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An archaeal CRISPR type III-B system exhibiting distinctive RNA targeting features and mediating dual RNA and DNA interference

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ABSTRACT

CRISPR-Cas systems provide a small RNA-based mechanism to defend against invasive genetic elements in archaea and bacteria. To investigate the *in vivo* mechanism of RNA interference by two type III-B systems (Cmr- α and Cmr- β) in *Sulfolobus islandicus*, a genetic assay was developed using plasmids carrying an artificial mini-CRISPR (AC) locus with a single spacer. After pAC plasmids were introduced into different strains, Northern analyses confirmed that mature crRNAs were produced from the plasmid-borne CRISPR loci, which then guided gene silencing to target gene expression. Spacer mutagenesis identified a trinucleotide sequence in the 3'-region of crRNA that was crucial for RNA interference. Studying mutants lacking Cmr- α or Cmr- β system showed that each Cmr complex exhibited RNA interference. Strikingly, these analyses further revealed that the two Cmr systems displayed distinctive interference features. Whereas Cmr- β complexes targeted transcripts and could be recycled in RNA cleavage, Cmr- α complexes probably targeted nascent RNA transcripts and remained associated with the substrate. Moreover, Cmr- β exhibited much stronger RNA cleavage activity than Cmr- α . Since we previously showed that *S. islandicus* Cmr- α mediated transcription-dependent DNA interference, the Cmr- α constitutes the first CRISPR system exhibiting dual targeting of RNA and DNA.

INTRODUCTION

Most archaea and ~40% of bacteria encode CRISPR-Cas (clustered regularly interspaced short palindromic repeats-CRISPR-associated) systems to defend themselves against invasive genetic elements. This system is comprised of two

genetic entities: CRISPR loci and *cas* gene cassettes. The former consist of short DNA repeats interspaced by unique sequences (spacers) derived from mobile genetic elements (1–3), while the latter encode proteins of different CRISPR adaptation and interference complexes, including architectural proteins, nucleases and helicases (4–6). Studies on diverse bacterial and archaeal CRISPR-Cas systems indicate that they all function in three distinct phases. First, a short DNA fragment derived from a mobile genetic element is captured as the first spacer in a CRISPR locus immediately after the leader sequence in the stage of 'spacer acquisition' (7–13). Then, transcription conferred by a promoter in the leader sequence expresses the entire CRISPR locus as a precursor CRISPR RNA (pre-crRNA), and pre-crRNA processing yields mature crRNAs in the process of 'crRNA biogenesis' (14–26). Finally, crRNAs form ribonucleoprotein (RNP) complexes with Cas proteins and guide the RNP to recognize invading nucleic acids by sequence complementarity between the crRNA and its target, and destroy the invading nucleic acids in the stage of 'CRISPR interference' (27–41).

There are three main classes of CRISPR-Cas system: types I, II and III (42). Studying model systems has demonstrated DNA interference activity for all tested CRISPR systems *in vivo*. These studies have also revealed two distinct mechanisms to recognize foreign DNA targets by these CRISPR systems. In types I and II systems, the RNP effector complexes identify a protospacer-adjacent motif (PAM) in the invading DNA to initiate the interference (31,43–45), and the action requires an important sequence region in crRNA named 'seed sequence' (46–51). For type III systems, the *Staphylococcus* III-A Csm mediates DNA interference in which self and non-self DNAs are distinguished by sequence complementarity between the 5' repeat handle of crRNA and its target, and the process is PAM-independent (52). Characterization of type III-B Cmr systems of *Pyrococcus furiosus*, *Thermus thermophilus* and *Sulfolobus solfataricus* has shown that each system mediates PAM-independent RNA cleavage *in vitro* (30,53–55).

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Moreover, it has been shown that the antisense transcript of spacer 1 in a CRISPR locus was cleaved *in vivo* in *P. furiosus* in a fashion matching the *in vitro* cleavage by the encoded Cmr system (56), and gene silencing has been demonstrated in *S. solfataricus* using plasmid-borne mini-CRISPR loci (57). However, we found that *Sulfolobus islandicus* III-B Cmr- α encodes a novel DNA interference activity that requires transcription of the protospacer (58). This raises an intriguing question as to whether the same Cmr system can also mediate RNA interference. Furthermore, as *P. furiosus* and *S. solfataricus* carry several active CRISPR systems (reviewed in (59,60)), *in vivo* RNA interference by a III-B Cmr system has not yet been demonstrated unambiguously in any organism.

We employed *S. islandicus* REY15A to further characterize III-B Cmr systems. This organism carries two CRISPR loci of identical repeats and three different CRISPR systems, including one I-A system and two Cmr gene cassettes (Cmr- α and Cmr- β) (61). Using versatile genetic tools developed for this model organism (62–64), we constructed mutants of *cmr* gene cassettes and studied crRNA-guided gene silencing by endogenous CRISPR systems in the mutants using plasmid-borne artificial mini-CRISPR loci. We have, for the first time, demonstrated that both type III-B CRISPR systems mediate RNA interference to target gene expression and that a trinucleotide sequence motif located at the 3'-end of crRNAs is crucial for the interference. We infer that Cmr- α probably forms a stable complex with its target, which functions as an intermediate in the Cmr- α -mediated DNA interference.

MATERIALS AND METHODS

Strains, growth conditions and transformation of *Sulfolobus*

The genetic host *S. islandicus* E233 and E233S1 (65) as well as deletion mutants of *cmr* gene cassettes (44) employed in this work are listed in Table 1. *Sulfolobus* strains containing a plasmid-expressed *pyrEF* gene cassette were grown at 78°C in a SCV (0.2% sucrose, 0.2% casamino acids plus 1% vitamin solution) medium (66) whereas the medium for the genetic hosts was supplemented with 20 μ g/ml uracil. *Sulfolobus* competent cells were prepared as previously described and transformed by electroporation (65).

Construction of plasmids

To facilitate construction of artificial mini-CRISPR loci, a plasmid vector pSe-Rp was constructed with pSeSD1, a *Sulfolobus* expression vector (67). pSe-Rp contained a DNA fragment of two tandem copies of CRISPR repeat that were separated by two oppositely oriented BspMI recognition sequences (Supplementary Figure S1). To clone a spacer to pSe-Rp, the vector was digested with BspMI, yielding a linear plasmid carrying protruding ends of 4 nt nucleotides at both ends. Spacer fragments were prepared by annealing oligonucleotides of each spacer by heating to 95°C for 10 min and subsequently cooling gradually down to the room temperature. Since the spacer DNA fragments carried 4 nt protruding ends that were complementary to those of the linearized pSe-Rp (Supplementary Table S1), ligation of the vector and the DNA fragment gave a plasmid carrying an

Artificial mini-CRISPR locus (pAC plasmid). The cloning procedure was shown in Supplementary Figure S1, with which a number of pAC plasmids were constructed (Table 2) using spacer oligonucleotides listed in Supplementary Table S1.

To construct a plasmid to express a small RNA complementary to ProtoS1 (anti-sense RNA) of the *lacS* gene (Figure 1A), a DNA fragment was prepared by annealing of the oligonucleotides of Antifwd and Antirev-SalI (Supplementary Table S1), and subsequent digestion with SalI and purified again. Ligation of the purified DNA fragment with pSeSD1 at StuI and SalI sites gave pAsRNA.

All the oligonucleotides were synthesized from TAG Copenhagen A/S (Copenhagen, Denmark) and the sequences of mini-CRISPR loci in all plasmid constructs were verified by DNA sequencing at MacroGene Europe (Amsterdam, The Netherlands).

RNA preparation and northern blot analysis

Total RNAs were extracted from *Sulfolobus* cells using Trizol reagent (Invitrogen) following the instruction of the manufacturer. Thirty micrograms of total RNA was denatured at 94°C for 5 min and fractionated on a 6% polyacrylamide-SDS gel (200 V, 2 h). The quality of RNA samples was examined under UV light after staining with ethidium bromide, and fractionated RNAs were transferred onto a nylon membrane using the BIO-RAD SD Trans-Blot Semi-Dry Transfer Cell (20 V, 45 min). RNAs were then immobilized on the membrane by UV cross-linking and analysed by northern hybridization using radioactive probes indicated in each experiment. Results were recorded by exposing the membrane to an X-ray film. To reuse the membrane, the probe was stripped off from the nylon membrane by washing twice with 0.5% SDS (m/v) at 60°C for 1 h.

β -Glycosidase assay

To determine β -glycosidase activity, different *S. islandicus* strains were grown to an optical density of 0.2–0.4 (600 nm). Cell mass was collected for each strain and used for preparing cellular extracts. Protein content of the cellular extracts was determined using BCA Protein Assay Reagent (Thermo Scientific) whereas the enzyme activity was determined by β -glycosidase assay using ONPG (ρ -nitrophenyl- β -D-galactopyranoside) method (67).

Evaluation of mRNA cleavage efficiency by quantitative PCR

Total RNA samples to be used for quantitative PCR (qPCR) analysis were treated with DNase I (Thermo Scientific) to eliminate any possible DNA contamination. Then 2 μ g RNA was withdrawn from each sample and polyadenylated using an *E. coli* Poly A polymerase (New England Biolabs) to generate a poly A tag for each RNA molecule. Reverse transcription of each polyadenylated RNA sample was conducted with the First Strand cDNA Synthesis Kit (Thermo Scientific) using an oligo(dT)₁₈ as the primer, yielding RNA/DNA hybrids. The RNA strand of the hybrids was removed by treating with RNase H (Thermo Scientific), resulting cDNAs for further analysis.

Table 1. *Sulfolobus* strains used in this work

Strains	Genotype and features	Reference
<i>S. islandicus</i> E233	$\Delta pyrEF$	Deng <i>et al.</i> (65)
<i>S. islandicus</i> E233S1	$\Delta pyrEF\Delta lacS$	Deng <i>et al.</i> (65)
$\Delta Cmr-\alpha$	Derived from E233S1, carrying deletion of III-B Cmr- α locus including six <i>cmr</i> genes	Peng <i>et al.</i> (44)
$\Delta Cmr-\beta$	Derived from E233S1, carrying deletion of III-B Cmr- β locus including seven <i>cmr</i> genes	Peng <i>et al.</i> (44)
$\Delta Cmr-\alpha\beta$	Derived from E233S1, carrying deletion of both III-B gene cassettes, Cmr- α and Cmr- β	This work

Table 2. Plasmids used in this work

Plasmid	Genotype and features	Reference
pSeSD1	A <i>Sulfolobus-E. coli</i> shuttle vector carrying an expression cassette controlled under a synthetic strong promoter <i>P_{araS-SD}</i>	Peng <i>et al.</i> (67)
pAsRNA	Derived from pSeSD1, expressing the antisense RNA of protospacer S1 of the <i>S. islandicus lacS</i> gene	This work
pAC-SS1	An artificial mini-CRISPR locus plasmid derived from pSe-Rp, carrying an artificial CRISPR locus with 1 S1 spacer of the <i>S. islandicus lacS</i> gene (pAC plasmid)	This work
pAC-SS2	pAC plasmid carrying an artificial mini-CRISPR locus with 1 S2 spacer of the <i>S. islandicus lacS</i> gene	This work
pAC-SS3	pAC plasmid carrying an artificial mini-CRISPR locus with 1 S3 spacer of the <i>S. islandicus lacS</i> gene	This work
pAC-SS4	pAC plasmid carrying an artificial mini-CRISPR locus with 1 S4 spacer of the <i>S. islandicus lacS</i> gene	This work
pAC-SS1-LacS	Derived from pAC-SS1, carrying <i>lacS</i> of <i>S. islandicus</i> Rey15A with native promoter and terminator upstream of the <i>P_{araS-SD}</i> promoter	This work
pLacS-ck	Derived from pSeSD1, carrying <i>lacS</i> of <i>S. islandicus</i> Rey15A with native promoter and terminator	This work

qPCR was conducted with a CFX96 Touch™ real-time PCR detection system (Bio-Rad), using Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific), and the PCR condition was as following: denaturing at 95°C for 5 min, 40 cycles of 95°C 15 s, 55°C 15 s and 72°C 20 s. Relative amounts of RNAs were calculated using the comparative C_t method (68), and amplification efficiencies of the target and reference sequences were validated (Supplementary Figure S2).

Determination of cleavage sites on RNA target molecules by rapid amplification of cDNA ends (RACE)

RACE was conducted with primer RACE-fwd and the oligo(dT)₁₈, using cDNAs generated above as templates. Phusion® High-Fidelity DNA polymerase (New England Biolabs) was the enzyme for the amplification, and PCR was conducted under the following condition: denaturing at 95°C for 5 min, 35 cycles of 95°C 30 s, 55°C 30 s and 72°C 20 s and a final extension at 72°C for 5 min. After purified with a QIAquick PCR purification Kit (QIAGEN), the amplified RACE products were ligated with pJET1.2 (CloneJET PCR Cloning Kit, Thermo Scientific). The ligation was used to transform *E. coli* DH5 α , and transformants were characterized by colony PCR to amplify the RACE products, which were directly sequenced at MacroGen (Amsterdam, The Netherlands) using the pJet1.2 forward sequencing primer.

RESULTS

Studying RNA interference by endogenous CRISPR systems in *S. islandicus* using plasmid-borne artificial mini-CRISPR loci

We employed a strategy of constructing plasmids containing artificial mini-CRISPR loci (pAC plasmids) to study Cmr-mediated RNA interference. The principle of this assay is that mature crRNAs produced from mini-CRISPR loci will reprogram RNA interference activity of the endogenous CRISPR systems to reduce target gene expression in *S. islandicus*. The *lacS* gene encoding a β -glycosidase was chosen as the reporter gene since its enzymatic activity is highly specific and can be measured in the cellular extracts reliably even at a very low level (69). The *Sulfolobus* expression vector pSeSD1 was employed to clone mini-CRISPR loci and the strong promoter (*araS-SD*) on the vector (67) would ensue a high level of expression from plasmid-borne mini-CRISPR loci, generating large amounts of mature crRNAs.

However, there is a prerequisite to the assay; crRNAs generated from pAC plasmids should not reprogram endogenous DNA interference systems to destroy the reporter gene since doing so would result in cell death of transformants. Therefore, to obtain transformants of pAC plasmids for studying RNA interference, the two distinct DNA interference activities in *S. islandicus* Rey15A should be circumvented, including the PAM-dependent type I-A DNA targeting and the PAM-independent III-B Cmr- α DNA interference (31,44,58). We and others showed that including the pentanucleotide 5'-GAAAG-3' of the 8 nt repeat han-

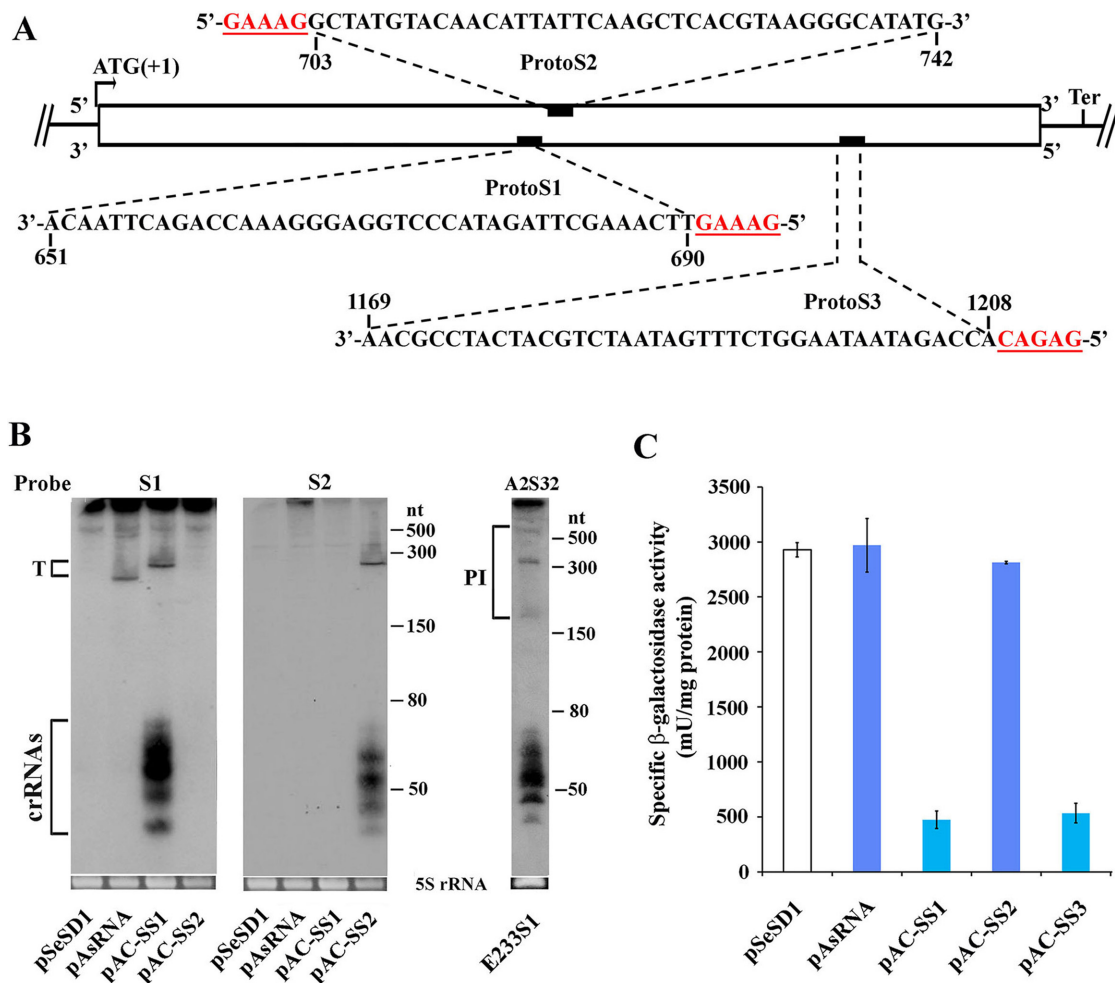


Figure 1. Plasmid-borne artificial mini-CRISPR locus-mediated gene silencing in *S. islandicus*. (A) Schematic of three sequence regions selected for studying gene silencing of *lacS* expression. Sequences of the antisense strand from 651 to 690 and 1169–1208 constitute protospacer 1 (ProtoS1) and ProtoS3, respectively, whereas ProtoS2 is the sense sequence from 703 to 742 nt, relative to the start codon (ATG, +1) of the *lacS* gene. The pentanucleotides (5'-GAAAG-3' or 5'-GAGAC-3') are underlined. Ter, terminator of the *lacS* gene. (B) Expression of mini-CRISPR loci from pAC-SS1 and pAC-SS2 and crRNA maturation. Northern analysis of total RNAs prepared from cells of *S. islandicus* E233 transformants harbouring pSeSD1, pAsRNA, pAC-SS1 and pAC-SS2. Probes used in these analyses are indicated individually (S1, S2, or A2S32). The two former detect crRNAs generated from the mini-CRISPR loci on plasmid whereas the latter identifies mature crRNAs generated from spacer 32 in the genomic CRISPR locus 2 (A2S32) of *S. islandicus* E233S1. 5S rRNA was used as a loading control. T – RNA transcripts; PI – processing intermediates; crRNAs – mature crRNAs. (C) Specific β -galactosidase activity in these transformants. Three independent transformants were analysed for each construct with bars indicating standard deviations.

dle efficiently protected the immediately downstream DNA against both DNA interference activities (37,44) and, furthermore, several other DNA motifs were found to protect their downstream sequences from DNA interference including 5'-GAGAC-3' (58). Thus DNA sequences downstream of these motifs could be regarded as non-DNA targeting protospacer in the host genome and used as spacers to construct artificial mini-CRISPR loci.

Analysing the coding region of the *S. islandicus lacS* gene identified three such sequence stretches. One is the sequence complementary to 5'-GAAAG-3' positioned at 691–695 on the non-coding strand, the other is located at 698–702 on the coding strand, and a third sequence is further downstream (complementary to 5'-GAGAC-3'), located at 1209–1213, relative to the start codon of *lacS*. Then, a stretch of 40 nt sequence immediately following each motif was selected as protospacer since this spacer size corresponded

to the length of most spacers in the *S. islandicus* REY15A genome (61). This led to the identification of three protospacers (ProtoS1-S3) in the *lacS* gene (Figure 1A). Furthermore, a sequence immediately followed a CCA PAM sequence was selected as protospacer 4 to test for DNA interference directed by plasmid-derived crRNA.

Oligonucleotides of all four protospacers, each with a 4 nt repeat sequence added at the 5'-end, were synthesized (Supplementary Table S1). Annealing of each pair of oligonucleotides generated the corresponding spacer DNA fragment, which was inserted into the cloning vector pSe-Rp, and this yielded artificial CRISPR plasmids, pAC-SS1 to pAC-SS4, each containing a mini-CRISPR locus of a single copy of spacer. These pAC plasmids were used to transform *S. islandicus* E233, a genetic host that retained the wild-type *lacS* gene (65). We found that transformation efficiencies with pAC-SS1 to pAC-SS3 were comparable to that of the

cloning vector pSeSD1, whereas >1000-fold of reduction in transformation rate was observed for pAC-SS4 (Supplementary Figure S3). These results were consistent with the prediction that only pAC-SS4 could yield DNA interference to the *lacS* gene of *S. islandicus* E233.

Mature crRNAs generated from pAC plasmids greatly reduced expression of the target gene in *S. islandicus*

We then examined the processing of RNA transcripts that were expressed from the plasmid-borne AC loci. Total RNAs were prepared from *S. islandicus* E233 strains carrying pAC-SS1 or pAC-SS2 and analysed by northern hybridization. We found that transcripts of the plasmid-borne S1 and S2 mini-CRISPR loci were processed into mature crRNAs that were comparable in size to those generated from spacer 32 of the chromosomal CRISPR locus 2 (A2S32) (Figure 1B). Interestingly, while the transcript of the antisense RNA (asRNA) and that of the S1 mini-CRISPR locus gave a similar intensity of hybridization, much stronger signals were observed for mature crRNAs derived from S1 mini-CRISPR transcripts (Figure 1B). Since both RNAs were expressed from the engineered promoter *P_{araS-SD}* derived from pSeSD1 (67), the elevated levels of crRNAs in pAC transformants indicated that there is a mechanism of crRNA protection in *S. islandicus*. For example, Cas proteins could bind mature crRNAs and protect them from degradation. More importantly, these results indicated that transcripts generated from plasmid-borne mini-CRISPR loci were processed into mature crRNAs in *S. islandicus*.

Next, we investigated whether crRNA produced from pAC plasmids could guide endogenous CRISPR interference complexes to reduce enzymatic activity of the *lacS* reporter gene (gene silencing). Since crRNAs generated from S1 and S3 are complementary to the *lacS* mRNA while crRNAs produced from S2 are identical to the transcript, gene silencing was only expected in transformants of pAC-SS1 and pAC-SS3. Indeed, while the specific activity of β -glycosidase in pAC-SS2 transformant was very similar to the activity in the reference strain (2927 and 2812 mU/mg, respectively), pAC-SS1 and pAC-SS3 transformants showed much lower activities (478 and 535 mU/mg, respectively), retaining only 16–18% of the activity in the reference strain (Figure 1C). Finally, transformants of pAsRNA and pSeSD1 showed very similar activities (Figure 1C) and this excluded the possibility that antisense activity of S1 mature crRNAs contributed to the observed gene silencing. Together, these results demonstrated that crRNAs produced from an artificial CRISPR plasmid were incorporated into ribonucleoprotein (RNP) interference complexes and mediated strong gene silencing to *lacS* expression in *S. islandicus*.

A trinucleotide motif important for Cmr-mediated RNA targeting was located at the 3'-end of S1 crRNA

Taking the advantage of the efficient gene silencing by the CRISPR systems in the above assay, we tested the importance of sequence complementarity between crRNA and the RNA target sequence on gene silencing. A number of mutated S1 spacers were obtained by annealing of pairs

of oligonucleotides listed in Supplementary Table S1 and cloning of these mutant spacers to pSe-Rp gave corresponding pAC plasmids. These plasmids were introduced to *S. islandicus* E233 strain by transformation and studied for gene silencing by measuring residual activity of the reporter gene in their transformants. We found that introducing individual mutations to the first half S1 spacer (positions 1–20) did not yield any effect on RNA interference because mini-CRISPR loci carrying each mutant S1 spacer showed >90% of gene silencing of the wild-type spacer (Figure 2). Then, quadruple mutations were introduced in the second half (position 21–40) of the spacer, and two of these spacer mutants (25–28, 29–32) failed to guide endogenous CRISPR systems to reduce *lacS* expression (Figure 2). Moreover, generating multiple mutations in the spacer region of 1–20, including sequence regions of 1–4, 5–12, 13–18, and 1–12, only yielded a modest decrease in gene silencing. For example, the 12 bp mutation (positions 1–12) still maintained ca. 50% of the interference activity by the wild-type spacer. Together, these results indicated that the crRNA sequence important for RNA interference was located within 8 nt sequence located within 25–32 of the spacer.

To investigate the importance of the octanucleotide sequence in detail, four dual mutations of S1 spacer were generated. Two of them, 27 + 28 and 29 + 30, reduced targeting activity by ~36% and ~81%, respectively, whereas the remaining two mutations did not impair the RNA interference (Figure 2). This experiment further narrowed down the important region to positions of 27–30 in the spacer.

Next we replaced each base with all three possible substitutions and tested the effect of each mutation on RNA interference. Whereas transition substitutions showed little or no influence, transversion mutations impaired the RNA targeting activity. Three of the mutant spacers, A28T, C29A and C30G, reduced gene silencing by more than 50% whereas the remaining three showed modest reductions, conferring 20–30% of gene silencing. This established that the trinucleotide 5'-ACC-3' at positions 28–30 in S1 constituted the most important sequence motif in Cmr-mediated RNA interference in *S. islandicus*.

Taken together, these results indicated that the CRISPR-mediated RNA interference in *S. islandicus* exhibits an extraordinary flexibility in base pairing complementarity between crRNA and its target with only a single trinucleotide motif being of crucial importance.

Both type III-B Cmr systems mediated gene silencing in *S. islandicus*

S. islandicus Rey15A encodes one type I-A and two type III-B CRISPR systems: Cmr- α and Cmr- β . Thus, it was important to investigate whether all CRISPR systems contribute to the RNA interference observed in *S. islandicus*. In a previous work, knockouts of each type III-B Cmr gene cassette were obtained with the genetic host *S. islandicus* E233S1, including strains carrying either Cmr- α , or Cmr- β (44), and a mutant lacking both type III-B systems was constructed in the present work (Table 1). All three strains were useful for studying which CRISPR system(s) contributed to the observed RNA interference in *S. islandicus*.

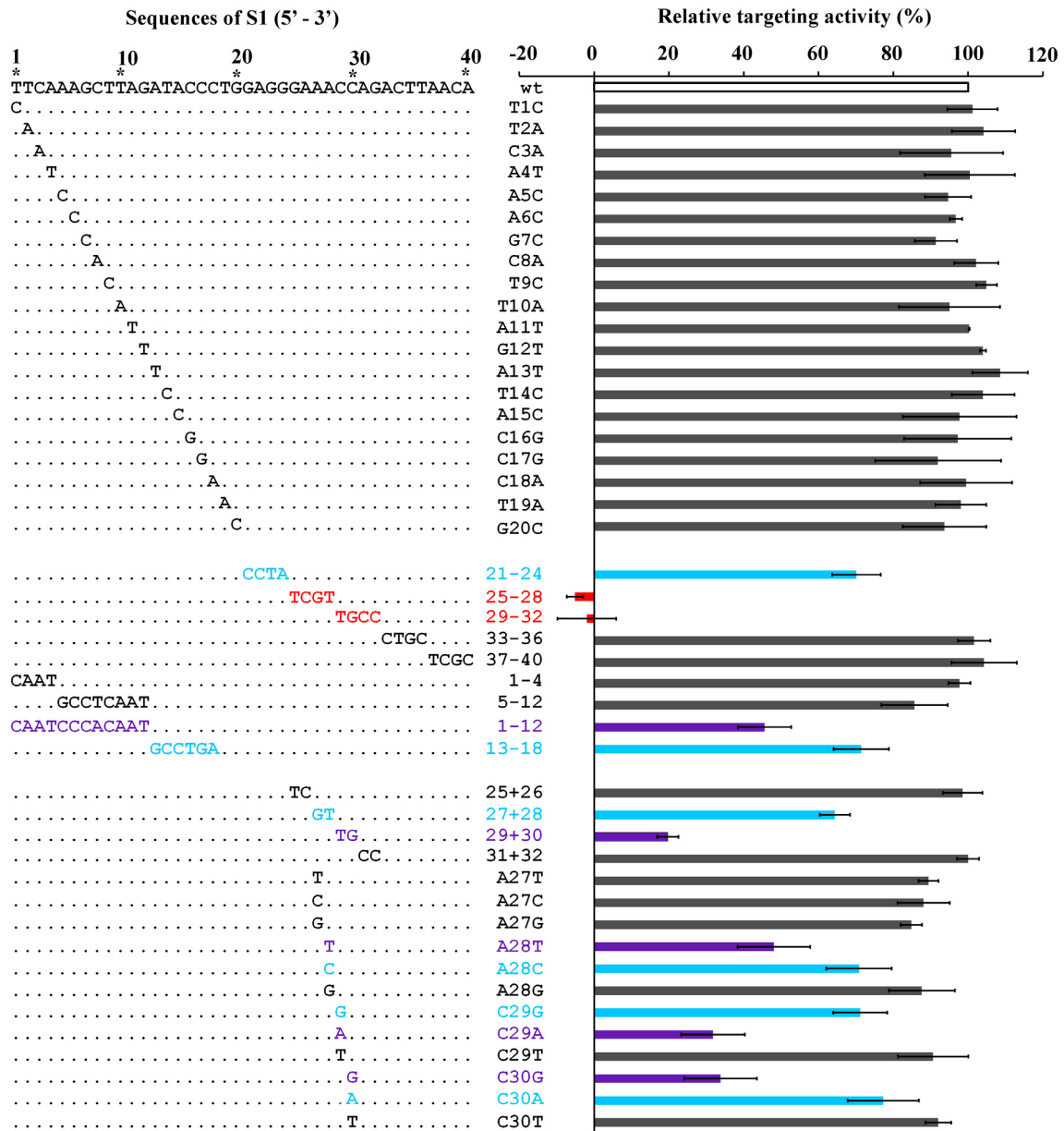


Figure 2. Identification of a trinucleotide sequence motif in crRNA important for RNA interference. Complete sequence of S1 is shown on the top of the left panel and numbered. S1 spacer mutants are annotated according to the positions of their mutations in the spacer and the mutated bases are shown in the left panel. The right panel illustrates RNA targeting activity of the wild-type S1 spacer (100%) and mutated derivatives relative to the wild-type spacer. Spacer mutants showing a greatly reduced RNA targeting activity are highlighted in colours. Three independent transformants were analysed for each construct with bars indicating standard deviations.

Since all three mutants of Cmr gene cassettes were derived from E233S1, a strain from which the *lacS* gene was deleted (65), it was necessary to introduce the target gene back into the host in order to enable the gene-silencing assay. We chose to put the reporter gene onto pAC-SS1, yielding a novel test plasmid pAC-SS1-LacS (Figure 3A). This test plasmid carried both the mini-CRISPR locus and its target gene *lacS*. After introducing the plasmid into each of the three *cmr* cassette mutants, as well as the genetic host E233S1, RNA interference activity in the transformants was evaluated by measuring the residual activity of β -glycosidase expressed from the plasmid-borne *lacS*. As

shown in Figure 3B, the mutant strain lacking both type III-B *cmr* gene cassettes showed activity comparable to that of the reference strain (9738 versus 9375 mU/mg), indicating that the type I-A CRISPR system did not confer detectable RNA interference. Reduced levels of the reporter gene activity were observed for all remaining *S. islandicus* strains, including E233S1, a Cmr- α strain lacking all *cmr*- β genes, and a Cmr- β strain devoid of all *cmr*- α genes. These results indicated that both Cmr- α and Cmr- β mediated RNA interference in *S. islandicus*. Interestingly, the Cmr- β system was more active in gene silencing than Cmr- α , showing gene silencing of 42% and 31%, respectively. In the presence of

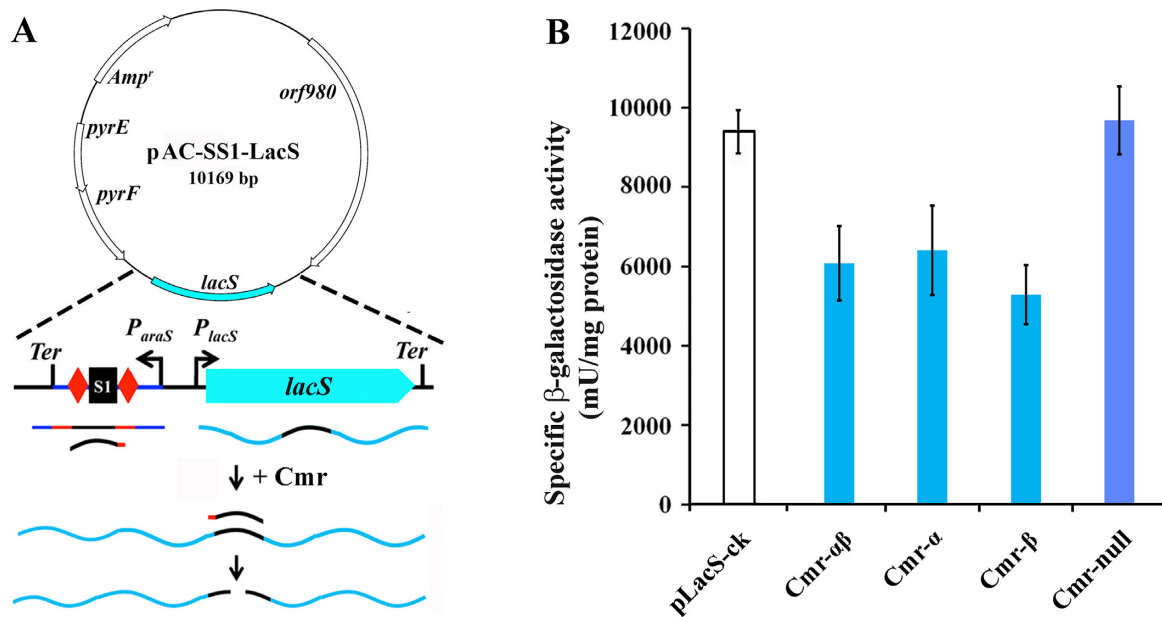


Figure 3. Each type III-B Cmr system mediated gene silencing in *S. islandicus*. (A) Schematic of pAC-SS1-LacS, an RNA interference test plasmid producing both S1 crRNA and its RNA substrate. After introducing pAC-SS1-LacS into *S. islandicus* E233S1 or mutants of *cmr* gene cassettes by transformation, mature crRNAs were to be produced from the SS1 mini-CRISPR locus and then guided the endogenous Cas proteins to silence *lacS* mRNAs, which were also produced from the *lacS* gene on the plasmid (3–5 copies per cell). (B) Specific β-glycosidase activity in *S. islandicus* Cmr mutants. Cmr-αβ – the wild-type strain carrying both *cmr* gene cassettes, Cmr-α – mutant retaining Cmr-α with *cmr*-β gene cassette deleted, Cmr-β – mutant retaining Cmr-β with *cmr*-α genes deleted, and Cmr-null – mutant with both *cmr* gene cassettes deleted. *S. islandicus* E233S1 strain carrying pLacS-ck was used as a control. Three independent transformants were analysed for each construct. Bars: standard deviations.

both Cmr systems, the gene silencing effect was 35%. These results were in consistent with the scenario that Cmr systems could compete for common crRNAs, resulting in the reduction of Cmr-β activity by the Cmr-α system in this archaeon.

Previously we showed that the same *S. islandicus* Cmr-α system exhibits a transcription-dependent DNA interference (58). Together, these results indicated that Cmr-α mediates both DNA and RNA interference, representing the first demonstration of a CRISPR system with dual targeting activity of DNA and RNA.

RNA cleavage by the two *S. islandicus* Cmr systems showing distinctive features

To investigate whether the Cmr systems mediated gene silencing of *lacS* expression by mRNA cleavage *in vivo*, we employed qPCR to determine the amount of uncleaved *lacS* mRNA present in *S. islandicus* transformants carrying a pAC plasmid. cDNAs were generated from total RNAs isolated from the *S. islandicus* strains and used for qPCR with two sets of primer, Qtar and Qref (Figure 4A). qPCR with the former set of primer would reveal the population *lacS* mRNAs containing the target sequence, representing the level of uncleaved RNAs, whereas qPCR with the latter was to amplify a DNA fragment downstream from the target site to estimate the total mRNA level. The yielded data were used to calculate RNA cleavage efficiency by the *S. islandicus* Cmr systems (Supplementary Figure S4).

In the *S. islandicus* E233 transformants carrying pAC-SS1, qPCR analysis revealed that >97% of mRNAs were cleaved in the RNA target sequence (Figure 3B), which was

higher than gene silencing at the enzyme level (>80%, Figure 1C). qPCR was also conducted on cDNAs generated from total RNAs prepared from cells of *S. islandicus* strains carrying the test plasmid pAC-SS1-LacS, including E233S1 and its derivative mutants lacking one of both *cmr* gene cassettes (Table 1). In this experiment, β-glycosidase activity in the reference strain was ~3-fold of that in *S. islandicus* E233 because the enzyme was expressed from the plasmid-borne *lacS* gene (Figure 1 versus Figure 3). We found that Cmr-α eliminated 30% of *lacS* mRNAs, Cmr-β removed 70%, but mRNA cleavage was 50% in the presence of both Cmr systems (Figure 4C).

Next, we studied *in vivo* cleavage of *lacS* mRNAs in an *S. islandicus* E233 transformant of pAC-SS1 using 3'-RACE. The strain contained both Cmr-α and Cmr-β systems, showing mRNA cleavage efficiency of >97% (Figure 4B) and enzyme activity silencing of >86% (Figure 1C). A total of 56 PCR clones were sequenced among which the cleavage sites of 31 clones were located within S1 protospacer and an additional five sites were located immediately upstream or downstream of the protospacer (Figure 4D, Supplementary Figure S4). Interestingly, the cleavage sites clustered into two distinct groups: (a) those with UA-like cleavage (indicated with black arrows) as reported for *S. solfataricus* Cmr, and (b) those of the two defined positions at a 6 nt interval (indicated with red arrows), resembling the cleavage products of the ruler mechanism proposed for Cmr systems of *P. furiosus* and *T. thermophilus*. We inferred that the UA-like cleavages resulted from Cmr-β activity while cleavages at the two defined positions at the 6 nt interval were produced by Cmr-α by the putative ruler mechanism

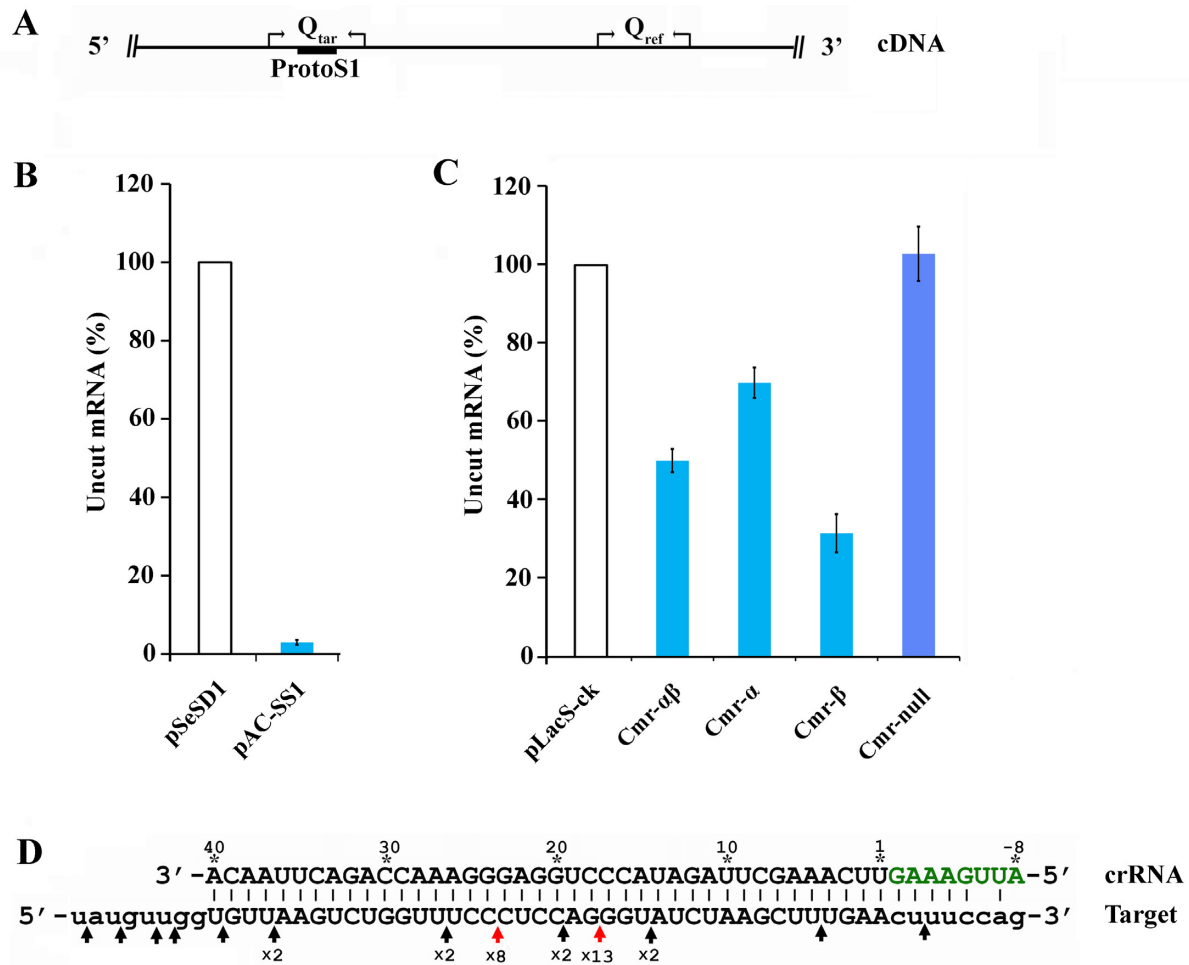


Figure 4. Target RNA cleavage analysis by real-time qPCR and 3'-RACE. (A) Strategy for detecting RNA cleavage at the S1 protospacer in mRNA. Q_{tar} primer set amplified a region containing the predicted RNA cleavage sites while Q_{ref} amplified a region positioned upstream the S1 protospacer in mRNA. (B) Quantification of mRNA cleavage in RNA targeting strains of *S. islandicus* E233 carrying the chromosomal target gene *lacS*. Total RNAs were prepared from *S. islandicus* E233 cells harbouring pAC-SS1 or pSeSD1 and used for real-time qPCR. The amount of uncut mRNA is expressed as a ratio of *lacS* mRNA levels in transformants of pSeSD1 versus pAC-SS1. Bars represent the standard deviations of triplicates. (C) Quantification of mRNA cleavage in RNA targeting strains derived from *S. islandicus* E233S1 carrying the plasmid-borne *lacS*. Strains used are the same as in Figure 3B. Uncut mRNA is calculated as the percentage of the mRNA level in each strain carrying pAC-SS1 and that in *S. islandicus* E233S1 containing pLacS-ck. Bars represent the standard deviations of triplicates. (D) Schematic of cleavage sites within the S1 protospacer identified by 3'-RACE. Both protospacer S1 and the crRNA are shown with positions are numbered on crRNA. On RNA target: protospacer is shown as capital letters whereas the flanking nucleotides are in lowercase letters. The repeat handle of crRNA appears in green. Cleavage sites are indicated with arrows. Red arrows indicate cleavage sites by Cmr- α while Black arrows show cleavage sites by Cmr- β . Numbers below arrows indicate PCR clones carrying each cleavage site.

because the *S. islandicus* Cmr- α and Cmr- β systems are related in their protein composition and sequences to the Cmr systems of *P. furiosus* and *T. thermophilus* and that of *S. solfataricus*, respectively. To affirm the ruler mechanism-like sites were indeed produced by Cmr- α , 3'-RACE was conducted for pAC-SS1-LacS transformants retaining either Cmr- α gene cassette or the Cmr- β cassette. Sequencing several PCR products revealed that the two predominant sites at 6 nt intervals were only present in the cDNA generated from total RNAs prepared from the cells carrying Cmr- α and were absent from the sample derived from the cells containing Cmr- β .

Furthermore, although the RNA cleavage efficiency of the two Cmr systems did correlate with their effects on enzyme activity silencing, there were important differences.

The Cmr- α system degraded 30% of mRNAs that resulted in gene silencing at a similar level (31%), indicating that the system effectively mediated silencing of enzyme activity. However, Cmr- β was very efficient in destructing mRNA molecules, eliminating 68% of *lacS* mRNA but the residual enzymatic activity was only reduced by 42%. These differences were reinforced by the observation that 50% of mRNA cleavage versus 35% gene silencing occurred when both Cmr systems were present. A plausible explanation of these results is that Cmr- α targets nascent RNA transcripts and the interference complex is not recycled whereas Cmr- β complexes could be recycled and reloaded for a new round of RNA interference.

DISCUSSION

Here, we report a genetic assay for investigating RNA interference by CRISPR systems and using this assay we have, for the first time, shown that two distinct type III-B systems, Cmr- α and Cmr- β of *S. islandicus*, are capable of mediating mRNA cleavage *in vivo*. The assay is based on *lacS* mRNA-matching crRNAs produced from artificial mini-CRISPR loci on pAC-SS1 or pAC-SS3 that guide the endogenous CRISPR interference complexes to mediate RNA cleavage and thereby silencing of target gene expression. These spacers are appropriate for *in vivo* analysis of RNA interference by Cmr effector complexes because the corresponding protospacers are preceded by 5'-GAAAG-3' or 5'-GAGAC-3', which allow the corresponding protospacers to escape from known DNA interference activities (44,58). Therefore, transformants of pAC plasmids carrying these spacers could be obtained for RNA interference study. We found that each pAC plasmid strongly reduced activity of the *lacS* gene, yielding >80% inhibition of enzyme activity and >90% cleavage of *lacS* mRNAs, and this demonstrates that the genetic assay is very efficient. Recently Zebec *et al.* reported that *in vivo* RNA interference in *S. solfataricus* was about 50% (57), much lower than observed here. This could be due to that crRNAs were produced to different levels from the two artificial mini-CRISPR loci: whereas a CRISPR leader sequence was employed to express artificial mini-CRISPRs in their work (57), we used *P_{aras-SD}*, a strong expression promoter for doing that.

Exploiting the simplicity of pACs, and their high efficiency in mediating gene silencing, we have performed systematic nucleotide mutagenesis on the spacer sequence and analysed the effect of complementarity between crRNA and the target RNA on RNA interference. The results demonstrated that a trinucleotide motif ACC at positions 28–30 of S1 corresponding to the 3' region of crRNA is of vital importance for RNA interference. This contrasts with the results obtained for DNA targeting with diverse type I and type II systems. For example, DNA interference in most known bacteria and archaea requires a longer seed sequence and functions together with the PAM sequence for the type I-B system of *Haloferax volcanii* (70), type I-E system of *E. coli* and type I-F system of *Pseudomonas aeruginosa* and *Pectobacterium atrosepticum* (45–47,51,71), and type II systems of *Streptococcus pyogenes* and *Streptococcus thermophilus* (48–50). It has been postulated that the PAM provides the entrance site for the RNP complex whereas the seed sequence region facilitates the formation of an R-loop structure (36). Interesting, DNA interference in *S. solfataricus* tolerates a wide range of mismatches (31,37). Since this archaeon encodes multiple DNA targeting complexes including a Csm (59,72), the broad target recognition could reflect a combinatorial effect of different CRISPR systems. Likewise, the broad target recognition in RNA interference in *S. islandicus* could represent a compilation of RNA targets by Cmr- α and Cmr- β systems.

Currently, it remains to be determined how Cmr complexes scan and complex with their RNA targets. Since the identified crRNA motif is located in the 3'-region of the crRNA and the targeting activity is PAM-independent, we postulate that the corresponding region of the protospacer

provides the entry site for the formation of Cmr RNA targeting complexes in *S. islandicus*. Crystal structural analysis of the Cmr RNP complex will yield further insights into recognition and cleavage mechanisms.

By using deletion mutants of each Cmr gene cassette, Δ Cmr- α and Δ -Cmr- β , as well as a mutant with both Cmr gene cassettes deleted (Δ Cmr- $\alpha\beta$), we have established unambiguously, that each III-B Cmr system possesses RNA targeting activity whereas the type I-A CRISPR system does not show any detectable gene silencing in *S. islandicus*. These results are consistent with the *in vitro* RNA cleavage activity demonstrated for Cmr systems of *P. furiosus* and *T. thermophilus* and also that of *S. solfataricus* (30,53–57). The interference complexes of *P. furiosus* and *T. thermophilus* contain six subunits, adopting a seahorse-like structure as for type I RNPs and they cleave the target RNA by a ruler-based mechanism (30,54–56) whereas the *S. solfataricus* Cmr complex has seven subunits, forming a RNP complex of crab claw-like structure which cleaves RNA substrates at UA or UU dinucleotides *in vitro* (53,57). Here, we have shown that the *S. islandicus* Cmr- α utilizes a ruler-like mechanism to cleave mRNA *in vivo* as was seen *in vitro* for Cmr systems of *P. furiosus* and *T. thermophilus* whereas Cmr- β cleaves RNAs by the mechanism demonstrated for the Cmr system of *S. solfataricus*. Furthermore, since the entrance site for the complex formation is located at 3'-end of the crRNA, it is more likely that the measurement of the ruler mechanism for the Cmr- α system starts from the same side, namely, 3'-end of the crRNA.

The distinctive patterns of RNA cleavage by the two Cmr systems raise a question whether the sequences of crRNAs will affect their efficiency of RNA interference. Since Cmr systems of *P. furiosus* and *T. thermophilus* (30,54) and *S. islandicus* Cmr- α employ a ruler mechanism to guide RNA cleavage and the active sites of *P. furiosus* Cmr interference complex was found to be present in Cmr4 subunits of the effector complex (73), the process is probably sequence-independent. However, the Cmr- β effector complex destructs its RNA substrates specifically at UA or UU dinucleotides on the RNA target as has been demonstrated for a homologous Cmr system of *S. solfataricus* (53). Therefore, it is interesting to determine RNA targeting efficiency with mini-CRISPR loci carrying spacers that contain different numbers of cleavage sites to gain additional insight into Cmr- β mediated RNA cleavage.

Although both Cmr systems exhibit RNA interference, our results suggest that *S. islandicus* Cmr- β provides the major apparatus for RNA targeting, and upon accomplishing the first round of RNA cleavage, the protein components could be recycled for another round of RNA interference. In contrast, the primary role of Cmr- α is unlikely to exert RNA interference since the complex probably targets nascent RNA transcripts primarily and remains associated with the RNA. Following lines of evidence support the hypothesis: (i) RNA cleavage efficiency mediated by Cmr- α is the same as enzyme activity silencing and this should only occur if Cmr- α targets nascent RNA transcripts. Targeting mRNAs post translation would explain the high residual β -glycosidase activity relative to mRNA content as observed with the exclusive Cmr- β activity. Moreover, the *S. solfataricus* Cmr complex was found to be very likely recycled

in an *in vitro* RNA cleavage assay (53). Therefore, Cmr- α is probably not recycled whereas Cmr- β is to be recycled. (ii) Analysis of RNA cleavage sites that fell within the protospacer revealed a ratio of 2:1 for Cmr- α to Cmr- β , indicating that the Cmr- α complexes associated with their targets were two-fold of the Cmr- β interference complexes. However, the RNA cleavage efficiency was found to be 30% for Cmr- α but 70% for Cmr- β . Together this also supports a stable association of Cmr- α with the target and a probable recycling of Cmr- β .

Our recent work on genetic analysis of Cmr- α DNA targeting using an invader plasmid assay indicates that the DNA interference relies on the *csxI* gene encoding a Cas-accessory protein and the transcription of the protospacer (58). While the involvement of transcriptional activity in the Cmr- α DNA targeting was initially surprising, this requirement fits well with the properties of Cmr- α RNA interference: the stable association of Cmr- α effector complex with the RNA substrate suggests that the complex probably serves as an intermediate in the Cmr- α -mediated DNA interference pathway. Events that are likely to occur during Cmr- α dual targeting of RNA and DNA include: (i) the Cmr- α system targets nascent RNA transcripts, and forms a stable RNA-RNA complex, (ii) Csx1 is to be recruited to the RNA interference complex and the interaction between Csx1 and Cmr- α induces a conformational change in Cmr- α , (iii) the complex can then identify the DNA target to execute DNA interference. At present, we cannot exclude the possibility that additional Cas proteins or Cas-accessory proteins are involved at the DNA interference stage. Furthermore, Cmr- α -like systems and *csxI* genes are widespread in archaea and bacteria (74,75), this suggests there could be more CRISPR systems that exhibit the dual targeting activity. Indeed, re-examination of the *S. epidermidis* type III-A Csm system has revealed that this CRISPR system also requires transcriptional activity at the target site for the DNA interference although it remains to be investigated whether the interference involves an RNA targeting intermediate and whether any Cas-accessory proteins are involved (76). Moreover, in light of the identification of several different classes of Cas-accessory proteins in diverse archaea and bacteria (75,77), these proteins provide a great potential to modulate CRISPR defence mechanisms and other CRISPR-mediated cellular processes.

Indeed, novel activities are emerging for CRISPR systems. For example, *Francisella novicida* Cas9 uses a unique, small, CRISPR Cas-associated RNA to repress an endogenous transcript encoding a bacterial lipoprotein (78), and a Cas9 protein shows RNA cleavage activity *in vitro* when short DNA molecules containing a PAM are provided (79). In the present work we show that the two Cmr systems display extraordinary broad target recognition in RNA interference in *S. islandicus*, and this suggests that CRISPR systems could play a more general role in regulating genome expression. Undoubtedly, investigations of different CRISPR systems in archaea and bacteria will further unravel functional diversification of CRISPR systems and the involved mechanisms.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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