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ARTÍCULO DE REVISIÓN

Factors affecting phage development and anti-phage defence systems in Staphylococcus aureus

Igor Eduardo Astudillo-Skliarova (1) igor.astudillo@espoch.edu.ec

(1) Carrera de Nutrición y Dietética. Escuela Superior Politécnica de Chimborazo, Riobamba, 060101, Ecuador.
Electronic address: tannia.carpio@espoch.edu.ec
* Correspondence to igor.astudillo@espoch.edu.ec

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ABSTRACT

Staphylococcus aureus is one of the most common human pathogens worldwide. The emergence of antibiotic-resistant strains of *S. aureus* has prompted the development of alternative therapeutic approaches such as phage therapy. Recent clinical trials have proven the efficacy of phage therapy. However, the selection pressure has led to the emergence of phage-resistant phenotypes or novel bacterial anti-phage defence systems. In a recent study, through wide-scale screening and genome-wide association study (GWAS) techniques, six novel genes affecting bacterial growth and phage development were reported in *S. aureus*, but yet more studies are required to explain how exactly these genes affect phage development. Anti-phage defence systems, on the other hand, are not required for bacterial growth and target specifically incoming phage DNA. So far, in *S. aureus* only two such systems have been well characterised: clustered regularly interspaced short palindromic repeats (CRISPR-Cas) and restriction-modification (R-M) systems. Novel systems were recently discovered in *E. coli* and Bacilli species. Among these systems, homologues for Thoeris, Hachiman, Gabija and Lamassu have been found in certain strains of *S. aureus*. The knowledge of factors affecting phage infection will improve the design of phage therapies or the formulation of phage cocktails. Furthermore, drugs inhibiting those factors could be developed and implemented in phage adjunctive therapies. Here, we summarise recent advances regarding factors affecting phage development in *S. aureus* and anti-phage defence systems that are either ubiquitous in *S. aureus* or are present only in certain strains.

Keywords: *Staphylococcus aureus*, phage, anti-phage defense systems, phage therapy.

1. Introduction

Staphylococcus aureus is one of the most common human pathogens worldwide. The emergence of antibiotic-resistant strains of S. aureus has prompted the development of alternative therapeutic approaches such as phage therapy. Recent clinical trials have proven the efficacy of phage therapy. However, the selection pressure has led to the emergence of phage-resistant phenotypes or novel bacterial anti-phage defence systems. In a recent study, through wide-scale screening and genome-wide association study (GWAS) techniques, six novel genes affecting bacterial growth and phage development were reported in S. aureus, but yet more studies are required to explain how exactly these genes affect phage development. Anti-phage defence systems, on the other hand, are not required for bacterial growth and target specifically incoming phage DNA. So far, in S. aureus only two such systems have been well characterised: clustered regularly interspaced short palindromic repeats (CRISPR-Cas) and restriction-modification (R-M) systems. Novel systems were recently discovered in E. coli and Bacilli species. Among these systems, homologues for Thoeris, Hachiman, Gabija and Lamassu have been found in certain strains of S. aureus. The knowledge of factors affecting phage infection will improve the design of phage therapies or the formulation of phage cocktails. Furthermore, drugs inhibiting those factors could be developed and implemented in phage adjunctive therapies. Here, we summarise recent advances regarding factors affecting phage development in S. aureus and anti-phage defence systems that are either ubiquitous in S. aureus or are present only in certain strains.

Keywords: Staphylococcus aureus, phage, anti-phage defense systems, phage therapy.that involved 1307 patients with different types of suppurative bacterial infections caused by different species of multidrug-resistant bacteria such as Pseudomonas aeruginosa, S. aureus and Klebsiella spp. (12). Furthermore, the efficacy and safety of phage therapy was demonstrated in a randomised, double-blind, placebo-controlled clinical trial which involved 24 patients suffering from chronic otitis caused by multidrug resistant (MDR) Pseudomonas aeruginosa (13).

Despite our vast current knowledge of bacteriophages and the success of recent clinical trials of phage therapy, there are still many concerns that need to be considered before phage therapy can be re-introduced into clinical practice, for instance, the ability of phages to spread virulent genes among bacterial populations or their immunogenicity within the human body (14). Furthermore, this therapeutic approach could be compromised by the development of phage-resistant bacterial strains. Currently, there is very limited information on phage resistance mechanisms for S. aureus, but recent studies have unravelled these mechanisms in E. coli and Bacilli species (15) which are genetically close to S. aureus. We review here the bacterial host factors that affect or might affect the development of phages or lead to phage resistance in S. aureus.2.

2. Methodology

In the present non-systematic review article, the search for information was performed in the following databases: Pubmed, Google Scholar, bioRxiv and so forth. For the searching method, the conjunction “AND” and the disjunction “OR” were used in addition to key words, such as, “anti-phage defense systems”, “Staphylococcus aureus”, “phage”, “GWAS”. The following inclusion criteria were used: original research articles, non-systematic review articles and preprint servers in English of the last ten years. In contrast, the exclusion criteria comprised irrelevant articles for the present review article, outside of the established timeframe or language.

3. Results

We revised 150 sources of information related to S. aureus phages and anti-phage defense mechanisms, among which 86 scientific articles were selected by author. All of these articles complied with the established inclusion and exclusion criteria.

4. Discussion

4.1. Phage-host interaction

4.1.1. Phage adsorption – Recognition of bacterial receptors

The process of phage adsorption is the first critical step in a viral infection cycle. The cell wall of the vast majority of S. aureus strains contain wall teichoic acid (WTA) chains made of repeated ribitol-phosphate (RboP) units. These units can be further substituted with either α-1,4-N-

acetylglucosamine (α-1,4-GlcNAc) or β-1,4-N-acetylglucosamine (β-1,4-GlcNAc) respectively introduced by the glycosyltransferases TarM and TarS (16). Additionally, some strains from the sequences types (STs) 398 and 5 express the alternative glycosyltransferase TarP (17) which introduces a β-1,3-N-acetylglucosamine (β-1,3-GlcNAc) modification instead. GlcNAc modifiifed RboP-type WTA chains constitute the main component of the receptor recognised by S. aureus phages (18). S aureus siphophages specifically recognise glycosylated WTA chains with α-1,4-GlcNAc or β-1,4-GlcNAc (19). Myophages recognise the WTA main chain regardless of the presence of GlcNAc modifications (7). Furthermore, O-acetylation of peptidoglycan, introduced by the peptidoglycan acetyltransferase OatA, was previously shown to facilitate the adsorption of phage ϕ11 (20).

Currently, one strain of S. aureus is known to produce WTAs composed of repeated units of glycerol-phosphate (GroP) units instead of RboP. This is the case of S. aureus strain ST395 clone PS187 (a pneumonia isolate) which has a different arrangement of the WTA-biosynthetic genes cluster within its genome. A very similar genetic arrangement is found in coagulase-negative Staphylococci (CoNS) such as S. epidermidis and S. carnosus. This unique S. aureus strain can be infected only by the siphophage ϕ187, which can also infect CoNS and even species from other genera such as Listeria monocytogenes (21).

4.1.2. Post-adsorption stages of phage replication cycle

The post-adsorption stage comprises the events that take place once the bacteriophage particle has established a firm contact with the bacterial surface (22). Immediately after adsorption, the phage digests the cell wall until the tip of the tail reaches the cytoplasmic membrane (23). Subsequently, a possible signal triggers the ejection of the phage genome in the host cell (23, 24).

Once the genome has been ejected into the host cell, the phage may pursue either the lytic or lysogenic cycle (25). The precise molecular mechanism underlying both the lytic and lysogenic cycles of S. aureus phages is still poorly understood. Most of the studies on lytic and lysogenic cycles are based on phages infecting Gram-negative bacteria such as phage λ or T4. In general, it is known that during the lytic cycle the viral genome triggers the expression of its genes in order to continue with the cycle of reproduction, whereas the lysogenic cycle involves the integration of the viral genome into the bacterial chromosome. The integrated state of the viral genome is referred to as prophage.

Based on the selected route, phages are classified into two groups: virulent or temperate; virulent phages may only undertake the lytic cycle while temperate phages may choose any of both routes. In phage ϕ11, the decision-making process directed towards the establishment of lysogenic or lytic pathways is regulated by a mechanism similar to that of the E. coli phage λ (26). This process is essentially a race between two proteins encoded in the lysogenic module of the genome – Cro protein and cI repressor – for the control of specific promoters. If Cro protein prevails, the lytic cycle will be promoted; whereas the predominance of cI repressor leads to lysogeny (8). The outcome of this process mainly depends on specific external environmental factors such as the abundance or scarcity of nutrients in the culture media. Nutrient-rich culture media will promote the lytic cycle, whereas the insufficiency of nutrients in culture media will more likely result in the establishment of lysogenic pathway. One of the possible explanations for this could be that abundance of nutrients promotes metabolic pathways including the production of proteases. The cI repressor is susceptible to proteases and thus its amount will decrease allowing the protein Cro to bind its specific promoter and trigger the lytic cycle (27).

Once the lysogenic state has been established, this process can be stabilised by internal factors such as sigma factor H which interacts with the promoter region of the integrase (int) gene (8). In contrast, the lysogenic state can be reverted by exposing the lysogen to factors that cause DNA damage including UV light or mutagens such as mitomycin C. These factors trigger the SOS response which arrests the cell cycle and induces DNA repair (28). The SOS response activates RecA protein which binds to the cI repressor and causes its self-cleavage. This in turn leads to the expression of genes whose products are involved in lytic cycle induction (Fig. 1) (29).

The lytic cycle in S. aureus phages has been

studied to some extent in the myophage ϕK. Studies using bioinformatics tools demonstrated that the lytic cycle of myophage ϕK is very similar to phage T4. Like phage T4, myophage ϕK has the potential to produce all the components of the replisome: polymerase, helicase, DNA binding proteins, primase, RNase H and ligase (30). Myophage ϕK, during infection, in the same manner as phage T4, halts host's DNA replication and causes the degradation of the host DNA, which provides the raw material for phage DNA replication (31, 32). Previously, a host replication inhibitor was identified in another phage, S. aureus siphophage 77, as a putative host helicase loader DnaI (33).

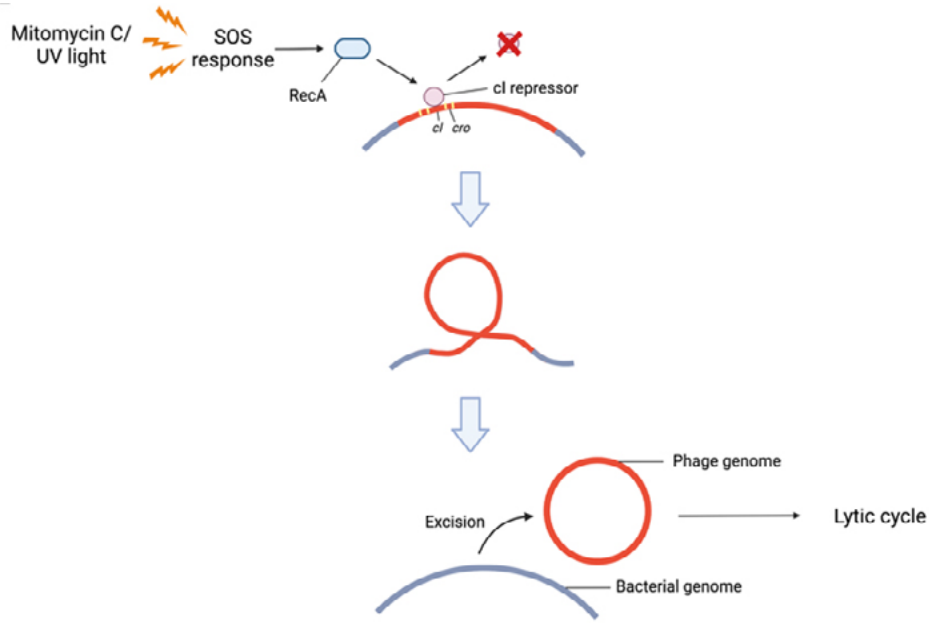

Figure 1: Schematic representation of the prophage induction mechanism. A specific DNA damaging agent (mitomycin C or UV-light) triggers the SOS response. This activates the RecA protein which then binds to the cI repressor and causes its self-cleavage. As a consequence, the prophage separates from the host chromosome and the phage switches to the lytic cycle.

Along with DNA replication, myophage ϕK synthesises its own sigma factors and uses the bacterial RNA polymerase for the transcription of its genes (30). The process of DNA replication occurs inside rapidly sedimenting complexes (as described by Rees and Fry in 1981 (34)), which are stabilised by lipids and proteins surrounding them (34, 35). The assembly of the phage head has been studied in phages ϕ80α and ϕ68 (36, 37). This process is initiated by a portal protein which forms a nucleus around which major capsid protein and scaffold proteins build the prohead. The scaffold also contains a protease which removes scaffolding proteins and allows the head to expand facilitating the process of DNA packaging (36, 37). At the same time, phage DNA is synthesised as a long DNA molecule containing many copies of the genome. This long DNA molecule is referred to as a concatemer (38). This concatemeric DNA is then recognised and cleaved at specific *pac* or *cos* sites by a terminase complex (TerLS). *pac* and *cos* sites are typical of phages ϕ11 and ϕSLT, respectively (39). The terminase complex then translocates the phage DNA into the prohead through the portal protein (40, 41). The head further expands as the DNA is packaged to allow the DNA to fill the whole space within the prohead. Once the DNA translocated is completed, the terminase complex cleaves the DNA at a non-specific site. Finally, neck proteins and tail proteins attach to the portal forming the complete phage particle (38).

The final stage of the lytic cycle implies the lysis of the cell. To achieve this, phages usually employ a holin-endolysin system. This system has been identified and characterised in the *S. aureus* podophage ϕ68. Holins create pores in the cytoplasmic membrane allowing endolysins to reach the peptidoglycan (PG) and degrade it. Endolysins are a big group of enzymes bearing muralytic activity: amidases, lysozymes 6or endopeptidases (42).

4.2. Factors affecting phage infection

Phages (in a similar fashion as antimicrobial drugs) create selective pressure which has forced bacteria to develop several strategies in order to overcome phage infection at any stage of replication cycle (43).

4.2.1. Factors affecting phage adsorption

Bacterial cells can overcome phage adsorption by blocking or modifying the phage receptor or synthesising an extracellular matrix (43). S. aureus employs some of these strategies to decrease the affinity of the phage receptor. For instance, the β-1,3-GlcNAc modification WTA. confers resistance to siphophages and podophages (17) (Fig. 2).

Earlier studies have suggested that the S. aureus protein A (Spa) (43) and lipoteichoic acids (LTAs) (44) play a role in phage adsorption. However, recent studies demonstrated that Spa and LTAs do not affect phage adsorption or infection (17, 18). In a recent study, S. aureus strain SA003R11 (ST352) mutants resistant to myophage ϕSA012 were selected. One of these mutants had a missense mutation in the gene rapZ, encoding the RNase adapter protein. It was suggested that the observed phenotype was due to excessive capsular polysaccharide production since RapZ controls the expression GlmS (45), which is required for the synthesis of uridine diphosphate N-acetylglucosamine (46), an essential precursor of peptidoglycan and capsular polysaccharide in S. aureus (47). Moreover, other organisms use this mechanism to inhibit phage adsorption, for instance Pseudomonas spp., Azotobacter spp. or Streptococcus pneumoniae (43).

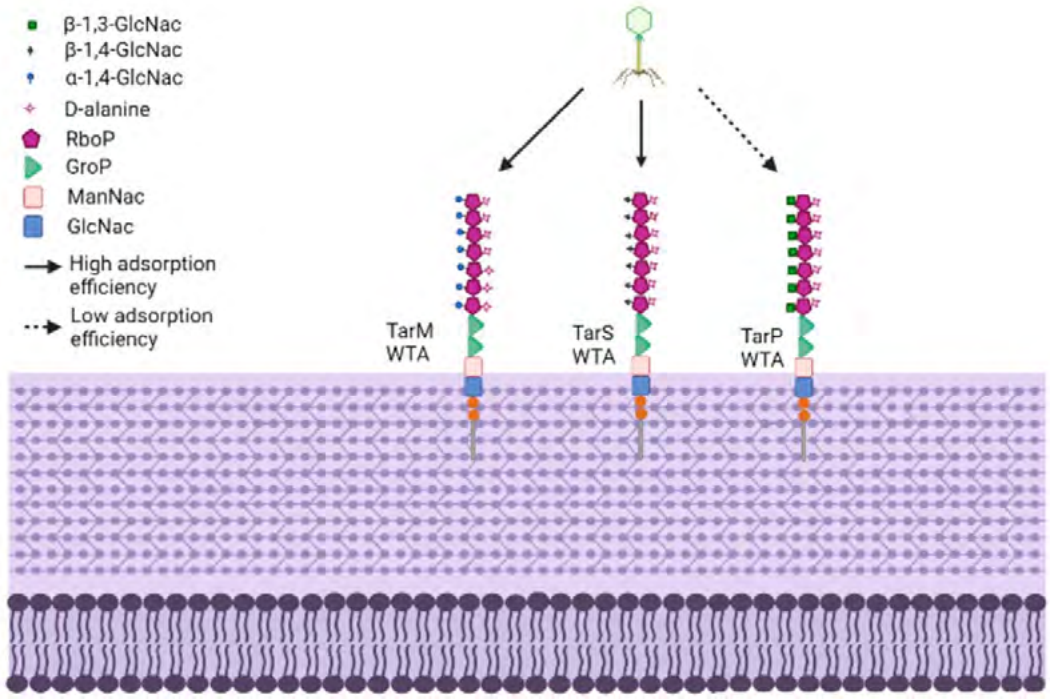

Figure 2: Bacterial surface receptors recognised by S. aureus siphophages. WTA chains together with the sugar modifications α-1,4-GlcNAc or β-1,4-GlcNAc (introduced respectively by the glycosyltransferases TarM and TarS) are recognised as receptors by siphophages. Certain S. aureus strains, such as those belonging to the ST5 and ST398, encode the glycosyltransferase TarP which introduces an alternative modification to the WTA chain: β-1,3-GlcNAc. This modification significantly decreases the efficiency of adsorption of the siphophage.

4.2.2. Post-adsorption factors affecting phage development

Many bacterial strains have developed or acquired mechanisms that affect bacterial growth and at the same time prevent phage development by limiting the resources available such as nucleic acids or amino acids (48). For instance, recently, spontaneous mutations in two genes inhibiting phage multiplication have been identified in S. aureus SA003R11. One of these genes encodes the enzyme guanylate kinase, whereas the other encodes DNA-directed RNA polymerase (45). It is known that guanylate kinase is important for the synthesis of nucleic acids and for GMP recycling (49). On the other hand, DNA-directed RNA polymerase is an important enzyme for the process of transcription (50). Most likely, Twort-like phages such as phage G1 inhibit the transcription of S. aureus by blocking the bacterial RNA polymerase with the aid of the protein gp67 and, at the same time, promote the transcription of their own genes (45).

In a recent study, six genes (trpA, phoR, sodM, isdB, fmtC and relA) affecting phage development were discovered through the screening of 259 diverse S. aureus strains from over 40 sequence types for sensitivity to siphophages p0045, p0017S, p002y, p003p and p0040, myophages p0006 and pyo, and podophage p0017. These specific genes were identified with the aid of genome-wide association study (GWAS) techniques (48) and validated by challenging the respective transposon mutants from the Nebraska Transposon Mutant Library (NTML) (51) with the same phages (48).

TrpA is involved in the last step of the biosynthesis of L-tryptophan. The disruption of trpA leads to the accumulation of intermediates such as indole-glycerol which could sensitise S. aureus to phage infection. Furthermore, it was hypothesised that a reduction in the total tryptophan intracellular concentration could increase the ratio of tryptophan used for the synthesis of phage proteins relative to host proteins. Therefore, the phage will produce its proteins at the host's expense (48).

The phosphate regulon sensor protein (PhoR) is part of the phosphate regulon (PhoPR) two-component system which regulates the phosphate uptake systems (ABC transporters) to overcome phosphate deficiency (48). It has been previously proposed that in other Gram-positive bacteria such as Bacillus subtilis (and probably S. aureus) phosphate deficiency leads to a surge in the intracellular levels of WTA intermediates, resulting in the activation of the sensor kinase PhoR (52). This sensor kinase, in turn, represses the WTA biosynthetic pathways, which leads to reduced WTA and phage-resistant phenotypes (48).

The superoxide dismutase M (SodM) is an antioxidant enzyme that catalises the breakdown of the superoxide, converting it into oxygen and hydrogen peroxide (48). Previous studies have shown that the absence of SodM affects phage infectivity in Campylobacter jejuni (53) and, probably, S. aureus (48). This more likely occurs because the oxidative stress has a negative effect on phage development (53).

The iron-regulated surface determinant (IsdB) is a cell wall-anchored surface receptor that mediates the scavenging of iron from haemoglobin (48). IsdB seems to promote phage development; however, its mechanism of action remains unclear especially because phage experiments were conducted using rich medium (48).

The gene fmtC encodes the phosphatidylglycerol lysyltransferase/multiple peptide resistance factor (FmtC/MprF) (54). FmtC/MprF mediates the transfer of a lysyl group to the membrane-bound phosphatidylglycerol, thereby conferring the bacterial membrane a positive net charge (55). It is thought that FmtC/MprF decreases phage infectivity by interfering with holins (48), which mediate cell lysis (56). This probably occurs because the depletion of FmtC/MprF confers the membrane a more negative net charge, which probably affects the activity of holins. Alternatively, the absence of FmtC/MprF may affect the endolysin activity or phage adsorption (48).

The bifunctional (p)ppGpp synthase/hydrolase (RelA) is a stringent response protein that is triggered mainly as a result of amino acid starvation (57). Additionally, the stringent response is mediated by the protein Guanosine-3',5'-bis(diphosphate)-3' (SpoT) which is activated during fatty acid, carbon and phosphate starvation (57). In S. aureus, the stringent response is mediated by a RelA/SpoT homologue (SAUSA300_1590). This homologue was previously shown to be upregulated in S. aureus biofilms when infected with a lytic phage phiIPLA-RODI. Therefore, the deletion of RelA/SpoT homologue leads to a reduced phage infectivity (58). This probably occurs because the absence

of this gene results in slow bacterial growth and formation of persister cells where phages cannot efficiently replicate (48).

It is known that several factors encoded by S. aureus pathogenicity islands (SaPIs) can interfere with the assembly of phage particles (59). SaPIs are mobile genetic elements (MGEs) that steadily reside within the bacterial chromosome. Their size ranges from 14 to 27 kb and they contain genes that are typical of phages such as terminase, integrase and phage-like repressor genes; however, they lack genes encoding structural proteins. Moreover, SaPIs bear additional genes encoding virulence factors and antibiotic resistance cassettes (8). SaPIs integrate into the host chromosome by a similar mechanism as prophages. In this integrated state, the stability of SaPIs is provided by the Stl-repressor, which represses the expression of SaPIs genes including those genes that are involved in the excision of SaPIs (60). Unlike the CI repressor of temperate phages, the Stl-repressor is not inactivated by the bacterial SOS response and thus this mechanism will not trigger SaPI excision (61). The excision of SaPIs requires the participation of a prophage (helper phage). The induction of the prophage leads to the expression of dUTPase encoded by dut gene and can bind and inactivate the Stl repressor, thereby inducing SaPI excision (60).

SaPIs can interfere with phage assembly with the aid of different mechanisms. SaPIs encode phage packaging interference proteins which inhibit the helper phage small subunit of terminase (TerSp) but not the cognate terminase of the SaPI particle (TerSs), thereby promoting the packaging of SaPI DNA instead of phage DNA (59, 62).

4.2.3. Anti-phage defence mechanism

One of the most common anti-phage strategies used by bacteria is the cleavage of phage DNA. The cleavage of phage DNA can be achieved by two mechanisms: the restriction-modification (R-M) system and the clustered regularly interspaced short palindromic repeats–CRISPR-associated proteins (CRISPR-Cas) system (43).

R-M systems are the most common anti-phage defence systems and have been identified in 75% of prokaryotic genomes (48). The R-M systems use restriction endonucleases and methyltransferases. Restriction endonucleases recognise the foreign DNA at a specific site or at a certain distance from this site and, subsequently, cleave the DNA molecule. The host DNA is not affected because it is protected by the addition of methyl groups to sites that are recognised by the restriction endonucleases, which is performed by methyltransferases (63).

Currently, R-M systems are classified into four types (type I-IV) and all of them have been found in S. aureus (64). Type I R-M systems are hetero-oligomeric complexes made of three different subunits of the host specificity determinant (Hsd): HsdS, HsdM and HsdR; HsdS recognises the specific DNA sequence, HsdM methylates host DNA and HsdR cleaves foreign DNA. This system uses two functional complexes: a methyltransferase complex which is made of one HsdS subunit and two HsdM subunits, and a complex that acts as both methyltransferase and endonuclease which is made of one HsdS subunit, two HsdM subunits and two HsdR subunits. HsdS is composed of two target recognition domains; each of these domains recognises a half of a bipartite sequence separated by a gap (64) (Fig. 3a). One of the most common type I R-M systems in S. aureus is SauI (65). Type II R-M systems often use two separate subunits: a methyltransferase (MTase) and a restriction endonuclease (REase). These systems recognise specific (usually palindromic) sequences (Fig. 3b) (64).

In S. aureus, Sau3AI is one of the best studied type II R-M systems. Sau3AI recognises specifically the GATC sequence (66). Type III R-M systems are hetero-oligomeric complexes composed of Res (restriction) and Mod (modification). The Mod subunit can act as a dimer (Mod2) or in a complex with one or two Res subunits (Mod2Res1 or Mod2Res2). The modification complex methylates only one DNA strand (64) (Fig. 3c). The restriction complex recognises short asymmetric sequences and cleave a non-specific sequence located 25-28 nucleotides 3' away from the recognition site (67). There is little information about type III R-M systems in S. aureus since they are difficult to discern (68). Finally, type IV R-M systems are methylation-dependent restriction enzymes. In these systems, the restriction enzyme cleaves DNA substrates only when specific nucleotides are methylated (64) (Fig. 3d). In S. aureus, the type IV R-M system SauUSI has been studied. SauUSI predominantly cleaves DNA containing the modified nucleotides 5-methylcytosine and 5-hydroxymethylcytosine (69). Some phages have developed mechanisms that

overcome R-M systems. For instance, myophage ϕK lacks the sequence GATC, which renders this phage resistant to the type II restriction-modification (R-M) system Sau3A (30).

Different from the R-M system, the CRISPR-Cas system is an adaptive anti-phage system. This system contains a CRISPR array and a Cas operon. The CRISPR array is composed of repeats with intercalating between them spacer DNA. Each spacer sequence derives from foreign DNA of phage or plasmid origin, which is integrated into the CRISPR array through a process referred to as adaptation, i.e. the foreign sequence is stored within the bacterial genome. The CRISPR array is then transcribed, resulting in the production of CRISPR RNA (crRNA), which is complementary to the sequence of foreign DNA that had been previously injected or introduced into the bacterial cell (70).

Following phage infection, the crRNA together with RNA guided nuclease activity recognises the phage genome sequence. As a consequence, the phage DNA is cleaved by the CRISPR associated nucleases (Cas). One example of such nucleases is Cas9 which is synthesised by Streptococcus pyogenes (71).

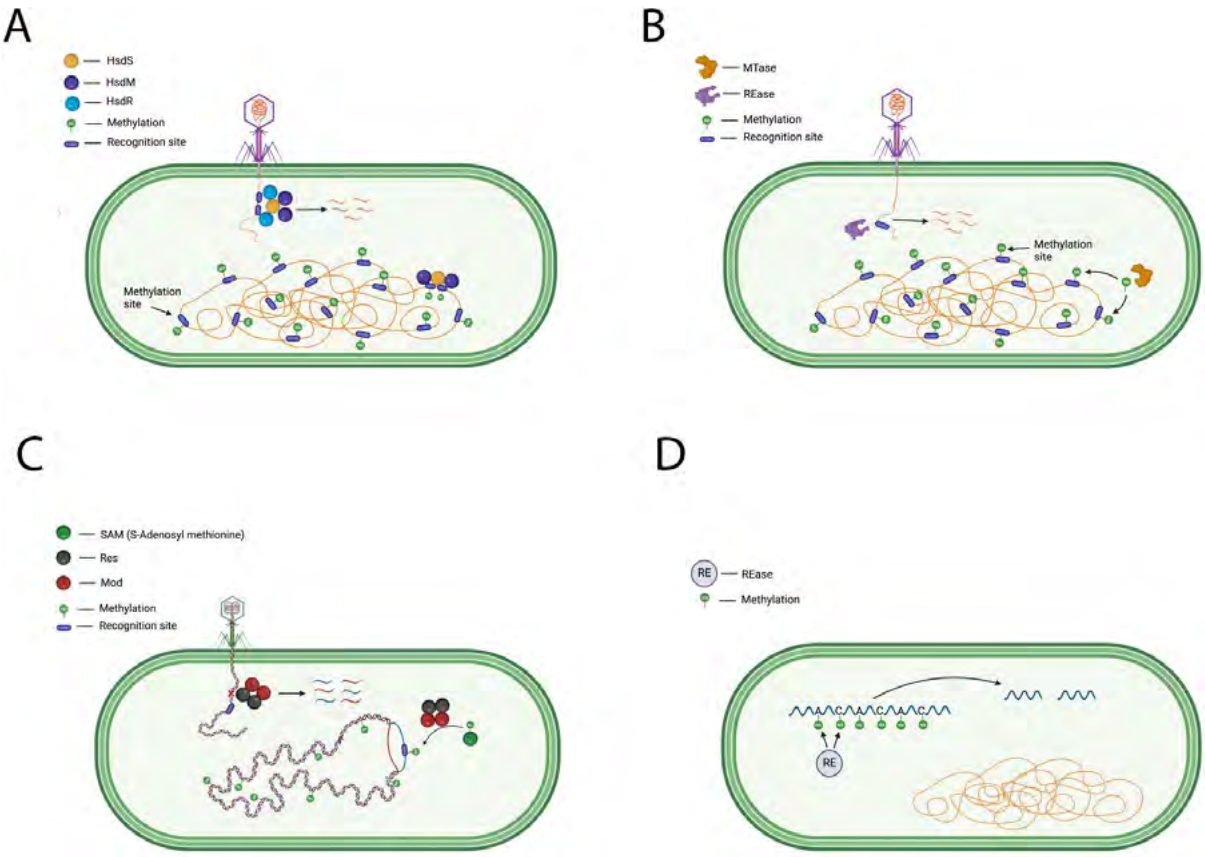

Figure 3: Schematic representation of the main features of the four types of R-M systems. (a) The type I R-M systems contain three components: HsdS, HsdM and HsdR. The DNA methyltransferase complex is a trimer made of one HsdS subunit and two HsdM subunits, whereas the restriction complex is a pentamer made of one HsdS subunit, two HsdM subunits and two HsdR subunits. (b) The type II R-M systems contain two separate components: the restriction enzyme (REase) and the methyltransferase (MTase). (c) The type III R-M systems contain two components: Res and Mod. The methyltransferase complex maybe composed of two Mod subunits (Mod2) or two Mod subunits and two Res subunits (Mod2Res2). The restriction complex is made of Mod2Res2. (d) The type IV R-M systems contain one restriction enzyme (REase) which uses modified nucleotides (usually adenine or cytosine) as target.

The CRISPR-Cas system is widespread among bacterial genomes and was identified in approximately 40% of them (72); depending mainly on which cas signature gene is present, CRISPR-Cas systems are classified into four types (I, II, III, IV) and ten subtypes (73). Recently, three subtypes of CRISPR-Cas systems have been described in S. aureus: I-C CRISPR, II-A CRISPR and III-A CRISPR, which were identified in S. aureus strains 08BA02176, MSHR1132 and GCF_001611345, respectively (74).

Many bacterial species have developed mechanisms that prevent DNA entry into the host cell, such as superinfection exclusion (Sie) systems (75), or inhibit key metabolic pathways such as replication, transcription and translation, such as abortive infection (Abi) systems (43). These mechanisms have been well characterised in lactococci phages, but not yet in staphylococci phages. So far, the only Abi system characterised in Staphylococci is the eukaryote-like serine/threonine kinase Stk2 (76). During phage infection, a specific PacK phage protein activates Stk2, which subsequently phosphorylates essential host proteins. This induces cell death and blocks phage propagation. Stk2 was originally discovered in Staphylococcus epidermidis, but homologues have been identified in many S. aureus strains (76).

Recently, additional and less predominant anti-phage factors were discovered including: bacteriophage exclusion (BREX), defence island system associated with restriction–modification (DISARM) and prokaryiotic Argonautes (pAgos). These systems are found in less than 10% prokaryote genomes (15). These systems are usually located within defence islands (77). Unlike R-M and CRISPR-Cas systems, BREX and pAgos are not that common in prokaryotic genomes and have been found in approximately 10% of them (15).

BREX is a phage exclusion system. This system targets the DNA of lytic and temperate phages and, like R-M systems, methylates the host DNA but using a different pattern. Unlike R-M systems, BREX does not cleave the incoming DNA and only blocks its replication (78). Interestingly, in some organisms such as Streptomyces coelicolor the BREX system expresses the serine/threonine kinase PglW (79) which shares 23% homology with the Staphylococci Stk2.

A more recent study has described novel anti-phage systems in Bacillus subtilis: “Thoeris”, “Hachiman”, “Shedu”, “Gabija”, “Septu”, “Lamassu” and in E. coli: “Zorya”, “Kiwa” and “Druantia” (15). These anti-phage systems were discovered by investigating gene families located next to previously described defence islands (15). Among these anti-phage systems only the mechanism of action for Theoris and Zorya can somehow be explained or at least hypothesised. Zorya system rarely occurs in Gram positive bacteria, whereas Theoris is more universal and is present in many Gram positive bacteria including certain Bacilli and Staphylococci strains (15).

Thoeris (protective ancient Egyptian goddess of childbirth and fertility) is an anti-phage system that targets myophages. This system has two essential components: ThsA and ThsB (15). The first Thoeris component, ThsA, is composed of an N-terminal sirtuin (SIR2)-like domain and a C-terminal SLOG domain (80) and, depending on the organism, can be membrane-associated or cytoplasmic (15). The SIR2-like domain exhibits a nicotinamide adenine dinucleotide (NAD+) cleavage activity (15, 80), whereas the SLOG-domain is a putative nucleotide-derived signalling-binding molecule (81). The second Thoeris component, ThsB, has a TIR domain and is predicted to be intracellular. It has been hypothesised that the TIR domain of ThsB senses phage infection by recognising a specific feature of the phage, which could be a phage protein or phage DNA. As a result, ThsB becomes enzymatically activate and converts NAD+ into an isomer of cyclic adenosine diphosphate ribose (v-cADPR) which acts as a signalling molecule. The SLOG domain of ThsA senses v-cADPR and then activates the SIR2-like domain, which depletes the cell of intracellular NAD+, resulting in abortive infection and cell death (81) (Fig. 4).

Gabija was discovered as an antiphage system in Bacillus cereus and homologues of its components have been found in other Gram-positive bacteria including certain strains of S. aureus (15). This system contains two proteins: GajA and GajB. GajA is a sequence-specific DNA nicking endonuclease which is strictly regulated by the concentration of nucleotides. GajA is

composed of an N-terminal ATPase-like domain and a C-terminal TOPRIM domain which was characterised as an ATP-dependent endonuclease domain. The nucleotides NTP and dNTP, at physiological concentrations, allosterically inhibit the endonuclease activity of GajA by binding the ATPase-like domain which in turn maintains the TOPRIM domain inactivated. The replication and transcription of phage DNA causes the depletion of NTP and dNTP, which subsequently results in the loss of nucleotide-binding of the ATPase domain. As a consequence, this domain activates the TOPRIM domain which will then degrade the phage DNA.

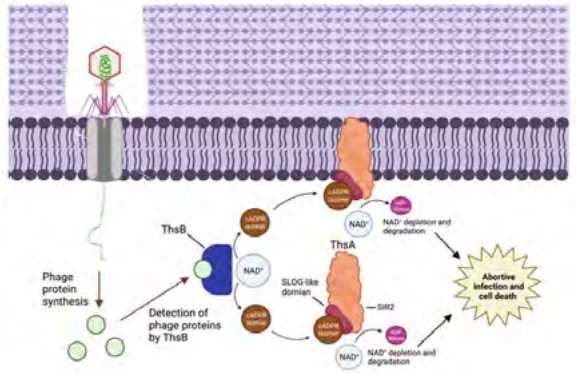

Figure 4: Schematic representation showing the possible mechanism of action of the anti-phage defence system Thoeris. This system contains two components: ThsA and ThsB. ThsA is made of an N-terminal SIR2-like domain and a C-terminal SLOG domain and can be either membrane-associated or cytoplasmic. ThsB contains a TIR domain and is cytoplasmic. The TIR domain of ThsB initially senses specific phage components (more likely proteins) and subsequently becomes active. Next, ThsB converts NAD+ into v-cADPR, which acts as a signalling molecule. v-cADPR is then recognised by the SLOG domain of ThsA, which, as a consequence, activates the SIR2 domain. Finally, the latter domain depletes the intracellular NAD+ molecules, leading to abortive infection and cell death.

Alternatively, it has been hypothesised that the TOPRIM domain might also cleave the host DNA and promote an abortive infection, which results in cell death (Fig. 5). GajB was predicted to be a UvrD-like helicase. Its function is still unclear, but it has been suggested that it contributes to the activation or cleavage of GajA (82).

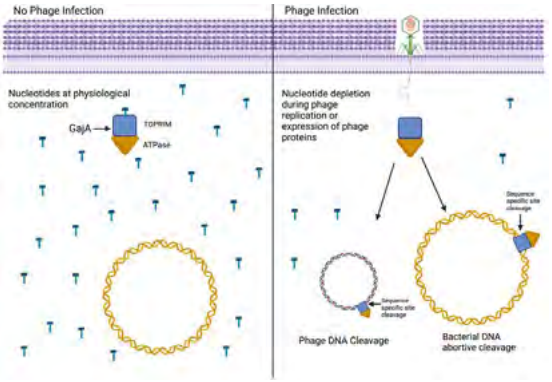

Figure 5: Schematic representation showing the putative mechanism of action of the anti-phage defence system Gabija. The main component of this system is GajA. This protein is composed of an N-terminal ATPase-like domain and a C-terminal TOPRIM domain which has a sequence-specific DNA-nicking endonuclease activity. In the absence of phage, the ATPase-like domain of GajA is allosterically inhibited by NTP and dNTP molecules. During phage infection, the replication and transcription of phage DNA deplete the intracellular NTP and dNTP molecules. This leads to the activation of the ATPase domain of GajA, which in turn activates the TOPRIM domain. Finally, this domain mediates the cleavage of either phage DNA or host DNA, leading in the latter case to abortive infection and cell death.

5. Conclusion

So far, several adsorption factors and post-adsorption factors affecting phage development, as well as anti-phage defence mechanisms have been identified in different S. aureus strains. Further screening of different S. aureus strains with different phages and analysis tools such as GWAS will reveal genes that might either affect phage development or be part of an anti-phage system. Furthermore, a screening of the whole NTML will also shed light on such genes.

Future studies will also reveal the mechanism of action for the novel anti-phage systems found in Bacilli: Hachiman, Shedu, Septu, Lamassu, Druantia and Kiwa. So far, it is known that Shedu and Septu contain proteins that were predicted to have endonuclease or HNH nuclease activity; Septu and Lamassu contain a protein that was predicted to have an ATPase activity similar to Gabija; Hachiman and Druantia contain one protein that was predicted to act as a helicase

Among these systems, Hachiman and Druantia were shown to be the most potent anti-phage systems (83). Homologues for the components of Hachiman and Lamassu have been identified in certain S. aureus strains. For instance, Hachiman was identified in two S. aureus ovine strains O11 and O46 (15), which cause severe and mild mastitis, respectively (84); Lamassu was identified in the S. aureus strains TCH130 and CN1 (15), both of which are methicillin-resistant S. aureus (MRSA) (85, 86). Since some anti-phage systems such as Thoeris seem to be specific to a group of phages (such as myophages), whereas other systems such as Hachiman confer broader resistance to phages (15).

Understanding the diversity of these factors could improve design of phage therapies or the formulation of specific phage cocktails. Furthermore, certain anti-phage factors could be used as targets for specific drugs, which could theoretically improve phage adjunctive therapies. Taken altogether, the discovery of novel factors affecting phage development or anti-phage systems will establish a basis for the development of more accurate phage therapies alone or with a combination with an antibiotic.

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