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Special Focus CRISPR-Cas

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A Sophisticated Defense System: CRISPR-Cas

Six years ago a new sophisticated prokaryotic defense system was identified that was termed CRISPR-Cas (CRISPR, clustered regularly interspaced short palindromic repeats; Cas, CRISPR associated).[1-5] Parts of it, the mysterious repeat sequences, were already detected in 1987[6] but their function remained unknown for the next 20 y. The CRISPR-Cas system is an adaptive, heritable defense system that prokaryotes use to protect themselves against invaders such as viruses.[3-5] Three highly diverse types of CRISPR-Cas that display major functional and structural differences exist (type I–III); and these three major types have been further divided into 10 subtypes, that also show considerable differences (subtypes IA-IF, IIA-B, IIIA-B).[7] The key elements of these defense systems are the Cas proteins and the CRISPR RNA. The CRISPR RNA consists of short repeat sequences interspersed with spacer sequences derived from invader DNA.

Since its discovery, much has been learned about this system: we now know that there are a variety of CRISPR-Cas subtypes[7] and that the Cas proteins may have multiple functions.[8] Biotechnological applications were implicated early after the discovery of this system,[4] and a new avenue of potential applications was recently reported.[9-18] However, our understanding of this system is far from complete. In this special issue, the state of this system is summarized in three reviews[19-21] and three commentaries,[22-24] and new data are reported in 19 research articles.[25-43]

The evolution of this system and its components is described in the review by Koonin et al.[19] They describe an interesting connection between the CRISPR-Cas system and the toxin-antitoxin systems.

New Details About the Archaeal Defense Systems

Although 90% of all archaeal genomes contain a CRISPR-Cas system, this defense system has been studied in only a handful of archaea. This special issue features two reviews and six original articles about archaeal CRISPR-Cas systems. Manica et al. summarize the current knowledge about the archaeal CRISPR-Cas system in *Sulfolobales*.[20] *Sulfolobus* is the only Crenarchaeon in which the CRISPR-Cas system has been studied, it comprises a complex CRISPR system with several Cas modules. New details regarding the *Sulfolobus* system are reported in the research article by Peng et al.[38]

A detailed overview of the CRISPR-Cas systems and genetic elements in the hyperthermophilic euryarchaea is provided by Norais et al.[21] New data concerning the hyperthermophilic archaeon, *Thermococcus kodakarensis*, a genetically tractable organism that allows for in vivo studies, are reported by Elmore et al.[30] Two original studies provide previously unidentified aspects about the archaeal CRISPR-Cas systems in *Methanosarcina mazei* and *Haloferax volcanii*.[34,36] Nickel et al. describe the I-B and III-B system in *M. mazei*.[36] They identified the Cas6 proteins for both modules and showed that crRNA generation is induced at high salt concentrations. The requirements for an efficient interference reaction of a type I-B system in *H. volcanii* are reported by Maier et al.[34] They show that the *Haloferax* I-B system requires a seed sequence for efficient interference, similar to what has been described for the *E. coli* I-E system. Using a plasmid-based invader methodology, the authors show that not all crRNAs are effective at triggering the degradation of invader plasmids. In addition, they report that the interference does not seem to be influenced by the copy number of the invader plasmid.

The Cascade effector complex that is involved in target DNA cleavage in type I systems, has been studied in detail in *E. coli*.[44] Malcolm White and his group recently provided details about the archaeal Cascade complex,[45] and in this issue they report the structure of an additional subunit of the archaeal Cascade complex, the Csa5 protein, that interacts weakly with the core complex proteins (Cas5 and Cas7).[39] Their structural analysis indicates that the small subunits of the CRISPR effector complexes have a shared evolutionary history.

New data regarding the archaeal CRISPR-RNA processing endonuclease, the Cas6 protein, are reported by the Randau group.[40] The authors show that the spacer sequence and length may influence the processing of spacer-crRNA-spacer molecules by the Cas6 protein from *Methanococcus maripaludis* (type I-B).

Cyanobacteria and Their Complex CRISPR-Cas Systems

The complex CRISPR-Cas systems of cyanobacteria are described in two original reports.[27,31] Hein et al. compared and analyzed the CRISPR-Cas systems of two closely related cyanobacteria.[31] Both strains examined contain three distinct CRISPR arrays: one is conserved between the strains, but the other two systems differ significantly from each other between the strains. Additionally, Hein et al. found that the subtype I-D system is negatively regulated by a transcriptional repressor and that accumulation of crRNAs is influenced by environmental factors.

A systematic study of the CRISPR/Cas systems in cyanobacteria is presented by Kerfeld and colleagues who investigated the 126 cyanobacterial genomes currently accessible in public

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databases.[27] The authors show that most cyanobacteria contain a CRISPR-Cas system, and many have several different CRISPR-Cas modules. In addition, data are presented that suggest that the absence of the *cas1/2* genes might be indicative of the first step in the complete loss of the CRISPR-Cas system. The authors also report numerous new repeat sequences that are not currently present in the Rfam database.

Regulation of CRISPR-Cas Expression

The regulation of CRISPR expression is reported in the manuscript by Pul and colleagues.[25] Pul's group has pioneered studies on the transcriptional activation of the *E. coli* CRISPR system and reports here that the activation of Cascade gene transcription is necessary but not sufficient to induce CRISPR-mediated immunity. The reported results suggest a complex regulation of the type I-E CRISPR-Cas system in *E. coli*.

Additional Functions of Cas Proteins

A new function for a Cas protein outside of the CRISPR-Cas system is reported for Cas3 by Ivančić-Baće et al.[32] The authors report that ectopic expression of Cas3 leads to an increase in the ColE1 plasmid copy number. The authors show that this increase in copy number depends on the helicase activity but not the nuclease activity of Cas3. This work provides evidence that Cas3 may have regulatory roles in the cell in addition to its involvement in CRISPR-Cas.

The Dynamic Type II System

Two articles in this issue describe new discoveries that have been made regarding type II systems.[28,33] The CRISPR-Cas type II system is the minimal system in which the Cas9 endonuclease targets the invader DNA guided by the crRNA and tracrRNA with the help of RNase III. Applications using type II systems to downregulate gene expression and perform genome editing have recently been published.[9-18] The two articles about the type II systems in this issue represent significant contributions to the further development of novel molecular tools for genome editing and other applications. Charpentier's group identified new CRISPR-Cas type II systems in many bacteria by searching for Cas9 protein homologs in the available bacterial genomes.[28] They clustered the Cas9 proteins into three groups. Novel tracrRNAs were predicted for most of the novel Cas9 proteins. A biochemical analysis of the Cas9 protein from *Streptococcus thermophilus* is described in the manuscript by Karvelis et al.[33] They report data that further specify the molecular basis for crRNA-based programming of Cas9 for genome editing applications.

Motifs for Spacer Acquisition and Target Recognition

Our current understanding of protospacer-adjacent motifs is summarized and discussed in the commentary by Garrett and colleagues.[23] The PAM sequences have been reported to be essential for the processes of spacer acquisition and the interference reaction, which employ different molecular mechanisms. Because there is increasing evidence to suggest that the recognized sequence motifs are overlapping but unlikely to be identical, they propose a new nomenclature for the protospacer-adjacent motifs. They suggest that the term protospacer-associated motif (PAM) should be used for the conserved DNA sequence, and the terms "spacer acquisition motif" (SAM) and "target interference motif" (TIM) should be used to refer to the acquisition and interference recognition sites, respectively.

News About the Adaptation Process

New aspects of the adaptation process are reported by Díez-Villaseñor et al.[29] Adaptation is the first step in the immune defense reaction, and it is also the step that is the least understood. Díez-Villaseñor et al. report the establishment and application of a new experimental system to study spacer acquisition in *E.coli*, which is based on positive selection and allows for the fast identification of spacer acquisition events. They used this tool to confirm the critical requirement for the Cas1 and Cas2 proteins in spacer acquisition.

Two articles report new findings regarding spacer acquisition in the type I-E system of *E. coli*.[8,41] Savitskaya et al. used a plasmid-based interference system to trigger spacer acquisition and used high-throughput sequencing to investigate the newly acquired spacers.[41] Their results indicate that spacer uptake has a strong preference for the protospacer-adjacent motif AAG and is inconsistent with a sliding mechanism of the acquisition machinery along the target DNA as the primary mechanism for strand bias during primed spacer acquisition.

Westra et al. report on spacer acquisition from a conjugative plasmid.[43] They found that protospacer selection is determined by the plasmid's mobilization type.

A new tool for the prediction and analysis of CRISPR targets is presented in the paper by Biswas et al.[26] The program developed by the authors predicts the most likely targets of CRISPR RNAs and can be used to discover targets in newly sequenced genomic or metagenomic data.

In their technical paper, the Marraffini group reports the use of a CRISPR decoy as a tool to disrupt CRISPR immunity.[35] This tool is based on the successful use of miRNA sponges to inactivate miRNAs. The CRISPR decoy can likewise knockdown the CRISPR-Cas system for a single specific invader or for several invaders.

The Unknown Invader: Prokaryotic Viruses

Viruses play major roles in promoting gene transfer and controlling microbial populations.[46] Unfortunately, we know very little about prokaryotic viruses: It is estimated that only 1% of all viruses are known and have been sequenced. We know even less about viruses that infect archaea. This issue contains two articles reporting studies with archaeal viruses and infections. The research article by Sencilo et al. describes the comparison and annotation of 10 recently isolated haloarchaeal virus genomes.[42] Many genes and genomic features were identified that are shared

with tailed bacteriophages. This is consistent with the hypothesis that haloarchaeal viruses and bacterial tailed viruses share common ancestry and that a viral lineage containing archaeal viruses, bacteriophages and eukaryotic viruses predates the division of the three major domains of non-viral life. However, archaeal viruses also contain a considerable number of predicted genes of unknown function.

Okutan et al. report changes in the transcriptome of *Sulfolobus* cells upon infection with a virus.[37] They showed that the earliest expressed genes were located mainly at the termini of the linear viral genome, and the genes expressed later were concentrated in the central region of the genome. Seventy-two host genes were downregulated, and 76 host genes were upregulated during infection. The altered transcriptional patterns suggest that the virus reprograms the host cellular machinery to facilitate its own DNA replication and to inhibit cellular processes required for defense against viruses.

Wiedenheft comments on a recently published *Nature* article[47] that showed that phages invented a new tool in the arms race by encoding anti-CRISPR proteins.[24] The author discusses viral suppressors of CRISPR (VSC) and compares viral defense mechanisms in various systems, including eukaryotes.

The commentary by Mick et al. discusses CRISPR biology in the human gut microbiome.[22] The observation that older spacers in the CRISPR locus are conserved between bacteria present in unrelated, geographically separated individuals is discussed as is the regulatory role of CRISPR-Cas in the interplay between bacteria and lysogenic phages.

The collection of articles in this issue presents the current knowledge of this defense system. However, there is still much to learn about the CRISPR-Cas system. The mechanism of the adaptation process and many of the molecular details of the other steps are unknown. Future research into the CRISPR-Cas system promises many exciting new discoveries.

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