BIOLOGY LETTERS

rsbl.royalsocietypublishing.org

Research

Cite this article: Pleška M, Guet CC. 2017 Effects of mutations in phage restriction sites during escape from restriction–modification. *Biol. Lett.* **13**: 20170646.
http://dx.doi.org/10.1098/rsbl.2017.0646

Received: 17 October 2017
Accepted: 15 November 2017

Subject Areas:
evolution, molecular biology

Keywords:
restriction–modification systems, restriction site avoidance, phage–bacteria interactions, mutation effects

Author for correspondence:
Călin C. Guet
e-mail: calin@ist.ac.at

Electronic supplementary material is available online at https://dx.doi.org/10.6084/m9.figshare.c.3938953.

THE ROYAL SOCIETY PUBLISHING

Molecular evolution

Effects of mutations in phage restriction sites during escape from restriction–modification

Maroš Pleška and Călin C. Guet

Institute of Science and Technology Austria, Am Campus 1, Klosterneuburg 3400, Austria

CCG, 0000-0001-6220-2052

Restriction–modification systems are widespread genetic elements that protect bacteria from bacteriophage infections by recognizing and cleaving heterologous DNA at short, well-defined sequences called restriction sites. Bioinformatic evidence shows that restriction sites are significantly underrepresented in bacteriophage genomes, presumably because bacteriophages with fewer restriction sites are more likely to escape cleavage by restriction–modification systems. However, how mutations in restriction sites affect the likelihood of bacteriophage escape is unknown. Using the bacteriophage λ and the restriction–modification system EcoRI, we show that while mutation effects at different restriction sites are unequal, they are independent. As a result, the probability of bacteriophage escape increases with each mutated restriction site. Our results experimentally support the role of restriction site avoidance as a response to selection imposed by restriction–modification systems and offer an insight into the events underlying the process of bacteriophage escape.

1. Introduction

Bacterial viruses, also called bacteriophages (phages), are the most abundant biological entities on Earth and as such, they represent a major driving force of bacterial evolution [1]. While temperate phages can, with a small probability, enter genomes of their hosts and potentially contribute genes that increase bacterial fitness [2], the vast majority of infections by both temperate and non-temperate (virulent) phages are lethal for infected bacteria. To protect themselves, many bacteria use a wide variety of phage resistance mechanisms, a large group of which are based on recognizing and destroying heterologous phage DNA [3]. Restriction–modification (RM) systems represent the first discovered [4], the simplest and one of the most prevalent [5] of such mechanisms.

Most RM systems are composed of two enzymatic activities: the restriction activity of a restriction endonuclease (R) and the modification activity of a methytransferase (M). Both R and M typically recognize and act on well-defined, short (4–8 bp) DNA sequences termed restriction sites. Upon infection, R recognizes the restriction sites on the phage DNA as non-self and cleaves it, thus aborting the infection. However, there is a non-zero probability that instead of being restricted, the phage restriction sites will be erroneously modified by M, whose primary role is to methylate restriction sites contained in the bacterium's own DNA and prevent self-restriction [6]. What causes a fraction of phages to escape restriction is not understood, as are not the factors determining the size of this fraction itself.

Interestingly, genomes of many phages display a significant underrepresentation of restriction sites [7–10]. This underrepresentation, also termed restriction site avoidance, is thought to result from natural selection favouring

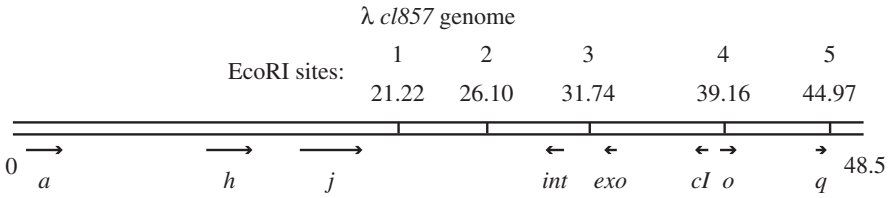

Figure 1. Genetic map of λ *cl857*. Locations (in kb) of five restriction sites are specified. Locations of characteristic λ genes are shown for reference.

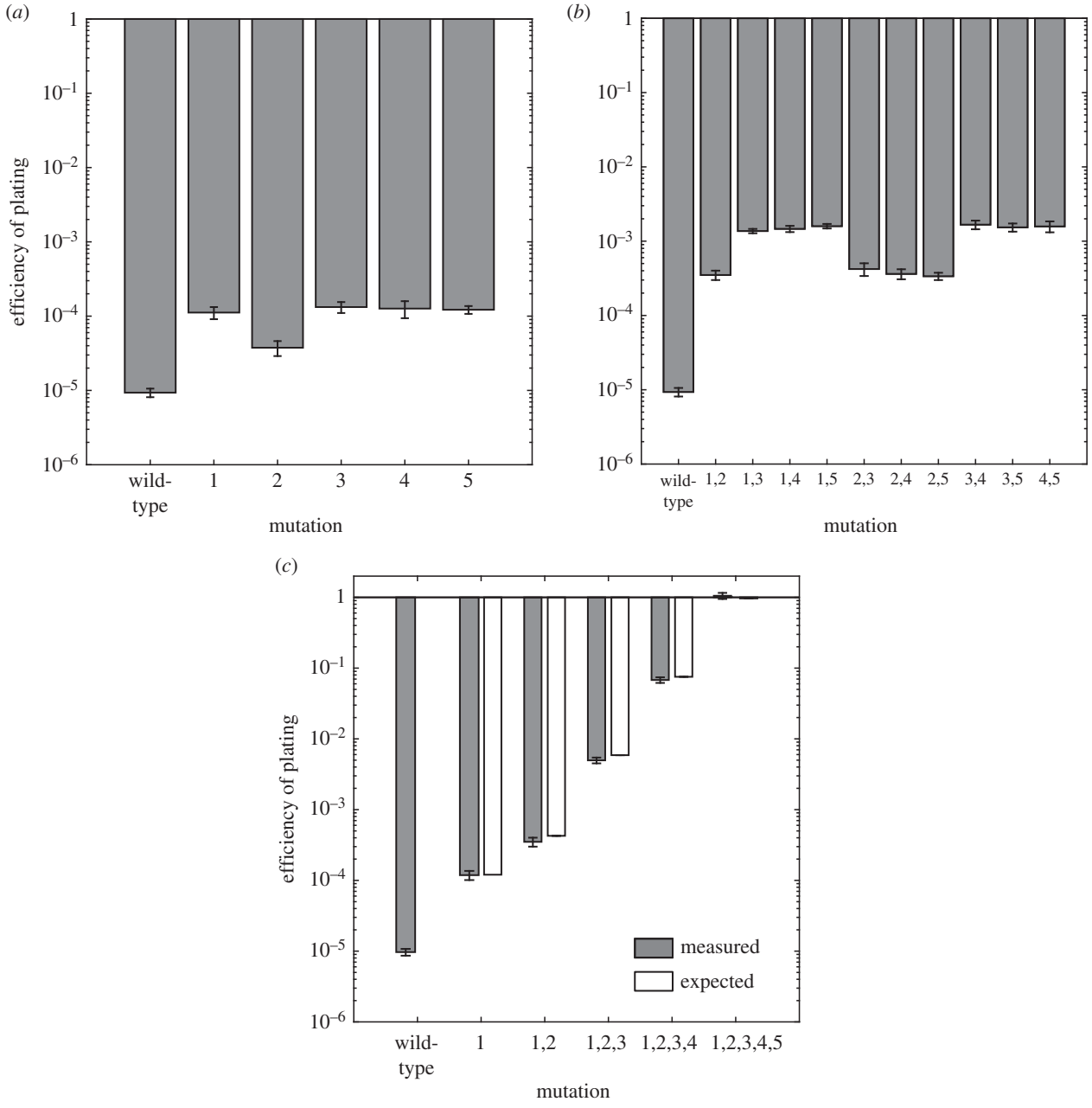

Figure 2. Independence of effects of mutations in phage restriction sites. (*a*) Efficiencies of plating of wild-type λ *cl857* and five single mutants. The numbering of restriction sites corresponds to that shown in figure 1. (*b*) Efficiencies of plating of wild-type λ *cl857* and all double mutants. (*c*) Efficiencies of plating of the wild-type λ *cl857* and mutants with consecutively added mutations. Experimentally observed values are shown as grey bars. White bars represent expected efficiencies of plating calculated based on effects of individual mutations (table 1), assuming complete independence of mutation effects. In all panels, means of six independent biological replicates from three separate experiments are shown except for wild-type, which shows 18 independent biological replicates from three separate experiments. Error bars represent 95% confidence intervals.

phages with mutations in restriction sites owing to their increased probability of escape. We test this hypothesis experimentally by studying the relationship between mutations in restriction sites and the probability of phage escape.

2. Material and methods

As a model system, we used the classic RM system EcoRI and bacteriophage λ variant *cI857*, which carries five EcoRI restriction sites (GAATTC) (figure 1). λ *cI857* is a temperature-sensitive

mutant, which behaves as an obligatory lytic phage at temperatures above 30°C [11]. All our experiments were performed at 37°C. *Escherichia coli* strain MG1655 was used in all experiments. The EcoRI RM system was carried on a plasmid pBR322ΔPtet EcoRI (R^+M^+) [12]. As a RM reference, we used a strain carrying the pBR322ΔPtet plasmid.

Mutations were introduced into the phage genome using recombineering [13]. In order to minimize any negative impact of mutations on phage fitness, a single synonymous point mutation was introduced into each EcoRI restriction site. The method of constructing and verifying the phage mutants is described in detail in the electronic supplementary material, material and methods.

As a measure of the probability of escape, we used the efficiency of plating (eop) defined as $\text{eop} = \frac{\text{pfu}_{\text{RM}}}{\text{pfu}_{\text{total}}}$, where pfu_{RM} is the number of plaque forming units (pfu) obtained on lawns of bacteria carrying the EcoRI RM system and $\text{pfu}_{\text{total}}$ is the total number of pfu obtained on the reference RM strain. For each measurement, 10 µl of serially diluted lysates was mixed with 0.1 ml of bacterial culture in 3 ml of soft agar and spread on phage plates such that 100–300 pfu per plate were obtained. In each measurement, $\text{pfu}_{\text{total}}$ was at least an order of magnitude lower than the total number of bacteria plated (approx. 10^8) so that the vast majority of infections correspond to a single phage infecting a single bacterium. Method of preparation of lysates and bacterial cultures is described in the electronic supplementary material, material and methods.

The effects of mutations and their interactions were calculated by fitting a single multivariate linear regression model with interaction terms to the data shown in figure 2*a*,*b*, with log(eop) as the dependent continuous variable and presence/absence of each of the five restriction sites as five categorical independent variables. Normal distribution of errors was verified by residual analysis.

3. Results

(a) Effects of mutations in restriction sites are unequal

To study how mutations in phage restriction sites affect the probability of phage escape, we first constructed five λ *cI857* mutants, each with a single point mutation in one of the five EcoRI restriction sites (figure 1). The probability of phage escape, measured as the efficiency of plating (eop) (Material and methods), of all five mutants was considerably higher than the probability of escape of the wild-type λ *cI857* (figure 2*a*). Each mutation thus increased the likelihood of phage escape. While mutations at sites 1, 3, 4 and 5 all increased eop by approximately an order of magnitude, the effect of mutation at site 2 was considerably smaller. This result was in accord with previous studies, showing that this particular EcoRI restriction site is cleaved with a lower efficiency both *in vitro* [14] and *in vivo* [15]. The lower likelihood of cleavage is possibly a result of the significantly reduced GC content in an approximately 5 kb long region, in which the restriction site is located [14].

(b) Effects of mutations in phage restriction sites are independent

We next asked whether effects of mutations in restriction sites change when they occur in combinations, for example as a result of relative positioning of individual restriction sites [16]. To this end, we constructed a set of 10 λ *cI857* mutants, each with a different pairwise combination of mutations. The eop of all 10 double mutants was higher than the wild-type, although apparent differences among mutants were observed (figure 2*b*). Namely, the eop of phages including a mutation at site 2 was lower when compared with other mutants. We tested whether interactions alter the effect of mutations occurring in combinations by fitting a linear regression model with interaction terms to data obtained for both single and double mutants. The effects of all five individual mutations were highly significant, with the effect at site 2 being the smallest (table 1). By contrast, all interaction terms were below the significance threshold ($\alpha = 0.05$) (table 1), implying that mutation effects are independent.

Table 1. Estimates of mutation effects and their interactions. wt, wild-type.

	est. effect	s.e.	*p*-value
main effects			
intercept (wt eop)	9.45×10^{-6}	1.05	<0.001
RS1	12.43	1.11	<0.001
RS2	3.56	1.11	<0.001
RS3	13.87	1.11	<0.001
RS4	12.81	1.11	<0.001
RS5	12.81	1.11	<0.001
interaction effects			
RS1 : RS2	0.84	1.16	0.234
RS1 : RS3	0.84	1.16	0.27
RS1 : RS4	0.97	1.16	0.856
RS1 : RS5	1.06	1.16	0.704
RS2 : RS3	0.90	1.16	0.455
RS2 : RS4	0.84	1.16	0.235
RS2 : RS5	0.78	1.16	0.108
RS3 : RS4	0.99	1.16	0.945
RS3 : RS5	0.91	1.16	0.541
RS4 : RS5	1.00	1.16	0.983

(c) Effects of mutations in restriction sites are multiplicative

To elucidate the dependence of the phage escape probability on the number of restriction sites, we created a third set of mutants by introducing mutations consecutively. As shown in figure 2*c*, each additional mutated restriction site considerably increased the eop. The mutant carrying all five mutations formed plaques on bacteria carrying EcoRI with the same probability as on bacteria devoid of the RM system (eop = 1). This result indicated that point mutations in restriction sites were sufficient to completely abolish cleavage. We compared the measured eop values with the expected values calculated based on individual mutation effects estimated in table 1, assuming complete independence of mutation effects. The measured and the expected eop values were in good agreement, further supporting the independence of mutation effects in phage restriction sites.

4. Discussion

Our results demonstrate that point mutations in phage restriction sites can substantially increase the probability of phages escaping restriction, thus providing direct experimental support for restriction site avoidance as an adaptive response to selection imposed by RM systems. Phages with complete or near-complete avoidance of restriction sites are commonly found in nature [17]. Notably, the mutations studied here were synonymous and thus minimized any negative influence on phage fitness. In a more general context, evolution of restriction site avoidance could be constrained by the availability of non-deleterious mutations, making particular restriction sites difficult to mutate.

The independence of mutation effects in restriction sites presented here is likely characteristic for RM systems such as EcoRI, which recognize and act on individual restriction sites. More complex dynamics of avoidance could occur in the context of RM systems recognizing multiple sites. For example, EcoRII, a type IIE RM system, cleaves the DNA only if it recognizes two proximate recognition sequences [18], whereas EcoP1I, a type III RM system, cleaves the DNA upon recognition of two opposing asymmetric recognition sequences [19]. Accordingly, in the genome of phage T7, recognition sequences of EcoRII are distantly apart, whereas those of EcoP1 are all facing the same direction [20].

In addition to evolution of restriction site avoidance, our results offer an insight into the molecular events underlying phage escape as a probabilistic process. The multiplicative nature of mutation effects suggests that each of the infecting phage restriction sites can be either restricted or methylated with a specific probability and that the fate of each site is determined independently of all other sites. Such stochasticity, also called 'molecular noise', is a common phenomenon at the level of single bacteria, where biochemical reaction events often involve a small number of interacting molecules [21]. Our results indicate that phage escape is an inherently stochastic process, and thus further highlight the importance of molecular noise in the biology of RM systems [12].

Data accessibility. The datasets supporting this article have been uploaded as part of the electronic supplementary material.

Author contributions. C.C.G. and M.P. conceived the research; M.P. performed the experiments and analysed the data; C.C.G. and M.P. wrote the paper. Both authors gave final approval for publication and agree to be held accountable for the work performed.

Competing interests. The authors have no competing interests.

Funding. This work was funded by an HFSP Young Investigators' grant RGY0079/2011 (C.C.G.). M.P. is a recipient of a DOC Fellowship of the Austrian Academy of Science at the Institute of Science and Technology Austria.

Acknowledgements. We thank D. Refardt for the wild-type λ *cI857* phage, M. Lang, F. Jesse and members of the C.C.G. laboratory for in-depth discussions.

References

1. Koskella B, Brockhurst MA. 2014 Bacteria–phage coevolution as a driver of ecological and evolutionary processes in microbial communities. *FEMS Microbiol. Rev.* **38**, 916–931. (doi:10.1111/1574-6976.12072)
2. Canchaya C, Fournous G, Brüssow H. 2004 The impact of prophages on bacterial chromosomes. *Mol. Microbiol.* **53**, 9–18. (doi:10.1111/j.1365-2958.2004.04113.x)
3. van Houte S, Buckling A, Westra ER. 2016 Evolutionary ecology of prokaryotic immune mechanisms. *Microbiol. Mol. Biol. Rev.* **80**, 745–763. (doi:10.1128/MMBR.00011-16)
4. Luria SE, Human ML. 1952 A nonhereditary, host-induced variation of bacterial viruses. *J. Bacteriol.* **64**, 557–569.
5. Oliveira PH, Touchon M, Rocha EPC. 2014 The interplay of restriction-modification systems with mobile genetic elements and their prokaryotic hosts. *Nucleic Acids Res.* **42**, 10 618–10 631. (doi:10.1093/nar/gku734)
6. Bickle TA, Krüger DH. 1993 Biology of DNA restriction. *Microbiol. Rev.* **57**, 434–450.
7. Krüger DH, Bickle TA. 1983 Bacteriophage survival: multiple mechanisms for avoiding the deoxyribonucleic acid restriction systems of their hosts. *Microbiol. Rev.* **47**, 345–360.
8. Karlin S, Burge C, Campbell AM. 1992 Statistical analyses of counts and distributions of restriction sites in DNA sequences. *Nucleic Acids Res.* **20**, 1363–1370. (doi:10.1093/nar/20.6.1363)
9. Rocha EPC, Danchin A, Viari A. 2001 Evolutionary role of restriction/modification systems as revealed by comparative genome analysis. *Genome Res.* **11**, 946–958. (doi:10.1101/gr.GR-1531RR)
10. Sharp PM. 1986 Molecular evolution of bacteriophages: evidence of selection against the recognition sites of host restriction enzymes. *Mol. Biol. Evol.* **3**, 75–83.
11. Oppenheim AB, Salomon D. 1972 Studies on partially virulent mutants of lambda bacteriophage. II. The mechanism of overcoming repression. *Mol. Gen. Genet.* **115**, 101–114. (doi:10.1007/BF00277289)
12. Pleška M, Qian L, Okura R, Bergmiller T, Wakamoto Y, Kussell E, Guet CC. 2016 Bacterial autoimmunity due to a restriction-modification system. *Curr. Biol.* **26**, 404–409. (doi:10.1016/j.cub.2015.12.041)
13. Oppenheim AB, Rattray AJ, Bubunenko M, Thomason LC, Court DL. 2004 In vivo recombineering of bacteriophage λ by PCR fragments and single-strand oligonucleotides. *Virology* **319**, 185–189. (doi:10.1016/j.virol.2003.11.007)
14. Berkner KL, Folk WR. 1983 An assay for the rates of cleavage of specific sites in DNA by restriction endonucleases: its use to study the cleavage of phage λ DNA by EcoRI and phage P22 DNA containing thymine or 5-bromouracil by HindIII. *Anal. Biochem.* **129**, 446–456. (doi:10.1016/0003-2697(83)90575-4)
15. Murray NE, Murray K. 1974 Manipulation of restriction targets in phage λ to form receptor chromosomes for DNA fragments. *Nature* **251**, 476–481. (doi:10.1038/251476a0)
16. Rau DC, Sidorova NY. 2010 Diffusion of the restriction nuclease EcoRI along DNA. *J. Mol. Biol.* **395**, 408–416. (doi:10.1016/j.jmb.2009.10.049)
17. Korona R, Korona B, Levin BR. 1993 Sensitivity of naurally occurring coliphages to type I and type II restriction and modification. *J. Gen. Microbiol.* **139**, 1283–1290. (doi:10.1099/00221287-139-6-1283)
18. Krüger DH, Kupper D, Meisel A, Reuter M, Schroeder C. 1995 The significance of distance and orientation of restriction endonuclease recognition sites in viral DNA genomes. *FEMS Microbiol. Rev.* **17**, 177–184. (doi:10.1111/j.1574-6976.1995.tb00200.x)
19. Meisel A, Bickle TA, Kriiger DH, Schroeder C. 1992 Type III restriction enzymes need two inversely oriented recognition sites for DNA cleavage. *Nature* **355**, 467–469. (doi:10.1038/355467a0)
20. Tock MR, Dryden DTF. 2005 The biology of restriction and anti-restriction. *Curr. Opin. Microbiol.* **8**, 466–472. (doi:10.1016/j.mib.2005.06.003)
21. McAdams HH, Arkin A. 1999 It's a noisy business! Genetic regulation at the nanomolar scale. *Trends Genet.* **15**, 65–69. (doi:10.1016/S0168-9525(98)01659-X)