Title

Evolutionary ecology of prokaryotic innate and adaptive immune systems and their interplay

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Abstract

Like many organisms, bacteria and archaea have both innate and adaptive immune systems to defend against infection by viruses and other parasites. Innate immunity most commonly relies on endonuclease cleavage of any incoming DNA that lacks a specific epigenetic modification, through a system known as Restriction-Modification. CRISPR-Cas adaptive immunity relies on the insertion of short DNA sequences from parasite genomes into CRISPR loci on the host genome to provide sequence-specific protection. The discovery of each of these systems has revolutionized our ability to carry out genetic manipulations, and, as a consequence, the enzymes involved have been characterized in exquisite detail. In comparison, much less is known about the importance of these two arms of the defence for the ecology and evolution of prokaryotes and their parasites. Here we review our current ecological and evolutionary understanding of these systems in isolation, and discuss the need to study how innate and adaptive immune responses are integrated when they coexist in the same cell.

Main Text

Introduction

Prokaryotes face infection by a wide range of genetic elements, from lytic viruses to plasmids and integrative elements that can confer fitness benefits. Prokaryotes thus experience selective pressures to defend themselves against parasitic threats, while, if possible, retaining the ability to associate with benign symbionts. Although prokaryotes have a large repertoire of defence systems [1], by far the most widespread defences are Restriction-Modification (RM) and CRISPR-Cas immune systems. RM systems are present in over 90% of sequenced bacterial and archaeal genomes [2], while CRISPR-Cas systems are found in approximately 30-40% of bacterial and >90% of archaeal genomes [3].

The mechanisms of RM and CRISPR immunity have been extensively studied [1], in part due to their value as tools for genetic manipulation. However, the importance of these systems for the ecology and evolution of prokaryotes and their parasites is less well understood. We briefly summarize the mechanistic basis of RM and CRISPR immunity, which has been covered in detail in several recent reviews [4–9]. We discuss the factors governing the distribution of these innate and adaptive immune systems, and their consequences for prokaryotic ecology and evolution. We highlight that despite their frequent co-occurrence [10], most studies have been carried out on individual systems in isolation, and emphasise the need to examine how these systems interact when they coexist in the same cell.

Overview of immune mechanisms in prokaryotes

RM systems: innate immunity based on detecting DNA modification states

RM systems are innate immune systems that recognise structural features on specific sequences of DNA bases, and target DNA identified as “foreign”. RM systems are divided into Types (I, II, III and IV), largely based on mechanistic properties rather than evolutionary relatedness [2]. Within Types I and III, the relatedness is greater and mechanistic properties more similar compared to Types II and IV. The common feature of Types I, II and III RM systems is specific DNA sequence recognition by an endonuclease activity which triggers a dsDNA break on foreign DNA while self-DNA is protected from cleavage by covalent methylation of the same

(cognate) sequences on the host genome producing N6-methyladenine, 5-methylcytosine or N4-methylcytosine (Figure 1A). Type IV enzymes lack methyltransferase activity since they target modified nucleotides on foreign DNA (Figure 1A). Distinguishing self from non-self is thus based on DNA modification (Types I to III) or its absence (Type IV).

Type I enzymes form multiprotein complexes that undertake both DNA methylation and cleavage. The HsdS subunit provides the recognition site specificity. Target sites are bipartite, comprising semi-specific sequences 3-6 bp separated by a 4-9 bp non-specific spacer. DNA cleavage occurs at distant non-specific sites and requires interaction of two enzyme complexes and ATP hydrolysis [5] (Figure 2). Type ISP enzymes comprise a single polypeptide with recognition, methyltransferase, translocase and endonuclease activities [5].

Type II enzymes are the largest and most diverse group [6]. Classical Type IIP enzymes comprise separate endonuclease and methyltransferase proteins. DNA cleavage occurs within or close to the recognition sites which are 4-8 bp. Several subclasses have the methyltransferase and endonuclease proteins fused as a single polypeptide. Types IIE, IIF and IIS bind two sites and capture a DNA loop to activate cleavage (Figure 2).

Type III enzymes form multiprotein complexes that undertake both DNA methylation and cleavage [2]. Target sites are asymmetric and 5-6 bp, and cleavage occurs ~2.5 DNA turns downstream of one site but requires a pair of recognition sites in inverted repeat and ATP to initiate the reaction [11] (Figure 1B).

Some phages have evolved metabolic pathways to modify bases or have acquired methylation due to avoiding restriction in a cell with a Type I – III RM system. To counter phages carrying these modifications, bacteria evolved Type IV enzymes [4]. These have an endonuclease activity that targets DNA but lack a cognate methyltransferase (Figure 1A). A diverse range of mechanisms have evolved, some of which appear to require interaction with multiple modified sites and some of which require an input of chemical energy (ATP or GTP). Type IV enzymes are the least well-understood at both mechanistic and ecological levels.

CRISPR-Cas, an adaptive immune system

Acquisition of CRISPR-Cas adaptive immunity requires exposure to an MGE. At initial exposure, a "memory" of infection can be recorded on the host DNA by recombining short sequences from the foreign genome. These sequences are then used to detect and destroy the MGE in subsequent infections.

CRISPR arrays are the immunological memory of the CRISPR-Cas immune system, and consist of repeating sequences (repeats, typically 20-40bp) interspersed with variable sequences (spacers, typically 20-40bp) that are complementary to MGE sequences (Figure 3). *Cas* genes encode proteins responsible for immunity which occurs in three stages: spacer acquisition (often referred to as "adaptation"), expression, and interference [7]. During acquisition, a protein complex including the conserved Cas1 and Cas2 enzymes inserts MGE sequences (protospacers) into the leader end of CRISPR arrays and duplicates the repeats, forming new spacers [12] (Figure 3A). During the expression stage, CRISPR arrays are transcribed and processed into CRISPR RNAs (crRNAs) and loaded onto Cas proteins. Finally, during interference, crRNAs guide Cas effectors towards complementary MGE nucleic acids, triggering cleavage (Figure 3B). Primed adaptation, in which effector recognition triggers further spacer acquisition [13], has been demonstrated in a subset of Type I systems and recently a similar mechanism has been proposed for Type II systems [14] (Figure 3A). To ensure that the system targets only infectious DNA, and not the CRISPR array on the host genome, CRISPR-Cas effector complexes use the sequence flanking the complementary target for discrimination (Figure 4): in a CRISPR array the flanking sequence consists of a CRISPR repeat sequence, whereas targets in an infectious genome are often selected such that they are flanked by the conserved protospacer adjacent motif (PAM) [15,16].

CRISPR-Cas systems have been classified into 2 main classes, 6 types and >30 subtypes, based on the phylogeny of Cas1, a conserved spacer acquisition protein, as well as signature genes and gene synteny [18]. However, some of these types and subtypes are relatively rare and concentrated in a few clades [19]. In this review we focus on the more frequent types I (30% of all genomes), II (8%) and III (6%) (Figure 4).

Types I and III belong to Class 1 and encode crRNA-Cas effectors composed of a single crRNA and multiple protein subunits (Figure 4A). Type I systems encode the Cascade ribonucleoprotein complex and a separate ATP-dependent helicase-nuclease Cas3 [20]. Cascade first scans DNA for PAMs. The DNA is then unwound to allow base-pairing between the crRNA and the complementary protospacer (R-loop). Full R-loop zipping recruits Cas3, which cleaves the non-targeted strand within or close to the protospacer. This process in turn provides substrates for spacer acquisition, resulting in more efficient "primed adaptation", even if targets contain mutations in the protospacer or PAM (Figure 3A)[12]. The majority of Type III systems form Csm or Cmr ribonucleoprotein complexes, which share structural similarities [21] (Figure 4A). These complexes use crRNA to bind complementary RNA transcripts, which

triggers Cas10-mediated DNA and Cas7-mediated RNA cleavage activities. Cas10 also produces cyclic oligoadenylates which activate a non-specific RNase activity [22].

Type II systems belong to Class 2, which encode a single effector protein, known as Cas9, which forms a complex with the crRNA and a trans-activating CRISPR RNA, tracrRNA. Cas9 effectors scan DNA for PAMs and form an R-loop with the target DNA sequence. Full complementarity activates the nuclease domains to cut both strands close to the PAM [23,24]. The RNA-guided dsDNA break activity of Cas9 proteins has been widely used for genome editing [9].

Costs and benefits of prokaryotic immune systems

Immune systems commonly carry both fitness costs and benefits which will affect system prevalence (Figure 5). Quantifying these, and understanding how they depend on their environment, can help explain the observed distribution of immune systems in nature.

Immune systems provide strong benefits in the presence of lytic phage

In the absence of parasites, costs should favour hosts without immune systems; on the other hand, hosts will experience strong selection for immunity when lethal parasites are present. Indeed, bacteria with RM [25] or CRISPR-Cas [26–28] immunity can increase rapidly in frequency in the presence of virulent phages. However, the selective benefits will depend on the level of protection they confer, which varies depending on the system, the phage, and the number and position of sequences targeted [29–34]. In addition, the rate of spacer acquisition can severely limit the benefits of CRISPR-Cas immune systems: if this is low – as is often the case in laboratory culture – the benefits of carrying an adaptive immune system are marginal [35].

Immune systems can be maladaptive in the presence of temperate phages

Temperate phages can replicate through both the lytic and lysogenic cycle. Targeting of incoming phages is beneficial, but targeting integrated prophages leads to immunopathological effects, since cleavage of the phage genome results in a break in the host chromosome (Figure

3B). Indeed, type I and II CRISPR-Cas systems cause cell death when they are programmed to target integrated prophages [36]. Consistent with these findings, carrying a CRISPR immune system was shown to be maladaptive during temperate phage infection of phage-sensitive, but primed, cells (i.e. cells carrying spacers that imperfectly match the temperate phage) [37]. Type III CRISPR-Cas systems, which rely on active transcription (Figure 4), can target phages that replicated through the lytic cycle, whilst tolerating prophages that repress their transcription [38]. However, low levels of transcription of integrated prophages can still lead to a high fitness cost of immunity [39].

RM systems can provide clear benefits in the context of temperate phage infection. During infection with phage λ, experiments with a large panel of RM systems showed that each system favoured lysogeny at the population scale, even though these systems are unable to discriminate between phages that enter the lytic or lysogenic cycle. Instead, this effect was due to RM immunity delaying successful infections until most cells are near to stationary phase, a state in which the probability of lysogeny is greater [32]. This might help explain why temperate phages avoid restriction sites to a lesser extent than lytic ones [40].

Targeting the mobile gene pool entails opportunity costs that can be mitigated

Ideally, immune systems provide protection against parasitic MGEs, but allow for the association with beneficial ones. Yet, immune systems can also limit acquisition of plasmids and prophages, which often confer environment-specific fitness benefits to their hosts [41,42]. Both RM and CRISPR-Cas systems can provide immunity against plasmid conjugation [43,44], transformation [45,46] and transduction [47,48]. While immunity can be beneficial in the presence of costly plasmids [49], both RM and CRISPR-Cas are disadvantageous when targeting beneficial plasmids [44,46,50], causing selection for inactivated RM or CRISPR-Cas systems [50]. When hosts are simultaneously exposed to parasitic and mutualistic elements there may be a trade-off between immunity and access to mutualists.

However, defence systems may discriminate between beneficial and parasitic MGEs. CRISPR-Cas immune systems can do so owing to their high sequence specificity (20-40 nucleotide protospacer targets), which is more limited for restriction enzymes (typically recognition sites of 4-8 nucleotides). Indeed, CRISPR-Cas immunity can lead to elevated levels of generalized transduction, because it protects cells from phage infection but does not cleave encapsulated host DNA of transducing particles [48]. The spacer content of CRISPR arrays can specialize

198 on parasitic sequences over time, through selection and primed adaptation. Indeed, most
199 identified spacers in sequenced genomes are from phages, and a smaller proportion from other
200 MGEs [51].

201 While RM systems lack the ability to discriminate between beneficial and parasitic MGEs
202 based on their sequence, they will favour MGE exchange among closely related strains over
203 more distantly related ones. Indeed, plasmids are more efficiently transferred among kin than
204 non-kin, a pattern explained partially by shared RM systems [52]. For beneficial MGEs, this
205 preferential transfer among kin is favoured by kin selection, because it allows host cells to
206 restrict MGE benefits to clonemates [53].

207 Finally, immune systems may use the entry route of MGEs and whether the nucleic acid is
208 single or double stranded for target discrimination. In conjugation and natural transformation,
209 DNA enters the cell single stranded, and ssDNA results in less restriction compared to lytic
210 phage infection [43,54]. CRISPR-Cas systems also acquire spacers preferentially from free
211 dsDNA ends [17], which favors spacer acquisition during dsDNA phage injection [31].
212 Restriction can also be alleviated when competence is induced [55], and some RM systems
213 even protect DNA entering through natural transformation by dedicated ssDNA methylation
214 [56].

215 The effect of immunity on MGEs, if consistent over time, could have longer-term
216 consequences on horizontal gene transfer (HGT). RM indeed limits HGT, but specifically
217 among strains bearing non-cognate systems [57]. The role of Type IV systems may be more
218 limited, but this will depend on the epigenetic status of MGEs in the environment, which has
219 not been sufficiently examined. Conclusions vary about CRISPR-Cas effect on long term HGT
220 [58–60]. Some lineages with strong signatures of HGT are depleted in CRISPR-Cas systems
221 [61], but across lineages there are no clear correlations between HGT and CRISPR-Cas activity
222 [59]. Perhaps the transient presence of immune systems in lineages, combined with the high
223 frequency of anti-immune genes born on MGEs [57,58,62,63] obscures these signatures.

224

225 ***Immune systems entail costs linked to activity and self-targeting***

226

227 Immunity is based on enzyme functionality, which entails a metabolic cost. This cost can be
228 constitutive, due to constitutive enzyme expression [64], or present only upon phage infection
229 [26,64]. For instance, translocation of Type I and III RM enzymes can consume as much as

one ATP for each base pair [65], but occurs only upon phage infection. The activity of immunity systems can also affect other cellular processes, for instance through the effect of RM methylation on global epigenetic patterns [66], or CRISPR-Cas interference with DNA repair [67]. The consequences of these pleiotropic effects will depend on the environment.

Immune systems can also present autoimmunity costs. DNA repair and HGT of RM systems create unmodified sites potentially targeted by Types I-III restriction (Figure 1A). Failure to repair DNA damage can result in a new recognition site while an existing methylated site may become demethylated due to repair. Although occurring at low frequency, these events are sufficient to generate toxic dsDNA breaks [68]. When a naïve strain acquires a new RM system by HGT, thousands of unmodified host recognition sites become targets, which can also result in recipient cell death [69]. Whether modified DNA-dependent Type IV RM systems suffer similar autoimmunity issues is unclear (Figure 1A). CRISPR-Cas systems can also cause autoimmune issues by self-targeting (Figure 3B). A small proportion of spacers target loci of the host genome [70,71], which can lead to chromosome dsDNA breaks, growth inhibition and filamentation [72].

Regulation of the expression and activity of immune systems limits their metabolic and auto-immune costs [73]. In Type I RM systems, nuclease activity is downregulated upon translocation events on the host genome but not on invading DNA, a phenomenon called Restriction Alleviation [5]. Host DNA translocation can trigger ClpXP-dependent proteolytic digestion of the nuclease subunit [68,74], or be inefficient compared to efficient translocation/cleavage of foreign DNA [74]. For CRISPR-Cas systems, upregulation of CRISPR-Cas expression frequently occurs following infection [75], through the activation of stress responses or detection of changes in cell metabolism that follow infection. CRISPR-Cas immunity can also be induced by quorum sensing, which anticipates infection by indicating cell densities are high [76,77]. Other mechanisms can also bias spacer acquisition towards foreign sequences. Spacer acquisition is particularly high at stalled replication forks, which are more abundant on foreign DNA, and limited by Chi sites on the chromosome [17] (Figure 3C). The phenomenon of priming in type I systems will then create a positive feedback, amplifying spacer acquisition from previously encountered threats.

In addition, autoimmunity is also limited due to past negative selection of self-targeting. Self-targeting CRISPR-Cas spacers are rare, and they are enriched at the leader end of arrays, suggesting they are recent and strongly selected against [70]. In genomes containing Type II RM systems, restriction site avoidance can also be detected [78]. Chromosomal avoidance is

even stronger than observed on phage genomes, suggesting auto-immunity represents a strong selective pressure [40]. Still, some degree of autoimmunity appears an unavoidable trade-off of maintaining efficient immunity. RM systems with higher restriction efficiency also have higher self-restriction [79]. That many RM enzymes must bind two sites to activate cleavage (Figure 2) may be an evolutionary adaptation to prevent autoimmunity where a single unmodified site arises [80], but will also limit immunity. Reliance on sites containing PAMs to limit auto-immunity also limits the choice of spacers available for efficient CRISPR-Cas immunity. On the other hand, the likely absence of auto-immunity in Type IV restriction, at least while modification targets are absent from the host, might explain relatively degenerate sequence context of modification, as it is free to evolve without that trade-off [4].

Selfish behaviour promotes immune system maintenance at a cost to their hosts

Over longer evolutionary timescales, immunity genes are part of the few gene categories to be on average negatively selected [81]. This suggests that costly parasites are not encountered often enough to balance the assorted costs of immune systems, and/or that immunity is not efficient enough (Figure 5). Importantly, long-term immune system maintenance in prokaryotic populations thus requires HGT [82]. HGT decouples immune system fitness from the one of their hosts, allowing them to act as selfish genetic elements as they can spread despite increased costs to the host.

Type II RM systems can exhibit particularly strong selfish behaviour, leading to host killing [83] (Figure 5). In these systems, loss of the M gene eventually leads to toxic dsDNA breaks as methylation patterning is diluted [84]. Even when R and M genes are lost simultaneously (commonly by failed segregation of plasmids carrying the system), endonuclease activity is usually more stable than methyltransferase activity, leading to post-segregational killing similarly to other toxin-antitoxin systems [85]. For the more mechanistically-complex Type I and III RM systems, gene loss does not cause detectable viability problems [68,86]. This may reflect the assembly of these systems into higher order RM machines where the loss of the M genes causes failure of the complete complex (Figure 2), or more stringent control and restriction alleviation. Type II systems thus appear to be the most selfish RM variants. Accordingly, they are also the systems most abundant on MGEs, experiencing frequent HGT [10]. The toxic effects associated with Type II loss do not occur upon entry in a new host due

to regulation by associated C protein transcriptional regulators which delay restriction until *de novo* methylation of the host [87].

Competition between systems can harm or benefit the host: when two RM systems with the same sequence specificity coexist within a cell, each system's modification protects the host from restriction by the other [88]. Alternatively, when two Type II R genes are regulated by the same C protein, entry of the second RM system causes upregulated restriction activity before the genome is fully methylated, causing cell death [89].

Host death or dormancy can benefit the host in the presence of parasites

Although CRISPR-Cas systems do not have such clear selfish addiction behaviour, several Cas components are evolutionary related to toxins and could arrest growth when induced, similarly to toxin-antitoxin systems [90]. Growth arrest or even death following phage infection can benefit the host population if it stops phages from completing their lytic cycle. Such "Abortive Infection" (Abi) has been demonstrated in some CRISPR-Cas type IF systems [91], and in type VI systems, where RNA targeting leads to growth arrest [92]. Type III systems also activate promiscuous RNAse activity [22,93] (Figure 4), which might also lead to Abi [8]. Competition among RM systems, despite best understood as a selfish behaviour, might similarly benefit host populations if a resident system stops the spread of a more deleterious invasive system.

Because cells engaging in abortive infection stop reproducing, the success of an abortive infection strategy requires the benefits from decreased phage encounters being directed at individuals that are related to the ones paying the cost [94]. However, it has been suggested that dormancy following phage infection might also benefit individual host cells by slowing down metabolism and phage reproduction, giving the host time to mount an immune response [90]. This might particularly be critical to allow spacer integration and CRISPR-Cas adaptive immune response [95].

Overall, bearing an immune system can translate into large benefits when providing defence against parasites, but also presents costs arising from immune activity, targeting the chromosome or beneficial MGEs, or selfish behaviour. Consequently, net fitness effects of immune systems will depend on the balance between all these factors (Figure 5). Moreover,

the efficacy of immunity is not a fixed parameter, but evolves as part of an arms race between immune systems and parasites.

Eco- evolutionary dynamics of immune systems and parasites

Parasites escape rapidly from immunity

Parasites commonly escape immunity by genetic or epigenetic mutation of the targeted sequence. For Type I-III RM systems, phage escape occurs through accidental methylation by the host [96], with probabilities ranging from 10^{-1} to 10^{-6} per infection [32] depending on relative restriction and modification efficiencies and the number of restriction sites [30]. High probability of phage escape means that the advantage of carrying a RM system is short-lived [25], and other mechanisms of resistance become more relevant after phages overcome restriction [96,97]. Phages can similarly escape CRISPR-Cas immunity by mutating their target sequence or PAM [15] (Figure 3B). If a single site is targeted, type I and II CRISPR-Cas escape by mutation is easier to achieve than for RM systems (commonly targeting multiple sites per MGE) [28]. However, because different bacteria in a population often acquire different spacers, it becomes increasingly hard to overcome CRISPR immunity (discussed below) [98–101]. Escape is even more limited against type III CRISPR-Cas systems because mismatches do not totally suppress interference [102]. Overall, promiscuous immune systems - which can cleave imperfect target sequences - are less susceptible to phage escape. KpnI, a restriction endonuclease that can cleave at non-canonical sequences, confers higher protection against phage than a more specific variant, because it still restricts a fraction of modified phages [103]. However, immune promiscuity also increases self-targeting [79,102], highlighting the trade-off between protection and auto-immunity.

Immune systems can evolve new immune specificities

CRISPR-Cas immune systems rapidly evolve new specificities through spacer incorporation. Type II RM systems are generally more inert: having functionally distinct DNA recognition elements in the separate methyltransferase and endonuclease, evolution of new recognition sites must require convergent evolution. On the other hand, Type I RM systems are particularly adept at evolving new DNA recognition specificity. The specific half-sites of a Type I

359 recognition site are recognised by two target recognition domain (TRD) folds of the HsdS
360 subunit that are separated by a coiled-coil linker that acts as a molecular ruler, setting the non-
361 specific DNA spacer length (Figure 6A). HsdS subunits show structural plasticity: TRDs can
362 be swapped within and between bacteria to generate new recognition sites [104], variation of
363 ±4 amino acids in the first alpha helix of the linker changes the spacer length [105] (Figure
364 6B), and two half HsdS subunits can dimerise to recognise a palindromic site [106] (Figure
365 6C). So-called "Shufflons" are Type I operons that exploit HsdS structural plasticity [107]
366 (Figure 6D) and have been identified in many species [108]. They can form replacement
367 recognition subunits using site-specific recombination to "flip" and rearrange HsdS genes, at
368 timescales similar to the ones of spacer acquisition [109].

369 Immune specificities can also be acquired through HGT [10,82]. Whole systems can be
370 transferred, but transfer of the subunits encoding specificity can be sufficient, as with *hsdS*
371 subunits encoded on plasmids [111], or CRISPR array spacers: the recombination of spacers
372 with the phage protospacers they target can even lead to specialized transduction of CRISPR
373 elements [112]. HGT might be a significant factor in generating immune variability,
374 particularly when other mutational pathways are less active [113].
375
376 ***Group-level immunity can counteract parasite escape***
377
378 If immune hosts differ in target specificity, a phage that overcomes immunity of one host
379 genotype remains sensitive to others. CRISPR-Cas systems often present high diversity in
380 spacer content [26–28,114], that is suggested to lead to 'distributed immunity' [115,116].
381 Diversity benefits were demonstrated by manipulating individual CRISPR-Cas immune clones
382 each with a single targeting spacer. Increasing population-level diversity led to faster extinction
383 of virus populations, which was associated with a reduced evolution and spread of escape
384 mutants [98–101]. These benefits depend on population structure: in spatially structured
385 populations, interactions between clones are strongly reduced, limiting the benefits of diversity
386 [117].

387 RM immunity diversity could similarly benefit host populations. A rare strain with a different
388 immune specificity should gain a short-term fitness advantage in the presence of phages that
389 escaped restriction from a dominant strain, leading to negative frequency-dependent selection
390 among strains [25]. In *S. pneumoniae*, a strain bearing an active shufflon generating high levels
391 of diversity appears to have increased resistance to phages compared to 'locked' forms not able

392 to undergo phase variation [108,118]. Additionally, phenotypic diversity can arise from
393 variation in the expression of RM systems in a population. Some, particularly Type III, contain
394 repeated sequences that lead to phase variation in ON/OFF expression with subpopulations not
395 expressing any RM function [119]. A recent survey found that 17% of *mod* genes contain
396 sequence repeats with potential for phase variable expression [120]. Variation in expression of
397 RM immunity has been proposed to benefit host populations because the presence of sensitive
398 hosts would reduce the abundance and weaken the selective pressure for escape phages
399 [121,122]. However, experimental tests of the dynamics and benefits of RM diversity are still
400 lacking.

401 When multiple systems coexist in a genome, this "within-individual" diversity in specificity
402 also increases the efficacy of targeting, making escape less probable. Bacteria often do not need
403 to encode multiple full-blown RM systems in order to encode multiple specificities, as multiple
404 HsdS subunits can combine with a single HsdM/HsdR complex [111]. Modelling suggests that
405 individual-based diversity might drive population-based diversity: bacteria first accumulate
406 diverse RM systems *within* cells – each new system providing additional immunity to phages
407 – until phage escape promotes the loss of immunity leading to the evolution of diversity *among*
408 lineages [123]. Genomes can also encode multiple CRISPR-Cas systems, and multiple spacers
409 targeting a single phage, which results in a strongly reduced probability of escape by point
410 mutation [98,115]. However, phages can still overcome multiple protospacer targeting by
411 insertions, deletions or recombination [29].

412

413 ***Short-term coevolution between parasites and immune systems***

414

415 The outcome of short-term coevolution depends on the interplay between parasite escape and
416 host response. For CRISPR-Cas systems, the outcome of coevolution depends on the number
417 and diversity of mutations that can be achieved on both sides [97]. The rate of spacer
418 acquisition against escape phages is greatly enhanced by priming [13,124]. *P. aeruginosa*
419 PA14, which is primed against phage DMS3vir, rapidly generates high spacer diversity,
420 leading to phage extinction [98]. In *S. thermophilus*, less spacer diversity is generated, and
421 longer-term coevolution can be observed [27,100]. Both immunity and infectivity increase over
422 time as hosts acquire more spacers and phages escape mutations, characteristics of an arms
423 race dynamics. Ultimately, the arms race is asymmetrical [100] because phages are limited by
424 mutation supply whereas hosts can acquire new spacers at low cost [64], and phages go extinct.

In natural environments, long-term coevolution can be observed [114,125]. However, CRISPR-Cas immunity can also be lost [126,127], due to loss of spacers or whole systems, or to the inactivation of CRISPR-Cas loci [37,50,126]. CRISPR-Cas loss favours host-phage coexistence if no other resistance mechanism is present [127] or if alternative mechanisms are less efficient in depleting phage [126].

In the case of RM immunity, evidence for short-term arms races has not been observed, although it is possible that the ability of shufflons to rapidly generate diversity leads to short-term coevolution with phages. However, phages cannot accumulate epigenetic modifications and will need to specialize on one RM type at a time (in contrast to the accumulation of escape mutations against CRISPR-Cas targeting) [128], preventing the appearance of generalist phages.

MGEs fight immunity by diverse mechanisms

In the long term, MGEs can avoid immunity by carrying fewer sequences that are recognized by immune systems. Carrying fewer restriction sites than expected by chance increases probability of parasite escape [30,129], and a total absence of target restriction sites explains the resistance of some natural phages to restriction [34]. Restriction avoidance is much rarer against Type I RM target sequences [129], possibly because other strategies can inactivate Type I systems. Some phages avoid Type III RM immunity by carrying all sites in the same orientation, as cleavage requires two inversely-oriented recognition sites [11]. Similar to restriction site avoidance, avoidance of PAM sequences can be detected for some CRISPR-Cas subtypes [130].

Another strategy to avoid immunity is to physically protect nucleic acids. A large variety of chemical modifications have been detected in the nucleic acids of virulent phages. Some common base modifications are C-5-methylcytosine, 5-hydroxylmethylcytosine (HMC), and sugar-derivatives such as glucosyl-HMC [131]. These modifications block the activity of Type I to III RM systems; however, they are targeted by Type IV systems (Figure 1A). Some chemical modifications also inhibit CRISPR-Cas immunity [33,132]. CRISPR-Cas9 is able to cleave methylated DNA [133], but is inhibited by larger modifications [132]. It is not clear how widespread such modifications are in the phage metagenome. Another physical barrier preventing nuclease access to DNA is the production of a nucleus-like structure during infection, which allows escape from type I CRISPR-Cas and RM immunity [134,135],

458 although phages remain sensitive to RNA targeting by type III CRISPR-Cas systems [134].
459 MGEs can also interfere with host regulation of immunity. Some phages activate host
460 methyltransferases [136] or possess their own [137], while others repress host CRISPR-Cas
461 systems by hijacking host regulators [138]. However, most anti-immune proteins inhibit
462 specific immunity enzymes and are likely to be an important part of phage-immune
463 coevolution.

464 Type I RM systems are targeted by diverse inhibitors that act through distinct mechanisms, for
465 instance occlusion of restriction sites [47], or competitive inhibition of DNA binding by DNA
466 mimics [62,139]. Anti-restriction proteins against Types II and III systems are not known; the
467 lack of Type II anti-restriction proteins may be due to their mechanistic and structural diversity.
468 Similarly to anti-RM proteins, anti-CRISPR proteins (Acrs) make MGEs able to infect and
469 replicate in hosts with active CRISPR-Cas systems [140]. Acr activity is usually restricted to
470 specific CRISPR-Cas subtypes [141–143]. They can interfere with target DNA recognition or
471 its destruction [140], by associating with Cas proteins and preventing either DNA binding or
472 cleavage. Like RM inhibitors, some Acrs carry negatively charged surfaces that mimic DNA.
473 Additionally, Acrs with enzymatic activity have recently been discovered. For instance, a
474 family of Acrs degrades cyclic nucleotides involved in type III CRISPR-Cas signaling [144].

475 Acrs bring large benefits to phages in the presence of CRISPR-Cas immune hosts [98]; and
476 costs of expression appear to be very low [145], possibly due to regulated expression. However,
477 Acrs vary in strength and do not totally antagonize CRISPR-Cas activity, requiring cooperation
478 between Acrs to overcome host immunity [146,147]. Because of this cooperative behaviour,
479 Acr phages can also be exploited by non-Acr phages. As this exploitation is costly for Acr
480 phages, it paradoxically increases the competitive fitness of weaker Acrs, less amenable to
481 exploitation [145]. Carrying anti-RM proteins is also likely to benefit MGEs in the presence of
482 restriction [47,62].

483 Anti-immune strategies impose strong selective pressures on hosts to find alternative ways to
484 defend themselves against phages. A conspicuous evolutionary example is Type IV restriction.
485 Phages bearing chemical modifications conferring RM resistance trigger restriction by Type
486 IV ENases. The best studied is McrBC, conferring immunity against HMC-modified phages
487 [148]. Fitness costs and benefits of Type IV systems have not been studied yet but are likely to
488 depend on the abundance and diversity of modified DNA. Nonetheless, known systems offer
489 a glimpse of multiple rounds of coevolution of restriction and anti-restriction systems: [4,149–
490 151]. No such arms race is known for CRISPR-Cas immunity in response to Acrs. Because

Acrs are usually restricted in host range, i.e. they antagonize a specific CRISPR-Cas subtype, switching to another subtype is likely sufficient to respond to Acr presence [152]. Accordingly, several CRISPR-Cas subtypes often coexist within a strain, which could be a way to overcome specific Acrs [19].

Interactions between immune systems

Defence systems are not present in isolation, but often cohabit within genomes, clustered into defence islands [3]. Yet, their interactions have been scarcely studied to date. RM systems can compete with each other [84]; Type IV systems are incompatible with a subset of Type I-III RM systems, as they can target methylated sites and can only coexist when methyltransferases do not create a modification target [4,153]. However, coexisting RM systems can also act in combination [111]. CRISPR-Cas subtypes can also cooperate, for instance type I derived crRNAs can be used by type III machinery, counteracting viral escape from the type I system [154]. Other positive interactions between CRISPR-Cas subtypes are suggested by preferential associations within genomes [19], but remain to be studied in detail.

Cooperation between innate and adaptive immune systems might also be widespread. In Vertebrates, innate and adaptive immunity act in synergy, with each system able to activate the other when detecting a threat. In prokaryotes, CRISPR-Cas and RM have mostly been studied in isolation despite their frequent genomic cooccurrence [10]. In *S. thermophilus*, the native type II CRISPR-Cas system and a type II RM from *Lactococcus* work additively, leading to high immunity against phage infection [133]. Both CRISPR-Cas interference and spacer acquisition also work on the methylated escape phage [133]. Two native systems in *E. faecalis* also work additively against plasmid conjugation [156]. Such additive effect might be enough to prevent MGE escape in many environments, and a simple way to extend the usually transient benefits provided by RM immunity. However, antagonism might also exist as some CRISPR-Cas subtypes are inhibited by DNA modifications [132].

One main challenge to developing CRISPR immunity against phages is spacer acquisition by a cell which is still susceptible to killing. In the same *S. thermophilus* system, it was demonstrated that restriction promotes spacer acquisition [157] (Figure 3C). Restriction inactivates most incoming phages, providing the CRISPR-Cas acquisition machinery with 'defective' phage DNA on which spacer acquisition can proceed [157]. Innate immunity thus allows adaptive immunity to develop, by protecting most hosts from death and increasing the

number of cells in which spacer acquisition can proceed. It remains to be seen if such synergy also occurs with other immunity subtypes. How efficiently RM and CRISPR-Cas cooperate could vary between types, for instance depending on the compatibility between the substrates generated by each RM Type, and the ones required by the CRISPR-Cas subtype. For example, Type I and ISP enzymes can liberate short DNA fragments [158,159] that may feed into adaptation (Figure 2). Synergy with RM immunity could be particularly important for naïve spacer acquisition, which can be very inefficient in the absence of priming. The common cooccurrence of CRISPR-Cas systems and RM systems would then increase the spacer acquisition rate, a critical bottleneck for efficient adaptive immunity [35]. It could also allow CRISPR-Cas spacer acquisition to benefit from RM ability to identify and target non-self DNA [73]. Synergy between immunity systems might also be particularly relevant for highly virulent phages. CRISPR-Cas systems confer reduced immunity against these phages, likely because rapid expression of early genes causes damage before spacer acquisition can happen [33,35]. Even inefficient RM immunity (with high rates of phage escape) will increase the probability that some hosts survive and acquire spacers, which might allow CRISPR-Cas immunity to take over.

Conclusions

Immune systems provide defence to prokaryotes against parasites, mostly thanks to their ability to generate high levels of diversity, which is a key element of effective defence against evolving parasites. In response, parasites also present a range of strategies to avoid or fight immunity mechanisms. We are only starting to identify the costs and benefits associated with immune and anti-immune strategies. Current knowledge is primarily based on controlled laboratory experiments with single defence systems. Understanding the costs and benefits and the population and coevolutionary consequences of bacterial immune systems in nature requires future studies that take into account the biotic and abiotic complexity of natural environments (such as interspecific interactions, diverse populations of MGEs, and spatial and social structures) as well as the coexistence of multiple defence mechanisms in the same host genome and the synergistic or antagonistic interactions that exist between them.

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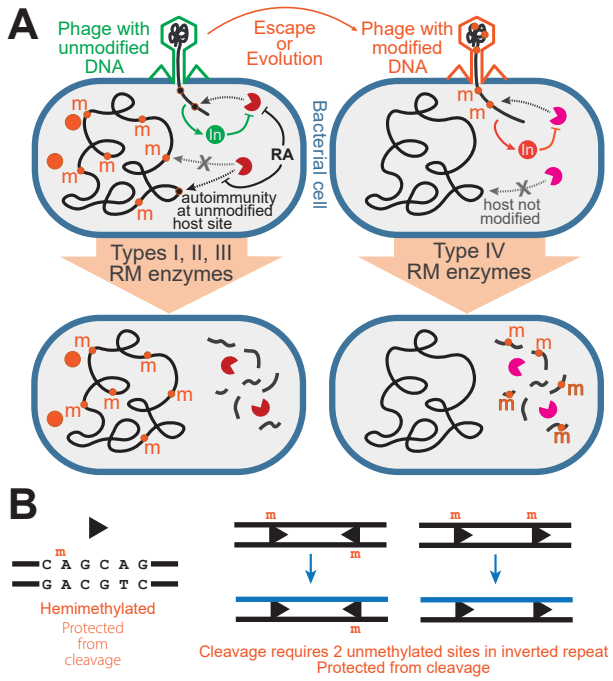
Figures

Fig. 1



567 **Fig. 2**

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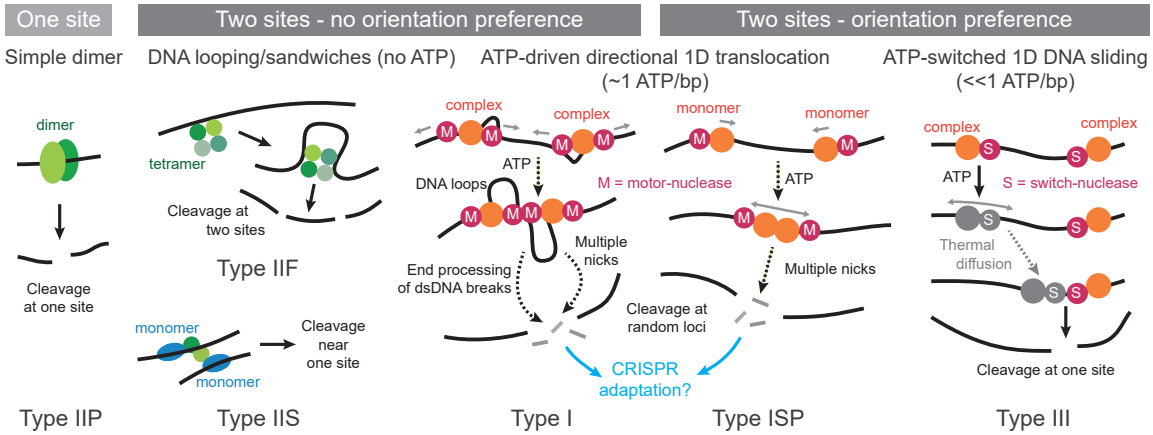

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571 **Fig. 3**

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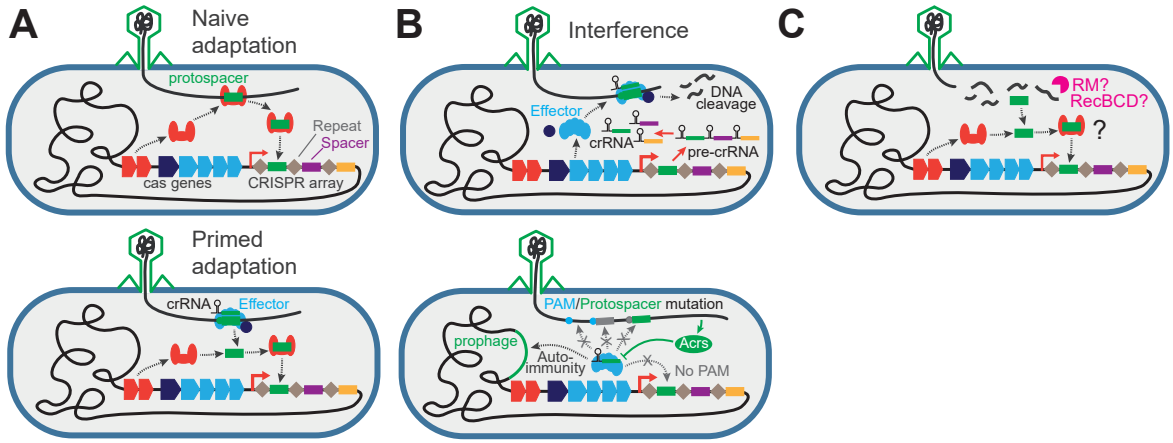

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575 **Fig. 4**

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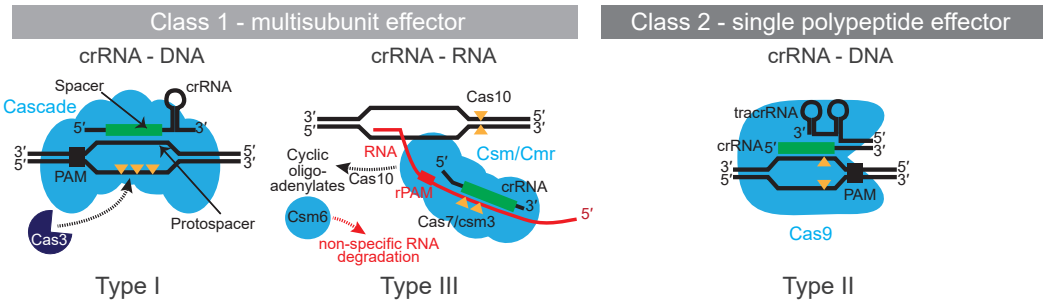

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580 **Fig. 5**

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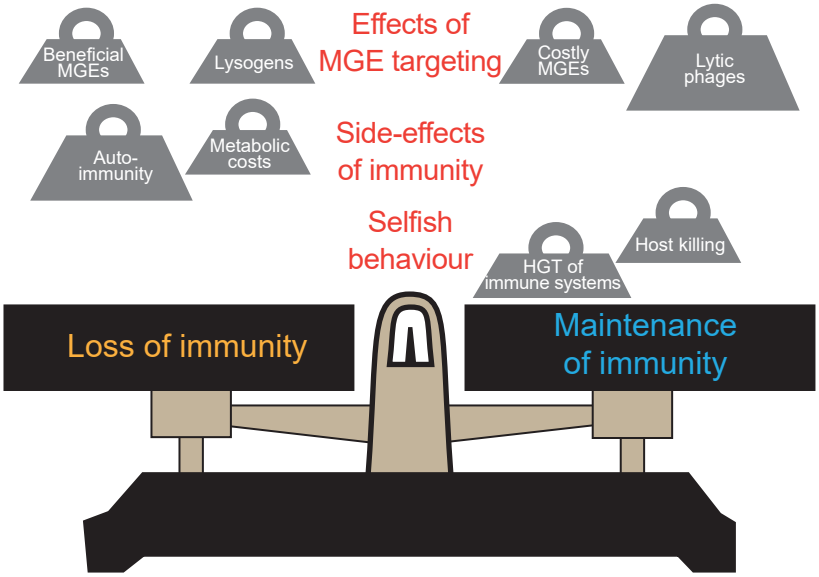

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584 **Fig. 6**

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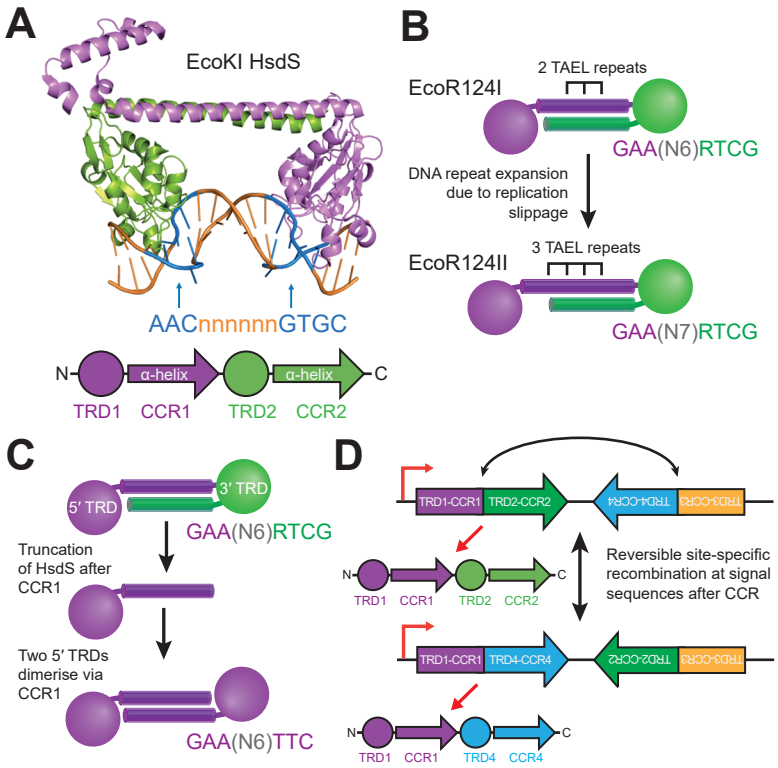

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Figure legends

Figure 1: Fundamental mechanisms of RM immunity. (A) Cartoons of bacterial cells infected by phage and the effect of Types I - III (left panel) and Type IV (right panel) RM systems. Types I - III comprise an endonuclease activity (Pacman) and methyltransferase (orange circle). DNA cleavage is targeted to specific sequences (circles) that are protected on the host genome by methylation (m). Appearance of unmethylated sites on the host leads to autoimmunity which is prevented for Type I systems by restriction alleviation (RA). Cleavage can be prevented by phage-encoded inhibitors (In). Type IV systems comprise only an endonuclease. (**B**) Asymmetric sites (arrowhead) that are only hemimethylated are protected following replication (one daughter DNA shown) by a necessity for interaction between two sites in inverted repeat to activate cleavage.

Figure 2. Mechanisms of DNA cleavage by Type I - III RM enzymes. The majority of RM enzymes require communication between two target sites to activate cleavage, using either energy-independent DNA looping or ATP-dependent mechanisms.

Figure 3. CRISPR-Cas adaptive immunity. (A) Adaptation is the uptake of MGE sequences by the Cas1-Cas2 complex (orange) into the leader of the CRISPR array. Primed adaptation is facilitated by the effector complex. (B) Interference is the specific recognition and cleavage of an MGE using crRNA processed from the CRISPR array. The process can be blocked by mutation of the PAM and/or protospacer sequences, or by phage-encoded inhibitors (Acrs). Autoimmunity is avoided by an absence of PAMs in the CRISPR array but may occur where foreign sequences are recombined e.g. prophage. (C) Nucleic acid processing by RM enzyme activity or repair nucleases working on stalled replication forks (e.g. RecBCD, [17]) may provide polynucleotide fragments that feed into adaptation.

Figure 4. Polynucleotide cleavage by type I, II and III CRISPR-Cas effectors. See main text for full details. Polynucleotide cleavage is shown by the orange arrowheads.

Figure 5. Summary of balancing factors that can affect immune system prevalence.

Figure 6. Generation of diversity in Type I RM systems by genetic recombination of the HsdS DNA recognition subunit. (A) Computational model of the EcoKI HsdS subunit bound to DNA (PDB:2Y7H, [110]) demonstrating how the target recognition domains (TRDs) and coiled coil region (CCR) allow HsdS to recognise an asymmetric bipartite DNA sequence. (B) Changes in the number of TAEL amino acid repeats in CCR1 of EcoR124I and EcoR124II changes the number of non-specific nucleotides in the spacer. (C) Dimerisation of half HsdS subunits produces a Type I enzyme that recognises a palindrome sequence. (D) Shufflon system. Reversible site-specific inversion between recombination sequences within two inverted *hsdS* genes produces HsdS subunit that recognise one of two sequences as one of the TRDs is swapped.

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