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CRISPR-based adaptive immune systems

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CRISPR–Cas systems are recently discovered, RNA-based immune systems that control invasions of viruses and plasmids in archaea and bacteria. Prokaryotes with CRISPR–Cas immune systems capture short invader sequences within the CRISPR loci in their genomes, and small RNAs produced from the CRISPR loci (CRISPR (cr)RNAs) guide Cas proteins to recognize and degrade (or otherwise silence) the invading nucleic acids. There are multiple variations of the pathway found among prokaryotes, each mediated by largely distinct components and mechanisms that we are only beginning to delineate. Here we will review our current understanding of the remarkable CRISPR–Cas pathways with particular attention to studies relevant to systems found in the archaea.

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Introduction

Small RNA-based defense systems that provide adaptive, heritable immunity against viruses, plasmids, and other mobile genetic elements have recently been discovered in archaea and bacteria. The RNA and protein components of these immune systems arise from the CRISPR (clustered regularly interspaced short palindromic repeat) and Cas (CRISPR-associated) genes, respectively. The CRISPR–Cas pathway functions in three phases — adaptation of CRISPRs to invaders, crRNA biogenesis, and invader silencing (Figure 1). It appears that nearly all archaea and approximately half of bacteria are equipped with CRISPR–Cas systems [1–3], which have been shown to provide protection from viral predation and plasmid invasion in both laboratory settings [4,5,6^{••},7[•],8[•],9] and natural environments [10–13].

The discovery of these prokaryotic immune systems has generated considerable excitement, and several excellent

reviews are available [2,14–20]. Here, we describe the components and mechanisms of CRISPR-mediated immunity with emphasis on the systems found in archaea. Advances in understanding the three key steps in the CRISPR–Cas pathway are described, including important contributions from studies done in archaea. Finally, we summarize the significant gaps that remain in our knowledge of the molecular mechanisms of CRISPR–Cas-based invader defense.

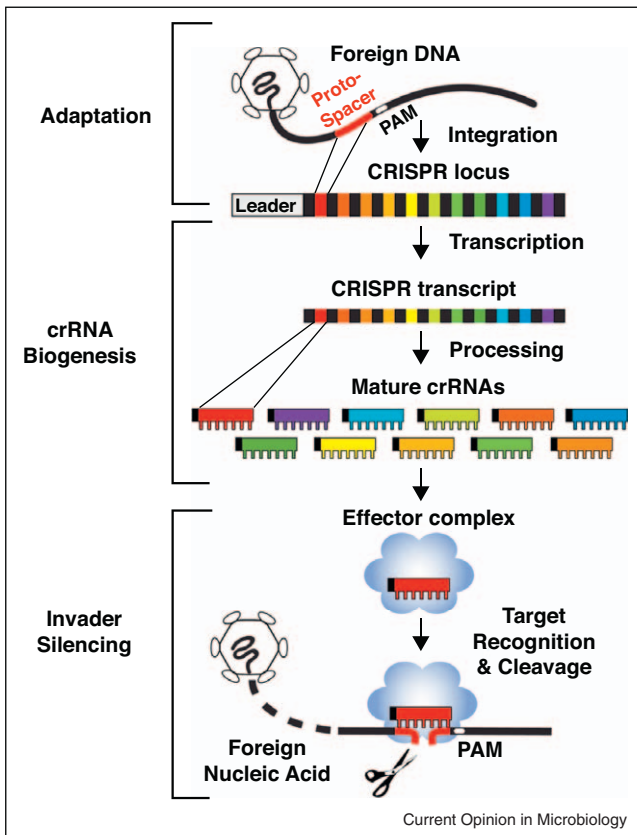
CRISPRs: genetic memory banks of past invasions and source of small invader-targeting RNAs

The hallmark feature of the CRISPR–Cas system is the CRISPR locus (see Figure 1). CRISPR loci are characterized by short, direct repeat sequences (typically 30–40 nts) that separate variable sequences of similar size. There are 12 families of CRISPR repeats based on sequence and predicted secondary structure [21]. The variable sequences (called spacers or guide sequences) are derived from viruses, plasmids, and other invaders [17,22–25] and, remarkably, confer immunity against the corresponding invader [4,5,6^{••},7[•],8[•],9,26^{••}]. CRISPR locus transcripts are processed to generate small crRNAs that contain individual invader-derived sequences and target invading nucleic acids for silencing ([5,9,26^{••},27,28[•]], and see Figure 1). Thus, CRISPRs capture and store fragments of invader sequence and give rise to small RNAs that impart heritable immunity against the invaders.

Cas proteins: hubs of CRISPR–Cas diversity

The cas genes are very tightly linked to CRISPR loci, both physically (location within genomes) and evolutionarily (cosegregation among genomes), consistent with the cofunction of crRNAs and Cas proteins. Over 45 cas gene families have been identified, but a given organism only possesses a subset of these [2,29–31]. A few ‘core’ cas genes (cas1–6) are present in a wide array of organisms [2,29–31]; however, most organisms have only some of these six genes, and only cas1 and cas2 appear to be universal (Figure 2). In a given organism, the core cas genes are supplemented by one or more of the nine sets of subtype-specific cas genes (Figure 2). These sets of two to six genes cosegregate among genomes as distinct cas gene modules. Eight of the modules are named for a prototypical organism where they are the only additional cas genes found [29]. For example, the cas subtype *Aeropyrum pernix* or csa genes are a set of six noncore cas genes found together in *A. pernix* as well as other organisms. The other Cas subtypes include: *Thermotoga* (cst), *Haloarcula* (csh), *Mycobacterium* (csm), *Desulfotribrio* (csd), *Yersinia* (csy), *Escherichia* (cse), and *Neisseria* (csn). Each of these eight

Figure 1

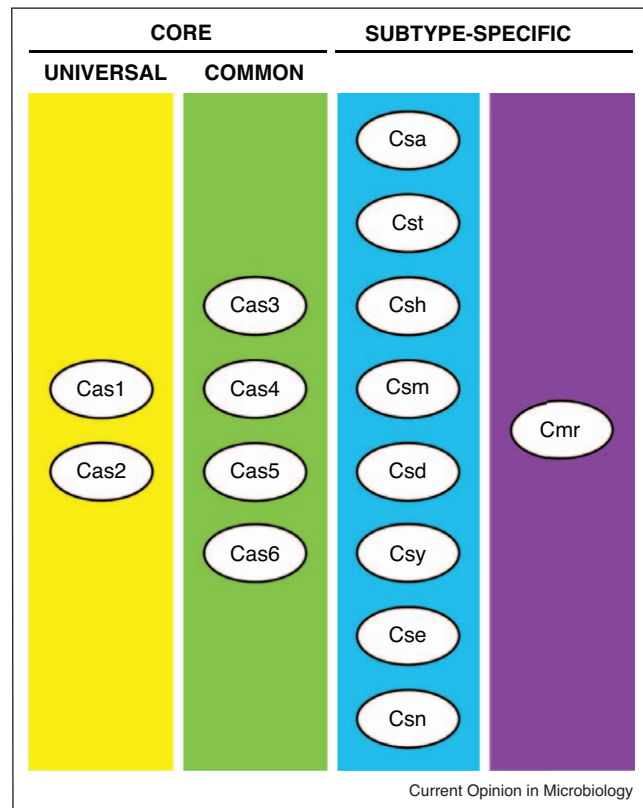


Overview of the CRISPR–Cas invader defense pathway. In the adaptation phase, a short fragment of foreign DNA (protospacer) is acquired from the invader and integrated into the host CRISPR locus adjacent to the leader. Protospacer adjacent motifs (PAMs) are found near invader sequences selected for CRISPR integration. The CRISPR locus consists of short direct repeat sequences (black) that separate similarly sized, invader-derived sequences (multiple colors). In the biogenesis phase of the pathway, CRISPR locus transcripts are processed to release individual mature crRNAs (each targeting a different sequence). Mature crRNAs typically retain some of the repeat sequence, which is thought to provide a recognizable signature of the crRNAs. In the silencing phase, crRNA–Cas protein effector complexes recognize foreign DNA or RNA through basepairing of the crRNA. The Cmr and Csn systems affect cleavage of target RNA and DNA, respectively. PAMs provide important auxiliary signals for the recognition of invaders for some DNA-targeting systems.

modules is associated with particular subsets of the core cas genes [18,21]. The ninth module, cas module RAMP (cmr), is only found in conjunction with other subtype-specific modules [29,31,32]. As more genomes are sequenced, new cas gene modules continue to emerge and relationships between components of the modules are recognized [2]. The limited available information confirms that cas genes are essential for the function of CRISPR–Cas systems [4,5,6,33,34].

The Csa, Cst, Csh, and Csm subtype Cas systems are common in archaea [2,29,31,32]. All of the Cas systems

Figure 2



Combinations of Cas proteins create diverse CRISPR–Cas systems. Cas1–6 are core Cas proteins found in many and diverse organisms. In addition, there are eight primary modules of subtype-specific Cas proteins (consisting of two to six proteins each), and the auxiliary Cmr module. A typical CRISPR–Cas system is composed of the nearly universal Cas1 and Cas2 proteins (yellow), a specific combination of the other core Cas proteins (green) and a set of subtype-specific Cas proteins (blue). A given organism may possess more than one CRISPR–Cas system, and may also have the Cmr module (purple). See Haft *et al.* [29].

are found in bacteria with the exception of Csa, which may be exclusive to archaea. In general, archaea tend to have multiple (or mixed) Cas systems. The diversity of Cas systems found among prokaryotes is illustrated in the cas genes present in some of the currently studied model organisms (Table 1). It is thought that the Cas systems are disseminated by horizontal gene transfer [32,35–38] with the result that closely related species can have completely different systems and highly divergent organisms can have very similar Cas systems.

The diversity of Cas proteins that populate CRISPR–Cas systems would suggest that there are multiple variations of the CRISPR–Cas pathway to be delineated and available information substantiates the expected diversity in the pathways (for example in the targeting of DNA versus RNA, see below). Cas protein sequences indicate potential functions as nucleases, helicases, RNA binding proteins, etc. [29,31]; however, most of the proteins have

Table 1

Core and subtype-specific Cas protein genes in model organisms being employed to understand CRISPR–Cas systems. Information from [1] except for *S. thermophilus* [15]. Cas nomenclature is from Haft et al. [29].

Organism	Core	Subtype-specific	Total#
Archaea			
<i>Pyrococcus furiosus</i> (DSM 3638)	Cas 1, 2, 3, 4, 5, 6	Csa, Cst, Cmr	27
<i>Sulfolobus sulfataricus</i> (P2)	Cas 1, 2, 3, 4, 5, 6	Csa, Csm, Cmr	53
Bacteria			
<i>Escherichia coli</i> (K-12)	Cas 1, 2, 3	Cse	8
<i>Psuedomonas aeruginosa</i> (PA14)	Cas 1, 2/3 fusion	Csy	6
<i>Staphylococcus epidermidis</i> (RP62a)	Cas 1, 2, 6	Csm	8
<i>Streptococcus thermophilus</i> (DGCC7710)	Cas 1, 2, 3, 6	Csn, Csm, Cse	25

not yet been biochemically characterized or assigned functions in the pathways. Exceptions are described below.

Three steps in the CRISPR–Cas invader defense pathway

Cas proteins function in each of the three steps required for CRISPR–Cas system function: firstly, adaptation of CRISPRs; secondly, crRNA biogenesis; and thirdly, invader silencing (Figure 1).

Adaptation: acquisition of new invader sequences in the CRISPR loci

In adaptation, a copy or fragment of invading nucleic acid termed a protospacer is generated and integrated into the CRISPR locus (Figure 1). Protospacers are typically inserted immediately adjacent to the leader sequence at one end of the CRISPR ([4,10,15,24,25]; see Figure 1) providing an approximately chronological record of past infections. Arguably, adaptation is the most unique and fascinating aspect of CRISPR–Cas biology, but there is scant information regarding the molecular mechanism.

Short (3–6 nt) sequence elements found adjacent to the protospacer in the foreign nucleic acid, termed PAMs [14,23,28*], are critical in the generation and/or integration of protospacers into CRISPR loci [4,6**,39]. The PAM is presumably recognized by the adaptation machinery. The trans-acting factors involved in novel spacer acquisition remain largely unknown. A central role for the universal Cas1 in invader DNA cleavage has been suggested based on observed cleavage of dsDNA *in vitro* [40]; however, Cas1 generates ~80 bp DNA fragments without a requirement for a flanking PAM. Compelling genetic evidence implicates Csn2 (also called Cas7) in acquisition in *Streptococcus thermophilus* [4,6**], suggesting that subtype-specific Cas proteins may act with Cas1 in adaptation. Moreover, at least in *Escherichia coli*, there is evidence that Cas1 may function with non-Cas proteins [41]. Mechanisms that limit the size of CRISPR arrays are also now coming to light. Loss of CRISPR length appears to occur via spontaneous homologous recombination between repeat sequences [7*,13,24,39].

CRISPR RNA biogenesis

Biogenesis entails production of numerous individual crRNAs from CRISPR locus transcripts ([5,9,26**,27,28*,33**]), and see Figure 1). ‘RNomic’ (RNA profiling) studies of the small RNAs from two thermophilic archaea, *Archaeoglobus fulgidus* and *Sulfolobus solfataricus*, revealed that CRISPR loci were transcriptionally active and yielded elaborately processed RNAs (before our understanding of CRISPR function) [42,43]. CRISPR loci are predominantly transcribed from promoters located at the leader ends [5,9,26**,27,28*,33**]. In most organisms analyzed thus far, CRISPR RNAs and Cas proteins are constitutively expressed, consistent with an immune system operating in ‘surveillance mode.’ However, there is evidence that the expression of the CRISPR–Cas system of specific *E. coli* strains is highly regulated [44] and that in *Thermus thermophilus*, the presence of an invader triggers elevations in the expression of CRISPR–Cas components [45].

Considerable progress has been made identifying and characterizing the primary crRNA biogenesis enzymes. In several CRISPR–Cas systems, RAMP [31] superfamily Cas proteins catalyze cleavage of CRISPR transcripts. For example, the core Cas protein, Cas6 (from *Pyrococcus furiosus*) [46,47,48*] and subtype-specific Cas proteins, Cse3 (*E. coli*) [5] and Csy4 (*Pseudomonas aeruginosa*) [49*] each recognize specific crRNA repeat sequences/structures and catalyze a single-cut within each repeat which liberates unit crRNAs (known as 1× processing intermediates [47]) containing 5′ and 3′ flanking repeat-derived sequences. The repeat sequences can be further trimmed by unknown mechanisms [26**,33**]. An 8-nucleotide, repeat-derived sequence is retained at the 5′ ends of CRISPR RNAs from several archaea and bacteria [5,9,26**,47] and likely plays an important role as a crRNA ‘identity tag’ that serves as a Cas protein binding site. Detailed biochemical and structural studies of the pre-crRNA biogenesis enzymes have provided a wealth of information on modes of cleavage and recognition of both palindromic and unstructured CRISPR repeat RNAs [46,47,48*,49*,50]. At the same time, a very distinct mechanism of pre-crRNA biogenesis has been found in bacteria with Csn subtype systems. A trans-

acting RNA encoded within the CRISPR–Cas region forms a duplex with the repeat sequence of the pre-crRNA that is processed by RNase III and perhaps Csn1 [33**].

Invader silencing

crRNAs are incorporated into effector complexes and guide the complexes to invading nucleic acid (via base-paired interactions). Silencing can occur at the DNA or RNA level, and DNA targeting requires a PAM in the DNA target for at least a subset of CRISPR–Cas systems [4,5,6**,7*,8*,9,26**,51].

CRISPR–Cas systems that target invader DNA

Evidence indicates that Cse [5], Csn [6**], and Csm [9] subtype systems directly or indirectly target the DNA of invaders. Cleavage of invader DNA has been observed in the case of the Csn system of *S. thermophilus* (but not yet in the others; [9]). It is not known whether Cas systems that target DNA employ silencing mechanisms other than cleavage or can also target RNA (DNA targeting can obscure identification of RNA targeting unless this is accounted for in the experimental design (e.g. as in [9])). The core Cas3 protein has been shown to be essential for invader defense *in vivo* [5], to degrade double-stranded DNA or RNA substrates *in vitro* [52], and likely catalyzes DNA cleavage in several CRISPR–Cas systems. By contrast, the predicted invader DNA nuclease in the Csn system is Csn1 (also called Cas5 [6**] or Cas9 [2]).

Systems to investigate silencing mechanisms *in vivo* have recently been developed in the crenarchaea *S. solfataricus* and *Sulfolobus islandicus* [7*,8*]. Results from studies in which artificial invaders containing sequences recognized by existing CRISPR spacers (i.e. protospacers) were introduced into cells indicate that invaders are targeted at the DNA level, though more work is required to understand which CRISPR–Cas system(s) (Csa, Csm or Cmr) are responsible for the observed activity and whether the targeted DNA is cleaved.

For DNA targeting systems, it is critical that the pathway not target the corresponding spacer in its own CRISPR (which matches the protospacer in the invader). PAMs provide one mechanism for distinction of self vs. nonself. PAMs are critical for silencing by several systems [6**,7*,51], and the PAM recognized by the CRISPR–Cas system in the invader is not present in the repeat sequence that flanks the potential target in the CRISPR. Another mechanism described in *Staphylococcus epidermidis*, which harbors a Csm-type system, is the requirement for mismatches between the target DNA and the 5' repeat tag sequences of the crRNA (which base-pair perfectly with the potential target in the CRISPR) [53**]. Interestingly, it appears that host sequences are occasionally integrated into CRISPR loci resulting in self-targeting

and cell death [54], and when self-targeting crRNAs were expressed from a plasmid-encoded CRISPR locus, *S. solfataricus* host cell survival was associated with homologous recombination that replaced the self-targeting CRISPR locus with the homologous chromosomal copy [8*].

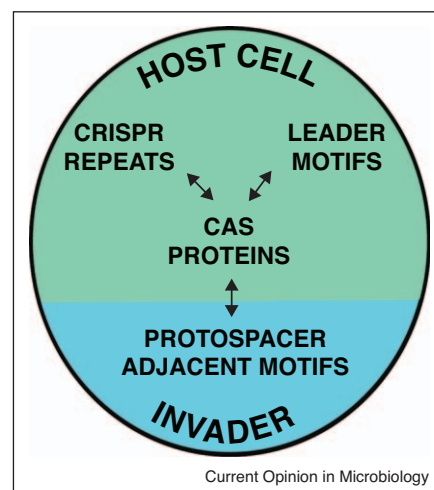
Target RNA cleavage by the CRISPR–Cmr complex

In *P. furiosus*, a complex comprising the six Cmr subtype proteins and mature crRNAs cleaves complementary RNAs (and not DNAs) [26**]. All six Cmr proteins are important for the function of the complex and the crRNAs direct cleavage 14 nucleotides upstream of their 3' ends [26**]. Approximately 70% of archaea and 30% of bacteria with CRISPR–Cas systems have the Cmr module in addition to other Cas systems [32] suggesting that this RNA-targeting branch of the CRISPR–Cas immune system plays an important role in the biological warfare against viruses and other mobile genetic elements.

Coevolving elements of a CRISPR–Cas system

Functional CRISPR–Cas systems include three coevolved components: the leader region of the CRISPR, the CRISPR repeat, and the cas gene collection (Figure 3). As described above, function of the system very likely requires specific interactions between Cas proteins and both the CRISPR leader (e.g. for integration of new invader-derived sequences) and crRNA repeat

Figure 3



CRISPR–Cas systems of various prokaryotes include three coevolved components: CRISPR repeats, CRISPR leaders, and associated Cas proteins that function on discrete PAMs (protospacer adjacent elements) present in the viruses and other mobile genetic elements that they encounter. Known and predicted interactions between Cas proteins and the host CRISPR RNA repeat sequences and leader DNA elements plus invader PAMs are indicated (arrows). The specificity of these RNA–protein and DNA–protein interactions likely contribute to the coevolution of the four components.

sequence (e.g. for crRNA biogenesis and cofunction in silencing; Figure 3), and recent studies indicate that these three elements cosegregate [14,21,28*,32,39]. Thus, sets of core and subtype-specific cas genes are associated with specific leader and repeat sequence families [21,28*,29,32,55]. Likely for the same reason (required Cas protein interaction), CRISPR–Cas systems are associated with specific PAM sequences in the targeted invaders (Figure 3). Extensive analysis of the CRISPR–Cas systems of *Sulfolobus* species, along with available viral and plasmid sequences, demonstrates the relationships between these elements [28*,32,55]. Not surprisingly, it appears that the selective pressure of PAM recognition and silencing by CRISPR–Cas systems is countered by viral evolution of the targeted PAM sequences [51].

Conclusions

Evidence indicates that CRISPR–Cas immune systems play a globally important biological role in host–parasite interactions and collectively shape the evolution and ecology of prokaryotes and viruses [10,11,13,56,57]. The early studies have revealed that there is a diverse series of CRISPR–Cas pathways that function through distinct components and mechanisms, which are dispersed throughout archaea and bacteria. Much of our still very limited knowledge has come from studies with a small set of model organisms that collectively do not encompass the known CRISPR–Cas modules, and further investigation in other organisms will help address this gap. In the near future, the concerted effort of numerous research groups is expected to provide answers to fundamental questions such as how novel protospacers are acquired from invaders and integrated into CRISPRs, what constitutes functional crRNAs and how they are generated, and how silencing is achieved for each of the CRISPR–Cas pathways, and should illuminate the molecular mechanisms governing the astonishing CRISPR–Cas-mediated prokaryotic immune pathways.

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