Biochimie 117 (2015) 119–128

Contents lists available at ScienceDirect

Biochimie

journal homepage: www.elsevier.com/locate/biochi

Review

The CRISPR-Cas immune system: Biology, mechanisms and applications

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ARTICLE INFO

Article history:
Received 19 January 2015
Accepted 31 March 2015
Available online 10 April 2015

Keywords:
CRISPR
Cascade
Cas9
Cas3
Cmr/Csm

ABSTRACT

Viruses are a common threat to cellular life, not the least to bacteria and archaea who constitute the majority of life on Earth. Consequently, a variety of mechanisms to resist virus infection has evolved. A recent discovery is the adaptive immune system in prokaryotes, a type of system previously thought to be present only in vertebrates. The system, called CRISPR-Cas, provide sequence-specific adaptive immunity and fundamentally affect our understanding of virus–host interaction. CRISPR-based immunity acts by integrating short virus sequences in the cell's CRISPR locus, allowing the cell to remember, recognize and clear infections. There has been rapid advancement in our understanding of this immune system and its applications, but there are many aspects that await elucidation making the field an exciting area of research. This review provides an overview of the field and highlights unresolved issues.

1. Introduction

For each cell on Earth there are about ten viruses [1]. Viruses are key factors in the ecology and evolution of life by acting as predators and facilitators of genetic exchange. Not surprising, an array of countermeasures can be observed in their hosts, generally grouped into innate and adaptive immune systems. Innate (non-specific) systems recognize certain generic features of infection. Restriction-modification and abortive infection are examples of such innate systems in prokaryotes. Adaptive systems, on the other hand, have the ability to learn to recognize specific features of pathogens. In humans, B and T cells can learn to recognize proteins and other structures in order to destroy pathogens and infected cells. Due to the complexity of that system, the demonstration of an adaptive immune system in prokaryotes [2] was a surprise. The prokaryotic system, based on a region of DNA called Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR; [3]), is a largely stand-alone system that is capable of functioning in an individual cell, a necessity for organisms that often display unicellular behavior. The CRISPR-Cas system targets DNA or RNA as a way of protecting against viruses and other mobile genetic elements [2,4].

The CRISPR locus, first observed in *Escherichia coli* [5], is present in about 84% of archaea and 45% of bacteria according to the most recent update of the CRISPRdb [6]. The difference in prevalence could be affected by sampling bias as almost twenty times more bacteria than archaea have been analyzed. The CRISPR is an array of short repeated sequences separated by spacers with unique sequences. The CRISPR can be found on both chromosomal and plasmid DNA. The spacers are often derived from nucleic acid of viruses and plasmids, an observation that gave rise to the idea that CRISPRs are part of an anti-virus system [7–9]. By adding new spacers new viruses can be recognized. The spacers are used as recognition elements to find matching virus genomes and destroy them.

CRISPR activity requires the presence of a set of CRISPR-associated (*cas*) genes, usually found adjacent to the CRISPR, that code for proteins essential to the immune response [2,10]. Since the genome is modified in the process of spacer acquisition, offspring inherit the protection. New spacers are usually added at one side of the CRISPR, making the CRISPR a chronological record of the viruses the cell and its ancestors have encountered.

The CRISPR-Cas mediated defense process can be divided into three stages (Fig. 1). The first stage, adaptation, leads to insertion of new spacers in the CRISPR locus (Fig 2). In the second stage, expression, the system gets ready for action by expressing the *cas* genes and transcribing the CRISPR into a long precursor CRISPR

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http://dx.doi.org/10.1016/j.biochi.2015.03.025

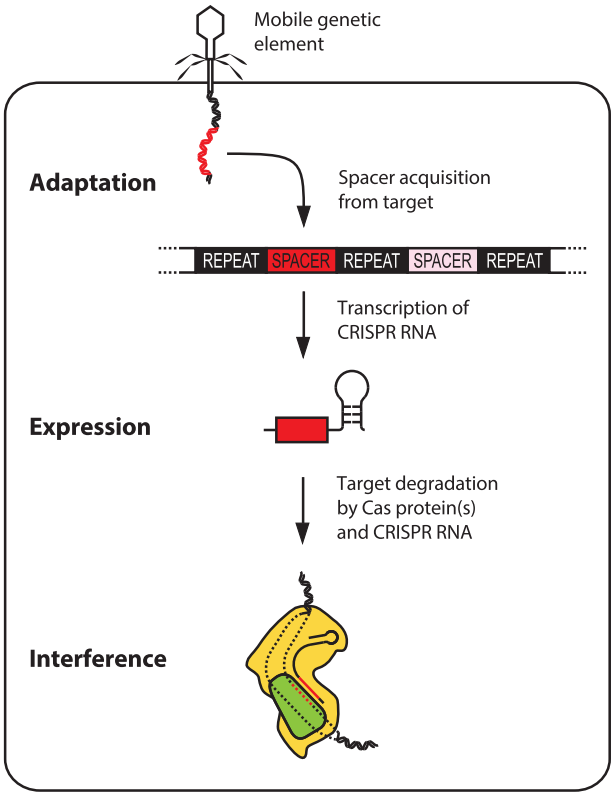

Fig. 1. The key steps of CRISPR-Cas immunity. 1) Adaptation: insertion of new spacers into the CRISPR locus. 2) Expression: transcription of the CRISPR locus and processing of CRISPR RNA. 3) Interference: detection and degradation of mobile genetic elements by CRISPR RNA and Cas protein(s).

RNA (pre-crRNA). The pre-crRNA is subsequently processed into mature crRNA by Cas proteins and accessory factors (Fig. 3). In the third and last stage, interference, target nucleic acid is recognized and destroyed by the combined action of crRNA and Cas proteins (Fig. 3).

This review provides an overview of the mechanism of the CRISPR-mediated immunity as well as its diversity, the virus countermeasures, evolution and ecology. Naturally occurring functions not related to immunity and the impressive recent development of genetic tools based on CRISPR-Cas and their applications are also covered.

2. Diversity, ecology and evolution of the CRISPR-Cas systems

The length and sequence of repeats and the length of spacers are well conserved within a CRISPR locus, but may vary between CRISPRs in the same or different genomes. Repeat sequences are in the range of 21 bp to 48 bp, and spacers are between 26 bp and 72 bp [3,6,7]. The observed variation is perhaps not surprising given how widespread the system is. The number of spacers within a CRISPR locus vary widely; from a few to several hundreds [6]. Genomes can have single or multiple CRISPR loci and in some species these loci can make up a significant part of the chromosome. In *Methanocaldococcus* sp. FS406-22 (with eighteen CRISPRs and 191 spacers) and *Sulfolobus tokodaii* str. 7 (with five CRISPRs and 458 spacers) the CRISPRs make up about 1% of the genome [11]. Not all CRISPR loci have adjacent *cas* genes and instead rely on trans-encoded factors. Another feature associated with CRISPR loci is the presence of a conserved sequence, called leader, located upstream of the CRISPR with respect to direction of transcription. The

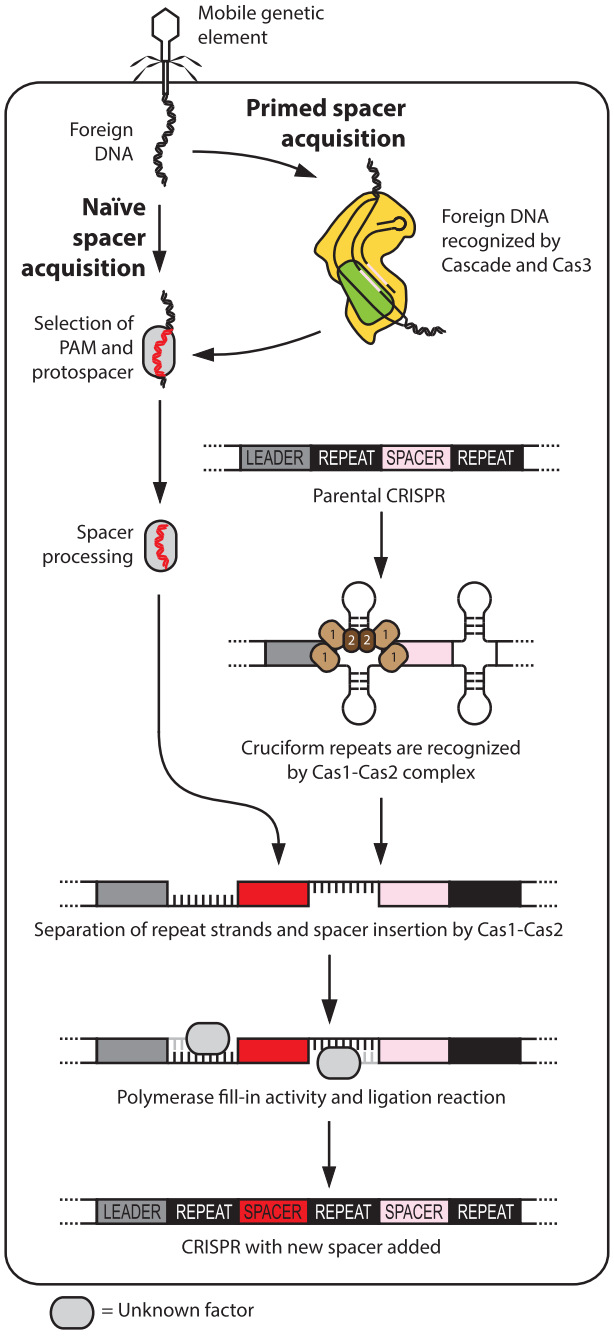

Fig. 2. Model of the adaptation in the Type I-E system. There are two types of spacer acquisition, naïve and primed. Both require the presence of a PAM and are dependent on the Cas1–Cas2 complex. The Cas1–Cas2 complex recognizes the CRISPR and likely prepares it for spacer integration. Naïve spacer acquisition occurs when there is no previous information about the target in the CRISPR. Primed spacer acquisition requires a spacer in the CRISPR locus that matches the target DNA and the presence of Cas3 and the Cascade complex. Primed acquisition results in insertion of more spacers from same mobile genetic element. PAM = Protospacer Adjacent Motif.

presence of these leader sequences was initially observed in *Methanocaldococcus jannaschii*, *Archaeoglobus fulgidus* and *Methanothermobacter thermautotrophicus* but has subsequently been found in many other species [3].

The Cas proteins are a highly diverse group. Many are predicted or identified to interact with nucleic acids; *e.g.* as nucleases, helicases and RNA-binding proteins [12]. The Cas1 and Cas2 proteins are involved in adaptation (see Section 3) and are virtually

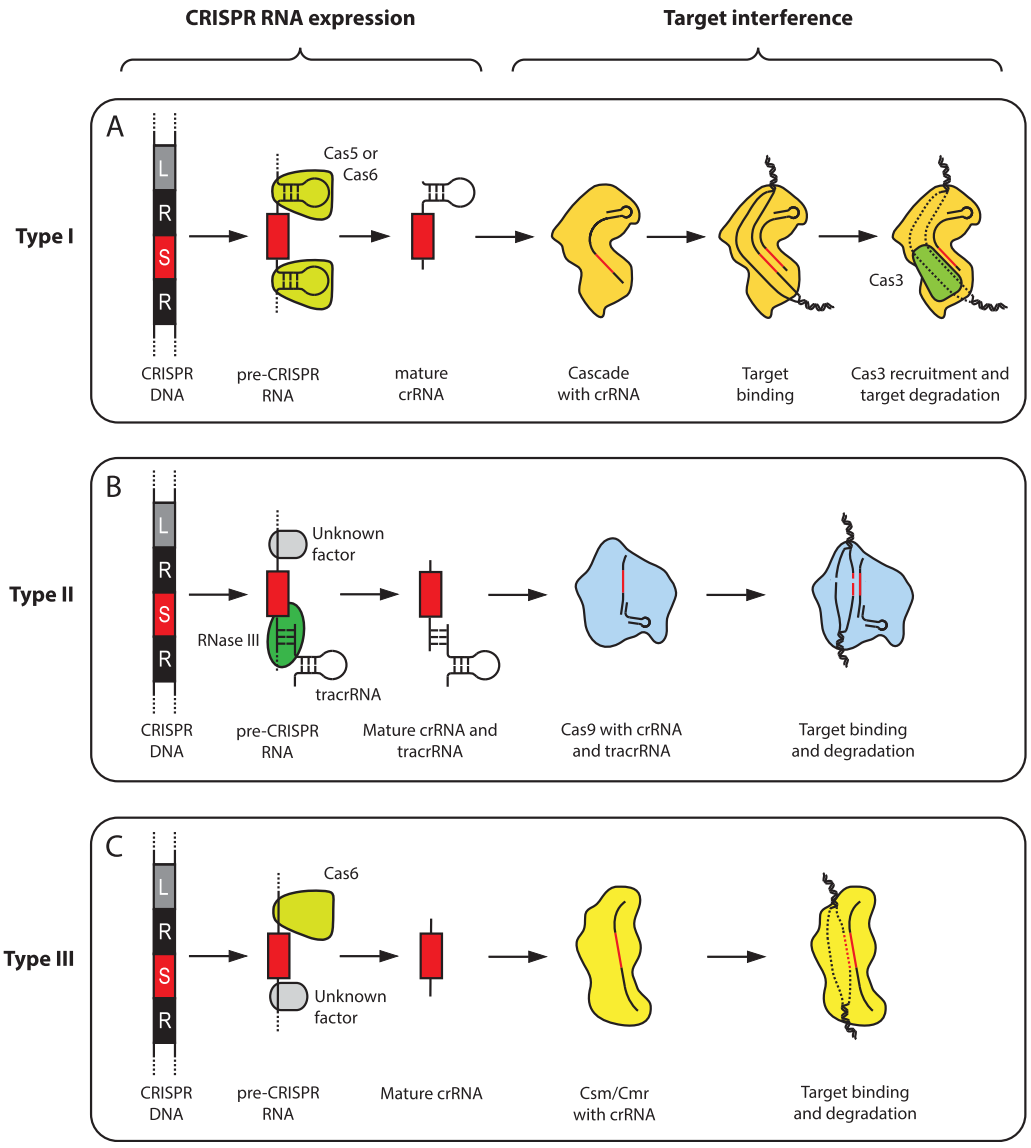

Fig. 3. Model of crRNA processing and interference. (A) In Type I systems, the pre-crRNA is processed by Cas5 or Cas6. DNA target interference requires Cas3 in addition to Cascade and crRNA. (B) Type II systems use RNase III and tracrRNA for crRNA processing together with an unknown additional factor that perform 5′ end trimming. Cas9 targets DNA in a crRNA-guided manner. (C) The Type III systems also use Cas6 for crRNA processing, but in addition an unknown factor perform 3′ end trimming. Here, the Type III Csm/Cmr complex is drawn as targeting DNA, but RNA may also be targeted.

universal for CRISPR-Cas systems. Other Cas proteins are only associated with certain types of CRISPR-Cas systems. The diversity of Cas proteins, presence of multiple CRISPR loci and frequent horizontal transfer of CRISPR-Cas systems make classification a complex task. The most adopted classification identifies Type I, II and III CRISPR-Cas systems, with each having several subgroups [13]. Different types of CRISPR-Cas systems can co-exist in a single organism. Recently, a Type IV system was proposed, which contain several Cascade genes but no CRISPR, *cas1* or *cas2* [14]. Type IV complex would be guided by protein-DNA interaction, not by crRNA, and constitutes an innate immune system preset to attack certain sequences.

The Type I systems are defined by the presence of the signature protein Cas3, a protein with both helicase and DNase domains responsible for degrading the target [15]. Currently, six subtypes of the Type I system are identified (Type I-A through Type I-F) that have a variable number of *cas* genes. Apart from *cas1*, *cas2* and *cas3*, all Type I systems encode a Cascade-like complex. Cascade binds crRNA and locates the target, and most variants are also responsible for processing the crRNA. Cascade also enhances spacer acquisition in some cases. In the Type I-A system, Cas3 is a part of the Cascade complex.

The Type II CRISPR-Cas systems encode Cas1 and Cas2, the Cas9 signature protein and sometimes a fourth protein (Csn2 or Cas4). Cas9 assists in adaptation, participates in crRNA processing and cleaves the target DNA assisted by crRNA and an additional RNA called tracrRNA [16–20]. Type II systems have been divided into subtypes II-A and II-B but recently a third, II-C, has been suggested [21,22]. The *csn2* and *cas4* genes, both encoding proteins involved in adaptation [2,19,20], are present in Type II-A and the Type II-B, respectively, while Type II-C lacks a fourth gene.

The Type III CRISPR-Cas systems contain the signature protein Cas10 with unclear function. Most Cas proteins are destined for the Csm (in Type III-A) or Cmr (in Type III-B) complexes, which are similar to Cascade [23–25]. Interestingly, while all Type I and II systems are known to target DNA, Type III systems target DNA and/

or RNA (see Section 5). So far, the Type II systems have been exclusively found in bacteria while the Type I and Type III systems occur both in bacteria and archaea [13].

The large number of genomes with detected CRISPRs could be used as an argument for its importance as defense mechanism. However, the CRISPR-Cas systems are probably mobile genetic elements that frequently transfers horizontally, which also contributes to their high prevalence. The CRISPR-Cas systems are not only beneficial, they have a maintenance and production cost and the risk of self-targeting (which could kill the host) reduces the value of the system. As an example, *E. coli* contains a CRISPR-Cas system that is efficiently silenced by H-NS [26], which could be interpreted as inactivation conferring a selective advantage. On the other side of the scale, in an analysis of acid mine drainage, extreme CRISPR diversity was observed where no two sampled individual cells had the same spacers [27]. The viruses in the acid mine drainage used recombination to diversify rapidly, making any but the most recent spacers lack a target [28].

Experiments on CRISPR dynamics have been performed mainly in *Streptococcus thermophilus*, and they indicate that spacer sampling is not random as a small number of spacers dominates and their relative abundances oscillate rapidly [29]. Other findings indicate that phages can still replicate in populations with one but not two spacers targeting them [30].

There is some light starting to be shed over the evolution of CRISPR-Cas systems. It has been hypothesized that the Type IV system is similar to an ancestral innate immune system that gained adaptive ability by associating with a transposon-like element containing *cas1* and *cas2* [14]. The transposon was domesticated but retained the terminal inverted repeats that formed the ancestral CRISPR repeats. Repeats were then duplicated and spacers added by the action of Cas1 [31]. The process eventually resulted in the formation of Type I and III CRISPR-Cas systems. The Type II systems are suggested to have been formed by a replacement of the Cascade genes by *cas9* (Fig. 4). Cas9 is also linked to mobile genetic elements as it resembles transposon-encoded proteins [14,21].

3. CRISPR adaptation

The adaptation phase provides the genetic memory that is a prerequisite for the subsequent expression and interference phases that neutralize the re-invading nucleic acids. The insertion of new spacers has been experimentally demonstrated in several CRISPR-Cas subtypes; Type I-A (*Sulfolobus solfataricus* [32], and *Sulfolobus islandicus* [33]), I-B (*Haloarcula hispanica* [34]), I-E (*E. coli* [35–37]) and I-F (*Pseudomonas aeruginosa* [38] and *Pectobacterium atrosepticum* [39]) and Type II-A (*S. thermophilus* [2,19,40] and a *Streptococcus pyogenes* system expressed in *Staphylococcus aureus* [20]). There are two types of spacer acquisition; naïve, when the invader has not been previously encountered, and primed, when there is a pre-existing record of the invader in the CRISPR (Fig. 2) [41].

Although spacer acquisition is observed, the mechanism is only partly understood. Conceptually, the process can be divided into two steps: protospacer selection and generation of spacer material followed by integration of the spacer into the CRISPR array and synthesis of a new repeat. Occasional deletion of spacers is required to limit the size of the CRISPR, but there is little knowledge of the mechanism or frequency of such events.

The key factors in spacer integration are Cas1 and Cas2. This function was suggested early as the proteins are ubiquitous but dispensable for interference [10]. This was later confirmed by overexpression of Cas1 and Cas2 from a Type I-E system in *E. coli*, which resulted in spacer integration even in the absence of all other Cas proteins [36]. Both Cas1 and Cas2 are nucleases [42–44] and mutations in the active site of Cas1 abolishes spacer integration in *E. coli* [36,37]. Cas1 and Cas2 from *E. coli* form a complex where one Cas2 dimer binds two Cas1 dimers. Formation of the complex is

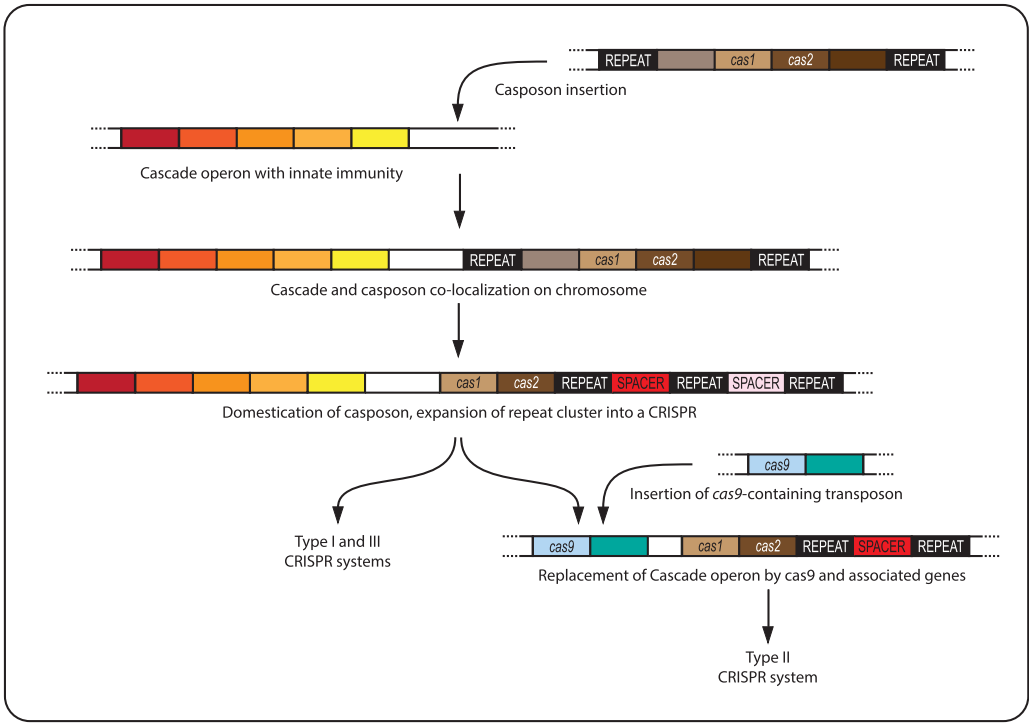

Fig. 4. Hypothesis for CRISPR-Cas system evolution. A casposon inserts adjacent to a Cascade operon with protein-based non-adaptive targeting ability. The casposon subsequently loses genes and the terminal inverted repeats (TIRs) expand into a CRISPR cluster to eventually form Type I and III CRISPR-Cas systems. The Type II CRISPR-Cas system development is initiated when a transposon containing *cas9* replaces the Cascade genes. Unlabeled genetic elements are of variable nature.

required for spacer acquisition but Cas2 nuclease activity is dispensable. Cas1 preferentially binds CRISPR DNA in a Cas2-dependent manner, further supporting a direct role in spacer acquisition [45]. It could be speculated that the Cas1-Cas2 complex both transport the spacer material and perform spacer integration, which would explain the need for the many Cas1 subunits in the complex. A few additional factors are known to be required for spacer acquisition: Cas9, Csn2 and tracrRNA in Type II-A [2,19,20] and Cas4 in Type I-B [34]. The roles of tracrRNA, Csn2 and Cas4 are unclear but Cas9 probably guides the integration machinery. Host polymerases, ligases and recombination proteins are likely to perform generic steps in the adaptation, as such factors can be found in every host cell.

Spacer selection appears guided by certain sequence elements in the target. Analysis of target sequences has revealed a short motif next to the target sequence called protospacer adjacent motif (PAM) that is crucial for discrimination between self and non-self [46] (see Section 5). While initially thought to be important only for interference, the PAM also has a role in spacer acquisition. This is supported by the fact that most newly acquired spacers have a PAM next to their protospacer [35,37]. In the Type II-A system, Cas9 is responsible for identifying the PAM as mutations that disable Cas9's PAM recognition result in acquisition from protospacers without PAM [20]. In Type I-E, PAM recognition during spacer acquisition may be different as they are indicated to be identified by Cas1-Cas2 alone [36]. However, Cascade increases the frequency of correct PAMs for inserted spacers [35,37]. In Type I-E, spacers are preferentially incorporated from extra-chromosomal elements [37], which is demonstrated to be a result of a connection between adaptation and replication [129]. In Type II-A, spacer acquisition may not be biased toward extra-chromosomal elements as cells with nuclease-deficient Cas9 demonstrate unbiased spacer sampling and an increased rate of spacer acquisition in one study [19]. Acquisition of self-targeting spacers would not readily be observed with functional Cas9, as the potential lethality of the events would result in the cells being lost from the population.

Once located, it is not known if the protospacer is copied or cut out of the target. The production of spacer material could be linked to other defense systems, such as the restriction-modification system [47], similar to primed spacer acquisition (see below). Such coupling may facilitate the systems' recognition of “foreign DNA” or provide the system with material suitable for new spacers. Infection by a phage incapable of reproduction, which *e.g.* only packaged a partial viral genome, could act as a vaccine and facilitate adaptation.

How are spacers actually integrated in the CRISPR array? Cas1 nuclease activity is required for nicking the CRISPR array in *E. coli*, and Cas1 is possibly responsible for the integration of the new spacer [48]. An *in vitro* study with Type I-E Cas1 and Cas2 confirm that the complex can insert DNA fragments into a CRISPR array by a mechanism reminiscent of retroviral integrases and transposases [31]. Whether or not the mechanism for spacer integration is conserved between the different systems remains to be determined.

In both the Type I-E and Type II-A system, it is demonstrated that parts of the leader and one repeat are required for spacer integration. Further, the leader-proximal repeat serves as template for synthesis of the new repeat [36,40], probably by a strand separation mechanism (Fig. 2). The leader dependence is likely the cause for the observed polar addition of spacers to the CRISPR [2], although there are reported exceptions [32]. The palindromic nature of many CRISPR repeats is important to determine the position and direction of spacer integration into the array [31]. It is indicated that palindromic repeats form cruciform DNA structures that recruits Cas1 and Cas2 (Fig. 2) [31,48], and such structures are known to be a target for Cas1 cleavage [42]. Interestingly, *in vitro* spacer integration can also be performed at other sequences predicted to form cruciform structures, in the absence of repeats [31]. Taken together, spacer integration is directed both by sequence and structure of the CRISPR.

Adaptation has been shown to be coupled to the interference machinery through primed spacer acquisition, which occurs when there is a targeting spacer already present in the CRISPR array. The interference machinery and a pre-existing spacer accelerate the acquisition of subsequent spacers from the same target. Primed spacer acquisition was first described in the Type I-E system in *E. coli* [37], but has subsequently been reported for I-B in *H. hispanica* [34] and I-F in *P. atrosepticum* [39], but so far not in any Type II or III system. Priming seems to occur by slightly different processes in the described cases but the exact molecular mechanisms remain unknown. In Type I-F systems, Cas2 is fused to Cas3 [13], further indicating a direct connection between the adaptation and interference processes. Interestingly, spacers with several mismatches that are incapable of providing protection against the target still induce primed spacer acquisition [49]. It should be noted that although Cas9 is required for spacer acquisition in the Type II-A system, this is not an example of primed spacer acquisition as the requirement is not dependent on a pre-existing spacer against the target [20]. The advantages of primed spacer acquisition are obvious: multiple spacers provide increased resistance against invading DNA, and make it more difficult for target to evolve escape mutants as several sites would need to be changed simultaneously.

4. Expression of CRISPR RNA and *cas* genes

The transcription of the CRISPR-Cas loci to generate a RNA-protein guide complex follows a general theme in most organisms but also displays several type-specific differences (Fig. 3). All systems transcribe the CRISPR locus; process the RNA with Cas ribonucleases and form a CRISPR ribonucleoprotein (crRNP) complex. In some species, *e.g. E. coli*, *Pyrococcus furiosus* and *Sulfolobus* sp., CRISPR transcription initiates in the leader region [26,50–52]. The leader contains promoter elements and binding sites for regulatory proteins, in addition to elements important for spacer integration (see Section 3). A long primary transcript, the pre-crRNA, is generated and may contain a series of secondary structures (hairpins) if the CRISPR contains palindromic repeats. The pre-crRNA is processed into smaller units corresponding to a single spacer flanked by partial repeats. The Cas protein responsible for the processing, and if that protein is part of a complex or not, varies with the subtype. Even when the three CRISPR-Cas types naturally co-exist they do not process each other's pre-crRNA [53].

Overall comparison of the three types of CRISPR-Cas systems shows that Type I and III systems share similarities in pre-crRNA processing as well as in the structures of the crRNP complexes formed (Fig. 3A and C). All Type I and III systems utilize a Cas6 protein for pre-crRNA processing except Type I-C, which employs Cas5d [54]. In Type I-E systems, *e.g.* in *E. coli*, the pre-crRNA is cleaved in a metal-independent mechanism by the Cas6e endoribonuclease [10].

The Cas6e and the crRNA are key components of the *E. coli* Cascade complex, which also contains one copy of Cse1, two copies of Cse2, one copy of Cas5e and six copies of Cas7 [55]. Cascade has a sea-horse shape where the Cas7 proteins provide a helical backbone along which the crRNA is displayed [56] held in place by β-hairpin thumbs extending from Cas7. Cas5e and Cas6e anchor the 5′ and 3′ ends of the crRNA to opposite sides of Cascade [57–59]. One study has demonstrated that Cascade could be assembled in cells lacking crRNA and subsequently loaded with crRNA *in vitro* [60].

The finding suggests that crRNA processing and Cascade assembly do not need to be an integrated process.

In Type III systems, pre-crRNA maturation also involves a sequence-specific processing step mediated by Cas6 [61,62], followed by a ruler-based sequence-unspecific crRNA trimming at the 3′ end to yield mature crRNAs with a defined 5′ end and variable 3′ end [61,63]. Structural analysis of Type III-A (Csm) and Type III-B (Cmr) complexes reveal a helical backbone, similar to that of Cascade, against which the crRNA is aligned. The 5′ end of the crRNA is likely anchored by Csm1-Csm4/Cmr2-Cmr3 and the 3′ end by Csm5/Cmr1-Cmr6 [23–25].

Type II systems employ a very different mechanism for crRNA biogenesis where processing is dependent on host RNase III and a trans-encoded small RNA (tracrRNA) that base pairs with the pre-crRNA [16] (Fig. 3B). In addition, Type II processing also requires the Cas9 protein [16,18] though its exact role is unclear. Another distinct feature of the Type II systems is the 5′ trimming of the crRNA by an unknown nuclease while the crRNA-tracrRNA remains bound to the Cas9 [18].

5. Interference

The principle of target interference by CRISPR-Cas systems is that crRNA bound to Cas protein(s) locates the corresponding protospacer to trigger degradation of the target (Fig. 3). The degradation is performed by specific Cas nucleases [10,17].

In Type I systems Cascade locates the target DNA but the Cas3 nuclease/helicase is needed for interference [10,13,64]. Cas3 can be recruited by Cascade upon target binding or, in the case of Type I-A, be a permanent part of Cascade. In the Type I-A system the Cas3 nuclease and helicase domains are encoded as separate genes [13]. Bioinformatics analysis also suggests a split *cas3* gene for I-B systems [13,65], but experimental data indicate that this is not always the case [34]. However, in all Type I systems the two domains act together to processively degrade the double stranded DNA target [66–68].

In Type I and II systems interference requires the presence of a PAM sequence and perfect protospacer-crRNA complementarity in the so-called seed region, located adjacent to the PAM [69–71]. The presence of a PAM triggers “non-self activation”, which prevents the systems from attacking its own CRISPR locus.

In Cascade, the Cas7 thumbs holding the crRNA kink every sixth base out of position and consequently mismatches at those positions do not affect target binding [49,57–59]. A few additional mismatches outside the seed region are tolerated and do not affect interference [49,69]. Cascade can interact non-specifically with DNA [55] and scans for PAMs and seed regions, with the PAM suggested to be detected by Cse1 [72]. Seed-region base pairing is followed by pairing the rest of the crRNA, leading to displacement of the non-bound DNA strand and formation of an R-loop [55] and also here Cse1 plays an important role [73]. The binding of the crRNA to the target causes conformational changes in Cascade and the target DNA [56,66] that could be the trigger for Cas3 recruitment. Cas3 then nicks the target DNA and proceeds with progressive degradation of the target [66] while Cascade presumably dissociates and is ready for action again.

Type II systems require only the Cas9 protein for interference but unlike Type I and III systems it needs not just crRNA, but also a tracrRNA bound to Cas9 and the crRNA to perform target recognition and degradation [16]. Cas9 structures from *S. pyogenes* and *Actinomyces naeslundii* reveal separate lobes for target recognition and nuclease activity, accommodating the crRNA-DNA heteroduplex in a positively charged groove at their interface [74,75]. The recognition lobe is important for binding crRNA and target DNA, and the nuclease lobe contains the HNH and RuvC nuclease domains that cleave the complementary and non-complementary strands of the target, respectively [74]. As the crRNA-induced reorientation of structural lobes facilitates DNA substrate binding, loading of crRNA is proposed as a key step in Cas9 activation [75].

The Csm complex in Type III-A typically includes six different proteins but the nuclease is not yet identified [23]. The Cmr complex in III-B includes six or seven different proteins [23,24,63] and the Cmr4 protein cleaves the target [76–78]. Early findings indicated that Csm complexes target DNA [62] and Cmr complexes targets RNA [4,25,79], but a more complex picture is now emerging. In *Thermus thermophilus* and *S. thermophilus* the Csm complex targets RNA, and in *T. thermophilus*, which harbors both Type III-A and III-B systems, the Csm and Cmr complexes share crRNA [80,81]. Targeting of RNA and DNA by the same Cmr complex has been demonstrated in *S. islandicus* [82,83]. No PAMs are detected for Type III systems, instead the discrimination between self and non-self is achieved by an extension of crRNA base pairing into the repeat region of host DNA, which results in “self inactivation”, a fundamentally different process to the PAM recognition used by Type I and II systems [84].

An additional aspect of interference has been demonstrated for a Type III-A system in *S. aureus*, where the system is prevented from attacking un-transcribed targets such as lysogenized phages [85]. Such a system makes sense since it prevents potential degradation of the host's own chromosome. The mechanism behind the system is not known and it remains to be determined if this is a widespread feature of CRISPR-Cas systems.

6. Anti-CRISPR mechanisms

Just as cells have developed multiple strategies to counteract viruses, the viruses have developed countermeasures to these strategies. Several distinct mechanisms for counteracting CRISPR-Cas systems have been described. The most basic way for viruses to escape CRISPR-Cas activity is by random mutagenesis that affects key bases in the interaction with the crRNA or the PAM recognition [69,86].

A more refined countermeasure has been discovered in *P. aeruginosa* phages that encode several proteins affecting the activity of Type I-E and I-F systems. The functions of the proteins are not clear but they do not appear to affect expression of Cas proteins or the crRNA. Likely, they interfere with activity of CRISPR-Cas complexes [87,88].

In an unusual turn of events, it has been demonstrated that the CRISPR-Cas system can be used by viruses to promote infection. *Vibrio cholerae* ICP1 phages carry a Type I-F CRISPR–Cas system that targets a host locus, PLE, containing an anti-phage system [89]. After entry of the phage genome into the cell, the viral crRNAs and *cas* genes are expressed to enable infection of the *V. cholerae* host. If the host or the CRISPR is engineered so that the viral CRISPR-Cas system no longer matches the PLE, the ability of ICP1 to infect is largely lost. The few phages that manage to infect do so by having picked up a new spacer targeting the host locus, demonstrating that the viruses can use the full adaptive potential of the CRISPR-Cas system.

7. CRISPR-Cas involvement in processes other than immunity

Although most studies on CRISPR-Cas systems points to its predominant role as protection against invading genetic material, its involvement in other cellular processes such as regulation of virulence, genome evolution and DNA repair is becoming increasingly evident. In most cases the processes behind the alternative functions are not well known and further investigations are required to bring clarity to the matter.

The Cas1 protein of *E. coli* has been demonstrated to process single-stranded and branched DNA species, replication forks and 5′ flaps. Further, Cas1 interacts with RecB, RecC and RuvB suggesting a role in DNA repair, [42] but the RecBCD complex also enhance spacer acquisition [129]. It has also been reported that CRISPR-Cas is triggered by accumulation of misfolded proteins in the membrane of *E. coli*, suggesting a role in handling accumulation of defective proteins [90].

Several examples of CRISPR-Cas involvement in gene regulation have been reported. In *Listeria monocytogenes*, a CRISPR without *cas* genes targets the host chromosome, and the crRNAs increase the level of target RNA by stabilizing it [91]. Another example is *Francisella novicida* where a Type II CRISPR-Cas system mediates repression of an endogenous lipoprotein gene. The repression is required for full virulence of *F. novicida* in mice as the lipoprotein otherwise triggers an innate immune response in the mouse. The repression is achieved by the combined action of Cas9, tracrRNA and a novel RNA, called scaRNA, and is suggested to work by binding a region spanning the target gene's start codon [92]. Expression of the Cas9 protein in *Campylobacter jejuni* lacking CRISPR loci has been demonstrated to increase virulence [93] and in *Legionella pneumophila* Cas2 is required for infection of amoebae in a CRISPR-independent manner [94]. In *P. aeruginosa*, CRISPR is involved in inhibition of biofilm formation. The inhibition requires *cas1*, a lysogenized DMS3 phage and a spacer matching the phage [95].

A CRISPR locus targeting the host chromosome can contribute to genome evolution. While such events are generally lethal, surviving mutants often have large-scale genomic rearrangements. For example, crRNA targeting a pathogenicity island in *P. atrosepticum* yielded mutants with ~100 kb deletions spanning the genomic island [96].

These reports raise interesting questions with regard to the evolution of CRISPR-Cas function. It is not clear if the additional roles developed prior to the function as an immune system, in parallel with it, or from it. Biological processes are usually in a state of flux, so the “additional roles” may be examples of evolution by tinkering.

8. CRISPR applications

CRISPRs were used for applied purposes before their functions in host defense were known, particularly by taking advantage of the heterogeneity of CRISPRs among isolates that are otherwise isogenic. This was the basis for a typing system for *Mycobacterium tuberculosis*, useful for determining diagnosis and epidemiology. An initial PCR-based method [97] was further developed into a hybridization-based method, called spoligotyping, suitable for routine use and high-throughput genotyping [98]. CRISPR typing has also been developed for *Yersinia pestis* [9,99], *Salmonella* [100,101] and *Corynebacterium diphtheriae* [102].

In dairy industry, Danisco (part of DuPont) are marketing starter cultures for improved cheese production and other applications. The cultures contain bacteria that have CRISPRs with improved resistance to phages that can cause production problems. The company realized the importance of CRISPR-Cas early, and was the first to demonstrate its function as an immune system [2].

However, the best known applications come from the development of the Type II system into powerful genetics tools for eukaryotic cells. Particularly the demonstration that crRNA and tracrRNA can be combined in to a single guide RNA (sgRNA) paved the way for this development [18]. Unlike Cas3, which processively degrades the target, Cas9 produces a single double-stranded break in the DNA, an important feature of a gene-editing tool. The method

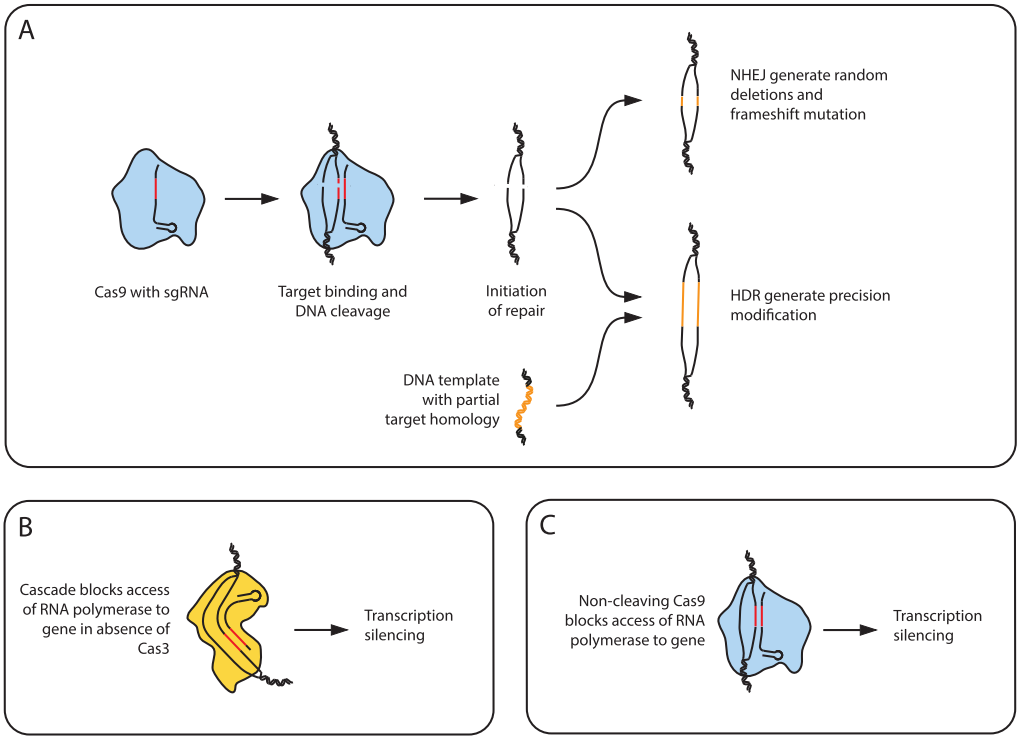

Fig. 5. Examples of basic CRISPR applications. (A) The sgRNA directs Cas9 cleavage of the corresponding target to initiate gene editing. In eukaryotic cells, two main pathways repair DNA damage: Non-Homologous End Joining (NHEJ) and Homology Directed Repair (HDR). NHEJ removes bases, often causing a frameshift and inactivation of the gene. HDR can be used to make specific changes to the target region by providing a designed repair template that becomes inserted in the damaged region. (B) and (C): Gene silencing using Cascade and Cas9. In absence of Cas3, Cascade can be used to block access of RNA polymerase to a gene, without damaging the target. Nuclease-deficient Cas9 can be exploited in a similar manner. sgRNA = single guide RNA.

makes use of DNA repair pathways in eukaryotic cells to provide two ways to make genetic alterations (Fig. 5A). The first relies on Non-Homologous End Joining (NHEJ) that joins the cut ends but in the process often deletes a few bases, which may cripple the gene product, or cause a frameshift that inactivates it. In the second, Homology Directed Repair (HDR) is used to repair the damaged allele using another piece of DNA with homology to the target. By providing a DNA element that can be inserted by recombination, any type of insertion, deletion or change in sequence can be achieved [103,104]. These approaches have previously been demonstrated using ZFNs and TALENs [105], but Cas9 is simple to use and re-target, and can easily be used to modify several targets simultaneously. The main limitation is the need for a PAM adjacent to the target. In the short time that has passed since the initial demonstration, Cas9 have been used for genome editing in virtually all commonly studied eukaryotes, from yeast to humans [106]. In some of those species precision genetic alteration has never been possible before. A suite of Cas9-based methods has been developed, *e.g.* genetic screening [107] and programmable RNA recognition and cleavage [108]. Off-target effects, where Cas9 interacts with an unintended target, are a concern [109] but strategies for prediction and prevention are being developed [110].

The therapeutical potential of Cas9 has been demonstrated. Cas9 has been developed as an antimicrobial agent that can be used to specifically target antibiotic-resistant and/or highly virulent strains of bacteria [111,112]. Gene therapy applications have also been demonstrated by repairing the *cftr* gene in cultured cells from human cystic fibrosis patients [113], by curing dominant cataract disorder and Duchenne muscular dystrophy by altering DNA in mouse germ-line cells [114,115], and by curing hereditary tyrosinemia in adult mice [116]. Cas9 also holds potential for treatment of viral infections, as demonstrated for HIV [117,118] and hepatitis B [119]. A short version of Cas9 have been demonstrated to be deliverable by Adeno-associated virus, greatly facilitating its use in somatic gene therapy [130]. Another important milestone was the first primate with precise genetic modifications [120], a result of gene editing in embryos. The finding allows for development of disease models in animals very similar to humans. A similar approach could be used to alter DNA in human embryos to prevent non-complex hereditary diseases, but also to attempt alteration of complex traits, which has triggered extensive ethical discussion [131].

CRISPR-Cas systems have also been developed for programmable gene regulation. Both Cascade and a nuclease-deficient Cas9 mutant (dCas9) can be used for gene silencing by interfering with RNA polymerase binding or elongation [121–124] (Fig. 5B and C). By fusing dCas9 with a transcriptional activation domain or a repressor, transcriptional activation or repression can be achieved [125–127]. By adding multiple activating domains strong induction can be achieved [128]. Genome-wide application of this approach can be used for screening, as demonstrated by the identification of genes that allow melanoma cancer cells to escape treatment.

9. Outlook

The field of CRISPR research has developed rapidly. Much knowledge has been gained, and the field is attracting an increasing amount of attention. However, for several key questions we have only partial knowledge, particularly with respect to the mechanism of spacer integration. Other outstanding questions are the connection between interference and adaptation in primed spacer acquisition. The reason for the frequent horizontal transfer of CRISPR also remains to be understood, as do the regulation of the CRISPR-Cas systems, and the evolution of the system. Other topics that deserve further attention are the ecological role of CRISPR, countermeasures to CRISPR-Cas systems and the role of CRISPRs in non-immunity processes. Applied use of Cas9 is now routine in many research labs around the world, and its popularity continues to increase. New and better applications will be developed, and in a not-so-distant future we will probably see medicinal use of CRISPR-Cas systems.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgments

This work was funded by Uppsala University, the Swedish Research Council, Bhabha Atomic Research Center, University of Mumbai and the Wenner-Gren Foundations. We thank Eva Berglund for insightful comments on text and figures.

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