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Some Reflections on the Origin of Lambdoid Bacteriophages

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1. Introduction

Monophyletic theory of the origin of life postulates that all cellular organisms have evolved from a common ancestor. This is based on nucleotide sequence analyses of rRNA genes, which all cellular organisms possess (Woese et al., 1975). On this basis and supported by other observations, the first common ancestor dates back to ~ 3.7 billion years. However, the scenario is not equivalent for the viruses, since they lack these genetic elements. In fact, there is such substantial diversity in viral genome structures (dsDNA, dsRNA, ssDNA, ssRNA) that it has proven extremely difficult to answer several key evolutionary questions. Do they co-evolve with their hosts? How do viruses first infect a new species? When was the first virus? Thus, a polyphyletic origin of viruses has been proposed (Bamford, 2003). It has been proposed that this could have been even before the appearance of the first cell. Although Boyer et al. (2010) have suggested that the eukaryote viruses may have appeared just after or simultaneously with the emergence of modern eukaryote lineages. However, there are other proposals which state that as new species appeared (of any of these three domains: Eukarya, Archaea and Bacteria), and after a certain period of time, their new infective viruses emerged. Nevertheless, information about common ancestor(s) related to viruses is still an enigma. Similarly, if we ask the same question for bacteriophages, or simply, when did the first lambdoid phage emerge? The answer is also unknown. Even with the support of bioinformatics and phage genomic knowledge, and quite possibly due to the lack of specific strategies and/or design methodologies, and phage genomic complexity the problem has not yet been resolved.

In this chapter, in an attempt to address this issue in *grosso modo*, we propose the analysis of two genomic regions of lambdoid phages, regions that are dissimilar regarding their nucleotide variability and stability: (1) The variable and not essential region that confers immunity to the lysogenic bacteria against phage superinfection counterparts, and is related

to repressor; (2) The conserved and essential region for development that is related to the gene encoding the "Receptor-Binding Protein" (RBP), and which is involved in the process of infection onset. To begin to understand even a fraction of what was the common ancestor of lambdoid phages and the changes that had to occur to generate the diversity of lambdoid phages could be informative both of lambda biology and virus evolutionary processes in general.

2. Diversity of immunity regions in lambdoid phages

We reported the isolation and characterization of a collection of 47 lambdoid phages from human fecal samples (Kameyama et al., 1999). To determine the immunity group to which each phage belonged to; their lysogenic strains (lysogens) were constructed and then challenged with each lambdoid phage. The physiological study of growth indicates that for two phages belonging to the same immunity group, each lysogen should prevent growth of the other phage. For example, as we know each lysogens A and B are resistant to their respective phages, and if the lysogen A is resistant to phage B infection, and lysogen B to phage A, then both phages (A and B) belong to the same immunity group (Fig. 1D).

A	phage	
	A	B
	-2 -4 -6 -8	-2 -4 -6 -8
lysogen (A)	- - - -	+ + + +
lysogen (B)	+ + + +	- - - -

B	phage	
	A	B
	-2 -4 -6 -8	-2 -4 -6 -8
lysogen (A)	- - - -	- - - -
lysogen (B)	+ + + +	- - - -

C	phage	
	A	B
	-2 -4 -6 -8	-2 -4 -6 -8
lysogen (A)	- - - -	+ + + +
lysogen (B)	- - - -	- - - -

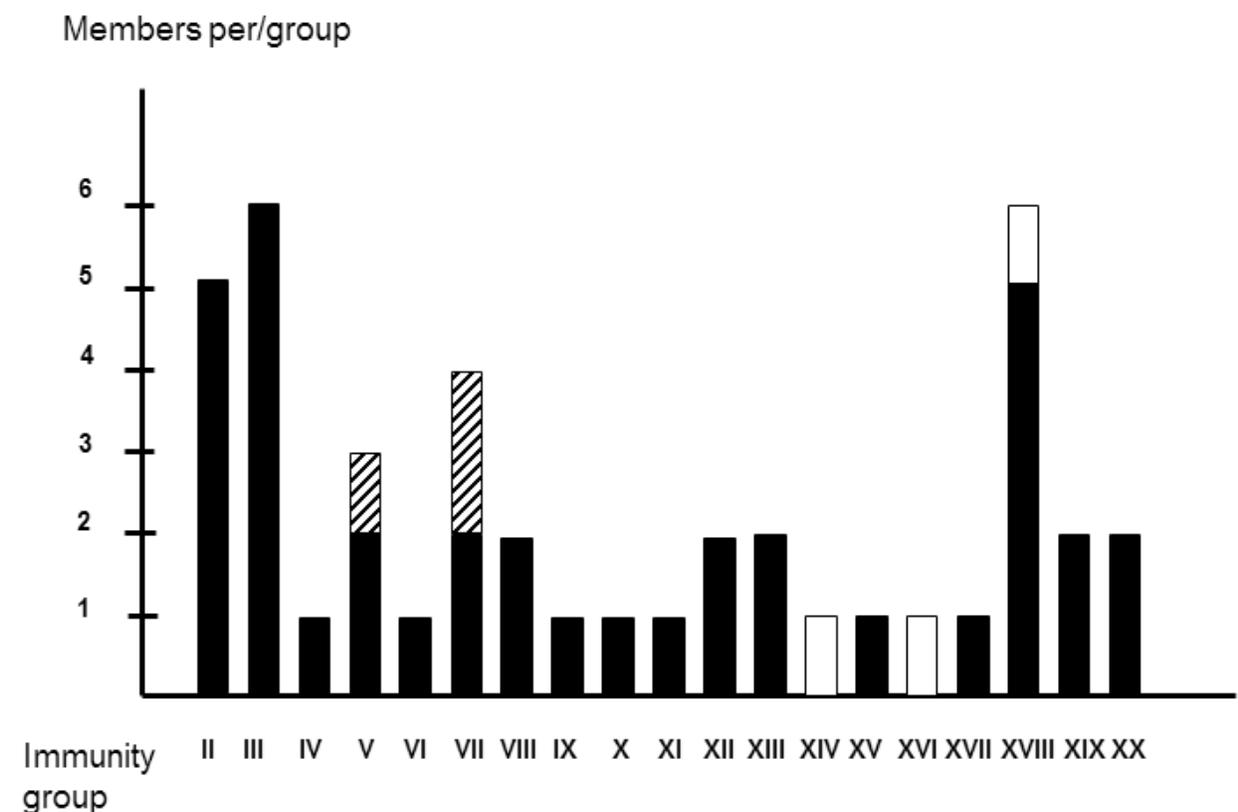
D	phage	
	A	B
	-2 -4 -6 -8	-2 -4 -6 -8
lysogen (A)	- - - -	- - - -
lysogen (B)	- - - -	- - - -

A) When lysogens are sensitive to the other phages but are resistant to their homologous phages they are referred as heteroimmune. B) The lysogen A is resistant to phage B, this indicates that lysogen A must have another exclusion system than the repressor. C) Similar example as B, but it is referred to the lysogen B. D) If both lysogens (A and B) are resistant to phages A and B infections, this result strongly suggests that they belong to the same immunity group. The numbers -2, -4, -6 and -8 represent different dilutions of phage lysate that can be tested on the strain. Presence (+) or absence (-) of phage growth can be determined by infecting with a series of phage lysate dilutions.

Fig. 1. Possible combinations between phages (A and B) and their lysogens.

It is simple to understand when two lysogens A and B are resistant to their respective homologous phages, but are sensitive to the phages B and A, respectively, this indicates that both phages (A and B) belong to different immunity group (Fig. 1A). A different scenario

would be if lysogen A is resistant to phage B, this would indicate that lysogen A must have another exclusion system different to the repressor (Fig. 1B). The same case is applied for lysogen B (Fig.1C). From this study (Kameyama et al., 1999), it was possible to classify 19 different immunity groups (Fig. 2), of which 9 out of 19 (~ 50%) had to represent a unique individual.



Phages were classified into nineteen immunity groups following the phage-lysogen cross test as previously reported by Kameyama et al. (1999). Phage groups were classified according to phage number per group. Black bars represent FhuA-receptor dependent phages; striped and white bars are phages that were unable to grow in strain MCR106 ($\Delta lamB$) and MH760 (*ompC*⁻) cells, respectively (Taken from Hernandez-Sanchez, et al., 2008).

Fig. 2. Frequency distribution of nineteen lambdoid phage-infection immunity groups.

It is noteworthy that the immunity group XVIII (lambda phage belongs to this group) is comprised by 6 individuals. To determine whether the lambda specific repressor CI was present in all of them, we proceeded to evaluate its physiological function. For this, the strain LK1683, derived from the *E. coli* W3110 with a cryptic lambda prophage and the genotypic main feature: *N::Kan*, *cI₈₅₇* (Kameyama et al., 1999) was used. As expected, lambda phage was not developed at 32 °C, since at this temperature the *CI₈₅₇* repressor is active, but lambda phage developed normally at 42 °C, as *CI₈₅₇* from lysogenic strain is heat-inactivated. If the 5 individuals of the group XVIII all had the same lambda CI repressor, one would expect that their behavior were similar to that shown by lambda. Indeed, 4/5 phages of this group were unable to develop at 32 °C, while at 42 °C they did. One phage did not develop because it is temperature-sensitive, since it did not develop even in the wild type strain at 42 °C. In addition, Degnan et al. (2007) sequenced more

than 5,000-bp of the immunity region of several lambdoid phages and among them the mEp234 and mEp332 (belonging to the immunity group XVIII, from our collection). They found that the mEp234 and mEp332 sequences coded for repressors almost identical to that of lambda CI with equivalent function, although respect to the Rex Region sequences they were different. The CI functional assay findings, are supported by genetic and sequencing data, therefore the classification of immunity lambdoid phage groups is reliable.

3. The gene coding for the "Receptor-Binding Protein" (RBP), which recognizes FhuA is borne by most lambdoid phages

The structural morphology of lambda and most lambdoid phages is characterized by a non-contractile and flexible tail, which is necessary for infection. At the onset and in order to carry out infection, gpJ lambda protein [equivalent to the Receptor-Binding Protein (RBP) for other phages], and located in the distal part of the tail, recognizes an outer membrane protein (OM), the trimeric maltoporin LamB (Gurnev et al., 2006). In other lambdoid phages, such as ϕ 80, HK022, mEp167, they all recognize the ferrichrome-Fe³⁺ receptor (FhuA) (Guihard et al., 1992; Uc-Mass et al., 2004). In an approach to identify the most common receptors FhuA, LamB and OmpC used by lambdoid phages from our collection, we used three different deficient *E. coli* mutants *fhuA*⁻, *lamB*⁻ and *ompC*⁻ for the physiological assay. It was found that 37 out of 43 phages (~85%) used FhuA, since they are not able to infect strain C600 (*fhuA*⁻), but they can whenever this strain is complemented with a plasmid expressing FhuA (Hernández-Sánchez et al., 2008). These results clearly indicate that most of the lambdoid phages require FhuA to penetrate into bacteria.

4. The *cor* gene is present in half of the lambdoid phages population

cor gene product is involved in phage exclusion, in those that require FhuA receptor for infection. Thus, *cor* excludes lambdoid phages ϕ 80, HK022, mEp167, etc., and non lambdoid phages T1, T5, etc. (Kozyrev & Rybchin, 1987; Malinin et al., 1993; Matsumoto et al., 1985; Uc-Mass et al., 2004), being all of them FhuA dependent. *cor* gene and the gene encoding RBP (gene p21 for phage N15 and gene p23 to HK022), are separated by two ORFs (Wietzorrek et al., 2006), and these are located in the cluster of genes that encode tail proteins. Because of this tight physical and functional association, we asked how many of the lambdoid phages contain *cor*? The answer was obtained by amplifying a 155 bp intragenic region of *cor* by PCR. We found that 25 out of 43 (~58%) phages bore it. To verify that the products corresponded to *cor* region, 4 PCR fragments were taken randomly and sequenced. Alignment analysis confirmed that the amplified region corresponded to *cor* gene (Hernández-Sánchez et al., 2008).

5. Identification of Nus-dependent non-lamdoid phages group

During the characterization of the first isolated phages from our collection, a new group of phages emerged. As part of the selection strategy, the potential lambdoid phages were challenged with 4 isogenic *nus* mutant strains *nusA1*, *nusB5*, *nusD026* and *nusE71*. Pre-selection of potential lambdoid phages was carried out considering those phages that failed

to grow at least in a couple of these mutants (Kameyama et al., 1999). Of these, 97 phages were selected. However, in the course of the characterization, a group of them (48 phages) did not recombine, nor hybridize with the lambda DNA, nor were recognized by antibodies directed against lambda structural proteins, nor their prophages were induced with light UV, and most failed to develop at 32 °C (Kameyama et al., 2001). However, this group of non-lambdoids shares an essential feature with lambdoid phages and that is the requirement for Nus factors to grow, suggesting that these phages may have an anti-termination mechanism homologous to that reported for lambdoid phages. Regarding growth cross-test assay, unlike the great diversity found in lambdoid phages, all of them had a single immunity! It is amazing how any of these lysogens has the ability to exclude any of the 48 phages of this group.

6. Discussion

We can infer from phage-lysogen cross test that lambdoid phage immunity groups are diverse and rich. If we consider that 9 out of the 19 groups had a unique representative, this could indicate that the number of different groups of immunity should be much larger. However, taking into account that the sample of the population of phage analyzed is small, it is not possible to infer probabilistically the number of possible different immunities in the region. A completely different scenario was obtained when testing the requirement of different bacterial receptors. It was found that 37 out of 43 require the *E. coli* FhuA receptor for infection. As mentioned above, the gene encoding RBP is essential, therefore nucleotide changes in this gene may be deleterious, and then it can be considered highly conserved. On the other hand, the bacterium *E. coli* use the FhuA receptor for iron assimilation through the ferrichrome-Fe³⁺ transport system. Interestingly, although the bacterium *E. coli* contains the genes *fhuA*, *fhuB*, *fhuC* and *fhuD* in an operon, for the ferrichrome-Fe³⁺ transport, it lacks of the genes for the biosynthesis of ferrichrome. Hence, in nature, the ferrichrome is produced by other species such as *Ustilago maydis*, and is taken in by *E. coli* for growth. This argument suggests that *E. coli* had to acquire the *fhu* operon at some stage of its evolution. Provided that most of lambdoid phages are FhuA dependent and considering that the gene encoding RBP would be highly conserved, as its product requires a perfect match with its receptor, it is likely that the first lambdoid phage had to require the FhuA receptor to infect its host *E. coli*. In addition, if we consider the argument that the *fhu* operon was acquired at some stage during *E. coli* evolution, then, the origin of the first lambdoid phage must not be older than that of its host *E. coli*! This idea though highly speculative, if true, it would support the proposal that viruses appeared after new cellular species emerged.

On the other hand, even having a great variety of phage immunity groups, it is still not possible to propose a putative origin of the first repressor, because the major constraint is present in the sample population. However, the dynamics of changes can be appreciated by the wide range of immunities provided.

Also it is interesting the analysis of *cor* gene implication. It has been proposed that *cor* is a moron that at some point of phage evolution was obtained (Juhala et al., 2000). Morons are autonomous genetic modules that are expressed from the repressed prophage probably

acquired by horizontal transfer (Juhala et al., 2000). However, given the proximity to the gene encoding RBP and the high percentage that is present in the population (more than 50%), it would be more likely that *cor* associated with the RBP gene were acquired together in the formation of new phage. Unlike the RBP gene, *cor* is not essential, making easier to explain why *cor* is missing in a sector of the population.

It is also interesting to take into account the other group of 48 phages that emerged as a new group with a unique immunity, and knowing that this region must be very variable (as well as has been indicated for lambdoid phages), this may suggest that this group was recently created. However, other explanations are possible. For example the acquisition of the unique immunity region as a possible recombination with a different phage, since its repressor must be different to that of lambdoid phages, in which this is not inducible by UV light. Based on the phage numbers, one can infer that they are successful as lambdoid phages in nature. It is also a notable observation that only a single group or family of phages in *Brucella abortus* has been observed (personal communication of Flores, V). This idea complements the proposal that viruses appeared after emerging of the species.

New data will be needed to generate more precise and convincing answers. It should be noted that host participation can be critical in certain tasks, and finally given the great diversity of the viruses, these studies should be carried out according to each one of the family or group of viruses concerned.

It is clear that this chapter would be subject to polemic, as would any different or relatively new idea proposed to explain viral evolution. Indeed, it will serve to enhance, refine, or change approaches to shed more precise answers in this topic.

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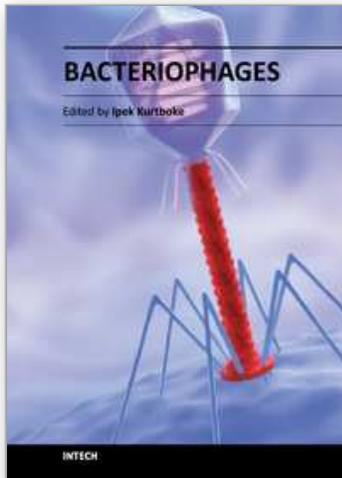
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Bacteriophages have received attention as biological control agents since their discovery and recently their value as tools has been further emphasized in many different fields of microbiology. Particularly, in drug design and development programs, phage and prophage genomics provide the field with new insights.

Bacteriophages reveals information on the organisms ranging from their biology to their applications in agriculture and medicine. Contributors address a variety of topics capturing information on advancing technologies in the field. The book starts with the biology and classification of bacteriophages with subsequent chapters addressing phage infections in industrial processes and their use as therapeutic or biocontrol agents. Microbiologists, biotechnologists, agricultural, biomedical and sanitary engineers will find Bacteriophages invaluable as a solid resource and reference book.

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