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The 936-group Lactococcal phage

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Summary

Since the discovery of bacteriophages in 1917, phages were already used to fight bacterial infections. Decades later phage research was at the center of understanding genes function, was instrumental in the rise of molecular biology, and a promising tools to fight antibiotic resistant bacteria. The amount of phages is immense, with about 10^{31} phage particles in the biosphere. Almost all phages have dsDNA genomes and tails. While phages have been very beneficial, in the dairy industry, phages cause big problems by infecting the milk fermenting Lactic acid bacteria (LAB). When this occurs the whole process has to be restarted, yielding big economic damages. Therefore, much research has been done into lactococcal phages. The most common phage group, isolated from different places all over the world, is the so called 936-group phage. This phage group is not only the most common, but also has advantages over other lactococcal phage groups. The 936 phages are more heat resistant, can increase its virulence, easily overcomes host-receptor changes, and can persist for a long time in the same location. Furthermore, many bacterial systems that prevent phage infection are active against the 936 phages. In this assay phages, their evolution, application, characteristic and phage-host interaction are discussed. Lactococcal phages, specifically the 936 phages and the advantages they have, from a phage and host point of view are discussed.

Contents

1. Introduction

1.1 Origins of phage research.

The first phage was discovered in 1917 by Félix d'Herelle at the Pasteur Institute, in Paris. d'Herelle discovered that these phages were useful as an antimicrobial agent that was way more potent against bacteria then any substance known at that time. He developed the idea of a phage therapy, which had the advantage of specifically killing pathogenic bacteria and not damaging eukaryotic cells. By employing phages, d'Herelle and colleagues could kill pathogenic bacteria, such as *Escherichia coli* and *Vibrio cholerae*. They administered phage suspensions and were able to successfully treat many illnesses like dysentery, cholera and typhoid. The phage treatments were even added to the water supply in high epidemic areas [1]. Furthermore, in 1924, d'Herelle proposed that these bacterial viruses were ancestral to cells. This view turned out not to be true, since phages are dependent on their hosts for survival [2]. Nevertheless, phages are ancient and were probably around even before the divergence of the bacteria from the archaea and eukarya [3].

Three decades later, in the 1950's, the *Escherichia* phage lambda was the first phage to be extensively researched. This phage was at the center of the golden age of molecular biology, which lasted until the mid-1980's. At that time the lambda phage was one of the only ''organisms'' that was experimentally feasible to be completely understood, due to its small genome size. Although it had only basic genomic information, it was still able to make drastic changes to its host's gene expression. Thereby giving huge insights into genetic and molecular understanding of gene expression. The MS2 phage, that also infects *E. coli*, was the first ''organism'' to be sequenced 1976 and had a genome size of 3.569bp [4].

1.2 Evolution of phages

The amount of phages is immense, the total phage population on the planet is estimated to be around 10^{31} particles. Since there are around 10 phages for each bacterial cell, this estimation is validated by independent estimates of 10^{30} bacterial cells in the biosphere. Experimental studies show that the population is highly dynamic, with an estimated 10^{23} infections per second [5].

The evolution that impacts organisms is driven by mutations, gene migrations, genetic drift, natural selection, nonrandom mutating and recombination. These genetic traits on the micro scale lead to differentiation on the species level, or macro evolution, and follows the Darwinian model of natural selection. Evolution in phages is not well understood, because phages require host organisms for their reproduction, and do not follow standard ideas of branching trees and linear genetic descent. Nevertheless, researchers have attempted to define phages in terms of their evolution. The main theory of phage evolution was, the theory of modular evolution, proposed by Botstein et al. in 1980. The theory states that not a given phage is the product of evolution, but rather a family of interchangeable genetic elements or modules. These modules of their genome come to a joint evolution of these genetically interchangeable elements. All phages in nature are a favorable combination of modules that work individually and together to infect a specific organism [6].

The theory of modular evolution was widely accepted for almost two decades. However, more recent whole genome comparisons rather suggest that phages indulge in horizontal gene exchange. These non-homologous recombination events take place at random points in the genome, and not at specific regions between the modules. This is a process driven by natural selection, where non-functional mutations in essential modules leads to an inactive phage, leaving phages with mutations predominantly in between essential genes. There are still many unknowns in respect to the evolution of phages, simply due to lack of genomic data [2].

1.3 Phage characteristics and applications

Out of all the viruses, except for a few small plant viruses, the bacteriophage is the most well studied regarding its physical and chemical properties. This is due to the easy availability of homogenous populations of uniform phage particles. The phage particles can be relatively easily obtained in large quantities in fermenters. Therefore, a wealth of knowledge is obtained about the structural origination of phages, phage-host interactions and phage DNA injection mechanisms. Phages have and continue to play an important role in bacterial genetics and molecular biology. Phages can transfer key phenotypes to their host, for instance, changing a non-pathogenic bacterium to a pathogenic one. Furthermore, they play important roles in the regulation of bacterial populations in all kinds of environments. Due to their small genome size, and their mechanisms of changing host gene expression, they have been essential for the development of molecular biology techniques. For example T4 ligase, the most widely used ligase in molecular cloning, was first isolated from the T4-phage [7]. The use of phages as anti-biotic treatment started out promising around the start of the previous century. Nevertheless, the discovery of antibiotics in 1928 shifted the focus of phage research towards the bio-molecular direction. However, in former soviet countries, phage therapy remained a widely used tool to battle bacterial infections. Nowadays, in many eastern European countries like Georgia, phage treatments are routinely given to fight bacterial infections. Due to the lack of published research out of the Soviet era, most western countries are still hesitant to use these phage therapies [8].

1.4 Phage classification

To date more than 5500 page species have been morphologically analyzed by electron microscopy [9]. Due to the differences in evolution of phages, illustrated earlier, in comparison to regular ''organisms'' it has been difficult to classify phages according to the standard taxonomy principles of shared characteristics. Systems of classification based on host range, proposed by Lwoff, Horne and tournier in 1962, never won acceptance. Even with the computer revolution, there is no accepted program for the classification of phages. Only for small groups, such as the T7-like phage or individual phage proteins, computer based classifications programs are accepted. Therefore, phage classification is still a complex issue. Nevertheless, International Committee of Taxonomy of Viruses (ICTV) has adopted the principle of classifying phages on the basis of their morphology, nucleic acid content, genomic data, and physiochemical properties [10]. This lead to four main types of phages based on the nucleic acid content, ordered from least to most occurring: dsRNA, ssRNA, ssDNA and dsDNA phages. Furthermore, classification of phages based on morphology lead to 12 types depicted in figure 1. The majority of known phages are tailed phages and belong to the order of *Caudovirales* (fig.1.5-1.7). This order is further sub-divided into three families according to tail morphology: *Siphoviridae* (long non-contractile tail) (fig.1.5), *Podoviridae* (short non-contractile tail) (fig.1.7) and *Myoviridae* (long contractile tail) (fig1.6) [11].

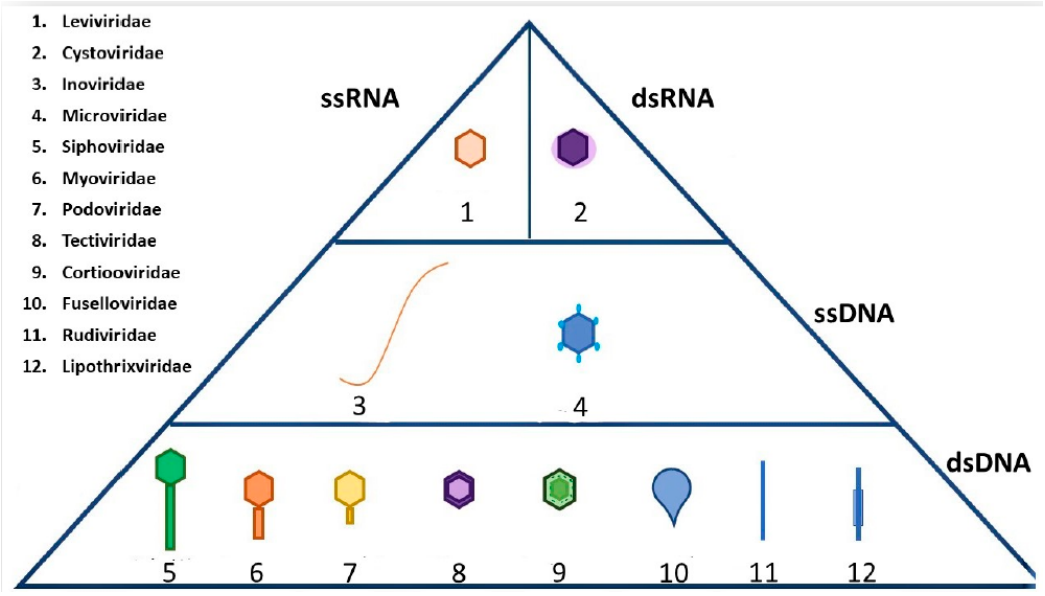

Figure 1: All phage families ordered by their nucleic acid content and morphology, over 95% of all known phages have dsDNA genomes [11].

1.5 Phage infection pathway

Phages have two main life cycles, the lytic and lysogenic cycle. The lytic cycle leads to direct phage particle production, while in the lysogenic cycle the phage integrates in the host genome (fig.2). Phages that follow the lytic cycle are called virulent phages. This cycle starts after the phage attaches to the bacterial cell when its DNA is injected. The phage DNA highjacks the cell towards the production of new phage particles. Lysin is produced, which breaks down the bacterial cell wall to burst out the new phages into the environment, killing the host (fig.2A). Phages that follow the lysogenic cycle, are called temperate phages. These phages can follow two pathways, the first results in the lytic cycle where new phage particles are produced and the host cell dies. The other pathway leads to the integration of the phage DNA into the bacterial chromosome (fig.2B). This integration can be beneficial to the host, since useful genes can be transferred. An integrated phage is also called a prophage. Under environmental stresses, such as temperature, UV radiation or chemical treatments, the prophage is excised from the genome. This again leads to the lytic cycle mentioned above. Another, less common cycle, is the chronic cycle. This only occurs in archaeal viruses, some filamentous and temperate phages. Where new phage particles are constantly produced and released from the cell. In comparison to the lytic and lysogenic cycles, the chronic cycle does not end in killing the host. The host is even capable of growing, but at a much slower rate [11].

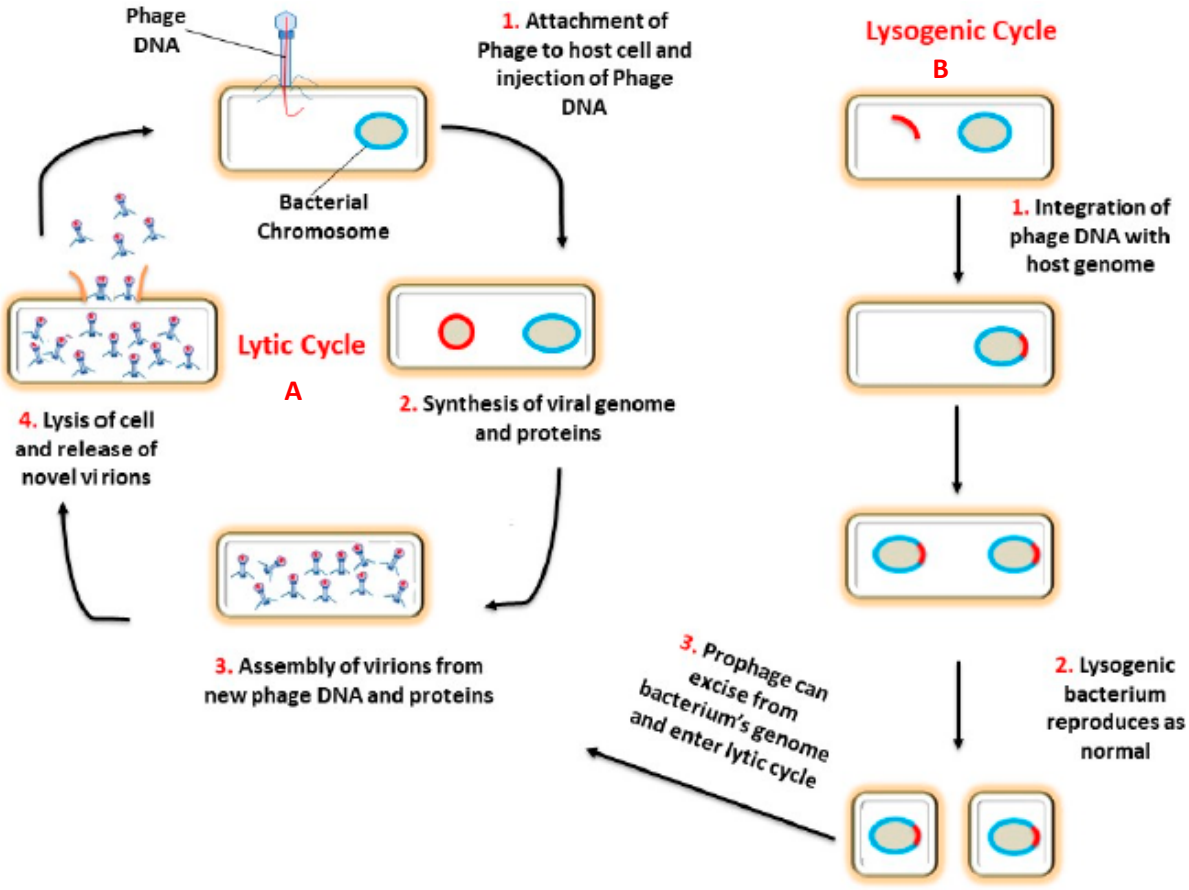

Figure 2: the lytic and lysogenic cycles of phages. A) Virulent phages follow the lytic cycle strictly, killing the host. B) Temperate phages can integrate in the host genome, eventually leading to the lytic- or chronic cycle [11].

1.6 Phage-host interactions

The phage infection is initiated by the attachment of the phage to the host cell via specific host cell surface receptors (fig.3). This is triggered by the recognition of the phage's Receptor Binding Protein (RBP), on the tip of the tail, with its specific receptors on the cell surface. Factors such as localization, volume and density of the receptors are vital for the recognition. Binding of the RBP to the receptor (fig.3A) leads to conformational changes in the baseplate, creating an interface between the phage and the membrane, and triggering tail contraction (fig.3B). Afterwards the phage DNA is injected into the host cell (fig.3C) [12].

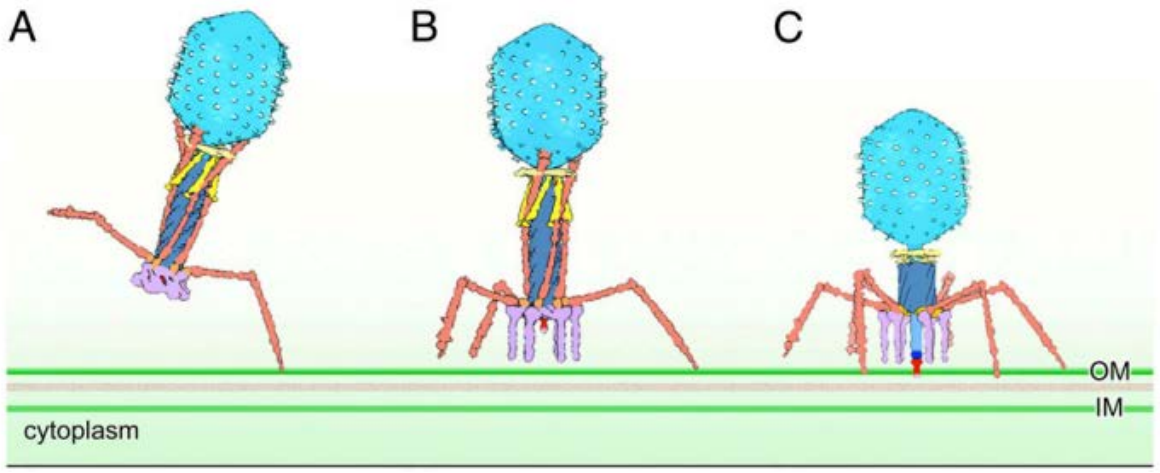

Figure 3: Schematic model of phage infection initiation. A) Phage binds to receptors on host-cell by its receptor proteins (pink). B) Binding to the host-cell leads to conformational changes in the baseplate (purple), which triggers the contraction of the tail proteins (dark blue). C) Tail proteins contract leading to injection of phage DNA into the target cell [13].

1.6.1 Gram-negative

The phage-host recognition, and subsequent infection process, of the *E. coli* phage T4 has been the model for phage-host interactions in Gram-negative bacteria [14]. The T4 phage is virulent and a member of the *myoviridae* family. The recognition of the host cell is mediated through long tail fibers (LTFs), which are attached to the phage base plate. These LTFs can bind to either a protein receptor membrane porin C (OmpC), or lipids on the surface of the *E. coli* host.

Other structures, that are not present on the cell wall, are also known receptors for phages that infect Gram-negative bacteria. These include pili, flagella and capsules. In Shin et al. 2012., such mechanisms of phage-host interaction were observed. In this study 23 phage isolates, infecting the *Salmonella typhimurium*, were analyzed for their adsorption mechanism. While some of these phages were able to adsorb to the host cell using the lipid bound O-antigen and BtuB outer membrane protein, most used the flagella as a receptor. Interestingly the bacterium contains other outer membrane proteins like OmpC, which are known to be *Salmonella*-phage receptors. The authors propose that the complexity of the *S. typhimurium* LPS might block access to these receptors. This forces the phage to utilize more complex receptors like the flagella [15].

1.6.2 Gram-positive

The mechanisms of phage adsorption in Gram-negative bacteria are extensively studied, while in Gram-positive bacteria the adsorptions are less well understood. The Gram-positive cell wall is a complex structure composed out of biopolymers, such as peptidoglycan (PG), sugars, and teichoic acids. Due to this thick layer of biopolymers, lipids and proteins are not accessible for phages infecting

these bacteria. Therefore, these phages adhere to the sugar complexes imbedded in the PG, rather than proteins and lipids. This will be further discussed in the following section.

2. Lactococcal phages

Phages have been important in knowledge and technical advancements in molecular biology, and are promising candidates for treatments of antibiotic resistant bacteria. Nevertheless, in the industrial setting, phages cause big problem. Lactic acid bacteria (LAB) are used in the manufacturing of fermented dairy products, such as yoghurt and cheese. When these bacteria are infected by phages during the process, the whole process has to be restarted causing big economic damages. In the industry mainly the bacterium *Lactococcus lactis* is used, which can be infected by a range of phages. Much research has been done to prevent these phage attacks, currently only *E. coli* phages have received more attention than lactococcal phages [16].

All known lactococcal phages have a dsDNA genome, and are part of the *Caudovirales* order. As mentioned earlier, this order comprises 95% of all known phages. Most Lactococcal phages are members of the *Siphoviridae* family, and a few are part of the *Podoviridae* family. Over two decades ago, 12 lactococcal phage groups were classified based on their DNA homology and morphology homologies. This made comparing lactococcal phage isolates from around the world easier [16].

It became clear that the vast majority belonged to one of three main groups, the c2-, P335- and 936-groups. Therefore, most research has been focused on these three groups [16]. Furthermore, multiplex PCR methods that are available now can rapidly assign new phage isolates in one on these groups [17].

Lactococcal phages use Cell Wall PolySaccharides (CWPS) structures as receptors. In LABs, five CPWS subtypes (C_1-C_5) were identified by structural and genetic analysis. The subtypes differ in the sugars that are incorporated in the PG structure. Furthermore, it was found that these specific CWPS subtypes contribute to phage-host range. This was illustrated in Ainsworth et al. 2014. Here the CWPS C_2-subtype of the phage infection model organism, *Lactococcus lactis* 3107, was incorporated in the parent strain *L. lactis* MG1363. By swapping the MG1363 C_1 subtype for the 3107 C_2 subtype, it was found that the C_2-subtype was the host-cell receptor of two P335-group phages. These phages were only able to infect MG1363 mutants harboring the 3107 CPWS C_2-subtype [18]. Furthermore, research was done on a specific part of the CWPS of 3107, the surface exposed polysaccharide pellicle (PSP). Mutants lacking most of this PSP, were able to avoid phage adsorption and become almost completely resistant against phage infection of the c2-, P335- and 936-groups [19].

3. 936-group phages

The virulent lactococcal 936-group phages are the most frequently encountered phages in dairy fermentations, and poses the biggest threat to the industry. 936 phages have been found in dairy factories all over the world. 22 phage isolates of the 936-group were isolated from cheese whey in Ireland. Also in a buttermilk facility 22 phage isolates were found belonging to the 936-group, which accounted for over 80% of the phage isolates in that facility. In the latter study raw milk was identified as the source of phages, since buttermilk fermentation occurs in closed barrels. Furthermore, 936 phages isolates from different regions showed significant similarities in host range and restriction profiles. In Canada, 30 lactococcal phage isolates were isolated in 1992. At that point, c2-group phages were the major group found. However, recent studies show that the 936 phages accounted for 74% of phages found [20], exhibiting a wide host range. The only region where 936 phages are not the main group isolated is Belarus, where only 4 out of 23 isolated phages belonged to this group [21].

Not only are 936-group phages the main isolated phages, they also have significant advantages over their fellow lactococcal phage groups. For instance, they are observed to be more heat resistant, can increase their virulence, persist for long periods in dairy facilities, and can rapidly change their RBP structure to overcome biological hurdles put up by the host organism. However, most of the molecular mechanisms that contribute to these advantages remain elusive. Nevertheless, to prevent negative effects of the phage infection in dairy factories, a few standard measures are utilized. Such as, biocidal and thermal treatments, not using the same strain multiple times in a row, and the use of bacteriophage insensitive strains [21].

3.1 936-group phage evolutionary relations

936 phages can be grouped by either genomic homologies [22] of modules, host range or morphology [21]. Much research has been done regarding the structure and phylogenetics of 936 Receptor Binding Proteins (RBPs), this lead to a more information about host range specificity of the RBPs. The data showed that RBPs of the 936 phages have a conserved N-terminus and variable C-terminus [23]. This explains observations of 936 phages being able to infect both *L. lactis* ssp. *lactis*, and ssp. *cremoris* (fig.4).

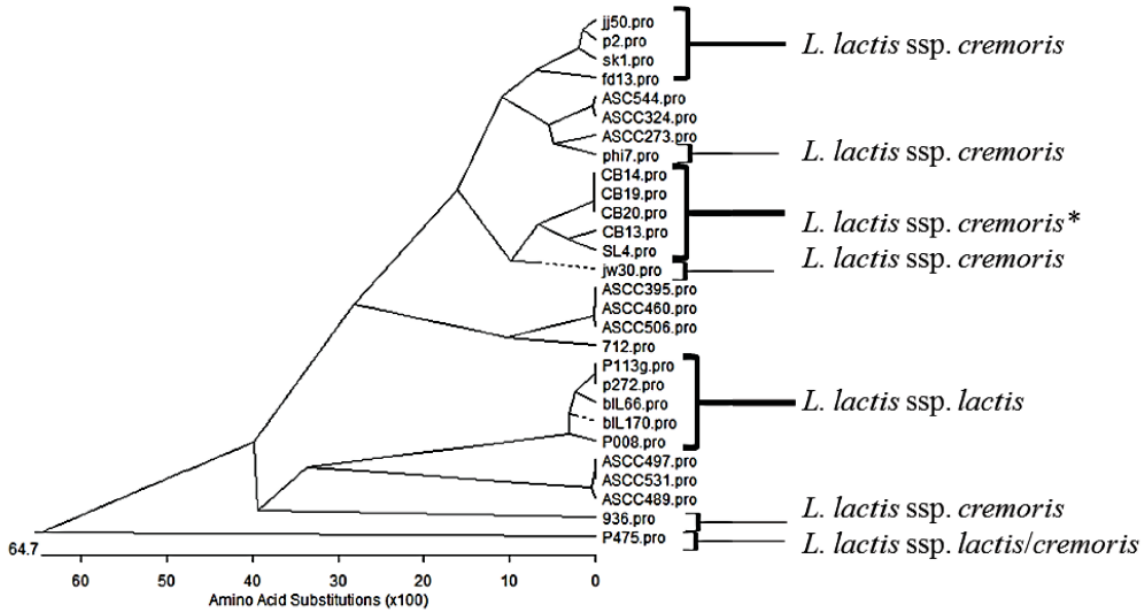

Figure 4: phylogenetic analysis of host-range of 936 phages in relation to AA substitutions of their RBPs [21].

This leads to two scenarios, either the RBP C-terminus is able to bind with multiple sugar substrates, or the RBP C-terminus is interacting with a sugar substrate that is not specific to one of the two subspecies. Genomic comparison of the lysin, tail tape measure, RBP and neck passage structure proteins show that the 936 phages seem to follow a parallel evolution pathway. This was illustrated in Mahony et al. 2006, where the sequences of multiple 936 phage species were compared. Interesting data was obtained from the sequence comparison of five 936 phage species, jj50, 712, P008, sk1 and bIL170. Although these phages originated from distinct geographic areas, high genetic homology was observed (fig.5). Furthermore, phage species bIL170 and P008 were isolated almost 20 years earlier than the other three phages, and infect different hosts. Due to this similarity, it seems that the more recent phages jj50, 712 and sk1 have evolved from the same genetic core as the older bIL170 and P008 phages. It was hypothesizes that, the phages used in the study and other 936 phages have a common ancestor and were able to infect the same host at some point in their evolution. Therefore, the phages would have had the advantage of genetic recombination amongst themselves, promoting diversification of these phages [22]. This is strengthened by the high frequency of homologous recombination of 936 phages observed in vitro, which is assumed to occur at the same frequency in nature [24].

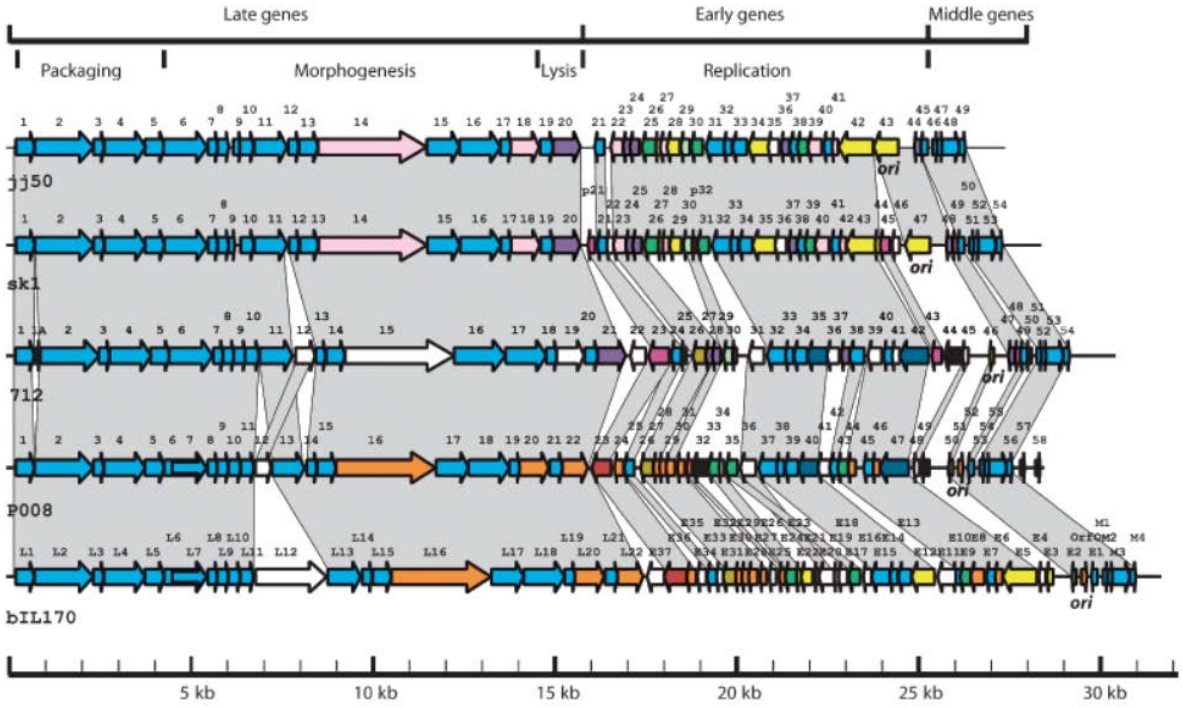

Figure 5: genetic comparison of five 936 phages, early isolated phages P008 and bIL170 still share high homology with more recent isolated 936 phages, jj50, sk1 and 712 [22].

3.2 Advantages of the 936-group phage

3.2.1 Increased virulence

From a phage point of view, there are multiple examples of advantages that the 936 phages have in order to successfully infect its host. A good example of the ability of a 936 phage to increase its virulence, was found in a Danish Cheddar cheese factory. Here a mixed undefined starter culture TK5 was used, for more than 11 years, without any decrease in acid production due to phage infections. The use of the same starter culture for this period of time, without any phage infection, is rare. The reason for this was most likely due to the fact there were at least 32 different LAB strains in the TK5 mixture, making it less susceptible to infection of a single phage species. Nevertheless, over time the phages that were always present in the whey, started to be able to attack multiple strains used in the process. ''New'' phages of the 936-group were found, which had a broader host range and larger burst size compared to the 936 phages isolated earlier in 1994 [25].

3.2.2 Persistence

The 936 phages have also been shown to persist in a specific dairy factory during an extended period of time, as demonstrated by the isolation and subsequent re-isolation of the 936 phage species CB13/GR7. These two phages share 100% genetic homology, but were isolated 14 month apart, illustrating the persistance of the 936 phages over an extended period of time [20].

3.2.3 RBP mutations

In Giesbers et al. 2019., 936 phage escape mutants able to overcome phage resistant *L. lactis* 3107 derivatives where sequenced. This revealed a distinct mutation in the RBP encoding gene, leading to the AA change V225I. Due to this mutation, the phage escape mutants were able to adsorb to the cell surface lacking most of its PSP receptors that are normally the target for the 936 phages. Interestingly, the V225I AA change was already observed by Tremblay et al. in 2006. In this article, the structures of

multiple 936 RBPs were solved [26]. Here, phages sensitive to anti-phage compounds were sequenced. The authors found that mutations in this binding crevice contributed to the binding of the phage to the compound. Moreover, AA's around this region were found to bind with high affinity to saccharides [27]. This illustrates the ease by which the 936 phages can change their RBP, to create affinity for other receptors if pressured.

3.2.4 Heat resistance

There are a number of temperature steps included in the processing of raw milk, called pasteurization. This includes both low temperature of 63°C for 30 minutes and higher temperatures of 72°C for 15 seconds. Those temperatures are too low for phage inactivation, and multiple studies support that 936-group phages are significantly more heat resistant than the other lactococcal phage groups. In Madera et al. 2004., lactococcal phages infecting starter cultures were analyzed for heat resistance. The three main groups, p335, c2 and 936 were analyzed. The P335 phages were found to be susceptible to this process, while c2 and 936 were not. However, the 936 phages were a lot more heat resistant then the c2 phages at 70°C. The decimal reduction time, in other words the time in which the amount of virus particles decreases 10 fold, differs. The c2 phages had a time of 1.3 minutes, while the 936 phages needed 3.35 minutes to decrease 1 decimal. The authors also indicated that this could be a reason why before pasteurization, c2 is often the most predominantly found phage group. While in processed whey, after pasteurization, the 936 is the most predominant phage group. Due to the limitations of temperature that can be used in the process, thermal inactivation is not a feasible technique to prevent further infections of the 936 phages [28].

3.3 Bacterial defense mechanisms in response to 936-infection

3.3.1 Superinfection Exclusion system

The bacterial cell has many systems to defend itself from phage attacks. The first layer of defense starts after the adsorption of the phage to the cell, where the DNA injection is blocked by the Superinfection Exclusion (Sie) system (fig.6A). Blocking proteins encoded by the system are predicted to be membrane anchored or associated with membrane components. The Sie system is not a system native to the host genome, but is encoded by prophage DNA in the genome, that encodes the Sie protein on the cell wall. In essence, the integrated phage is protecting the host from other phages and is a phage-phage interaction rather than a phage-host interaction. The most studied in *L. lactis* is the Sie_{2009} system, encoded by the temperate P335-group phage TUC2009. The sie_{2009} gene in the TUC2009 lysogenic module translates the membrane associated Sie_{2009} protein, which blocks DNA injection from multiple 936 phages, without affecting adsorption. A similar system in *S. thermophilus,* of the TP-J34 phage that encodes the ltp Sie protein, was observed. Interestingly this system was able to block the 936 phage P008, for which *S. thermophilus* is not a host, while P335 and c2 group phages were not affected [29].

3.3.2 Restriction/Modification system

If a phage is able to overcome the first layer of defense, and is able to inject its DNA into the host, the Restriction/Methylation (R/M) system can defend the cell by attacking the viral DNA (fig.6B). Most, if not all, bacteria possess such a system. When the unmethylated phage DNA enters the cell it is recognized by restriction enzymes or methylases, which rapidly degrades or methylate the viral DNA respectively [30]. Nevertheless, phages are able to overcome this system by incorporating methyltransferase (MTase)-encoding genes in their genomes. These MTases, function to methylate the phage DNA to avoid recognition and subsequent restriction of the host restriction enzymes. In Murphy et al 2014., this system of avoidance was also observed in 936-group phages. In this study, three 936 phage species, Phi93, Phi145 and Phi15 were isolated from a Gouda producing cheese factory. These phages contained predicted MTases in their genomes. Their genomes were isolated,

and cut with either DpnII (cuts unmethylated DNA) or DpnI (cuts methylated DNA). The phage DNA was not cut by DpnII, confirming methylation and resistance to the R/M system of its host [31].

3.3.3 Abortive infection system

When all defense measures mentioned above fail, there is one last system that the cell can utilize. This system is called the Abortive infection (Abi)-system. In contrast to the other systems mentioned above, the Abi-system does not lead to survival of the cell, but rather induces programmed cells death to prevent the production and or release of new phage particles. This system is called an altruistic defense mechanism, because the population is protected instead of the cell. Abi systems have been studied for over 50 years, but their modes of action are still not fully understood. The Rex system found in *E. coli* has been studied the most extensively. This two-component system requires RexA/B proteins to protect against phage infection. Phage DNA triggers the intracellular sensor RexA, which in turn activates the membrane bound RexB. RexB is an ion channel that, when activated, decreases membrane potential leading to a reduction in ATP. This halts macromolecule synthesis and cell division. Phage replication is also halted due to the lack of ATP (fig.6) [30].

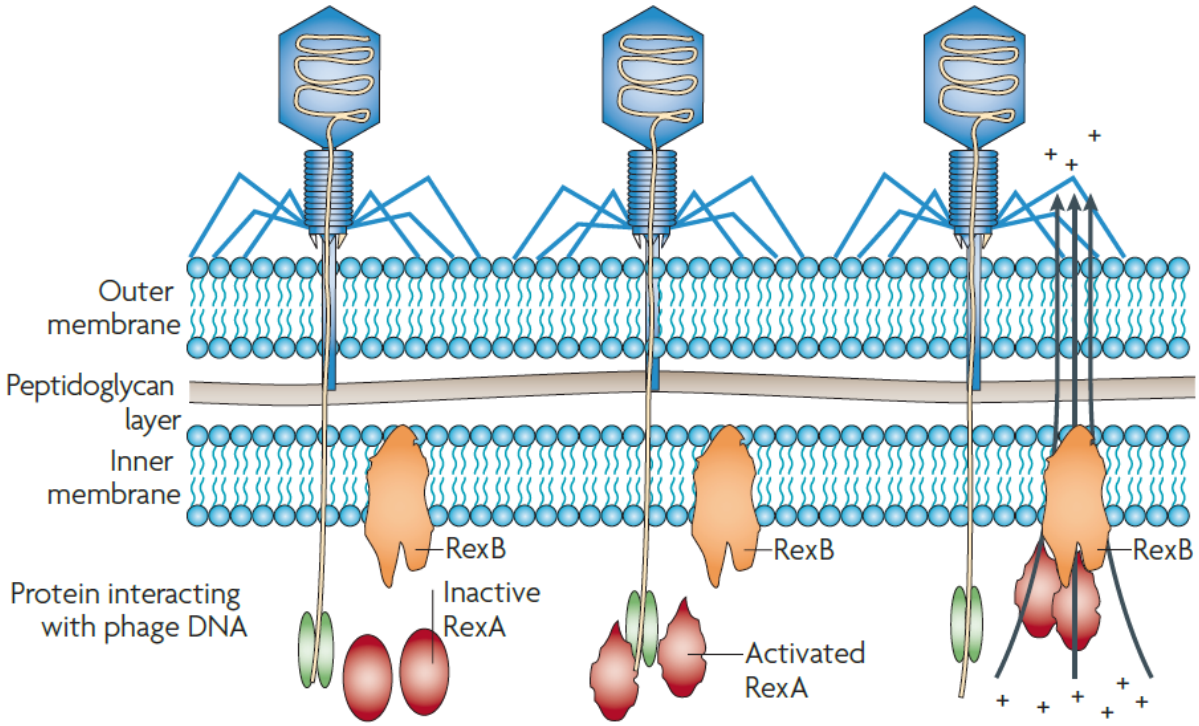

Figure 7: The Rex Abi system of *E. coli*. Phage DNA triggers the intracellular sensor RexA, which binds and activates the ion channel RexB. Activation of RexB leads to decrease in membrane potential and halts macromolecule synthesis and cell division, leading to cell death [30].

Abi systems have been researched in *L. lactis*, where 23 distinct Abi systems have been identified. Different steps of phage multiplication cycle can be inhibited by proteins of those systems. For instance, in phage DNA replication (fig.6C1), RNA transcription (fig.6C2), production of the major capsid protein, and packaging of phage DNA (fig.6C3) [30]. These proteins thereby also target the host replication, transcription and translation, stopping cell metabolism. Every Abi system described is active against 936 phages, while this is not the case for the c2 and P335 phages [32]. Nevertheless, there are many 936 phages that obtained resistance by simple single nucleotide mutations in the part of the genome, that is targeted or recognized by the system [32]. A more sophisticated method of obtaining resistance, was described in Dominques et al. 2004. The AbiP system is known to block 936

phage DNA replication, 10 minutes after DNA injection. Three 936 phage species, bIL40, bIL66M1 and sk1 are sensitive to this system, while bIL170 is not. By simply co-infecting bIL41 and bIL170 with the *L. lactis* strain that harbored this AbiP system, bIL41 obtained the AbiP resistance gene *e*6 of bIL170 by non-homologous recombination [33]. Illustrating the ease by which these phages obtain resistance to such systems.

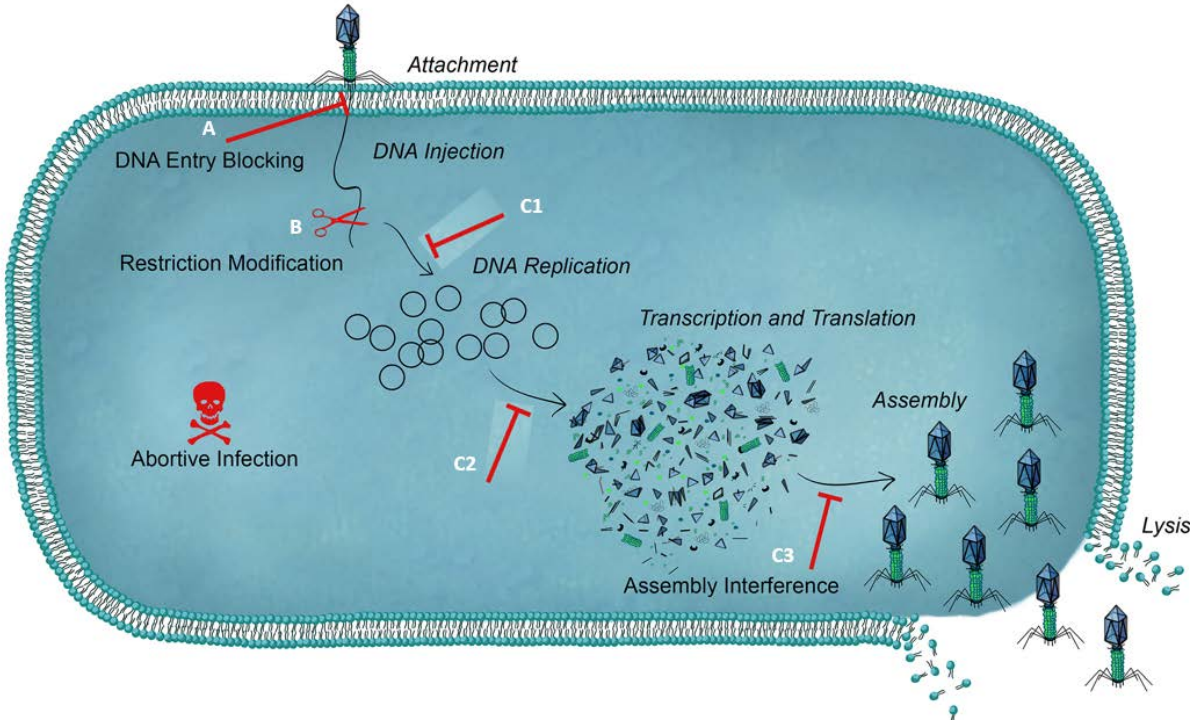

Figure 6: Schematic representation of bacterial systems in response to phage infection. A) Phage DNA injection is blocked by the membrane associated proteins of the Sie-system. B) Injected phage DNA is cut or methylated by proteins of Restriction/Modification systems. C1-3) Proteins of Abortive infection systems inhibit host and phage DNA replication (C1), RNA translation (C2) and protein assembly (C3), killing the host and preventing phage replication [34].

4. Discussion

Phage research has been instrumental in understanding genes function, the rise of molecular biology [7], and are promising tools to fight antibiotic resistant bacteria [8]. Nevertheless, in the dairy industry, phages cause big problems by infecting the milk fermenting LAB [16]. The most common phages, isolated from different places all over the world, are the so called 936-group phages. This phage group is not only the most common, but also has advantages over other lactococcal phage groups. The 936 phages are more heat resistant, can increase their virulence, easily overcomes host-receptor changes, and can persist for a long time in the same location. Although these observation are widely studied, the exact molecular mechanisms that create these advantages, remain elusive. One might wonder if this niche situation of the 936 phages with LAB bacteria, is a situation created by the industrial setting. Mostly because this virulent phage has no option to hide in the genome as a prophage, but has to constantly battle with its host for survival. As illustrated earlier, evolution of phages is difficult to retrace due to the lack of genetic lineage. Nevertheless, Mahony et al. 2012 proposed the idea of a common *L. lactis* ancestor that may already have been host to the 936 phages. This is strengthened by the isolation of 936 phages that infect both *L. lactis* ssp. *cremoris*, and *L. lactis* ssp. *lactis* [21]. Furthermore, all Abi-system described target members of the 936-group phage [32], even Sie-systems of *S. thermophilus* target 936 phages [29]. Moreover, the recently discovered CRISPR-Cas9 system in *S. thermophilus* contains protospacers targeting 936 phage DNA [35]. This strengthens the idea of an ancient battle, between the 936 phages and these gram-positive bacteria. This is not a consequence of the industrial setting, but rather highlighted by the research into preventing these phage infections.

5. References

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