16. Akam, M. (1989). Drosophila development: making stripes inelegantly. Nature *341*, 282–283.

17. Kanayama, M., Akiyama-Oda, Y., Nishimura, O., Tarui, H., Agata, K., and Oda, H. (2011). Travelling and splitting of a wave of hedgehog expression involved in spider-head segmentation. Nat. Commun. *2*, 500.

18. Ingham, P.W., and Martinez Arias, A. (1992). Boundaries and fields in early embryos. Cell *68*, 221–235.

19. Benton, M.A., Akam, M., and Pavlopoulos, A. (2013). Cell and tissue dynamics during Tribolium embryogenesis revealed by versatile fluorescence labeling approaches. Development *140*, 3210–3220.

20. Seaver, E.C., Yamaguchi, E., Richards, G.S., and Meyer, N.P. (2012). Expression of the pair-rule gene homologs runt , Pax3/7, even-skipped-1 and even-skipped-2 during larval and juvenile development of the polychaete annelid Capitella teleta does not support a role in segmentation. EvoDevo *3*, 8.

CRISPR–Cas: Spacer Diversity Determines the Efficiency of Defense

Anna Lopatina and Rotem Sorek*
Department of Molecular Genetics, Weizmann Institute of Science, Rehovot 76100, Israel
*Correspondence: rotem.sorek@weizmann.ac.il
http://dx.doi.org/10.1016/j.cub.2016.05.034

Bacterial CRISPR–Cas systems acquire short sequences, called spacers, from viruses and plasmids, leading to adaptive immunity. The diversity of spacers within natural bacterial populations is very high. New data now explain how spacer diversity strengthens resistance of the bacterial population to phage infection.

Viruses that infect bacteria (phages) shape bacterial population abundance and community structure in numerous habitats worldwide. The continuous evolutionary arms race between bacteria and phages resulted in the appearance of a broad range of defense mechanisms in bacteria and a high, mostly unexplored diversity of anti-defense strategies in phages. Bacteria have evolved two kinds of defense systems. Innate-immunity systems include restriction-modification systems [1], argonaute-based RNAi-like mechanisms [2], abortive infection [3], and other systems that serve to restrict incoming foreign DNAs [4,5]. Many bacteria also utilize CRISPR–Cas systems, which represent the adaptive defense system of prokaryotes. As part of CRISPR–Cas defense, bacteria acquire short sequences (spacers) from the genomes of phages or plasmids, and insert these spacers into the CRISPR array locus to build the immune memory. Transcription products of newly acquired spacers are then used as molecular guides by Cas proteins to degrade complementary foreign nucleic acids.

The natural diversity of spacers within bacterial populations is known to be very high. Thousands of different spacers can be found in natural bacterial communities, from the very simple communities in Antarctic snow samples [6] to more complex ones like the human gut microbiota [7]. This diversity leads to a population in which otherwise clonal bacteria contain different spacers against phage invaders [8]. The benefit of spacer diversity is now explained in a recent study by van Houte *et al.* [9], who, using experimental infection studies, have shown that phages cannot overcome CRISPR–Cas defense by point mutations in their genomes when spacer diversity in the population is sufficiently high.

van Houte *et al.* [9] established a co-evolutionary experimental system using *Pseudomonas aeruginosa* and its phage, DMS3vir, and monitored viral titers at certain time points after infection. Firstly, they showed that phages that infected bacteria lacking CRISPR–Cas persisted in the experimental system during 30 days of co-incubation, whereas phages that infected bacteria containing a type I–F CRISPR–Cas system went extinct at five days post-infection. They found that bacteria with CRISPR–Cas developed immunity through the acquisition of new spacers, whereas CRISPR-less bacteria developed immunity through mutations that led to loss or masking of the surface receptor recognized by the phage.

The authors were surprised by the rapid extinction of phages upon infection of CRISPR-containing bacteria because phages can in principle gain point mutations to rapidly escape CRISPR–Cas immunity. They suspected that spacer diversity within the population may be responsible for this effect, and therefore designed an experiment in which they generated bacterial populations with varying levels of spacer diversity: monocultures, in which all bacteria contain a single spacer against DMS3vir, and polycultures consisting of equal mixtures of clones each carrying one distinct, but different, spacer against the phage. The authors then experimented with populations containing either 1, 6, 12, 24 or 48 clones, with each clone carrying a different individual spacer against DMS3vir. All bacterial populations were infected by DMS3vir and viral titers were monitored over time. The results were quite striking: whereas populations with low initial spacer diversity led to phage persistence and escape from CRISPR immunity, phage became extinct in those populations with high spacer diversity (Figure 1).

van Houte *et al.* [9] further showed that after one day of co-incubation, phages infecting the monocultures developed

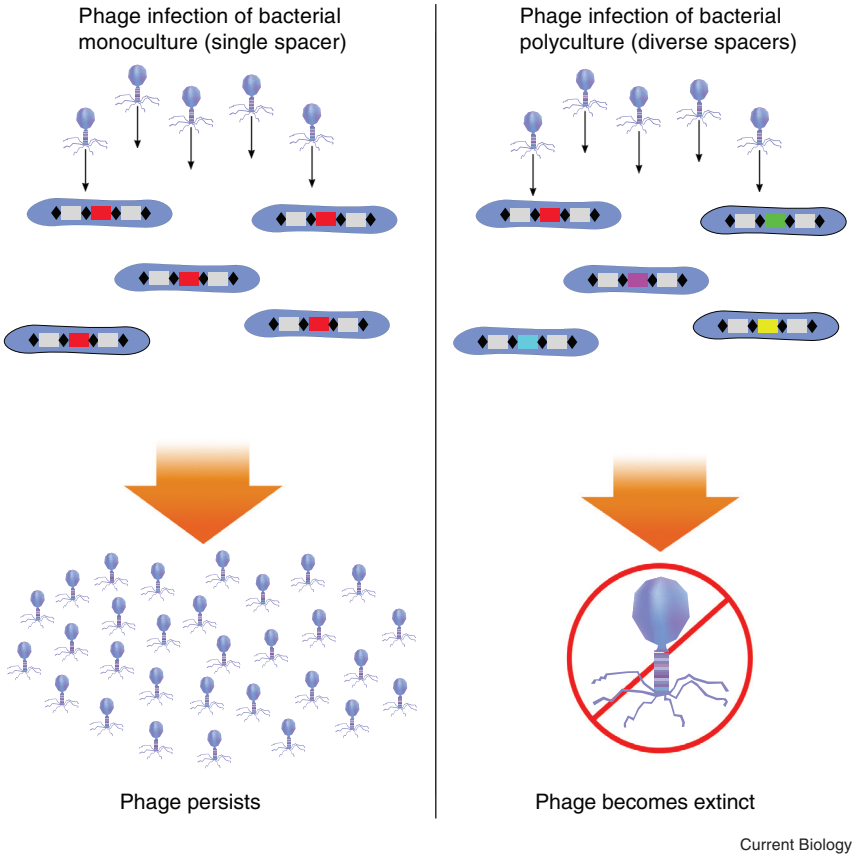

Figure 1. High spacer diversity causes viral extinction.
New work by van Houte et al. [9] reveals that phages can persist when infecting bacterial populations with low initial spacer diversity, whereas phages infecting bacterial populations with high spacer diversity go extinct.

mutants that escaped CRISPR–Cas defense, while in the polycultures no such escape mutants were observed. Thus, the population benefits from spacer diversity within its individuals because this diversity leads to the inability of the phage to evolve escape mutants. Finally, van Houte *et al.* [9] have shown that the same principle applies for other CRISPR–Cas systems, i.e. the type II-A CRISPR–Cas system of *Streptococcus thermophilus*.

The results of van Houte *et al.* [9] nicely conform to current theory on disease spread amongst infected hosts. Previous theoretical analyses suggested that host-genetic diversity synergistically reduces the spread of parasites (in our case, phages) when a specific parasite strain can infect only a restricted number of host strains and when a failed infection results in lack of parasite propagation [10]. In the case of the new study, when a bacterial population contains multiple spacers, a phage mutant that escapes one spacer will have lower chances of finding the host in which it can propagate. Since the initial number of escape mutants in the phage population is low, this reduced probability is detrimental to the overall success of phage persistence.

The observations of van Houte *et al.* [9] represent an important milestone in our understanding of the role of CRISPR–Cas in the co-evolutionary arms race between bacteria and phage. It would be interesting to examine, in the future, how additional important factors are woven into the matrix of phage–bacteria interactions. For example, in co-evolution experiments between *Streptococcus thermophilus* and its phages, it was shown that phage extinction occurred when a single phage was used, but the addition of a homologous phage into the experimental system extended phage persistence [11]. Apparently, co-infection by multiple phages enabled recombination-based formation of chimeric phage genomes in which sequences heavily targeted by CRISPR were replaced. Therefore, continuous immigration of new phages into natural bacterial populations may protect phages against extinction.

Differences in the specificity of target recognition by different types of CRISPR–Cas systems could also result in alternative co-evolutionary dynamics. Several mismatches between the CRISPR RNA (crRNA) and the protospacer sequence are tolerated during recognition by type I and II CRISPR systems; but, for type III CRISPR–Cas systems, many more mutations (up to 40% of the length of crRNA) in the protospacer can be tolerated without affecting CRISPR–Cas-mediated defense [12]. The relaxed specificity of type III CRISPR–Cas systems, as well as the absence of protospacer-adjacent motif requirements in these systems, could lower the probability of escape mutations and could lead to extinction of phages, even in the absence of spacer diversity in the population. Finally, an additional strategy of CRISPR–Cas systems in battling phage-escape mutations is primed adaptation (or priming) [13]. Priming occurs when escape mutations in invader DNA cause non-optimal binding of the Cascade (the effector crRNA–Cas proteins complex) to the target DNA, which stimulates rapid and efficient acquisition of additional spacers from the same foreign DNA. It would be interesting to compare the relative contributions of primed adaptation and of the population-spacer diversity strategy in mitigating the development of escape mutants in the phage population.

The co-evolutionary interactions between bacteria and phage are among the most complex long-term processes in nature, and hence are highly challenging to model. Simple model systems can provide important insights into the principles of these interactions, as nicely exemplified by the van Houte *et al.* [9] study. Many additional questions concerning phage–bacteria co-evolution — for example, the roles of lysogeny, phage anti-defense mechanisms, and phage–phage interactions — may benefit in the future from studies in similar model systems.

REFERENCES

1. Tock, M.R., and Dryden, D.T. (2005). The biology of restriction and anti-restriction. Curr. Opin. Microbiol. *8*, 466–472.
2. Swarts, D.C., Jore, M.M., Westra, E.R., Zhu, Y., Janssen, J.H., Snijders, A.P., Wang, Y., Patel, D.J., Berenguer, J., Brouns, S.J., *et al*. (2014). DNA-guided DNA interference by a prokaryotic Argonaute. Nature *507*, 258–261.
3. Chopin, M.C., Chopin, A., and Bidnenko, E. (2005). Phage abortive infection in lactococci: variations on a theme. Curr. Opin. Microbiol. *8*, 473–479.
4. Goldfarb, T., Sberro, H., Weinstock, E., Cohen, O., Doron, S., Charpak-Amikam, Y., Afik, S., Ofir, G., and Sorek, R. (2015). BREX is a novel phage resistance system widespread in microbial genomes. EMBO J. *34*, 169–183.
5. Wang, L., Chen, S., Vergin, K.L., Giovannoni, S.J., Chan, S.W., DeMott, M.S., Taghizadeh, K., Cordero, O.X., Cutler, M., Timberlake, S., *et al*. (2011). DNA phosphorothioation is widespread and quantized in bacterial genomes. Proc. Natl. Acad. Sci. USA *108*, 2963–2968.
6. Lopatina, A., Medvedeva, S., Shmakov, S., Logacheva, M.D., Krylenkov, V., and Severinov, K. (2016). Metagenomic analysis of bacterial communities of Antarctic surface snow. Front. Microbiol. *7*, 00398.
7. Stern, A., Mick, E., Tirosh, I., Sagy, P., and Sorek, R. (2012). CRISPR targeting reveals a reservoir of common phages associated with the human gut microbiome. Genome Res. *22*, 1985–1994.
8. Tyson, G.W., and Banfield, J.F. (2008). Rapidly evolving CRISPRs implicated in acquired resistance of microorganisms to viruses. Environ. Microbiol. *10*, 200–207.
9. van Houte, S., Ekroth, A.K., Broniewski, J.M., Chabas, H., Ashby, B., Bondy-Denomy, J., Gandon, S., Boots, M., Paterson, S., Buckling, A., *et al*. (2016). The diversity-generating benefits of a prokaryotic adaptive immune system. Nature *532*, 385–388.
10. King, K.C., and Lively, C.M. (2012). Does genetic diversity limit disease spread in natural populations. Heredity *109*, 199–203.
11. Paez-Espino, D., Sharon, I., Morovic, W., Stahl, B., Thomas, B.C., Barrangou, R., and Banfield, J.F. (2015). CRISPR immunity drives rapid phage genome evolution in *Streptococcus thermophilus*. mBio *6*, e00262–15.
12. Manica, A., Zebec, Z., Steinkellner, J., and Schleper, C. (2013). Unexpectedly broad target recognition of the CRISPR-mediated virus defence system in the archaeon *Sulfolobus solfataricus*. Nucl. Acids Res. *41*, 10509–10517.
13. Amitai, G., and Sorek, R. (2016). CRISPR-Cas adaptation: insights into the mechanism of action. Nat. Rev. Microbiol. *14*, 67–76.

Animal Evolution: The Hard Problem of Cartilage Origins

Thibaut Brunet* **and Detlev Arendt***
Developmental Biology Unit, European Molecular Biology Laboratory, Meyerhofstraße 1, 69017 Heidelberg, Germany
*Correspondence: brunet@embl.de (T.B.), arendt@embl.de (D.A.)
http://dx.doi.org/10.1016/j.cub.2016.05.062

Our skeletons evolved from cartilaginous tissue, but it remains a mystery how cartilage itself first arose in evolution. Characterization of cartilage in cuttlefish and horseshoe crabs reveals surprising commonalities with chordate chondrocytes, suggesting a common evolutionary origin.

Skeletons are misunderstood. Because of their resistance to decay, bones have become symbols of death; yet, they are intensely alive tissues, undergoing lifelong active remodeling. To the evolutionary biologist, the hard parts of animals are similarly double-faced: their endurance makes them the prime candidates for fossilization and provides paleontologists with a wealth of information on the skeleton of extinct animals. From the paleontologist's view, animal evolution is thus mainly the evolution of hard parts (plus what can be deduced from them). But for the same reason, the origin of the first animal skeletons, and the ancestral structures that gave rise to them in soft-bodied animals, remains mysterious; preservation of soft tissue is too rare to provide a clear-cut solution. For more than a century, morphologists have been debating, with precious little evidence, the hard questions of skeleton origins: When did animal skeletons first evolve? Did they appear once or several times independently? Which ancestral soft tissues first became rigid, and by what molecular mechanisms? A recent study by Tarazona and co-authors [1], comparing skeleton formation between invertebrates and vertebrates at the molecular level, sheds new light on these questions.

Historical attempts to compare vertebrate and invertebrate skeletons have not fared well. In the early 20th century, the zoologist William Patten proposed that the most ancient vertebrates known at the time — a group of armored jawless fishes called ostracoderms — had evolved from extinct marine chelicerates, the eurypterids, which were encased in an equally impressive exoskeleton (Figure 1A). To support his view, Patten pointed out that the modern horseshoe crabs and arachnids, the closest living relatives of eurypterids, showed evidence of an internal skeleton [2]. The central nervous system of horseshoe crabs is indeed supported by a cartilaginous piece, the endosternite, and other cartilage pieces underlay the animal's gills. To Patten, these structures were clear precursors to the vertebrate skull and branchial arches. Patten's views never gained broader acceptance and