutralizing antibodies	[68]
Isolation of a novel virulent bacteriophage (MSA6) from a cow with mastitis	Isolated phage was capable of infecting a wide spectrum of staphylococcal strains of both human and bovine origin	[69]
Isolation of bacteriophages virulent against *Staphylococcus aureus* associated with goat mastitis. Bacteriophages were isolated from soil and fecal samples	Three of the bacteriophage isolates, phage/CIRG/1, phage/CIRG/4 and phage/CIRG/5, exhibited lytic activity against over 80% of the staphylococcal isolates. All isolates were stable up to 3 months at 37°C, and for 16 months at 4°C but the stability of their respective endolysins only lasted for 12–23 days at 37°C and 6 months at 4°C. Lytic activity was determined *in vitro*	[70]
Isolation of a phage that infects *S. aureus* from bovine mastitis. SA phage was isolated from sewage water	Authors analyzed *in vitro* the sensibility to phage infection of five *S. aureus* strains with drug resistance. Phages were stable at wide temperature and pH ranges SA phage efficiently reduced bacterial growth in the bacterial reduction assay	[71]
Endolysins		
Fusion of endopeptidase domain from streptococcal endolysin SA2 with either lysostaphin or LysK endolysin and the recognition domain of endolysin LysK	In a mouse model of mastitis, chimeric SA2-E-Lyso-SH3B and SA2-E-LysK-SH3B reduce *S. aureus* CFUs by 1–3 log units in cow milk and by 0.63–0.8 log units in mammary glands. Synergism with lysostaphin reduced CFUs by 3.36 log units	[72]

Experiment	Observations/treatment	Reference
Engineering triple-acting staphylolytic peptidoglycan hydrolases. Both amidohydrolase/peptidase and amidase domains from LysK bacteriophage fused with the N-terminal domain of lysostaphin	Modification of the triple-acting lytic construct with a protein transduction domain significantly enhanced both biofilm eradication and the ability to kill intracellular *S. aureus* as demonstrated in cultured mammary epithelial cells and in a mouse model of staphylococcal mastitis shows that bacterial cell wall degrading antimicrobial enzymes can be engineered to enhance their value as potent therapeutics	[73]
Endolysin gene from novel bacteriophage IME-SA1 expressed in pET-32a fused with Trx-SA1	Each udder quarter suffering from mild clinical mastitis received the experimental treatment of intramammary infusion of 20 mg of recombinant endolysin once per day. Milk samples were taken on days 1, 2 and 3 from each infected udder quarter before treatment for SCC determination and microbiological analysis. Preliminary results of therapeutic trials in cow udders showed that Trx-SA1 could effectively control mild clinical mastitis caused by *S. aureus*	[74]

Table 3. Bacteriophages and endolysins therapy for treatment of *S. aureus* mastitis.

8. Animal models for treatment of other *S. aureus* infections

Animal models have been widely used to evaluate the performance of phage therapy in the treatment of a variety of infections caused by *S. aureus*, usually nosocomial infections in humans. **Table 4** presents the use of phages and/or their endolysins in infections by *S. aureus* in animal models.

Experiment	Observations/treatment	Reference
Bacteriophages		
Isolation of φMR11 phage, tested against *S. aureus* in mice causing bacteremia	Intraperitoneal administration of purified φMR11 can protect mice with bacteremia caused by methicillin-resistant *S. aureus*. Use of φMR11 did not cause any adverse effects	[75]
Isolation of Stau2 phage from hospital effluents. Tested in mice infected with *S. aureus* S23	*S. aureus* inoculated in an injection with 0.5 ml in intraperitoneal cavities of the mice. Protection by Stau2 from a lethal bacterial infection occurred in a dose-dependent manner. Immediate phage administration provided better protection than delayed administration. The surviving mice remained healthy during the 14-day observation period. Injection with a large amount of phage (7.5×10^{10} PFU) or SM buffer alone did not affect their physical condition during the same period. Injection with a mechanical bacterial lysate of strain S23 did not protect the mice from a lethal infection,	[76]

Experiment	Observations/treatment	Reference
	suggesting that bacterial components, such as bacteriocins, were not involved in protection	
S13 phage against lung-derived lethal septicemia by *S. aureus* strain SA27 in a mouse model.	Intranasal application of *S. aureus* strain SA27 induced 93% lethality in 3 days. S13 phage was done administered 6 h postinfection with 0.2 ml of solution of 15×10^{10} PFU/ml. The survival rates of phage administered and control groups were 67% and 10% on day 5, respectively. The administration of phage S13 reduced the *S. aureus* cell densities with significant phage replication in different tissues and it rescued the infected mice	[77]
Endolysins		
Endolysin LysGH15 derived from staphylococcal phage GH15 was used against MRSA *in vivo* using mice and *in vitro*	Mice were infected with 2× of the minimum lethal dose of MRSA. The bacterial growth in spleens was determined 1–24 h after the lethal infection. Although the number of bacteria in spleens decreased slightly 6–12 h after infection, it increased until death. In contrast, the number of MRSA cells in spleens declined by 2 log units at 5 h after LysGH15 treatment (50 μg/mouse) in the lethal MRSA-infected mice and continue decreasing to reach an undetectable level. Also, LysGH15 treatment could modulate inflammation reducing the levels of IL-6, IL-4 and IFN-γ mRNA in spleens	[78]
PlySs2 bacteriophage lysine derived from *Streptococcus suis* was used to treat MRSA which cause bacteremia in mice	Mice were infected i.p. with MRSA (MW2). PlySs2 protected mice and result in 89% survival in a bacteremia model, while in the control group without treatment with PlySs2 only 6% of mice survived	[79]
Nine endolysins within an homology group sharing SH3b domain but diverse classes of peptidoglycan hydrolyses (PGHs) from *S. aureus* were tested to determinate their antimicrobial activity	Proteins were expressed, purified and tested for staphylococcal activity *in vitro*. Cut sites from endolysins were determined. PGHs show different degrees of activity *in vitro*. Some PGHs can eliminate biofilms. Six of the nine PGHs protected from death at 100% of infected mice with MRSA	[80]

Table 4. Use of phages and endolysins against *S. aureus* infections using animals models.

9. A functional molecular epidemiology approach to isolate bacteriophages against specific genetic lineages of *S. aureus*

As stated previously, particular genetic lineages are related to host specificity and pathogenic strategies of *S. aureus*. In a previous work, we isolated and typed *S. aureus* isolates from bovine mastitis in backyard farms in México. Most of these isolates were related to CC5 subgroups ST97 and ST126 and present diverse *spa*-types [56,57]. An isolate of ST8 (CA, human-associated) genetic background was also found. Several isolates from different STs were selected

according to their *spa*-type or their antimicrobial resistance profile. **Table 5** shows examples of phages isolated using the selected molecular-typed *S. aureus* strains. All of the strains used for isolation belong to CC5 subgroup 97, but differed in their *spa*-type and their resistance profile, or belong to the ST8. Twenty-eight bacteriophages were isolated from 10 different *S. aureus* genetic lineages. Host ranges of isolated phages included strains from the same genetic lineage (CC5 subgroup 97). NST-1 corresponds to a new ST that is a single locus variant of ST126. None of these bacteriophages were active against the isolate with ST8 genotype. Restriction fragment length polymorphism with *XbaI* enzyme revealed only four different phage genotypes (data not shown). Phages MICHSAF5 and MICHSAF9 were clustered in the same RFLP group, whereas MICHSAF1 and MICHSAF15 were from different groups.

Phage	Strain for isolation	Genotype (ST and *spa*-type) and antibiotic resistance	Susceptible STs	Susceptible CCs
MICHSAF1	MRI-166	ST352/t267/GM	97, 352	5 subgroup ST97
MICHSAF5	MRI-150	ST97/t4570/NB, GM, FOX	126, NST-1, 97, 352	5 subgroups ST97 and ST126
MICHSAF9	MRI-150	ST97/t4570/NB, GM, FOX	126,NST-1, 97, 352	5 subgroups ST97 and ST126
MICHSAF15	MRI-151	ND/ND/NB, GM, FOX, C, CC, L, E, LZD	97, 352	5 subgroup ST97

NB, novobiocin; GM, gentamicin; FOX, cefoxitin; C, chloramphenicol; CC, clindamycin; L, lincomycin; E, erythromycin; LZD, linezolid; ND, not determined.

Table 5. Preliminary analysis of phage isolation using a molecular typing background.

It is interesting to note that phages MICHSAF5 and MICHSAF9 were isolated using the same strain as host, and both presented the same host range and RLFP pattern. Phages MICHSAF1 and MICHSAF15 were associated with strains with different STs and resistance patterns, and the genotypes of the susceptible *S. aureus* strains were similar. All strains used for the isolation of bacteriophages and the susceptible strains belonged to CC5 subgroups ST97 and ST126. These results suggest that genetic background of the strain used for isolation of the bacteriophage will determine the host range of the bacteriophage.

10. Conclusions

Bacteriophages and their endolysins in its natural or recombinant forms have proven to function in animals and animal models to control diverse forms of *S. aureus* infections. More structure-function studies of endolysins will contribute to design recombinant enzybiotics for the control of *S. aureus* infections. Functional molecular epidemiology is the applied use of the knowledge generated by molecular epidemiology to establish strategies for the control of infectious diseases [58] such as bacteriophage therapy. Bacteriophage selection using finely typed strains will help to properly select phages for therapy and to analyze the host range of the isolated bacteriophages. The strains typed by molecular approaches may also be useful to test ranges of activity of phage-derived endolysins. These, along with genetic engineering for

the study and expression of endolysins, will help to design better biotechnological approaches for the control of infectious diseases.

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