Type III CRISPR/Cas System: Introduction and its Application for Genetic Manipulations

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https://doi.org/10.21775/cimb.026.001

Abstract

Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) genes provide adaptive immunity against invasion of foreign nucleic acids in archaea and bacteria. The system functions in three distinct stages: adaptation, biogenesis, and interference. CRISPR/Cas systems are currently classified into at least five different types, each with a signature protein among which Type III systems exhibit a dual DNA/RNA interference activity. Structures of a few Type III surveillance complexes have been determined: they are composed of several different subunits and exhibit striking architectural similarities to Type I surveillance complexes. Here, we review the genetic, biochemical, and structural studies concerning CRISPR/Cas Type III systems and discuss their application for genetic manipulations, including genome engineering and gene silencing.

Introduction

Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) genes encode the adaptive immune system in prokaryotes, which protects the host against invasion by foreign mobile genetic elements such as virus and plasmid DNA (Barrangou *et al.*, 2007; Brouns *et al.*, 2008; Makarova *et al.*, 2011). CRISPR/Cas systems are widespread and are found in about 90% of archaeal and 40% of bacterial genomes (Grissa *et al.*, 2007; Kunin *et al.*, 2007). In the current classification based on signature protein families and gene synteny of *cas* gene loci, CRISPR/Cas systems comprise two main classes (Classes I and II), which are further divided into at least five different types. Whereas Class I systems (Type II and V) utilize a single Cas protein such as Cas9 to confer immunity, Class I systems employ multiple Cas proteins that form an effector complex to perform the same function (Makarova *et al.*, 2015; Shmakov *et al.*, 2015).

All known CRISPR/Cas systems function in three distinct stages: adaptation, biogenesis, and interference. First, immunity is acquired in a prokaryotic host by integrating short DNA fragments from the genome of an invading virus or plasmid DNA into a CRISPR locus, immediately after the first repeat (Cady *et al.*, 2012; Erdmann *et al.*, 2014; Li *et al.*, 2014; Liu *et al.*, 2015; Richter *et al.*, 2014; Yosef *et al.*, 2012). The CRISPR is transcribed as premature RNA and processed into short mature crRNA by distinct mechanisms, reflected in the diversification of CRISPR/Cas into various subtypes. In Type I and Type III systems, Cas6 family proteins (Carte *et al.*, 2008) or alternatively Cas5d (Garside *et al.*, 2012) catalyses processing within the repeats, and in Type II systems, a trans-acting small RNA directs the pre-crRNA processing by

endoribonuclease III and Cas9 within the repeats (Deltcheva *et al.*, 2011). At the interference stage, crRNAs in the effector complexes guide foreign nucleic acids recognition and confer target destruction. A number of recent review articles have been published for other CRISPR/Cas systems and their applications, with emphasis on Type II system (Jamal *et al.*, 2016; Mojica and Montoliu, 2016; Wright *et al.*, 2016). Here we present an overview of the functions of Type III systems and introduce their possible applications in genetic manipulations.

Functions of Type III CRISPR/Cas systems

Classification of Type III CRISPR/Cas systems

The composition and organization of type III CRISPR/Cas loci are diverse, suggesting that these systems could have undergone gene duplications and deletions, domain insertions and fusions during evolution. In addition, a distinct class of Cas-accessory proteins carrying poorly characterized domains have been identified, which could either be involved in crRNA–effector complex functions or in associated immunity (Makarova *et al.*, 2015). Type III CRISPR/Cas systems are characterized by the presence of the signature gene *cas10*, coding for a multidomain protein such as a HD domain and a Palm domain (a variant of the RNA recognition motif) both of which are involved in target nucleic acids interference. Type III CRISPR/Cas systems were first classified into two subtypes: III-A (previously known as Csm module or Mtube subtype) and III-B (also known as Cmr or Cas RAMP module), each of which has a signature protein, i.e. Csm2 for III-A and Cmr5 for III-B subtypes (Makarova *et al.*, 2011). The interference module Type III-A Csm loci usually contain *cas1*, *cas2*, and *cas6* genes, whereas many Type III-B Cmr loci lack these genes (Fig. 1.1), suggesting that they probably share certain functions with other CRISPR/Cas systems in the same genome, such as CRISPR processing and spacer acquisition (Makarova *et al.*, 2013).

Two type III variants (one from subtype III-A and one from subtype III-B) are upgraded to subtypes III-D and III-C, respectively (Makarova *et al.*, 2015; Vestergaard *et al.*, 2014). Both Type III-C and Type III-D lack the *cas1* and *cas2* genes; Type III-C loci encode a Cas10 with inactivated cyclase-like domain, and Type III-D loci typically encode a Cas10 that lacks the HD domain (Makarova *et al.*, 2015) (Fig. 1.1). All type III loci encode small subunit proteins, including one Cas5 protein (Csm4 in Type III-A; Cmr3 in Type III-B and C; and Csx10 in Type III-D) and typically, several Cas7 paralogue proteins (Csm3 and Csm5 in Type III-A; Cmr1, Cmr4, Cmr6 in Type III-B, and III-C; Csm3 in Type III-D) (Makarova *et al.*, 2015; Vestergaard *et al.*, 2014).

crRNA processing

CRISPR arrays are transcribed into long precursors containing spacers and repeats, and are processed into mature small CRISPR RNAs (crRNAs) that act as guides for a targeting complex, which cleaves the genetic elements of the invading virus or plasmid. Biogenesis of mature crRNAs requires a primary cleavage within the repeat sequences to generate intermediates and secondary maturation of these intermediates by additional cleavage. In Type III CRISPR/Cas systems, pre-crRNA processing is initiated by Cas6 endoribonuclease within the repeat sequences, thus generating crRNAs containing complete spacer sequences flanked by an 8-nt repeat sequence at the 5′ end and the rest of the repeat at the other end (Carte *et al.*, 2008; Hatoum-Aslan *et al.*, 2011). In the *Staphylococcus epidermidis* Type III-A system, Cas10 and Csm4 in addition to Cas6 were required for primary processing, to produce an intermediate crRNA of 71 nt. Additional nucleolytic cleavage of the 3′ end generates smaller mature crRNAs and the length of mature crRNA was determined by the 5′ primary processing site (Hatoum-Aslan *et al.*, 2011). Furthermore, Csm2, Csm3, and Csm5 could mediate crRNA maturation following primary processing (Hatoum-Aslan *et al.*, 2011). The crRNA from the *S. solfataricus* Csm complex showed a conserved 8-bp repeat derived sequence at the 5′ end and further processed sequences of 3 bp from the repeat sequences at the 3′ end of the mature crRNA (Rouillon *et al.*, 2013). Similarly, in the *S. solfataricus* P2 Type III-B system, 3′ termini of the crRNA extracted from the Cmr complex showed more variables that displayed

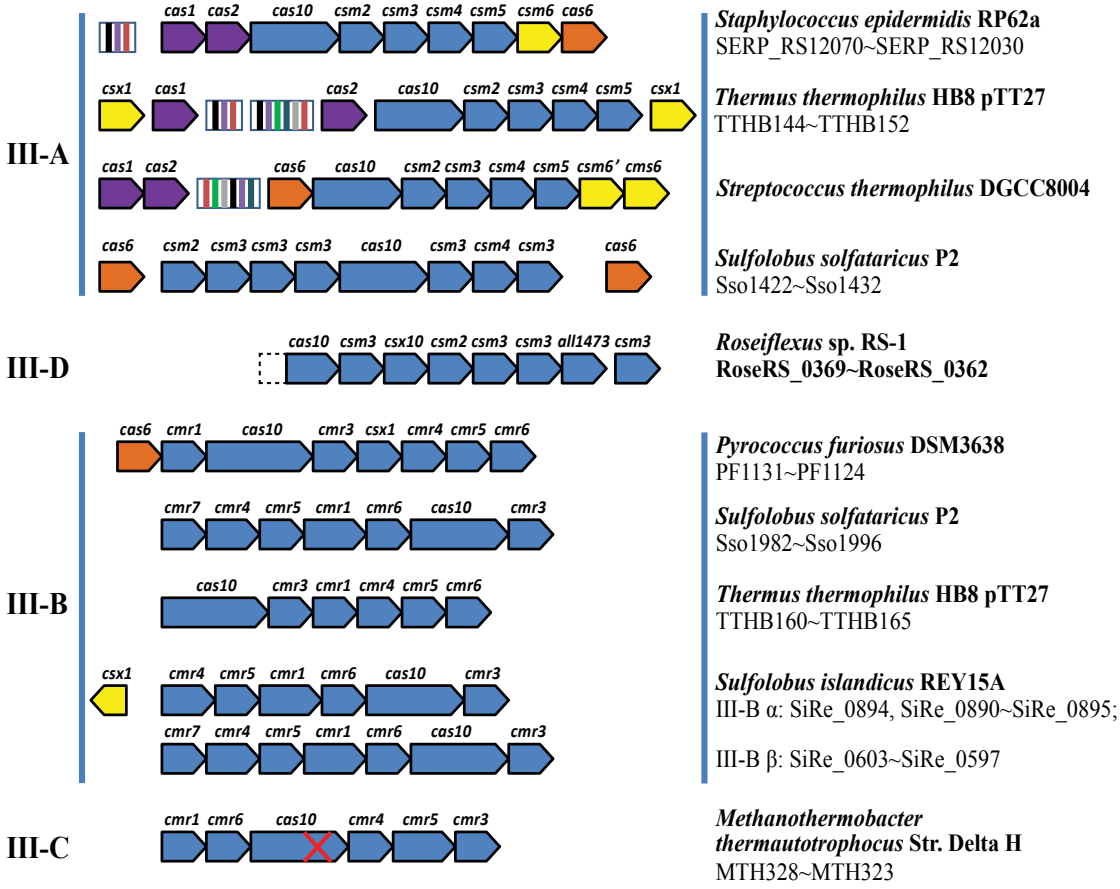

Figure 1.1 Architecture of the genomic loci for the typical Type III CRISPR/Cas systems. Gene organization is shown for the typical subtypes of Type III systems. The representative genomes and the corresponding gene locus tag names are indicated for each subunits, except for the *Streptococcus thermophilus* DGCC8004 strain. Rectangles represent the CRISPR arrays. Genes encoding the adaptation modules are in purple, genes encoding the components of the interference complex are in blue, and the genes encoding the nuclease for CRISPR RNA maturation are in orange. Type III-C loci encode Cas10 with an inactivated cyclase-like domain, and Type III-D loci typically encode Cas10 that lacks the HD domain.

a short handle, or had very few repeat-derived sequences (Zhang *et al.*, 2012). In contrast, crRNA isolated from the *S. solfataricus* P2 Cascade complex still includes the 3′ repeat derived sequence (Lintner *et al.*, 2011), suggesting that crRNAs were differently processed by the Cascade or Cmr complexes. All Type III Csm- or Cmr-bound crRNAs have a variable 3′ end suggesting that processing requires a nuclease for trimming these crRNAs (Hale *et al.*, 2009; Hatoum-Aslan *et al.*, 2011; Rouillon *et al.*, 2013; Staals *et al.*, 2013, 2014; Zhang *et al.*, 2012). Genetic analysis demonstrated that *S. islandicus cmr1α*, *cmr2α*, and *cmr3α* mutant strains lacked the smallest mature crRNA (~38 nt), and that the *cas6* mutant strain lacked both the ~38 nt and ~45 nt mature crRNA, suggesting the presence of secondary processing synergy between Cas6 and Cmr-α interference proteins (Deng *et al.*, 2013).

RNA cleavage activity

CRISPR crRNA-guided RNA cleavage was first reported for a purified native effector complex of the *Pyrococcus furiosus* Type III-B Cmr system (Hale *et al.*, 2009). The *P. furiosus* Cmr complex contains Cmr1, Cmr2 (Cas10), Cmr3, Cmr4, Cmr5, and Cmr6 subunits (Hale *et al.*, 2009). A distinct Type III-B Cmr complex has been purified from *S. solfataricus*, and this effector complex contains seven

subunits including Cmr1 to Cmr6, most of which show significant sequence similarity to the corresponding subunits of the *Pyrococcus* Cmr complex and an additional Cmr7 subunit (Zhang *et al.*, 2012). The *Thermus thermophilus* Cmr complex is composed of six different subunits and one crRNA with a stoichiometry of $Cmr1_1 2_1 3_1 4_4 5_3 6_1$:$crRNA_1$ (Staals *et al.*, 2013). The purified Cmr–crRNA complex cleaves complementary RNA but not single-stranded DNA (Hale *et al.*, 2009; Staals *et al.*, 2013; Zhang *et al.*, 2012). The riboendonuclease activity against the RNA complementary to Cmr-bound crRNA requires Mg^{2+} for *T. thermophilus* Cmr complex (Staals *et al.*, 2013) and Mn^{2+} for *S. solfataricus* Cmr complex (Zhang *et al.*, 2012).

The target RNA cleavage by Type III systems shows two distinct patterns. The *S. solfataricus* Cmr complex cleaves target ssRNA complementary to the crRNA at UA sites (Zhang *et al.*, 2012). However, the sizes of the cleavage products were found to be at 6-nt intervals, which has been identified *in vitro* in *T. thermophilus* Type III-A (Staals *et al.*, 2014), *S. thermophilus* Type III-A (Tamulaitis *et al.*, 2014), *P. furiosus* Type III-B (Hale *et al.*, 2014; Osawa *et al.*, 2015; Zhu and Ye, 2015), and *Thermotoga maritima* Type III-B (Estrella *et al.*, 2016), with the substrate initially cleaved at its 3′ end, followed by additional cleavage towards its 5′ end (Staals *et al.*, 2013). Both UA cleavage pattern and 6-nt interval pattern were found *in vivo* in *S. islandicus* Type III-B system (Peng *et al.*, 2015). *T. thermophilus* Cmr complex cleaves ssRNA target initially at its 3′ end, followed by additional cleavage towards to its 5′ end (Staals *et al.*, 2013). In *T. maritime*, target ssRNA cleavage by the Cmr–crRNA complex requires an intact 5′ handle from the repeat sequences of crRNA that cleaves the target at 6-nt intervals (Estrella *et al.*, 2016). In this system, the target was cleaved sequentially, starting predominantly at one site and then proceeding to other sites, and the 3′ end of the crRNA limited the cleavage boundary (Estrella *et al.*, 2016). *S. islandicus* REY15A encodes two type III-B systems (Cmr-α and Cmr-β) and the cleavage patterns clustered into two distinct groups: those with UA–like cleavage resulted from Cmr-β activity, and those of the two defined positions at the 6-nt interval produced by Cmr-α (Peng *et al.*, 2015).

RNA cleavage by the Cmr and Csm complex is not strictly dependent on complete complementarity between the crRNA and the RNA target *in vitro* (Estrella *et al.*, 2016; Staals *et al.*, 2014; Tamulaitis *et al.*, 2014) and *in vivo* (Peng *et al.*, 2015), and the matching or mismatching of 5′ repeat crRNA tag to the target RNA shows no difference in RNA targeting by the *S. epidermidis* Csm complex (Samai *et al.*, 2015). However, spacer mutagenesis identified a trinucleotide seed sequence in the 3′ region of the crRNA that was crucial for RNA targeting (Peng *et al.*, 2015).

The Cas7 family protein Cmr4 or Csm3 act as the catalytic subunit in the Cmr or Csm complex for target RNA cleavage. The structural model (see below) indicates that the Cmr4 helical backbone intercalate between the segments of duplexed crRNA:target RNA, thus distorting the crRNA:target RNA duplex after every 5-bp segment (Osawa *et al.*, 2015; Taylor *et al.*, 2015). The Cmr4 D26A and K46A mutants in *P. furiosus* Cmr complex impaired the target RNA cleavage activity (Benda *et al.*, 2014; Ramia *et al.*, 2014a). The effect of the Cmr4 K46A mutation on target RNA cleavage was accompanied by a loss of crRNA association/Cmr complex formation, indicating that K46 plays a critical role in crRNA interaction (Ramia *et al.*, 2014a). In contrast, the Cmr4 D26A mutation resulted in a specific loss of target RNA cleavages without loss of crRNA association/Cmr complex assembly or target RNA association, indicating that Cmr4 D26 is directly important for target RNA cleavage (Benda *et al.*, 2014; Ramia *et al.*, 2014a). The Csm complex shares similar structure with Cmr complex and the Csm3 subunit was inferred to form the helical backbone (see below). Single residue alanine replacement mutagenesis identified Csm3 is the ribonuclease in Csm complex, and the conserved D33 residue in *S. thermophilus* Csm complex (Kazlauskiene *et al.*, 2016; Samai *et al.*, 2015; Tamulaitis *et al.*, 2014) or D32 residue in *S. epidermidis* Csm complex (Samai *et al.*, 2015) is part of the catalytic site. Although Csm6, a subunit containing a Higher Eukaryotes and Prokaryotes Nucleotide-binding (HEPN) domain, was not found to be part of the Type III-A Cas10–Csm complex (Hatoum-Aslan *et al.*, 2013; Samai *et al.*, 2015; Staals *et al.*, 2014; Tamulaitis *et al.*, 2014), it is required for degradation of phage transcripts with nuclease activity-dead Csm3 that enables Type III-A CRISPR/Cas immunity that targets late expressed genes (Jiang *et al.*, 2016).

DNA cleavage activity

The DNA cleavage activity against cognate target DNA is first identified by genetic analysis. The *S. epidermidis* Type III-A system prevents plasmid conjugation and transformation, indicating that this system possess a DNA targeting activity (Marraffini and Sontheimer, 2008, 2010). The Type III-B DNA cleavage activity was identified by plasmid challenging assay in the archaeon *S. islandicus* REY15A, which encodes two Type III-B interference modules (Deng *et al.*, 2013) (Fig. 1.1). In their study, the challenging plasmid contained a protospacer which lacks the CCN PAM for recognition and cleavage by the Type I-A system and will be transcribed so that the produced transcripts could anneal to crRNA and be susceptible to RNA cleavage. It was assumed that if the protospacer transcript was targeted and cleaved by one or both type III-B interference modules, the transformation efficiency of the plasmid construct would not be impaired significantly. However, the challenging plasmid showed low transformation levels, suggesting the interference of plasmid DNA. Sequence analysis of the surviving transformants revealed that some transformants had mutations located in four different genes exclusive to the *cmr-α* gene cassette (*cmr1α*, *cmr2α*, *cmr3α* and *cmr5α*), and half of the transformants carried an identical 98 kb deletion between the CRISPR loci and the *cmr-α* gene cassette containing two CRISPR-related genes *csx1* and *csm6*; complementation with *csx1* restored interference activity in the deletion mutant (Deng *et al.*, 2013). Thus, *S. islandicus* Cmr-α constitutes the first CRISPR system that exhibits dual targeting of RNA and DNA with a single spacer (Deng *et al.*, 2013; Peng *et al.*, 2015).

The presence of protospacer adjacent motifs (PAMs) on the targets, licenses the DNA targeting in Type I and II systems (Gasiunas *et al.*, 2012; Westra *et al.*, 2013), while the 5′ handle derived from the repeat sequence is the signal for distinguishing self CRISPR DNA to avoid autoimmunity by the Type III system (Deng *et al.*, 2013; Kazlauskiene *et al.*, 2016; Manica *et al.*, 2013; Marraffini and Sontheimer, 2010). Moreover, *Sulfolobus* Type III-B systems require no PAM sequences to confer DNA interference (Deng *et al.*, 2013; Manica *et al.*, 2013). Like their RNA cleavage activity, the DNA cleavage activity of the *Sulfolobus* Cmr complex is not strictly dependent on complete complementarity between the crRNA and the DNA target (Manica *et al.*, 2013). Recently, transcription-dependent DNA targeting by the *P. furiosus* Type III-B Cmr system was found to require a functional rPAM (RNA protospacer-adjacent motif), including NGN, NNG, and NNA, in addition to the crRNA target sequence (Elmore *et al.*, 2016).

Cas10 family proteins are the signature and largest subunits in the Type III complex, and were initially considered to be a nuclease responsible for cleaving target DNA because of their ssDNA-specific cleavage activity (Jung *et al.*, 2015; Ramia *et al.*, 2014b). *P. furiosus* Cas10 alone showed Ni^{2+}-dependent ssDNA nuclease activity *in vitro*, and this activity was significantly attenuated in the Cmr complex *in vitro* (Elmore *et al.*, 2016). Both HD nuclease and Palm domains of the *P. furiosus* Cas10 subunit were required for *in vivo* plasmid DNA cleavage in a transcription-dependent manner (Elmore *et al.*, 2016); however, the GGDD motif in the Palm domain of Cas10 is required for DNA cleavage but not the HD domain in the *S. epidermidis* Type III-A system (Hatoum-Aslan *et al.*, 2014; Ramia *et al.*, 2014b; Samai *et al.*, 2015). Moreover, it was found that the GGDD motif in the Palm domain is involved in binding complementary RNA targets in the *T. maritime* Type III-B Cmr system, that activates ssDNA specific nuclease activity in the HD domain of Cas10 subunit (Estrella *et al.*, 2016). However, only the HD nuclease domain of Cas10 was essential for the DNA cleavage activity of *S. thermophilus* Type III-A Csm complex (Kazlauskiene *et al.*, 2016). The *S. solfataricus* Type III-D, a Cmr7 subunit-containing variant of III-B previously denoted as belonging to Type III-A, cleaves cognate RNA targets with a ruler mechanism *in vitro*, and cleaves plasmid DNA depending on both the cyclase and HD nuclease domains of the Cas10 subunit and independently in the presence of a cognate target sequence *in vitro* (Zhang *et al.*, 2016).

The *in vitro* DNA cleavage activity of Csm or Cmr complex requires target ssRNA. Binding of a target ssRNA activates the *in vitro* ssDNA targeting activity of the *T. maritime* Cmr complex (Estrella *et al.*, 2016) and the *S. thermophilus* Csm complex (Kazlauskiene *et al.*, 2016), and the dsDNA cleavage activity of the *P. furiosus* Cmr complex (Elmore *et al.*, 2016). Cleavage of the ssRNA target and dissociation of the resulting fragments then prevent

DNA cleavage; in addition, with uncleavable RNA targets, the complex gained stronger ssDNA cleavage activity *in vitro* than that of the wild type in *T. maritime* Type III-B system (Estrella *et al.*, 2016). Using an RNA cleavage activity-dead *S. thermophilus* Csm complex, 100% of the ssDNase activity was retained even after 70 min of pre-incubation, suggesting that the Csm complex is trapped in the ssDNase-active state (Kazlauskiene *et al.*, 2016). The ribonucleoprotein of the *S. epidermidis* Type III-A Cas10–Csm complex cleaved DNA at the 3′ flanking side of the target in a transcription-dependent manner in an *E. coli in vitro* transcription system (Samai *et al.*, 2015). Moreover, it was found that the ssDNA nuclease activity of the *T. maritime* Cmr complex was not sequence specific and that the Cmr complex cleaved ssDNA after every thymidine (Estrella *et al.*, 2016). However, the *S. thermophilus* Type III-A Csm complex cleaved the unpaired nucleotides of dsDNA at both strands with almost identical rates, and unpaired single-stranded fragments from 12 to 36 nt were cleaved relatively rapidly, 4–8 nt long were cleaved at a moderate rate, whereas 1–2 nt mismatches were resistant to cleavage (Kazlauskiene *et al.*, 2016).

Structural properties

CRISPR/Cas Type I and Type III systems are mechanistically distinct, in that Type I systems target double-stranded DNA (Hochstrasser *et al.*, 2014; Mulepati and Bailey, 2013; Sinkunas *et al.*, 2013) whereas Type III systems target single-stranded RNA and transcriptional active DNA (Deng *et al.*, 2013; Goldberg *et al.*, 2014; Hale *et al.*, 2009; Peng *et al.*, 2015; Samai *et al.*, 2015; Zhang *et al.*, 2012). However, the structure of the Type III surveillance complex is phylogenetically connected to that of the Type I Cascade complex which has been likened to a seahorse with subunits that represent its head, backbone, belly and tail (Jore *et al.*, 2011; Lintner *et al.*, 2011; Mulepati *et al.*, 2014; Wiedenheft *et al.*, 2011; Zhao *et al.*, 2014). The *T. thermophilus* Type III-B complex contained the Cmr component of $Cmr1_1 2_1 3_1 4_4 5_3 6_1$:$crRNA_1$, and electron microscopic analysis showed a sea worm structure composed of a repeating helical backbone of four Cmr4 subunits that are capped by three Cmr5 subunits juxtaposed with a 20-Å channel or groove for RNA duplex binding, and a 'head' containing Cmr1 and Cmr6 adjoins the other side of the proposed RNA-binding groove (Staals *et al.*, 2013). Near-atomic resolution cryoelectron microscopic reconstructions of the *T. thermophilus* native Type III-B Cmr complex with crRNA further reveal that the thumb-like β-hairpin domains of the Cmr4 subunits and the additional Cmr6 subunit intercalate between the segments of duplexed crRNA:target RNA, thus distorting the crRNA:target RNA duplex after every 5 bp segment and disrupt the formation of an extended A-form double helix, allowing cleavage by the catalytic residues of Cmr4 subunits in the *T. thermophilus* Type III-B Cmr complex (Taylor *et al.*, 2015) (Fig. 1.2).

The *P. furiosus* Type III-B Cmr complex showed similar structure with the *T. thermophilus* Cmr complex where three or four Cmr4 subunits formed the backbone and three Cmr5 subunits formed the belly as revealed by cryoelectron microscopy (Ramia *et al.*, 2014a; Spilman *et al.*, 2013). The Cmr4 and Cmr5 helical core is asymmetrically capped on each end by the Cas10 and Cmr3 at the conserved 5′ crRNA tag sequence and Cmr1 and Cmr6 near the 3′ end of the crRNA (Ramia *et al.*, 2014a; Spilman *et al.*, 2013). Formation of the *P. furiosus* Cmr complex requires both the 5′ tag sequence and 5′-OH group of the crRNA, while deletion or modification of the 5′ tag sequence disrupts binding of the Cas10-Cmr5 subcomplex (Hale *et al.*, 2014). The crystal structure of *P. furiosus* Type III-B Cmr complex shows that similar to the *T. thermophilus* Type III-B Cmr complex, the β-hairpins of three Cmr4 backbone subunits intercalate within the crRNA-target RNA duplex, causing nucleotide displacements with 6-nt intervals and thus periodically place the scissile bonds near the crucial aspartate of Cmr4 (Osawa *et al.*, 2015). In the *P. furiosus* Cmr complex, Cmr3 contacts the 5′ handle sequence of crRNA derived from the repeat sequence and Cmr1 previously found to be located at the head of the complex is absent (Osawa *et al.*, 2015). The crRNA in the Cmr complex flipped out at every sixth nucleotide to form an unwound ribbon-like structure of the duplex. Once the target RNA is captured in a base-complementary manner, the target sites are expelled from the duplex, due to steric clashes with the Cmr4 thumbs. Consequently, the target nucleotides become conformationally labile and are placed into the respective active sites in the Cmr4 (Cas7 family subunit) backbone. The Cmr complex then cleaves it at multiple sites at 6-nt

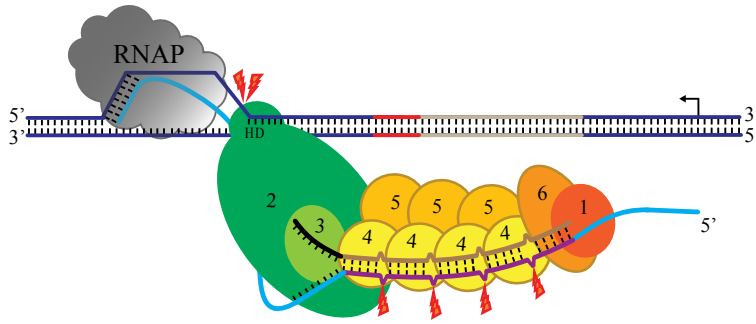

Figure 1.2 Model for RNA targeting and transcription-dependent DNA targeting by Type III CRISPR/Cas system (using the Cmr system as an example). Once RNA Polymerase transcribes through the protospacer, the Type III Cmr complex binds the RNA target mediated by crRNA:target RNA base pairing. The thumb-like β-hairpin domains of the Cmr4 (or Csm3 in Type III-A) subunits and the additional Cmr6 subunit intercalate between the segments of the duplexed crRNA:target RNA, thus distorting the crRNA:target RNA duplex after every 5-bp segment, disrupting the formation of an extended A-form double helix, and allowing cleavage by the catalytic residues of Cmr4 (or Csm3) subunits. The DNA nuclease activity of the HD domain from the Cas10 (Cmr2 or Csm1) subunit was activated by the binding of target RNA and cleaved the non-template DNA strand at the transcription bubble. The mismatch between the 5′ handle of the mature crRNA derived from the CRISPR repeat sequence and the 3′ target RNA sequence is required to avoid the targeting of CRISPR DNA.

intervals, which are specified by the 5′ ruler mechanism that defines the nearest cleavage site to be 5 nt downstream of the 5′ tag (Osawa *et al.*, 2015).

The Csm complex was first purified in *S. solfataricus* and contained Cas10 (Csm1), Csm2, Csm3, and Csm4 subunits (Rouillon *et al.*, 2013), and a Csm5 subunit was identified in the *T. thermophilus* and *S. thermophilus* Type III-A Csm complexes, but Csx1 was not identified (Staals *et al.*, 2014; Tamulaitis *et al.*, 2014). The Csm3 subunit was inferred to form the backbone (Fig. 1.2), Csm2 subunits formed the belly of the smaller filament, and the head of the complexes was capped by Csm5 whereas the foot contained Cas10 (Rouillon *et al.*, 2013; Staals *et al.*, 2014), and the pattern of the cleavage products at 6-nt intervals showed a striking resemblance to those observed with the Type III-B Cmr complex (Staals *et al.*, 2014; Tamulaitis *et al.*, 2014). These data suggest that the Csm complex is related structurally to Type I complexes, sharing a crRNA-binding helical backbone built from the Cas7-family RAMP domain proteins.

Genetic manipulation by Type III CRISPR/Cas system

Genome engineering

CRISPR/Cas system can specifically target DNA sequences by Type I, II, and III systems by crRNA:target nucleotide base-pairing mediated recognition. Among the three main types of CRISPR/Cas systems, DNA targeting by Type II systems requires a single Cas9 nuclease with multiple functions with a trans-acting RNA (tracrRNA) and the mature crRNA (Deltcheva *et al.*, 2011; Gasiunas *et al.*, 2012; Jinek *et al.*, 2012). Because of its simplicity, the Type II CRISPR/Cas9 system has been explored for genome editing immediately after its discovery (Cong *et al.*, 2013; Mali *et al.*, 2013). However, the Type II CRISPR/Cas9 system from mesophilic bacteria may not work in hyperthermophiles, and other CRISPR/Cas subtypes are needed for genetic manipulations in hyperthermophiles, requiring the construction of endogenous CRISPR/Cas systems as genetic tools. Type III CRISPR-Cmr or -Csm systems can target RNA via crRNA:target RNA base pairing, thereby activating DNA targeting activity on the non-template strand (Deng *et al.*, 2013; Estrella *et al.*, 2016; Samai *et al.*, 2015) (Fig. 1.2), thus conferring the possibility for gene silencing and genome editing by this system. Recently, we have employed the Type III-Bα system for genome editing in the hyperthermophilic archaeon, *S. islandicus* REY15A (Li *et al.*, 2016). A mini-CRISPR cassette under the control of an arabinose-inducible promoter allows cloning of any spacer sequences that matching the desired genomic sequences at the BspMI restriction sites in the *Sulfolobus* expression vector (Peng

et al., 2012, 2015) (Fig. 1.3). The mini-CRISPR was transcribed and processed by the endogenous Cas6 protein (Peng *et al.*, 2013), and mature crRNA base-pairing, for example, to the stop codon region of the non-coding strand of the *cmr2a* gene; the mismatches between the 5′ tag of crRNA derived from the repeat sequence and the 3′ flanking sequence of targeted sequence on *cmr2a* gene facilitated DNA interference by the Type III-B Cmr-α system in *S. islandicus* Δ*cas3* strain which lacked Type I-A DNA interference activity (Li *et al.*, 2016). Here, the mismatches between the target sequence and the 5′ tag of the mature crRNA derived from the repeat sequence is crucial for licensing the DNA nuclease activity. The DNA interference facilitated homologous recombination between the targeted genomic locus and the donor DNA cloned on the same plasmid resulting in the accurate insertion of a 6×His tag coding sequence at the 3′ end of the *cmr2a* gene. The plasmid carrying mini-CRISPR cassette and the *pyrEF* selection marker can be eliminated by counter-selection after genetic engineering. It seems that the *S. islandicus* Type III-B system did not require a PAM sequence for the discrimination of self and non-self DNA (Deng *et al.*, 2013), even though it is reported that the *P. furiosus* Type III-B Cmr system requires a non-strict rPAM for efficient DNA cleavage (Elmore *et al.*, 2016). Thus, Type III system-mediated genome editing allows the design of spacer (guide) RNAs from any DNA sequence without strict restriction by a PAM. The off-target effect was not determined in the *S. islandicus* Type III-B system; however, off-target effect will kill the cells of *Sulfolobus* hosts

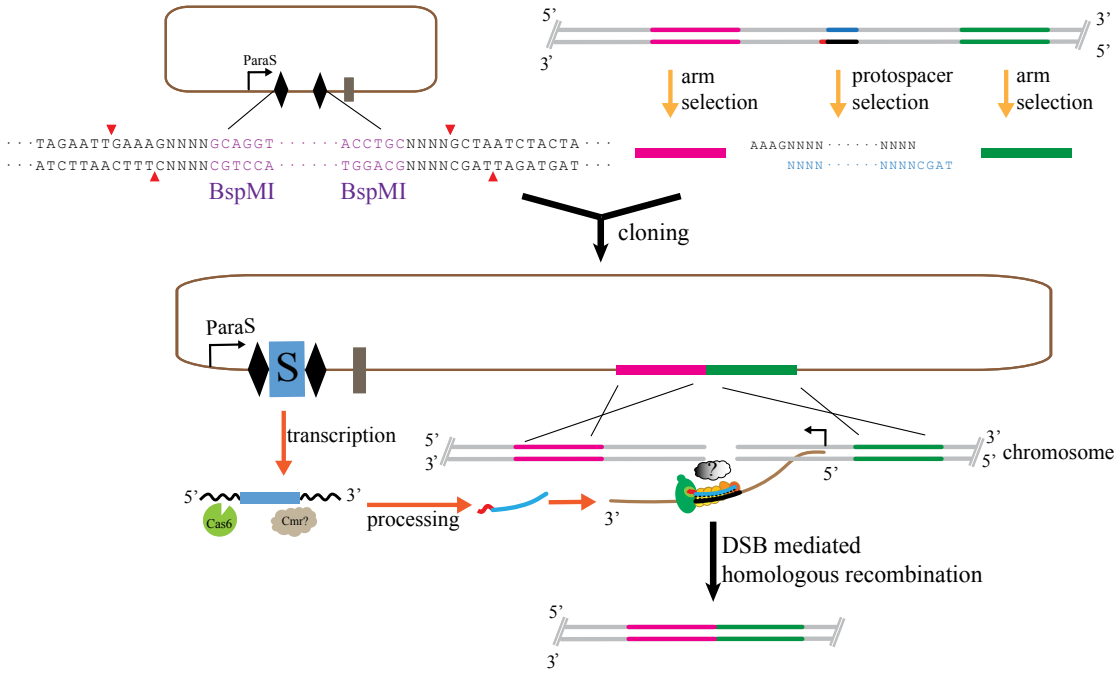

Figure 1.3 Architecture of CRISPR/Cas Type III system mediated genome engineering using the *Sulfolobus* Cmr system as an example. The 40-bp protospacer from the target DNA sequence with its 5′ adjacent sequence mismatches to 3′ segment of the repeat sequence is selected and cloned into the site between two repeats of the mini-CRIPSR through BspMI restriction sites. The mini-CRISPR will be transcribed by a inducible promoter (*araS* promoter) and the transcription will be stopped at a transcription terminator element. The left and right arms of the targeted sequence for homologous recombination are cloned into the same plasmid carrying the mini-CRISPR cassette. The plasmid carrying the mini-CRISPR cassette and the recombination arms is transformed into *Sulfolobus*. The mini-CRISPR is transcribed and processed by endogenous Cas6. The endogenous Cmr or Csm complex cleaves the target RNA mediated by mature crRNA:target RNA base pairing and cleaves the non-template DNA strand activated by the binding of target RNA with the Cmr or Csm complex. A DNA break at the target site enhances homologous recombination between the chromosomal DNA and the arms on the plasmid DNA, resulting in gene deletion, mutation, or insertion.

lacking the non-homologous End Joining pathway. Thus, only the cells repaired by homologous recombination with the donor sequences will survive. Furthermore, Type III Cmr or Csm complexes cannot process the CRISPR RNA itself, and so an endogenous Cas6 protein is required additionally (Carte *et al.*, 2008; Peng *et al.*, 2013).

Gene silencing

Genetic study of gene functions may encounter several problems, one of which is that essential genes cannot be knocked out or mutated. Especially in the hyperthermophilic archaeon that we study, even though we have developed several tools for gene editing in *S. islandicus* REY15A (Deng *et al.*, 2009; Zhang *et al.*, 2010, 2013), the *in vivo* function of essential genes are difficult to study. Type III-B Cmr systems have been shown to mediate PAM-independent RNA cleavage *in vitro* (Hale *et al.*, 2009; Spilman *et al.*, 2013; Staals *et al.*, 2013; Zhang *et al.*, 2012) and *in vivo* (Hale *et al.*, 2012). These results suggest that Type III CRISPR/Cas systems can be used as a gene silencing tool *in vivo*. Recently, gene silencing has been demonstrated in *S. solfataricus* (Zebec *et al.*, 2014) and *S. islandicus* (Peng *et al.*, 2015) using the β-galactosidase gene (*lacS*) as a reporter gene by the endogenous CRISPR Type III-B systems.

Construction of the knock-down plasmid is similar to the construction of the Type III type-based knock-out plasmid (Fig. 1.4). However, since the Type III-B Cmr system shows transcription-dependent DNA nuclease activity along with RNA targeting, the 3′ flanking sequences of the targets (or the 'protospacer') must match the 5′ handle tag (the pentanucleotide 5′-GAAAG-3′ or 5′-GAGAC-3′ of the 8-nt repeat handle in *Sulfolobus*) of the mature crRNA to avoid DNA targeting (Deng *et al.*, 2013;

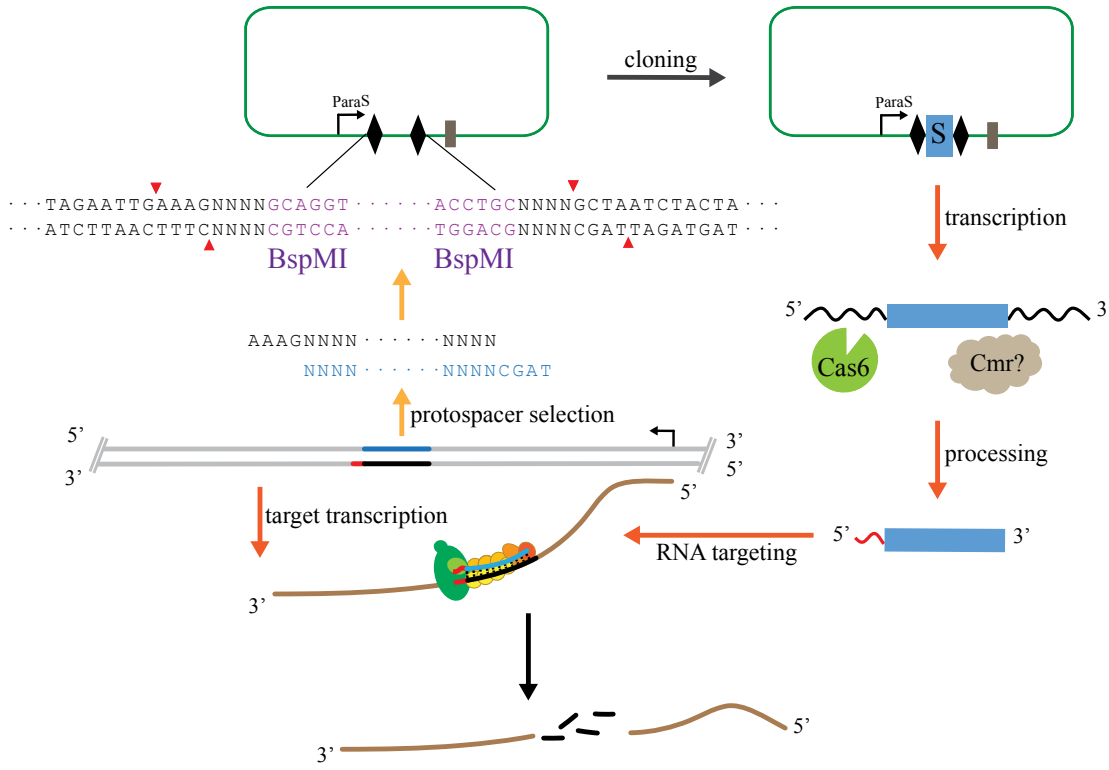

Figure 1.4 Architecture of CRISPR/Cas Type III system mediated gene silencing using the *Sulfolobus* Cmr system as an example. The 40-bp protospacer from the target DNA sequence with its 5′ adjacent sequence matches to 3′ segment of the repeat sequence is selected and cloned into the site between the two repeats of the mini-CRISPR through BspMI restriction sites. The mini-CRISPR will be transcribed by a inducible promoter (*araS* promoter) and processed by endogenous Cas6. The endogenous Cmr or Csm complex cleaves the target RNA mediated by mature crRNA:target RNA base pairing and avoids DNA targeting by base-paring between the 5′ tag of the crRNA and the 3′ adjacent sequence of the RNA target sequence.

Manica *et al.*, 2013). Thus, stretches of 40-nt sequences immediately following the pentanucleotide motifs can be selected as the protospacers to be targeted. However, these motifs will have a limited selection of protospacers from the target sequences for Type III-B-mediated gene silencing. It was reported that two selected spacers matching the middle or 3′ region of the chromosome-borne *lacS* gene coding region reduced ca. 85% of the β-galactosidase activity, but only reduced ca. 40% of the plasmid-borne LacS activity in *S. islandicus* (Peng *et al.*, 2015). This result suggests that Type III-B mediated gene silencing is efficient for the knock-down of expression of single-copy genes or less transcribed genes. In order to reduce the expression of high-copy genes or highly transcribed genes, the transcription of the mini-CRISPR needs to be enhanced by a strong promoter or multiple spacers complementary to the target RNA sequences need to be selected into the mini-CRISPR.

Conclusions

Recently, CRISPR/Cas systems have been classified into two classes (Class I and II) containing five types (Type sI to V). Among these types, the Type III system, further classified into Type III-A and III-B, as well as two less characterized III-C and III-D, is the only system showing both RNA and DNA cleavage activity. All Type III Cmr or Csm complexes have a similar structure where the Cas7 family subunits (Cmr4 in Type III-B systems or Csm3 in III-A systems) form a rigid backbone and the Cmr5 or Csm2 subunits form the belly. The head of the complex is capped by Cmr6/Cmr1 or Csm5, and the foot contains the signature Cas10 (Cmr2 or Csm1) protein and Cmr3. The target RNA is cleaved by Cmr4 or Csm3 mainly at 6-nt intervals, and the non-template ssDNA in the transcription bubble is cleaved by the HD domain and/or cyclase domain of the Cas10 subunit. The sequence-specific RNA targeting and the non-sequence-specific and transcription-dependent DNA targeting facilitates the Type III system-based gene silencing or genome editing tools. However, all well-studied Type III systems are from thermophiles. Characterization of Type III systems from mesophilic bacteria or archaea is important to explore scope of their application in genetic manipulation.

Acknowledgement

We would like to thank the National Natural Science Foundation of China (Nos. 31100050 and 31300042) for financial support.

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