Title: What the book of Lambda didn't tell us about lysogens from natural populations

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Abstract: Fundamental to the ecology of bacteria are bacteriophages (phages), a major source of bacterial mortality. Functionally, bacteria are protected from these viruses through resistance, where they are refractory, and immunity, where the phages infect but do not replicate. Immunity is the mechanism by which lysogenic bacteria are protected from temperate phage infections. This is what we know from studies of lysogens in the laboratory, but what about the real world? We address this question with mathematical models and experiments using *Escherichia coli*, λ-phage, and naturally occurring *E. coli* lysogens. Our study demonstrates that naturally occurring lysogens are resistant (refractory) to the phage coded by their prophage. We therefore postulate that lysogenic bacteria are likely resistant rather than only immune to temperate phages.

One-Sentence Summary: Naturally occurring lysogens are likely to be resistant, and not only immune, to bacteriophages encoded by their prophage.

Main Text:

Functionally and ecologically, the single most significant difference among bacteriophages is whether they are purely virulent (lytic) or temperate. Virulent phages, a major source of bacterial mortality (*1*), can only be transmitted horizontally, commonly with the death of the infected bacterium and subsequent release of phage particles (*2*). Temperate phages can also replicate and be transmitted horizontally, but with a low probability, their genome can become incorporated into that of the infected bacterium as a prophage (*3*), forming lysogens. The infected lysogen survives and the phage genome, borne by this bacterium, is then transmitted vertically during cell division (*3, 4*). For both temperate and virulent phages, selection favors bacteria that are not killed by the infecting virus. For bacteria infected by virulent phages, survival is achieved by resistance; commonly due to mutations in the receptor site, which prevent adsorption and make the host bacteria refractory to the phages (*5*). For lysogens infected by the temperate phages encoded by their prophages, survival is achieved by immunity, where the infection is aborted, and the infecting phage is eliminated (*5*). This distinction between resistance and immunity is drawn primarily from experiments with laboratory strains (*6, 7*).

Here, we investigate the validity of the interpretation that immunity and resistance are separate phenomena of temperate and lytic phages, respectively. Using a mathematical model and experiments with *Escherichia coli*, the phage lambda (λ), and naturally occurring lysogenic *E. coli* and their temperate phages, we explore the population and evolutionary dynamics of virulent and temperate phages and lysogenic bacteria. Contrary to the traditional interpretation, our findings demonstrate that lysogens are likely to be resistant as well as immune to the temperate phages coded by their prophage.

Results

In Figure 1, we present a diagram of a mathematical model of the population and evolutionary dynamics of virulent and temperate bacteriophages and their bacterial hosts. This model includes populations of non-lysogens (N) and lysogens (L), and free temperate phages (P), as cells and particles per mL, respectively. It also allows for a virulent phage population (V) that shares the same receptor as the temperate phage, and refractory mutants of the lysogens, L_R, as well as sensitive non-lysogens, N_R. Phage infection is a mass action process where the viruses adsorb to the bacteria at rates proportional to the product of their density and rate constants δ_p and δ_v for the temperate and lytic phages, respectively (*8*).

With a probability λ ($0 \leq \lambda \leq 1$), infections of sensitive bacteria with temperate phage form lysogens. The remaining fraction ($1-\lambda$) of infections by the temperate phages are lytic, and each infected bacterium bursts to yield β_P phage particles. Each infection of sensitive non-lysogenic and lysogenic bacteria with the virulent phages produces β_V phage particles. We neglect the lag phase of the bacteria and the latent periods for the phages, and assume that the likelihood of lysogeny is independent of the multiplicity of infection (*9, 10*)

The rate of growth of the bacteria is equal to the product of their maximum rates v, v_{nr}, v_l, v_{lr} (per hour) and a hyperbolic function, $\psi(R)$, of the concentration of a limiting resource (R, μg/ml) (*11*) (see Equation S1). The limiting resource is consumed at a rate equal to the product of the sum of the products of the density and maximum growth rate of the bacteria, and a conversion efficiency parameter, e, μg/cell (*12*). The rates of mutations $L \rightarrow L_R$, $L_R \rightarrow L$, $N \rightarrow N_R$, and $N_R \rightarrow N$ are, respectively μ_{lr}, μ_{rl}, μ_{nr} and μ_{rn} per cell per hour (*13*). To account for the decline in the physiological state as the bacteria approach stationary phase (R = 0), we assume that the rates of phage infection and mutation decline at rates proportional to $\psi(R)$. The seven coupled differential

equations for this model are presented in Equations S2–S8 and the values of the parameters used in our numerical solutions (simulations) are presented in Table S1. Although our primary focus in this study is temperate phages and lysogeny, this is a general model of the population and evolutionary dynamics of bacteria and their virulent and temperate phages.

We then explored the generality and fit of this model with the predictions made in our analysis of its properties, as presented in Figure S1, and the parallel experiments with *E. coli* and the phages λ and λ^{Vir}, presented in Figure S2.

We present the predictions from simulated serial transfer populations initiated with sensitive bacteria, N, free temperate phages, P, and a minority population of resistant non-lysogens, N_R (Figure 2A). As anticipated by the traditional view of lysogeny, within short order, lysogens were produced and ascended in density to become the dominant bacterial population. The resistant non-lysogens also increased in frequency and achieved a density similar to that of the lysogenic population. This was due to selection mediated by their resistance to the free temperate phages. The population of resistant lysogens (L_R) also increased in frequency but remained a minority.

We conducted parallel experiments where a low density of free λ temperate phages was inoculated into a population of λ-sensitive *E. coli* containing a minority population of λ-resistant *E. coli* (Fig. 2B, with replicas shown in Fig. S3). As predicted by our model, the resistant non-lysogens increased in frequency, as did a novel population that was both refractory to phage λ (Fig. S4) and lysogenic for the same phage, resistant lysogens.

To more extensively explore the conditions under which populations of resistant lysogens ascend and are maintained, we added to our simulations a virulent phage that shares the same receptor as the temperate phage. Under these conditions, the selection for resistance should be more intense. This can be seen in Figure 2C, where resistant lysogens (L_R), as well as resistant non-lysogens

(N_R), became the dominant bacterial populations. To explore the validity of this prediction experimentally, we initiated serial transfer populations with sensitive *E. coli* and both temperate and virulent λ (Fig. 2D). The results of this experiment are qualitatively consistent with the predictions of the simulations. Resistant non-lysogens, with *malT* mutations similar to what has been reported in other *E. coli* resistant to λ-phage ((*14, 15*); Fig. S5), which ascend and become the dominant bacterial population followed by lysogens and resistant lysogens (see Fig. S3 for the replicas of these experiments).

Our model and experiments make two predictions: (i) When a population of sensitive bacteria are infected by temperate phages, not only will lysogens be generated, but also resistant lysogens will be formed and increase in density; (ii) If the community includes virulent phages that share the same receptor as the temperate phage, resistant lysogens will ascend and become the dominant population of lysogens. In this interpretation, naturally occurring lysogens are likely to be refractory to the phage coded by their prophage. To test these predictions, we used a set of naturally occurring (wild) lysogenic *E. coli* with different prophages from sewage and the gut microbiomes of infants (*16*). We induced these wild lysogens to produce free temperate phages which we then used to infect the lysogens from whence they came (Table S2). Consistent with the prediction of our model and the above-described experiments with λ (Fig. 2), all ten naturally occurring lysogens were refractory to the free phages generated by induction (Figure 3A). As a control for these experiments, λ-sensitive *E. coli* C, λ lysogens, and λ^{Vir}-resistant bacteria were infected with a temperate λ-phage, coding for a kanamycin resistance gene, λ^{KAN}. As anticipated, the phages replicated on the sensitive non-lysogens but not the λ^{Vir}-resistant *E. coli*. However, not anticipated was the marked increase in the density of free phages in the control experiment with λ lysogens infected by temperate λ-phage (Figs. 3A and 3B, shaded areas).

To test the hypothesis that the resistance of naturally occurring lysogens to the phages coded by their prophages is a property of the bacteria rather than that of the prophages, we constructed lysogens by infecting *E. coli* C with each of the wild phages (Table S2). All ten wild phages formed lysogens with *E. coli* C. These lysogenic *E. coli* C did not appear refractory to their respective free phages (Fig. 3B). They behaved like λ lysogens infected with λ (Fig. 3B, shaded area). Most intriguingly, similar to the λ lysogens in the control experiment (shaded areas of Fig. 3), when the *E. coli* C lysogens were infected with a low density of free temperate phages, high densities of free phages were produced.

The observation that when infected with free phages, both the λ and *E. coli* C lysogens generated high densities of free phages (Fig. 3B) was unexpected. It suggested that lysogens were induced when infected with the temperate phages coded by their prophage. We postulated that infections with temperate phages induced lysogens by mounting an SOS response, as would be the case when exposed to ultraviolet light or other SOS-inducing insults (*17-19*). To test this hypothesis, a Δ*recA* *E. coli* construct obtained from the KEIO collection was lysogenized with λ and reinfected with free λ (*20*). Contrary to that observed with the $recA^+$ lysogen (Fig. 3B), infection of the Δ*recA* λ lysogen with free λ-phage did not generate a high density of free phage (Fig. S6A). To further test the hypothesis that infections with free λ induce λ lysogens by generating an SOS response, we transformed the Δ*recA* *E. coli* λ lysogens with plasmids either lacking or bearing *recA* (Fig. S7). With the cells bearing a functional *recA* in *trans*, the lysogens were induced at a high rate (Fig. S6B). This did not occur with the plasmid without *recA* (Fig. S6C).

Discussion

We set out to explore the validity of the generally assumed distinction between immunity and resistance – two mechanisms bacteria employ in response to phage infection. Contrary to the traditional assumption that lysogenic bacteria are solely immune to the phage coded by their prophage (*21*), the results of our study suggest that they are also likely to be resistant (refractory). Our mathematical models, computer simulations, and experiments with *E. coli* and the phage λ predicted this result. Our tests using naturally occurring lysogenic *E. coli*, support this prediction, as all ten wild lysogens tested were refractory to the phage coded by their prophages.

These models and experiments also predict that if the bacterial community includes virulent phages that share the same receptors as the temperate phage, the lysogenic population will be dominated by cells that are resistant (refractory) to the temperate phages. Overall, in this study, we predict that naturally occurring lysogenic bacteria will be both immune and resistant to the phages coded by their prophage.

From an ecological and evolutionary perspective, resistance to the phages coded by their prophage would be an asset. The prophage genome will be transmitted vertically, and the free temperate phages will be continually produced by induction. These free temperate phages will then be capable of infecting sensitive non-lysogens, thereby expanding the range of bacteria bearing that prophage. Simultaneously, these lysogens, and thereby the temperate phages they encode, will not be subject to extinction by virulent phages capable of adsorbing to the same receptors as those of the temperate phages.

The high rate of induction noted after the sensitive lysogens encountered the temperate phages coded by their prophage was unexpected. Prophage excision owing to phage infection by dissimilar infecting phages has been reported (*22, 23*). However, we were unable to find reports

of infections with temperate phages for which the bacteria are already immune leading to excision of prophages.

The molecular biologist Jacques Monod quipped that "What is true for *E. coli* is true for elephants, only more so" (*24*). While we appreciate this form of inductive inference, we understand that all of the experiments reported here were conducted only with *E. coli*. We postulate that naturally occurring lysogenic bacteria of any species that can produce resistant mutants will be resistant to the phages coded by their prophage.

References and Notes

1. F. Rohwer *et al.*, *Life in Our Phage World: A Centennial Field Guide to the Earth's Most Diverse Inhabitants*. (Wholon, 2014).
2. M. Adams, *Bacteriophages*. (Interscience, New York, 1959).
3. A. Lwoff, Lysogeny. *Bacteriological Reviews* **17**, 269 (1953).
4. M. Ptashne, *The Genetic Switch*. (Cold Spring Harbor Press, New York, 2004), vol. 3rd.
5. S. J. Labrie, J. E. Samson, S. Moineau, Bacteriophage resistance mechanisms. *Nature Reviews Microbiology* **8**, 317-327 (2010).
6. W. Arber, D. Dussoix, Host specificity of DNA produced by Escherichia coli: I. Host controlled modification of bacteriophage λ. *Journal of Molecular Biology* **5**, 18-36 (1962).
7. L. M. Malone, H. G. Hampton, X. C. Morgan, Peter C. Fineran, Type I CRISPR-Cas provides robust immunity but incomplete attenuation of phage-induced cellular stress. *Nucleic Acids Research* **50**, 160-174 (2021).
8. F. M. Stewart, B. R. Levin, The population biology of bacterial viruses: why be temperate. *Theoretical population biology* **26**, 93-117 (1984).
9. P. Kourilsky, A. Knapp, Lysogenization by bacteriophage lambda: III. - Multiplicity dependent phenomena occuring upon infection by lambda. *Biochimie* **56**, 1517-1523 (1975).
10. G. Li, M. H. Cortez, J. Dushoff, J. S. Weitz, When to be temperate: on the fitness benefits of lysis vs. lysogeny. *Virus Evolution* **6**, (2020).
11. J. Monod, The growth of bacterial cultures. *Annual Review of Microbiology* **3**, 371-394 (1949).

12. F. M. Stewart, B. R. Levin, Resource partitioning and the outcome of interspecific competition: a model and some general considerations. *American Naturalist* **107**, 171-198 (1973).

13. W. N. Chaudhry *et al.*, Leaky resistance and the conditions for the existence of lytic bacteriophage. *PLoS Biol* **16**, e2005971 (2018).

14. M. De Paepe *et al.*, Carriage of λ Latent Virus Is Costly for Its Bacterial Host due to Frequent Reactivation in Monoxenic Mouse Intestine. *PLOS Genetics* **12**, e1005861 (2016).

15. M. De Paepe *et al.*, Trade-Off between Bile Resistance and Nutritional Competence Drives Escherichia coli Diversification in the Mouse Gut. *PLOS Genetics* **7**, e1002107 (2011).

16. A. Mathieu *et al.*, Virulent coliphages in 1-year-old children fecal samples are fewer, but more infectious than temperate coliphages. *Nature Communications* **11**, 378 (2020).

17. N. Otsuji, M. Sekiguchi, T. Iijima, Y. Takagi, Induction of Phage Formation in the Lysogenic Escherichia coli K-12 by Mitomycin C. *Nature* **184**, 1079-1080 (1959).

18. A. Matsushiro, K. Sato, H. Miyamoto, T. Yamamura, T. Honda, Induction of prophages of enterohemorrhagic Escherichia coli O157:H7 with norfloxacin. *Journal of bacteriology* **181**, 2257-2260 (1999).

19. B. J. Barnhart, S. H. Cox, J. H. Jett, Prophage induction and inactivation by UV light. *Journal of virology* **18**, 950-955 (1976).

20. T. Baba *et al.*, Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol Syst Biol* **2**, 2006.0008-2006.0008 (2006).

21. M. Ptashne, *A Genetic Switch: Phage Lambda Revisited*. (Cold Spring Harbor Laboratory Press, 2004).

22. S. Campoy *et al.*, Induction of the SOS response by bacteriophage lytic development in Salmonella enterica. *Virology* **351**, 360-367 (2006).

23. I. Lamont, A. M. Brumby, J. B. Egan, UV Induction of Coliphage 186: Prophage Induction as an SOS Function. *Proceedings of the National Academy of Sciences of the United States of America* **86**, 5492-5496 (1989).

24. C. Zimmer, Microcosm: E. coli and the New Science of Life. 256 (2008).

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Data and materials availability:

The modeling programs and software used are available at ECLF.net.

The sequence data shown have been deposited at Genbank (NCBI, Bethesda, Maryland, USA).

All other data are available in the main text or supplementary materials.

Supplementary Materials

Materials and Methods

Tables S1 and S2

Figs. S1 to S7

Equations S1 to S8

Fig. 1. Model of population and evolutionary dynamics of temperate and lytic phages. Population and evolutionary dynamics of lytic and temperate phages with non-lysogenic and lysogenic bacteria are shown. There is a population of temperate phage, P; a population of virulent phage, V, that shares the same receptor with the temperate phage; a phage-sensitive non-lysogenic bacterial population, N; a refractory non-lysogenic bacterial population, N_R; a virulent-phage-sensitive lysogenic bacterial population, L; and a refractory lysogenic bacterial population, L_R. For the definitions, dimensions, and parameter values, see Table S1.

Fig. 2. Population dynamics for lysogeny in mixed populations with and without the presence of a virulent phage. (Top section) Simulation results with parameter values in the range estimated for *Escherichia coli* and lambda (λ) phage (Table S1). (Lower section) Experiments with sensitive and λ-resistant *E. coli*, λ^{KAN}, and λ^{Vir}. Changes in bacterial (colony-forming units per mL) and phage (plaque-forming units per mL) density in 24 h serial transfer populations with a 1/100 dilution factor are shown for: (**A**) Simulation initiated with 10^6 sensitive bacteria and 10^4 resistant non-lysogens with 10^5 temperate phage per mL; (**B**) experimental culture initiated with ~10^6 λ-sensitive *E. coli* and ~10^4 λ-resistant STR^R *E. coli*, and ~10^5 temperate phage with kanamycin resistance, λ^{KAN}; (**C**) simulation initiated with 10^6 phage-sensitive bacteria, 10^7 temperate phages, and 10^7 virulent phages that share the same receptor as the temperate phage; (**D**) experimental culture initiated with ~10^6 λ-sensitive bacteria, ~10^7 temperate phage with kanamycin resistance, λ^{KAN}, and ~10^7 virulent mutants of λ, λ^{Vir}.

Fig. 3. Susceptibility and resistance of naturally occurring and *E. coli* C constructed lysogens. Ratios of free phage density after 0.5, 1, and 3 h relative to that at 0 h (T_0). Means and standard deviations of the ratios of three replicas are presented. (Shaded regions) Infections with

phage lambda (λ) in three distinct bacterial states: λ-sensitive (λ-S), λ-lysogen (λ-L), and λ-resistant (λ-R). (A) Naturally occurring lysogens (W) infected with a low multiplicity of infection of their induced free phages for which they are lysogenic. (B) Constructed *E. coli* C lysogens (L) infected with a low multiplicity of induced free phages for which they are lysogenic. See Table S2 for designations and sources of the strains.

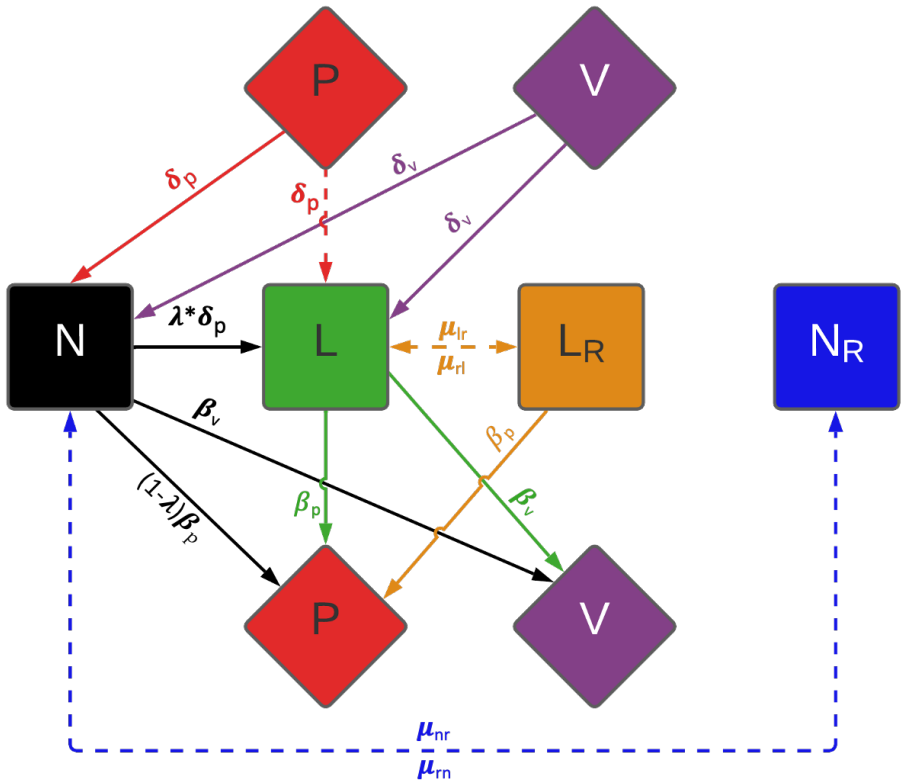

Figure 1

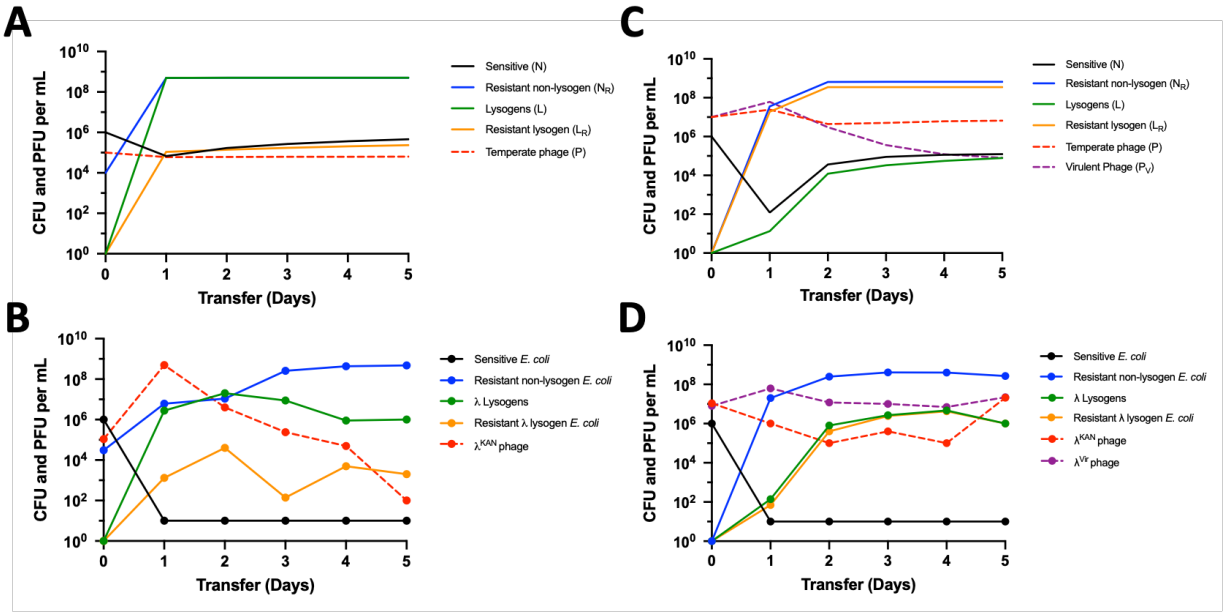
A
B
C
D
CFU and PFU per mL
Transfer (Days)
Sensitive (N)
Resistant non-lysogen (N_R)
Lysogens (L)
Resistant lysogen (L_R)
Temperate phage (P)
Virulent Phage (P_V)
Sensitive E. coli
Resistant non-lysogen E. coli
λ Lysogens
Resistant λ lysogen E. coli
λ^{KAN} phage
λ^{Vir} phage

Figure 2

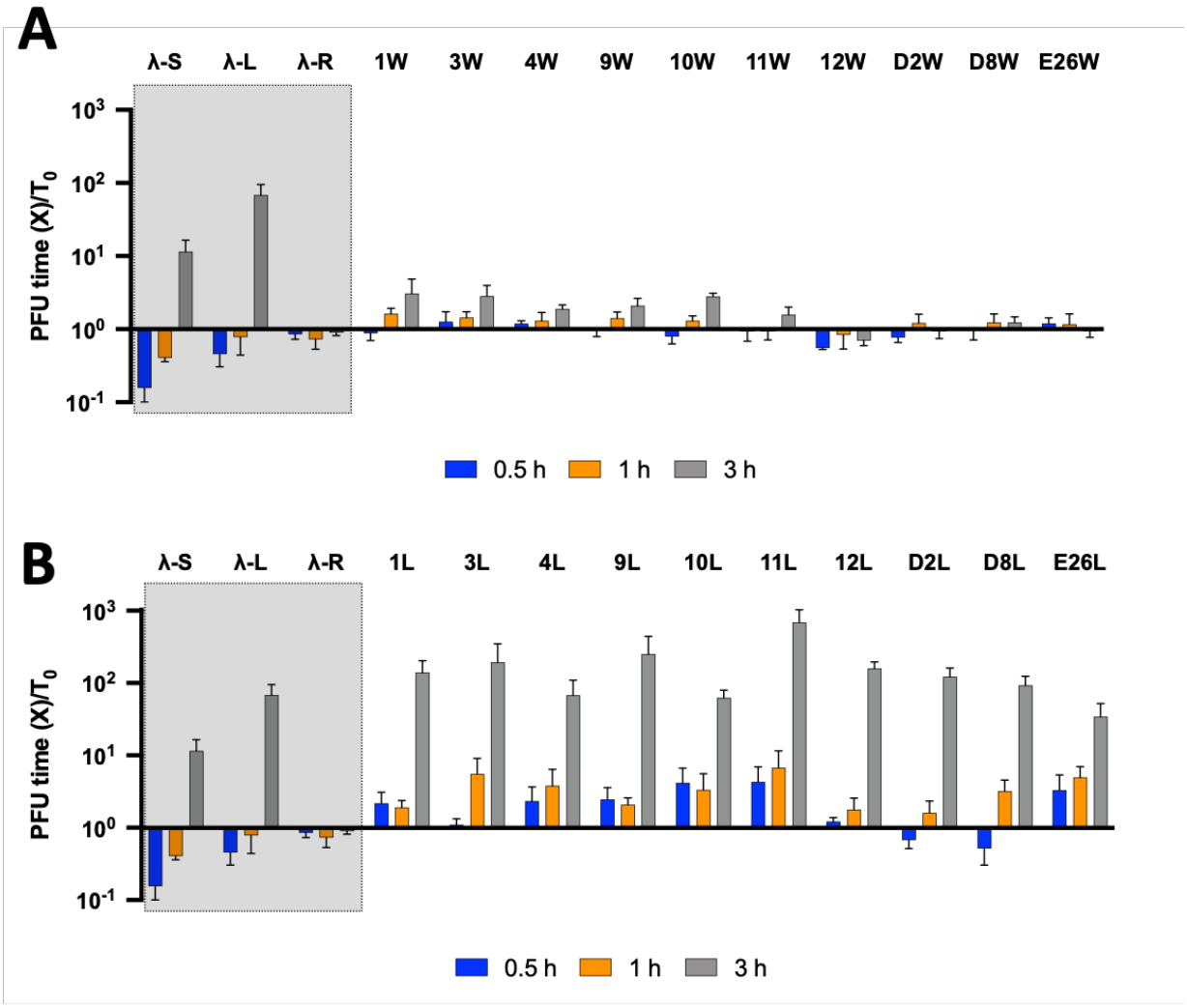
A
λ-S
λ-L
λ-R
1W
3W
4W
9W
10W
11W
12W
D2W
D8W
E26W
PFU time (X)/T0
10^3
10^2
10^1
10^0
10^-1
0.5 h
1 h
3 h
B
λ-S
λ-L
λ-R
1L
3L
4L
9L
10L
11L
12L
D2L
D8L
E26L
PFU time (X)/T0
10^3
10^2
10^1
10^0
10^-1
0.5 h
1 h
3 h

Figure 3