MICROBIOLOGY

How bacteria get spacers from invaders

Bacteria use CRISPR–Cas systems to develop immunity to viruses. Details of how these systems select viral DNA fragments and integrate them into bacterial DNA to create a memory of invaders have now been reported. SEE ARTICLES P.193 & P.199

IDO YOSEF & UDI QIMRON

Less than a decade ago, immunological memory was regarded as a feature unique to vertebrates — scientists ridiculed the idea that bacteria might be able to 'remember' viruses that attack them. Yet the almost inconceivable concept of bacterial immunological memory has since been shown to exist after all[1–3]. Two papers in this issue, by Heler *et al.*[4] (page 199) and Nuñez *et al.*[5] (page 193), report major advances in our understanding of the molecular mechanism of this phenomenon.

Bacteria remember their viral invaders by sampling short DNA sequences known as protospacers from the viruses' genetic material. These sequences become integrated into the bacterium's own DNA, specifically into an array of repeat sequences called clustered regularly interspaced short palindromic repeats (CRISPRs; Fig. 1); the integrated sequences are called spacers. When a bacterium is subsequently attacked by a recognized virus, the spacers are transcribed from the array and used to guide a complex containing CRISPR-associated (Cas) proteins, which cleave protospacers in viral nucleic-acid molecules[1].

Accidental destruction of the CRISPR array could occur if transcribed spacers guide Cas proteins to cleave it, leading to catastrophic degradation of bacterial genetic material. To prevent this potential autoimmunity, some bacteria have CRISPR–Cas systems that cleave DNA targets only if they are flanked by sequences known as protospacer adjacent motifs (PAMs)[6]. The repeat sequences that flank spacers in such CRISPR arrays lack PAMs and therefore cannot be cleaved (Fig. 1).

The mechanism by which spacers are chosen so that they target only PAM-associated protospacers has remained elusive. Heler and colleagues show that the Cas9 protein in two species of *Streptococcus* bacterium selects for spacers that have the correct PAM. Before now, the protein's main known role was cleaving targeted DNA.

When the authors exchanged Cas9 proteins for others that had different PAM specificities, they found that the PAM sequence of the acquired spacers changed accordingly. These CRISPR–Cas systems therefore efficiently use Cas9's ability to recognize PAM sequences for memory as well as for cleavage, instead of having dedicated memorizing proteins develop PAM recognition from scratch. In other types of CRISPR–Cas system, such as that found in *Escherichia coli*, the memorizing proteins have an intrinsic ability to select, at least partially, for PAM-encoding protospacers through an as-yet-unknown mechanism[3].

Heler and co-workers went on to show that Cas9 is required not only for determining the PAM sequence of the acquired spacers, but also for integrating spacers into CRISPRs. This feature is peculiar to Cas9 — the proteins that cleave nucleic acids in other CRISPR–Cas systems studied are not required for integration[3], but may enhance it under certain conditions[7].

These findings add crucial details to the mechanism of molecular memorization revealed by *in vivo* studies[3,7–9]. Each spacer is integrated into the CRISPR array with a new repeat; it is known that newly integrated repeats maintain the sequence of the existing repeat on the other side of the new spacer[3], and that the integration of new spacers probably occurs by separation of the two DNA strands of this repeat[9]. But more information is needed, and an *in vitro* system that allows further mechanistic details to be uncovered has long been awaited.

Nuñez and colleagues have established just such a system: it is composed of *E. coli* memorizing proteins, a supercoiled plasmid DNA as the spacer-acceptor molecule and a double-stranded (ds) DNA that serves as a spacer-donor molecule. The researchers first demonstrated the validity of their system by using it to corroborate many of the *in vivo* characteristics of the CRISPR memorization process. They went on to analyse high-throughput sequencing of spacers inserted *in vitro*, and show that the memorizing proteins integrate spacers in the correct orientation by recognizing a specific nucleotide base in the PAM.

Importantly, their system allows the spacer donor to be easily replaced with DNA that has different sequences, end modifications and strand compositions, and thus enables the influence of these features on spacer integration to be studied. In this way, Nuñez *et al.* show that hydroxyl (OH) groups at the 3′ ends of dsDNA substrates are essential for integration. On the basis of this requirement, and of characterization of intermediates identified *in vivo*[9], the authors propose a highly plausible model for spacer insertion. In this model, the memorizing enzymes catalyse bond formation between the 3′ end of a preferred strand of the spacer and a particular strand at the end of a repeat. This is followed by the formation of another bond between the 3′ end of

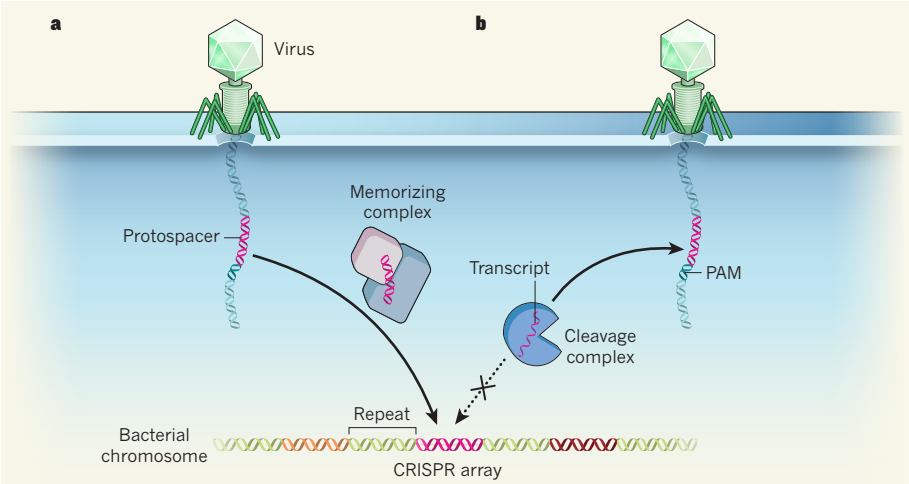


Figure 1 | The bacterial immune response. **a**, When bacteria are invaded by viral DNA, memorizing protein complexes of the CRISPR–Cas immune system select short sequences (protospacers) of the foreign DNA and integrate them as spacer sequences into their own chromosome. The spacers are integrated into an array of repeat sequences called clustered regularly interspaced short palindromic repeats (CRISPRs); different spacers are shown in orange, pink and red. **b**, If the bacterium is subsequently exposed to previously encountered DNA, a transcript of the spacer guides a cleavage protein complex to cut out the protospacer. In some types of CRISPR–Cas system, cleavage occurs only if the protospacer is flanked by a protospacer adjacent motif (PAM), and the CRISPR array is not cleaved because the spacers lack PAMs. Heler *et al.*[4] and Nuñez *et al.*[5] report details of the molecular mechanisms by which CRISPR–Cas systems select and integrate spacers into bacterial DNA.

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the complementary strand of the spacer and the complementary strand at the other end of the repeat (see Fig. 5 of the paper).

The strength of *in vitro* approaches to studying biological systems is that all the components are artificially added to the reaction; the requirements and features of each component can therefore be defined and manipulated. But differences from physiological activity may occur, stemming either from the use of a different chemical environment from that found *in vivo* or from the absence of regulatory elements. Such elements may not be essential for the generation of the end product of a reaction, but might have a key role in the physiological process.

Nuñez and co-workers report just such a difference. *In vivo* studies have revealed that spacer integration occurs predominantly at the first repeat of the CRISPR array[3,7]. By contrast, the authors observe that spacer insertion in their *in vitro* system is also distributed near other repeats, and even outside the CRISPR array. The researchers suggest that this might represent a physiological way of generating new arrays. This is a valid possibility, but regulatory elements *in vivo* or in physiological conditions probably often restrict this distribution and direct integration in a specific location.

The authors also report that PAM-encoding spacer donors are not preferred substrates for integration *in vitro*, as opposed to what has been seen *in vivo*[3]. Moreover, they observe that the length of integrated spacers may vary substantially, whereas spacers in naturally occurring arrays have a strictly defined length. These differences might be explained by the fact that the *in vitro* system simulates only the last stage of spacer integration; earlier steps in the natural process probably account for the PAM preference and for defined spacer lengths observed *in vivo*.

The differences in the *in vivo* and *in vitro* studies nevertheless highlight the cardinal question of what determines the constant length of newly acquired spacers *in vivo*. Is it dictated by a protein complex that hands the processed spacer to the memorizing enzymes? If so, then what are these proteins? An *in vitro* system composed of all of the elements that catalyse every step of the reaction is needed to address these issues.

PAMs prevent autoimmunity against the CRISPR array, but autoimmunity could also occur if the CRISPR–Cas system accidentally cleaves other DNA sequences. So how is this prevented? It is known[3,10] that foreign DNA molecules are sampled by CRISPR–Cas systems more frequently than the host's chromosome. Future work should investigate the mechanism underlying this selective sampling. ■

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1. Barrangou, R. *et al. Science* **315,** 1709–1712 (2007).
2. Brouns, S. J. J. *et al. Science* **321,** 960–964 (2008).
3. Yosef, I., Goren, M. G. & Qimron, U. *Nucleic Acids Res.* **40,** 5569–5576 (2012).
4. Heler, R. *et al. Nature* **519,** 199–202 (2015).
5. Nuñez, J. K., Lee, A. S. Y., Engelman, A. & Doudna, J. A. *Nature* **519,** 193–198 (2015).
6. Mojica, F. J. M., Díez-Villaseñor, C., García-Martínez, J. & Almendros, C. *Microbiology* **155,** 733–740 (2009).
7. Datsenko, K. A. *et al. Nature Commun.* **3,** 945; http://dx.doi.org/10.1038/ncomms1937 (2012).
8. Swarts, D. C., Mosterd, C., van Passel, M. W. & Brouns, S. J. J. *PLoS ONE* **7,** e35888 (2012).
9. Arslan, Z., Hermanns, V., Wurm, R., Wagner, R. & Pul, U. *Nucleic Acids Res.* **42,** 7884–7893 (2014).
10. Nuñez, J. K. *et al. Nature Struct. Mol. Biol.* **21,** 528–534 (2014).

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CLIMATE CHANGE

Black carbon and atmospheric feedbacks

Climate simulations show that interactions between particles of black carbon and convective and cloud processes in the atmosphere must be considered when assessing the full climatic effects of these light-absorbing particulates.

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Black carbon[1], often referred to as soot, is emitted during the incomplete combustion of fossil fuels, biofuels or wood. In contrast to other particulates emitted into the atmosphere by human activities, black carbon absorbs sunlight efficiently. This absorption leads to local heating of the atmosphere, warming the planet. Black carbon has received particular interest recently[2] in the context of changes in climate policy. It remains in the atmosphere for only a few days, so cutting black-carbon emissions may be a viable way to reduce global warming over the next few decades, alongside measures to mitigate

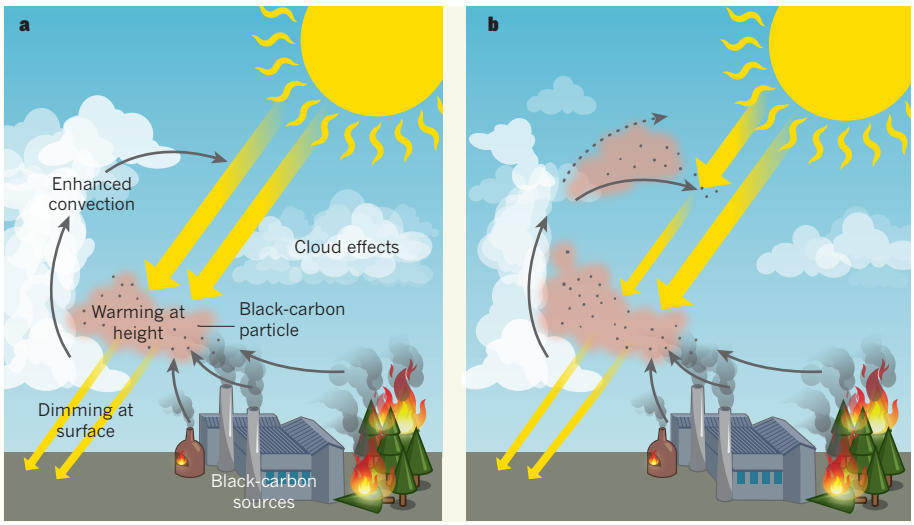


Figure 1 | A typical black-carbon feedback loop. **a**, Black-carbon emissions from sources such as industrial processes, brick kilns and forest fires have numerous influences on the atmosphere. The details of these influences can be strongly dependent on time and location, but the emissions generally lead to a net surface dimming (thin yellow arrows) alongside enhanced warming at height (pink shading). The latter factor is often associated with enhanced vertical convection and effects on clouds that have a net result, according to Sand and colleagues' climate simulations[3], of reducing cloud at altitude (not shown). **b**, Sand *et al.* suggest that roughly half of the total climate impact of black-carbon emissions is apparent only if these atmospheric responses feed back to the distribution of black carbon, lofting it to height and increasing its atmospheric lifetime and spatial extent. These changes enhance any surface dimming (extension of thin yellow arrow), and lead to greater warming at height (upper pink shading), black-carbon transport into the upper atmosphere (dotted black arrow) and further changes to clouds. Although, locally, greater warming can generate some deepened convective clouds, the net impact is a further reduction of cloud at altitude.

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