Introduction

Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) proteins constitute the adaptive and inheritable immune system in almost all archaea and more than 40% of bacteria. The antiviral nature of these systems was predicted based on bioinformatic analyses of bacterial and archaeal genomes and their genetic elements, which predicted that unique DNA sequences (spacers) of CRISPR arrays were derived from invasive genetic elements and it was further hypothesized that the defense could occur via an RNA interference-like mechanism. Subsequently, in vivo CRISPR activity has been studied in different microorganisms demonstrating that immune response by CRISPR/Cas systems exerts through three distinct stages: (1) Pieces of DNA sequence of invading genetic elements are incorporated into a CRISPR locus at a position between the leader and the first spacer, generating new spacers; (2) Transcription of CRISPR arrays from their leaders gives precursor CRISPR RNA molecules (pre-crRNAs) that are processed to yield mature CRISPR RNAs (crRNAs) and (3) crRNAs and Cascade (CRISPR-associated complex for antiviral defense) form ribonucleoprotein complexes, exerting silencing of invasive nucleic acids.

CRISPR/Cas systems are very diverse and their reevaluation has led to the classification of three main classes, type I, II and III, which are further divided into subgroups. Recently, Cas1 and Cas2, the only universally conserved Cas proteins, formed a complex with Csa1 and Cas4 in Thermoproteus tenax, which was implicated in acquisition of new spacers, and for this reason they were classified as a new Cas module, CRISPR-associated cluster for integration of new spacers (Cascis). Following the first demonstration of spacer acquisition in Streptococcus thermophilus, insertion of new spacers between the leader sequence and the first spacer of CRISPR arrays has been shown in Escherichia coli and Sulfolobus solfataricus. Moreover, it has been shown that Cas1 and Cas2 are sufficient to yield spacer insertion in E. coli.

Small RNA products generated from CRISPR arrays were first detected a decade ago in Archaeoglobus fulgidus and later were found to be widespread in microorganisms. CRISPR arrays often consist of a large cluster of spacer-repeat units. Transcription of an entire CRISPR array gives pre-crRNA to be processed into effector crRNAs. Two distinct mechanisms are known to generate
An important point in the antiviral defense is how CRISPR systems distinguish self vs. non-self DNA. Most known CRISPR systems recognize an invasive genetic element by identifying a protospacer-adjacent motif (PAM) located either at 5′- or at 3′-flanking position to a protospacer of the genetic element.\(^9\) PAM motifs were first recognized from genome sequence analyses,\(^20,39\) and subsequently experimentally demonstrated by virus infection in bacteria\(^40,41\) or by interference plasmid assay in archaea.\(^42-44\)

The other type of self vs. non-self discrimination involves repeat protection of chromosome-encoded CRISPR arrays from the DNA interference by the Csm module in \(S.\) epidermidis.\(^45\)

All known \(Sulfolobus\) strains encode multiple CRISPR interference modules belonging to type IA or type IIIA or IIIB, whereas the number and types of modules carried in each strain vary from one to another.\(^46\) In the recently completed genome of \(S.\) islandicus \(R\) \(E\) \(Y15\)\(A, \(^47\) there are two arrays of CRISPR elements with the same repeat, a \(C\) \(a\) \(s\) \(s\) \(is\) \(locus\) with five genes, a type IA CRISPR interference system and two distinct type IIIB \(Cm\) \(r\) \(s\) \(systems\) (Fig. 1).

**Figure 1.** \(S.\) islandicus Cas modules and construction knockouts lacking a Cas module or a \(c\) \(as\) gene. (A) Schematic of CRISPR arrays and Cas modules in \(S.\) islandicus \(R\) \(E\) \(Y15\)\(A.\) (B) PCR verification of mutant alleles of Cas modules and \(c\) \(as\) genes in the mutants constructed with the MID procedure, described previously.\(^66\) PCR was conducted with specific primers for each Cas module or \(c\) \(as\) gene with genomic DNAs extracted from the genetic host \(S.\) islandicus \(E23\) \(wt\) and corresponding deletion mutant (\(Δ\)). M, DNA size marker. Cascis, CRISPR-associated cluster for integration of new spacers; IA, type IA interference module; \(Cmr-α\) and \(Cmr-β\), two type IIIB modules containing 6 and 7 \(c\) \(m\) \(r\) genes, respectively.\(^47\)

crRNAs. The first is endonuclease Cas6, a processing enzyme for type I and type III CRISPR systems,\(^23-27\) which binds to a repeat of a pre-crRNA and cleaves it. In fact, the enzyme remains bound to the RNA after cleavage.\(^25,26\) The second is RNase III that does the cleavage under the guidance of a trans-acting tracrRNA in type II systems.\(^29\)

At the final stage, it is believed that Cascade complexes containing crRNAs recruit Cas3 helicase/nuclease and exert invader silencing,\(^25,30-32\) during which a target nucleic acid is recognized by complementarity between the protospacer and the corresponding effector crRNA. DNA targeting activity has been demonstrated for representative systems belonging to each of the main types of CRISPR systems, including the \(E.\) coli type IE crRNA-Cas3 ribonucleoprotein complex in vitro,\(^33\) DNA targeting activity of type II systems in \(S.\) thermophila in vivo\(^34\) and in vitro,\(^35\) and the \(Staphylococcus\) \(e\) \(p\) \(i\) \(d\) \(m\) \(i\) \(d\) \(is\) type IIIA system Casm.\(^36\) Furthermore, \(P.\) \(f\) \(u\) \(r\) \(i\) \(o\) \(s\) and \(S.\) \(s\) \(o\) \(f\) \(t\) \(a\) \(t\) \(a\) \(r\) \(i\) \(c\) \(i\) \(c\) \(us\) type IIIB CRISPR repeat-associated mysterious proteins (RAMP),\(^31\) also named Cmr modules, mediate RNA targeting activity.\(^32,37,38\)

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This organism is one of the genetically tractable archaeal models.\(^46\)
with which a novel gene knockout method has been established and plasmid interference assays were developed for testing interference activities. This has enabled us, for the first time, to conduct genetic analyses of cas genes in this microbe carrying complex CRISPR/Cas systems. Deletion mutants lacking Cascis, Cmr-α or Cmr-β module and those deleting each of the genes encoding components of the putative IA-Cascade complex were constructed and analyzed pre-crRNA processing and CC PAM motif-mediated targeting. We show that type IA module is responsible for the PAM-dependent interference. Evidence has been provided to demonstrate that other Cas proteins also play a role in modulating pre-crRNA processing by Cas6.

Results

Cascis, Cmr-α and Cmr-β modules are dispensable for pre-crRNA processing and CC PAM motif DNA targeting. Previously, it was shown that CRISPR arrays were transcribed and processed into short RNA molecules in different Sulfolobus species and that Sulfolobus CRISPR systems conferred DNA targeting activity. However, as Sulfolobus contain multiple Cas interference modules, functions of each Cas module and individual cas genes in these molecular processes have not been studied. Here, we systematically studied possible functions of four Cas modules in S. islandicus REY15A, i.e., a type IA interference module, a Cas cluster for integration of new spacers (Cascis) and two type IIIB modules Cmr-α and Cmr-β modules (Fig. 1A). Mutant strains lacking each entire module of Cascis, Cmr-α or Cmr-β were constructed, from which total RNAs were prepared and subsequently analyzed by northern hybridization as described in Materials and Methods.

Results of the northern hybridization of RNAs prepared from the wild-type strain were very similar to those of the northern analyses of the mutants (Fig. 2A and B). The radiolabeled repeat oligonucleotide probe hybridized to RNAs of 60–70 nt in size. These RNAs are comparable in size with the mature crRNAs produced by an S. solfataricus Cas6, the primary product of pre-cRNAs was processed by Cas6, yielding spacers carrying 8 nt 5′-handle and 16 nt 3′-handle of the repeat. A few processing intermediates were also hybridized by the repeat probe showing the sizes equivalent to the RNAs carrying 3, 5 and 7 spacers (Fig. 2A). When analyzed with an oligonucleotide of array 2 spacer 32 as probe, an RNA of ca. 62 nt as well as RNAs < 60 nt were identified (Fig. 2B). The former could represent a primary processing product of Cas6 cleavage and the latter should be resulted from further processing/maturation of the primary product. Again, the probe identified the same RNA bands in ΔCascis, ΔCmr-α and ΔCmr-β, comparing with the wild-type strain except for the smallest crRNA species (ca. 40 or 41 nt) that is absent from ΔCascis, ΔCmr-α and ΔCmr-β, a result consistent with the functional analysis of Cmr-α in this crenarchaeon. Furthermore, none of these bands was present in the hybridization of the total RNAs prepared from ΔCRISPR, a strain lacking both arrays of CRISPR, indicating that the hybridization was specific for both probes. Taken together, this indicated that whereas ΔCmr-α is responsible for the maturation of the smallest crRNA, none of these Cas modules play an important role in pre-cRNA processing in this archaeon.

These strains were then tested for DNA targeting mediated by CC PAM motif using two different interference plasmids, pCC-A2S32 and pCC-A1S6. These plasmids carry the artificial protospacer sequence of array 2 spacer 32 (A2S32) or that of array 1 spacer 6 (A1S6) where each spacer is preceded by the PAM motif (5′-CCA-3′) of Sulfolobus.
Functions of Cas6, Cas7, Cas5 and Csa5, components of the aCASCADE complex in *S. islandicus*. Recently, an archaeal Cascade complex was isolated from *S. solfataricus*. It contained Cas7, Cas5, Cas6 and Csa5 and was loaded with crRNAs of 60–70 nt, which correspond to primary cleavage products by Cas6. Each of these Cascade proteins has a homolog in *S. islandicus* REY15A showing 82–93% amino acid sequence identity. Since both *S. solfataricus* and *S. islandicus* exhibited PAM-dependent DNA targeting activity, we were interested in studying reference plasmids, each PAM motif was replaced with 5 or 8 nt of the repeat sequence, yielding a structure resembling the genetic organization of CRISPR arrays in the chromosome. As shown in Figure 2C, when each mutant was employed as host for transformation, hundreds to thousands fold lower rates of transformation were yielded with pCC-A2S32 and pCC-A1S6 comparing with their reference plasmids, indicating none of these modules is essential for the CC motif-mediated DNA targeting activity.

Functions of Cas6, Cas7, Cas5 and Csa5, components of the aCASCADE complex in *S. islandicus*. Recently, an archaeal Cascade complex was isolated from *S. solfataricus*. It contained Cas7, Cas5, Cas6 and Csa5 and was loaded with crRNAs of 60–70 nt, which correspond to primary cleavage products by Cas6. Each of these Cascade proteins has a homolog in *S. islandicus* REY15A showing 82–93% amino acid sequence identity. Since both *S. solfataricus* and *S. islandicus* exhibited PAM-dependent DNA targeting activity, we were interested in studying reference plasmids, each PAM motif was replaced with 5 or 8 nt of the repeat sequence, yielding a structure resembling the genetic organization of CRISPR arrays in the chromosome. As shown in Figure 2C, when each mutant was employed as host for transformation, hundreds to thousands fold lower rates of transformation were yielded with pCC-A2S32 and pCC-A1S6 comparing with their reference plasmids, indicating none of these modules is essential for the CC motif-mediated DNA targeting activity.

![Figure 3. Functions of Cas proteins of type IA interference module in pre-crRNA processing and PAM-dependent DNA silencing.](image-url)
possible functions of these Cas proteins in this process. In-frame deletion mutants were constructed for all four \textit{cas} genes, giving \textit{Δcas}7, \textit{Δcas}5, \textit{Δcas}5 and \textit{Δcas}6. Their complementation plasmids were constructed with a highly efficient expression vector pSeSD\textsuperscript{22} and transformed into corresponding mutants to yield complementation strains. Both mutants and complementation strains were used for northern analysis of pre-crRNA processing and for the interference plasmid assay.

First, we examined pre-crRNA processing in \textit{Δcas}6 and its complementation strain. As shown in Figure 3, depleting Cas6 completely inactivated pre-crRNA processing since neither intermediates nor mature crRNAs were present in the mutant strain. These bands appeared again in the complementation strain where the Cas6 protein was expressed from the expression plasmid pSe-cas6. These results indicated that \textit{cas}6 is essential for crRNA production in \textit{S. islandicus} and that it is the only enzyme responsible for pre-crRNA processing in this organism.

Next, we analyzed processing intermediates and crRNAs in \textit{Δcas}5, \textit{Δcas}5 and \textit{Δcas}7 from which different results were obtained. Northern analysis of \textit{Δcas}5 and its complementation strain showed the same hybridization pattern and RNA band intensities that were essentially identical to the results of the wild-type strain. Hybridization signals of processing intermediates were absent or greatly weakened in \textit{Δcas}7, while crRNAs were maintained at very similar levels. The exactly opposite results were obtained for \textit{Δcas}5 where mature crRNAs were hardly detectable but normal levels of processing intermediates accumulated. Furthermore, genetic complementation of mutant deficiency by episomal expression of each corresponding gene from a plasmid restored the hybridization pattern of the wild-type strain (Fig. 3). These results indicated that \textit{cas}7 is essential for stabilizing RNA intermediates of pre-crRNA processing and it could function in the absence of \textit{Csa}5 or \textit{Cas}5, and that \textit{Csa}5 is essential for maintaining mature crRNAs whereas \textit{Csa}5 does not appear to have a role in pre-crRNA processing and maturation.

These mutants were also tested for PAM motif-mediated DNA targeting. As shown in Figure 3C, whereas \textit{Δcas}7 and \textit{Δcas}5 lost the capability of conferring DNA interference to pCC-A2S32 and pCC-A1S6, depleting \textit{Csa}5 did not compromise the interference activity.

Taken together, we have demonstrated that only three of the four putative Cascade proteins, i.e., \textit{Cas}6, \textit{Cas}7 and \textit{Cas}5 are essential for the PAM-mediated DNA interference. All these Cas proteins function in pre-crRNA processing in which Cas6 is the primary processing enzyme in vivo whereas Cas7 and Cas5 stabilize processing intermediates and crRNAs, respectively. However, Cas5 does not appear to have a role in either process, suggesting that Csa5 may not represent an integral part of the Cascade that mediates PAM-directed DNA silencing.

**Cas3 helicase/nuclease functions both in processing and targeting.** Cas3 is required for all known type I CRISPR systems for interference. In some CRISPR systems such as those in \textit{Sulfolobus} species, Cas3 is divided into two parts: Cas3’ containing a helicase domain and Cas3” carrying a nuclease domain. To reveal their role in DNA interference, \textit{Δcas}3’ and \textit{Δcas}3” were investigated for silencing of pCC-A2S32 and pCC-A1S6 interference plasmids. As shown in Figure 3C, both mutants showed high transformation efficiencies with all tested plasmids, including both interference and control plasmids, indicating that the interference activity was inactivated in the mutants.

When pre-crRNA processing was studied by northern analysis, both mutants showed increased levels of processing intermediates compared with the wild-type strain and a much elevated level of mature crRNA was observed for \textit{Δcas}3’ (Fig. 3). Interestingly, while genetic complementation of the mutant deficiency by episomic expression of \textit{cas}3’ or \textit{cas}3” reversed the strong accumulation of the intermediates, a few new RNA species (60 < crRNAs < 80 nt), which first appeared in \textit{Δcas}3’ and \textit{Δcas}3”, persisted in their complementing strains. These results suggest that the helicase and nuclease activities of Cas3 function in resolving the processing intermediates and that the nuclease domain could play a role in crRNA degradation.

**Discussion**

In this work, we have studied functions of different interference modules in pre-crRNA processing and PAM-mediated DNA targeting in \textit{S. islandicus} \textit{REY}15A. Mutants lacking each module were constructed and analyzed for mature crRNA and their processing intermediates and for DNA interference. This has revealed that the type IA interference module mediates PAM-dependent DNA targeting and it functions independently from other Cas/Cmr modules that co-exist in \textit{S. islandicus} \textit{REY}15A. We also show that depleting Cas6 inactivates pre-crRNA processing and that inactivation of Cas7, Cas5, Cas3’ or Cas3” impairs processing of pre-crRNAs. This unravels an important difference between in vivo and in vitro results since biochemical characterization of a \textit{S. solfataricus} Cas6 protein, which is 93% identical to this \textit{S. islandicus} Cas6 in amino acid sequence, demonstrated that Cas6 was capable of generating mature crRNAs by itself.\textsuperscript{23}

A Cascade complex (IA-Cascade) isolated from \textit{S. solfataricus} was reported consisting of at least four components, Cas7, Cas5, Cas3 and Cas6.\textsuperscript{23} Characterization of other type I Cascade complexes, including the IE-Cascade of \textit{E. coli} and the IF Cascade of \textit{Pseudomonas aeruginosa}, revealed five components (Cas1, Cas2, Cas7, Cas5 and Cas6e) for type IE\textsuperscript{19,30} and four components Csy1, Csy2, Csy3 and Csy6 (Csy4) for type IF.\textsuperscript{33} Importantly, all Cascade components and Cas3 are shown to be essential for the DNA-targeting activity in IE and IF systems.\textsuperscript{19,30,54} Here, we show that Csa5, one of the Cascade components, is dispensable for the PAM-directed DNA targeting in \textit{S. islandicus}. It is important to point out though that a functional \textit{S. solfataricus} Cascade complex remains to be demonstrated since the isolated one did not show any cleavage activity.\textsuperscript{25}

Interestingly, although non-essential to DNA interference, Csa5 is encoded in all known archaea carrying a type IA CRISPR system, including all known \textit{Sulfolobus} species,\textsuperscript{47-58} \textit{Methanothermobacter jannaschii},\textsuperscript{39} \textit{Thermoproteus tenax}\textsuperscript{12} and four \textit{Pyrobaculum} species,\textsuperscript{60} whereas Csa5 is absent from all known type IB CRISPR systems, including \textit{Haloflexx volcanii}.\textsuperscript{43}
Clostridium thermocellum and Methanococcus maripaludis. 37 Currently, the role of Csa5 in type IA CRISPR system remains to be demonstrated.

In the E. coli type IE system, cleavage of pre-crRNAs by Cas6e yields mature crRNAs that bind to IE-Cascade to form ribonucleaseprotein complexes. 33 It has been shown that Cse1 component of the IE-Cascade recognizes the PAM motif of a protospacer of invasive DNAs 61 and complementarity between the crRNA and the protospacer then guides the cleavage of foreign DNA by Cas3. 31 Currently, the counterpart of Cse1 remains to be identified in the Sulfolobus IA-Cascade complex if this complex functions in analogy to the E. coli IE-Cascade.

Examination of crRNA processing in S. islandicus by northern hybridization showed that the processing was inactivated by Cas6 depletion and the inactivation only occurred in Δcas6 (Fig. 3). This is consistent with the results that P. furiosus and S. solfataricus Cas6 proteins are capable of generating crRNA in vitro. 25, 62 Together, these studies demonstrate that Cas6 is both necessary and sufficient for pre-crRNA processing in these archaea.

Furthermore, our study of pre-crRNA processing in the cas gene knockouts of S. islandicus has revealed three interesting features: (1) mature crRNAs are stabilized in Δcas5, (2) processing intermediates are stabilized in Δcas5 and (3) processing intermediates are accumulated in Δcas3 and Δcas7. Interestingly, Cas7 forms protein filaments in S. solfataricus. 25 Putting all these facts together, possible mechanistic details are revealed for pre-crRNA processing in S. islandicus. (1) Immediately after transcription, pre-crRNAs are bound by Cas7 protein filaments, protecting the large RNA molecules from degradation; (2) Cas6 proteins bind to repeat sequences of pre-crRNAs and make a cut at each repeat, (3) as both Cas7 and Cas6 proteins remain bound to the processing products, interaction between Cas3’ and Cas3” and the complex will facilitate the remodeling of new smaller complexes and (4) single spacer unit complexes can only be stabilized by recruiting Cas5, which either constitute the Cascade or a basis for generating Cascade complexes. Possibly, Cas5 may also modulate Cas3 activities that could lead to crRNAs degradation.

The proposed strategy is apparently not used in the type I systems of E. coli and P. aeruginosa because relative band intensities of intermediates and crRNAs did not change upon depletion of any component of the Cascade complexes in these organisms. 39, 54 Neither has it been reported that the E. coli Cas7 forms protein filaments. In fact, these type I systems are further diverged, whereas only Cas6 is essential in pre-crRNA processing in S. islandicus, two protein factors are reported to be essential for in vivo pre-crRNA processing in the bacterial systems, namely Cas5 and Cas6 in E. coli 25, 63 and Cas6f (Csy4) and Csy2 in P. aeruginosa. 54

Then, the question is why it is relevant for S. islandicus to employ such a strategy in crRNA processing. This archaean contains three distinct interference modules that are presumed to employ different types of mature crRNA for their interference. The type IA system most likely utilizes 60–70 nt crRNAs, which are components of the S. solfataricus Cascade complex. 25 The Cmr-β module most likely uses 30–50 nt crRNAs, which are associated with the S. solfataricus type IIIB Cmr reported by Zhang et al., 38 because the two Cmr systems share high-sequence similarity and exhibit the same gene synteny. Although the Cmr-α complex has not been isolated thus far, here we show that depleting the complex eliminates the smallest crRNA, which is ca. 40–41 nt crRNA for A2S32. More recently, interference activity has been demonstrated for the S. islandicus Cmr-α, which confers a protospacer transcript-dependent DNA interference. 50 Further investigation of the distinct Cas interference modules in S. islandicus will yield important insights into regulation of pre-crRNA processing and maturation as well as mechanisms of coordinating different interference activities that co-exist intracellularly.

Materials and Methods

Sulfolobus strains, growth conditions and transformation. All experiments were performed with the model organism of Sulfolobus islandicus REY15A originally isolated from an enrichment culture obtained from samples collected from hot springs in Iceland. 64 Its genetic host E2335 is double deletion mutant carrying a large deletion in pyrEF genes and a complete deletion of lacS gene. 65

All Sulfolobus strains used in this study are listed in Table 1. The strains that carry pyrEF genes either on the chromosome or on a plasmid were grown at 76°C in the SCV (0.2% sucrose, 0.2% casamino acids plus 1% vitamin solution) selective medium, whereas the medium was supplemented with 20 μg/ml uracil (SCVU) when growing pyrEF-deficient strains. Sulfolobus competent-cells were prepared as described previously 65 and plasmids were introduced into the cells by electroporation and transformed cells were allowed to form colonies on SCV plates.

Construction of deletion mutants of S. islandicus. A novel gene knockout method named marker insertion and unmarked target gene deletion (MID) developed by Zhang et al. 66 was employed to construct cas gene knockouts. In MID manipulation, each plasmid vector contains three homologous sequence arms, i.e., left, right and target gene arms. These homologous arms were carefully designed such that the insertion of a marker cassette would yield a functional target gene at the first step of recombination. Primers for amplification of these arms for each Cas module or cas gene by polymerase chain reaction are listed in Table 3. Ligation of these arms to the E. coli vector pUC19 gave pHID-Cascis, -Cmr-α, -Cmr-β, -cas7, -cas5, -cas6, -csa5, -cas3’ and -cas3”. These knockout plasmids were linearized with Pvu II before transformation. Transformants were obtained on SCV plates, whereas mutant cells were recovered by counter selection of pyrEF with 5’-fluoroorotic acids (5’-FOA). After three rounds of single colony purification, the mutants were analyzed for their mutant alleles by PCR with specific primers for each target gene or gene region and their mutant genotypes were verified (Fig. 1B).

Complementation plasmids were constructed with pSeSD, a highly efficient expression vector recently reported for Sulfolobus. 52 The expression is under the control of P aerS-SD, a synthetic promoter that confers up to one-third of the fully induced level of expression in a sucrose medium. 52 Each cas gene was amplified by PCR from S. islandicus total DNA with a Pfu DNA polymerase using specific primers listed in Table 3. After cleavage with the enzymes indicated in each PCR primer, the DNA fragments were

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RNA preparation and northern analysis. Total RNAs were extracted using the Trizol reagent (Invitrogen) following the protocol provided by the manufacturer. Thirty μg RNA was loaded on a 6% polyacrylamide-SDS gel for each sample and fractionated. RNAs in the gel were then transferred onto a nylon membrane and immobilized. Procedures for pre-hybridization, end labeling of complementary nucleotides and hybridization in northern analysis were described earlier and the probes used in this work included a probe of the repeat sequence (5’-CTT TCA ATT CTA TAG TAG ATT AGC NNN N-3’) for detecting intermediates of pre-crRNA processing and a probe of spacer 32 of array 2 (5’-GCG GGA TTA GTA GGG ATT CCC ATA GGG CTC TAT GAA CT-3’) hybridizing to mature crRNAs.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

cloned to pSeSD at the compatible sites, yielding pSe-csa5, -cas5, -cas7, -cas3’, -cas3” and -cas6.

Plasmid interference assay of PAM-mediated DNA targeting. Artificial interference plasmids were constructed with two spacers, spacer 6 of CRISPR array 1 (A1S6) and spacer 32 of array 2 (A2S32). Oligonucleotides containing the complete sequences of the two spacers that were preceded with 5’-CCA-3’ or with the last five or eight nucleotides of the repeat were synthesized from TAG Copenhagen (Table 3). Each pair of primers was annealed with each other by heating to 95°C for 10 min and then cooling down gradually to room temperature, yielding double-stranded DNAs with one blunt end and one protruding end, the latter of which matched the DNA ends after Mlu I digestion. These DNA fragments were cloned to pSeSD at Stu I and Mlu I sites individually, yielding plasmids containing an artificial protospacer (interference plasmids) and those containing a repeat-spacer module (reference plasmids) (Table 2).

### Table 1. Strains used in this study

| Strain   | Genotype and features                                      | Source                  |
|----------|------------------------------------------------------------|-------------------------|
| S. islandicus E233S ΔpyrEFΔlacS | CRISPR arrays are not detectable by PCR                      | Deng et al.65 |
| ΔCRISPR  | All genes in Cascis module, including csa3, cas1, cas2 and cas4 are deleted | Gudbergsdottir et al.62 |
| ΔCascis  | Deletion of the type IIIB Cmr locus with 6 genes           | This work               |
| ΔCmr-α   | Deletion of the type IIIB Cmr locus with 7 genes           | This work               |
| Δcsa5    | In-frame deletion of csa5                                   | This work               |
| Δcas7    | In-frame deletion of cas7                                   | This work               |
| Δcas5    | In-frame deletion of cas5                                   | This work               |
| Δcas3’   | In-frame deletion of cas3’                                  | This work               |
| Δcas3”   | In-frame deletion of cas3”                                  | This work               |
| Δcas6    | Carrying deletion of the entire cas6 gene plus 277 bp downstream flanking sequence | This work               |

### Table 2. Plasmids used in this study

| Plasmid    | Features                                                                 | Source                  |
|------------|--------------------------------------------------------------------------|-------------------------|
| pSeSD      | A Sulfolobus-E. coli shuttle vector carrying an expression cassette controlled under a synthetic strong promoter P araS-SD | Peng et al.52 |
| pCC-A1S6   | An artificial interference plasmid; pSeSD carrying the interference module of the sequence of spacer 6 of array 1 preceded by the 5’-CCA-3’ motif | This work               |
| pRt-A1S6   | Reference plasmid of pCC-A1S6; the PAM motif (5’-CCA-3’) was replaced with the last 5 nt of the repeat sequence (5’-GAA AG-3’) | This work               |
| pCC-A2S32  | An artificial interference plasmid; pSeSD carrying the interference module of the sequence of spacer 32 of array 2 preceded by the 5’-CCA-3’ motif | This work               |
| pRt-A2S32  | Reference plasmid of pCC-A2S32; the PAM motif (5’-CCA-3’) was replaced with the last 8 nt of the repeat sequence (5’-ATT GAA AG-3’) | This work               |
| pSe-Csa5   | pSeSD carrying the S. islandicus csa5 gene                            | This work               |
| pSe-Cas7   | pSeSD carrying the S. islandicus cas7 gene                            | This work               |
| pSe-Cas5   | pSeSD carrying the S. islandicus cas5 gene                            | This work               |
| pSe-Cas3’  | pSeSD carrying the S. islandicus cas3’ gene                           | This work               |
| pSe-Cas3”  | pSeSD carrying the S. islandicus cas3” gene                           | This work               |
| pSe-Cas6   | pSeSD carrying the S. islandicus cas6 gene                            | This work               |
### Table 3. Oligonucleotides used in this study

| Oligonucleotide          | Sequence (5’-3'; PAM motif or repeat sequence underlined, restriction site in bold) |
|--------------------------|----------------------------------------------------------------------------------|
| A1S6fwd-MluI             | CGC GTC CAG TTG GCA TAA TCC TCA ACA TAC TTT CTT GTG TCG GTT ATG C                 |
| A1S6rev                  | GCA TAC CCG ACA CAA GAA AGT TGG AGG ATT ATG CCA ACT GGA                           |
| ReA1S6fwd-MluI           | CGC GAA AGG TTG GCA TAA TCC TCA ACA TAC TTT CTT GTG TCG GTT ATG C                 |
| ReA1S6rev                | GCA TAC CCG ACA CAA GAA AGT TGG AGG ATT ATG CCA ACC TTT (C)                       |
| A2S3fwd-MluI             | CGC GTC CCA AGT TCA TAG AGC CCT ATG GGA ATC CCT ACT AAT CCC GC                    |
| A2S3rev                  | GCG GGA TTA GTA GGG ATT CCC ATA GGG CTC TAT GAA CTC TTT GGG A                     |
| ReA2S3fwd-MluI           | CGC GAT TGA AAG AGT TCA TAG ACC CCT ATG AGA ATC CCT ACT AAT CCC GC                |
| ReA2S3rev                | GCG GGA TTA GTA GGG ATT CCC ATA GGG CTC TAT GAA CTC TTT GGG A                     |
| Csa5fwd-Mluul            | CCA CGC GTT GGC ACA ACA AGT AAA AGA AG                                         |
| Csa5rev-Stul             | AAG GGC TTT TCT TCT CAC CAC CAC TTT G                                            |
| Cas7fwd-Ndel             | GTT GTT CCA TAT GAT AAG TGG TTC AGG TAG ATT T                                     |
| Cas7rev-Mluul            | CGA CGC GTC TAC TTT TCT TTT AAT TTA CC                                         |
| CasSfwd-Mluul            | GGA CGC GTT GAT CTA CTC TAA GGT CTT TTT                                         |
| Cas5rev-Stul             | GAG GCC TGC TAA AGA CAA CAT ATT TTC C                                           |
| Cas3’fwd-Ndel            | CCC AAA GCA TAT GTT GTC TTT AGC TGA TCT CTA T                                   |
| Cas3’rev-Mluul           | CGA CGC GTT CAA TAC ACA CCA CCT ATT TC                                         |
| Cas3”fwd-Ndel            | AAT AAA TCA TAT GAT CAA GCC TTG CGC TTA TGA G                                   |
| Cas’rev-Mluul            | CGA CGC GTT TAT AAA GGT GAA CAA CCT C                                           |
| Cas6fwd-Ndel             | GGG ACC GCA TAT GCC ATT AAT TTA CAA CAT A                                       |
| Cas6rev-Mluul            | GTA CGC GTA CTT TTA AAG TCT GAG GA                                              |
| Cmr-α-Gfwd-Sall          | AAT TGT CGA CGC AGG AAT GGT GTT AGA GTC T                                       |
| Cmr-α-Grev-Mluul         | CCA CGC GTC CTC CCC ACT TGA ATA CTA CC                                         |
| Cmr-α-Lfwd-Ncol          | AAT TCC ATG GCA GGA AAG CAG TAG AGA AGG A                                       |
| Cmr-α-Lrev-Xhol          | ATC CTC GAG AAC TTG TAA CCC TAC GTC GTT                                       |
| Cmr-α-Rfwd-Xhol          | ATC CTC GAG ACT AAG ATG ACT AAG GAA GGC                                        |
| Cmr-α-Rrev-Sphl          | AAT TGC ATG CAC AAA AAC ATG AAC GTA TGA G                                        |
| Cmr-β-Gfwd-Sall          | AAT TGT CGA CAT AGT CTT CAT CGG CAC AT A                                       |
| Cmr-β-Grev-Mluul         | CCA CGC GTT CAT CTG TAG AGG AGG AAA TA                                         |
| Cmr-β-Lfwd-Ncol          | AAT TCC ATG GTC AGG CAG TAA AGG AAG A                                           |
| Cmr-β-Lrev-Xhol          | ATC CTC GAG CAA TGG GTA AGG TGG ATA AGA                                        |
| Cmr-β-Rfwd-Xhol          | ATC CTC GAG TGT CAT AAG CCT CAT TCC                                            |
| Cmr-β-Rrev-Sphl          | AAT TGC ATG CAG AGG GTG GGT AGG TGA TGA T                                       |
| Casis-Gfwd-Sall          | AAT TGT CGA CAT GTT CTC TAT CAT GCC GTT T                                      |
| Casis-Grev-Mluul         | CCA CGC GTG TCC GTT CTA ATA CCG TCC CT                                        |
| Casis-Lfwd-Ncol          | AAT TCC ATG GTT CAT CAA GAG GAA AAT CGT C                                      |
| Casis-Lrev-Xhol          | ATC CTC GAG TTC GTT AGA AAC ACT CGC TAG                                        |
| Casis-Rfwd-Xhol          | ATC CTC GAG TGT ATT CAT AAG CCT CAT TCC                                         |
| Casis-Rrev-Sphl          | AAT TGC ATG CCA AGC GAA ATA GTA GGG AAC A                                       |
| Cas3’-Gfwd-Sall          | AAT TGT CGA CAA GGT AAC AAG ACT AAA GAC T                                       |
| Cas3’-Grev-Mluul         | CCA CGC GTC ACA CCA CCT ATT TCA CTA TT                                         |
| Cas3’-Lfwd-Ncol          | AAT TCC ATG GGC TTA CAC TGA GGA CAT TGT T                                      |
| Cas3’-Lrev-Xhol          | ATC CTC GAG ACC ATG ACG AGT TAT GAA ATC                                       |
| Cas3’-Rfwd-Xhol          | ATC CTC GAG TGA TCA AGC CTT GCG CTT ATG                                       |
| Cas3’-Rrev-Sphl          | AAT TGC ATG CTA CCC TAA TTG CTT TCA G                                          |
| Cas3’-Gfwd-Sall          | AAT TGT CGA CCG TGA TTG ATT CGA TCT TGG C                                       |
| Cas3’-Grev-Mluul         | CCA CGC GTA GTG GAA CCT CCA TTT AAC TC                                         |
Table 3. Oligonucleotides used in this study

| Oligonucleotide | Sequence |
|-----------------|----------|
| Cas3*-L fwd-Ncol | AAT TCC ATG GGG TGA TTG ATT CGA TTC TGG C |
| Cas3*-R fwd-Xhol | ATC CTC GAG CTC ATA AGC GCA AGG CTT GAT |
| Cas3*-R rev-sphl | ATC CTC GAG TTA TAT ATT TTG AGA TAA |
| Cas5-G fwd-Ssphl | AAT TGC ATG CTG ATG CA AAT ACT GTA GAG AAT A |
| Cas5-G rev-G rev-MluI cc | ATC TCT GAG TTA GCT GAC TTA TAT AAC GAT |
| Cas5-G rev-sphl | ATC TCT GAG TTA GCT GAC TTA TAT ATT TTG AGA T |
| Cas5-L fwd-Ncol | AAT TCC ATG GGT TAG AAA GGC AAA AGG CTC G |
| Cas5-L rev-Xhol | ATC CTC GAG TAA CTA CGG AAA AAC CCC AAP |
| Cas5-R rev-sphl | ATC CTC GAG TTA GCT GAC TTA TAT AAC GAT |
| Cas5-R rev-sphl | AAT TGC ATG CGT CTT TAG CAC CA AAT TCT T |
| Cas5-R rev-sphl | AAT TGC ATG CGT CTT TAG CAC CA AAT TCT T |
| Cas5-R rev-sphl | AAT TGC ATG CGT CTT TAG CAC CA AAT TCT T |
| Cas6-G fwd-Ssphl | AAT TGC ATG CCG AGG GGA GAG GTT CCG AGC T |
| Cas6-G rev-G rev-MluI cc | ATC TCT GAG CCT CAG ACT TTA AAG GTT AAG |
| Cas6-G rev-sphl | AAT TGC ATG CCG AGG GGA GAG GTT CCG AGC T |
| Cas6-L fwd-Ncol | AAT TCC ATG GGT TAG AAA GGC AAA AGG CTC G |
| Cas6-L rev-Xhol | ATC CTC GAG TAA CTA CGG AAA AAC CCC AAP |
| Cas6-R rev-sphl | ATC TCT GAG TTA GCT GAC TTA TAT AAC GAT |
| Cas6-R rev-sphl | AAT TGC ATG CGT CTT TAG CAC CA AAT TCT T |
| Cas6-R rev-sphl | AAT TGC ATG CGT CTT TAG CAC CA AAT TCT T |
| Cas6-R rev-sphl | AAT TGC ATG CGT CTT TAG CAC CA AAT TCT T |
| Cas7-G fwd-Ssphl | AAT TGC ATG CGT CTT TAG CAC CA AAT TCT T |
| Cas7-G rev-G rev-MluI cc | ATC TCT GAG CCT CAG ACT TTA AAG GTT AAG |
| Cas7-G rev-sphl | AAT TGC ATG CGT CTT TAG CAC CA AAT TCT T |
| Cas7-L fwd-Ncol | AAT TCC ATG GGT TAG AAA GGC AAA AGG CTC G |
| Cas7-L rev-Xhol | ATC CTC GAG TAA CTA CGG AAA AAC CCC AAP |
| Cas7-R rev-sphl | ATC TCT GAG TTA GCT GAC TTA TAT AAC GAT |
| Cas8-G fwd-Ssphl | AAT TGC ATG CGT CTT TAG CAC CA AAT TCT T |
| Cas8-G rev-G rev-MluI cc | ATC TCT GAG CCT CAG ACT TTA AAG GTT AAG |
| Cas8-G rev-sphl | AAT TGC ATG CGT CTT TAG CAC CA AAT TCT T |
| Cas8-L fwd-Ncol | AAT TCC ATG GGT TAG AAA GGC AAA AGG CTC G |
| Cas8-L rev-Xhol | ATC CTC GAG TAA CTA CGG AAA AAC CCC AAP |
| Cas8-R rev-sphl | ATC TCT GAG TTA GCT GAC TTA TAT AAC GAT |
| Cas8-R rev-sphl | AAT TGC ATG CGT CTT TAG CAC CA AAT TCT T |
| Cas8-R rev-sphl | AAT TGC ATG CGT CTT TAG CAC CA AAT TCT T |

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Author Contributions

Conceived and designed the experiments: Q.S., Y.X.L., W.P. and N.P. performed experiments: W.P., H.L. and S.H. analyzed data: Q.S., Y.X.L., W.P., N.P., H.L. and S.H. wrote the paper: Q.S. and W.P.

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