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***In vitro* reconstitution of Cascade-mediated CRISPR immunity in
*Streptococcus thermophilus***

Tomas Sinkunas¹, Giedrius Gasiunas¹, Sakharam P Waghmare², Mark J Dickman², Rodolphe Barrangou³, Philippe Horvath⁴, and Virginijus Siksnys^{1*}

¹*Institute of Biotechnology, Vilnius University, Graiciuno 8, LT-02241, Vilnius, Lithuania*

²*Department of Chemical and Biological Engineering, ChELSI Institute, University of Sheffield, Sheffield, UK*

³*DuPont Nutrition and Health, 3329 Agriculture Drive, Madison, Wisconsin 53716, USA*

⁴*DuPont Nutrition and Health, BP 10, F-86220 Dangé-Saint-Romain, France*

* Corresponding author

Mail: Institute of Biotechnology, Vilnius University, Graiciuno 8, Vilnius LT-02241, Lithuania

Tel: +370-5-2602108; Fax: +370-5-2602116; E-mail: siksnys@ibt.lt

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Abstract

CRISPR-encoded immunity in Type I systems relies on the Cascade ribonucleoprotein complex, which triggers foreign DNA degradation by an accessory Cas3 protein. To establish the mechanism for adaptive immunity provided by the *Streptococcus thermophilus* CRISPR4-Cas system (St-CRISPR4-Cas), we isolated an effector complex (St-Cascade) containing 61-nucleotide CRISPR RNA (crRNA). We show that St-Cascade, guided by crRNA, binds *in vitro* to a matching proto-spacer if a proto-spacer adjacent motif (PAM) is present. Surprisingly, the PAM sequence determined from binding analysis is promiscuous and limited to a single nucleotide (A or T) immediately upstream (-1 position) of the proto-spacer. In the presence of a correct PAM, St-Cascade binding to the target DNA generates an R-loop which serves as a landing site for the Cas3 ATPase/nuclease. We show that Cas3 binding to the displaced strand in the R-loop triggers DNA cleavage, and if ATP is present, Cas3 further degrades DNA in a unidirectional manner. These findings establish a molecular basis for CRISPR immunity in St-CRISPR4-Cas and other Type I systems.

Abbreviations:

bp, base pair; BSA, bovine serum albumin; cas, CRISPR-associated; Cascade, CRISPR-associated complex for antiviral defense; CRISPR, clustered regularly interspaced short palindromic repeats; crRNA, CRISPR RNA; ds, double-stranded; EDTA, ethylenediaminetetraacetic acid; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PNK, T4 polynucleotide kinase; SDS, sodium dodecyl sulfate; ss, single-stranded; Tris, tris(hydroxymethyl)aminomethane; WT, wild type

Introduction

Bacterial viruses (bacteriophages) provide a ubiquitous and often deadly threat to bacterial populations. To survive in hostile environments, bacteria have developed a multitude of antiviral defense systems (Labrie et al, 2010; Sturino & Klaenhammer, 2006). Clustered regularly interspaced short palindromic repeats (CRISPR), together with CRISPR-associated genes (*cas*) constitute an adaptive immune system which provides acquired resistance against viruses and plasmids, in bacteria and archaea (Barrangou et al, 2007). The CRISPR-Cas system hijacks short fragments of invasive DNA, integrates them as spacers within the CRISPR array, and subsequently uses them as templates to generate specific small interfering CRISPR RNA molecules (crRNA) that combine with Cas proteins into effector complexes that trigger degradation of matching foreign nucleic acids, thereby preventing their proliferation and propagation (Al-Attar et al, 2011; Bhaya et al, 2011; Terns & Terns, 2011; Wiedenheft et al, 2012).

CRISPR-Cas systems have been categorized into three main types that differ by the structural organization and function(s) of nucleoprotein complexes involved in crRNA-mediated silencing of foreign nucleic acids (Makarova et al, 2011). In Type I systems (as exemplified by the CRISPR-Cas system of *Escherichia coli* K12), crRNAs are incorporated into a multisubunit ribonucleoprotein (RNP) complex called Cascade (CRISPR-associated complex for antiviral defence), which binds to matching invasive DNA and triggers degradation by an accessory Cas3 protein (Brouns et al, 2008; Sinkunas et al, 2011; Westra et al, 2012). In Type II systems (as exemplified by the CRISPR1-Cas and CRISPR3-Cas systems of *Streptococcus thermophilus*), CRISPR-mediated immunity solely relies on the signature Cas9 protein which associates with crRNA to form an effector complex which specifically cleaves matching target dsDNA (Deltcheva et al, 2011; Garneau et al, 2010; Gasiunas et al, 2012; Jinek et al, 2012; Sapranaukas et al, 2011). In Type III systems (as exemplified by *Sulfolobus solfataricus* and *Pyrococcus furiosus*) Cas RAMP module (Cmr) in association with crRNA recognize and cleave RNA *in vitro* (Hale et al, 2012; Zhang et al, 2012), whereas the CRISPR/Cas system of *Staphylococcus epidermidis* targets DNA *in vivo* (Marraffini & Sontheimer, 2010).

The *S. thermophilus* DGCC7710 model organism (St), for which CRISPR-Cas interference has been demonstrated against phages and plasmids, contains four distinct CRISPR-Cas systems (Horvath & Barrangou, 2010). Direct spacer acquisition and interference activities have been demonstrated for two distinct Type II systems, namely St-CRISPR1-Cas and St-CRISPR3-Cas (Barrangou et al, 2007; Deveau et al, 2008; Garneau et al, 2010; Sapranaukas et al, 2011). However, until now, no spacer acquisition nor interference activity has been reported for the St-

CRISPR2-Cas or St-CRISPR4-Cas systems, which belong to Type III and Type I systems, respectively. Therefore, we investigated whether the St-CRISPR4-Cas system is functionally active and has the ability to provide immunity against invading DNA.

The St-CRISPR4-Cas system of *S. thermophilus* DGCC7710 and *E. coli* CRISPR-Cas system are orthologous (Type I-E) and share genetic structural organization (Horvath & Barrangou, 2010; Sinkunas et al, 2011). In the St-CRISPR4-Cas system, five *cas* genes are arranged into a cluster (*cse1-cse2-cas7-cas5-cas6e*) (Figure 1A) analogous to the *E. coli cas* genes, suggesting that corresponding Cas proteins may assemble into a homologous St-Cascade complex. Whilst *in vivo* functional activity has not been observed for the St-CRISPR4-Cas system, indirect *in vitro* evidence suggests that it may be active, at least at the interference step (Young et al, 2012). Indeed, we have previously shown that the Cas3 protein of the St-CRISPR4-Cas system is an active nuclease/helicase which may play a key role in DNA degradation (Sinkunas et al, 2011).

Here we report the isolation and biochemical characterization of the St-Cascade complex which consists of CasABCDE proteins (Cse1, Cse2, Cas7, Cas5 and Cas6e, respectively) and a 61-nt CRISPR RNA (crRNA). We further demonstrate that crRNA bound to the St-Cascade complex serves as the guide sequence which specifically recognizes a matching sequence (proto-spacer) in the target DNA. We show that similarly to other Type I systems, St-Cascade binding to the proto-spacer in the invading DNA requires an additional DNA sequence element, a specific proto-spacer adjacent motif (PAM). However, in contrast with other CRISPR systems, the PAM for St-CRISPR4-Cas is promiscuous and limited to a single nucleotide. Furthermore, we show that St-Cascade and St-Cas3 form a functional effector complex which cleaves target DNA *in vitro*.

Results

Cloning, expression and isolation of St-Cascade

In *E. coli*, CasABCDE proteins (Cse1, Cse2, Cas7, Cas5, and Cas6e, respectively) (Makarova et al, 2011), and crRNA form a Cascade complex (Ec-Cascade) (Brouns et al, 2008) which, together with Cas3, provide interference against invading foreign DNA. We tested the hypothesis that homologous *S. thermophilus* Cas proteins (Figure 1A) may assemble into a similar St-Cascade complex, and designed the following strategy for complex isolation. First, three compatible heterologous plasmids containing, respectively, a *casABCDE* cassette, the C-terminal His-tagged variant of *casC* (*casC-His*), and six copies of the repeat-spacer-1 unit (6xSP1) of the *S. thermophilus* CRISPR4 region, were engineered. Next, all three plasmids were co-expressed in *E. coli* BL21 (DE3) strain and the St-Cascade complex was purified by subsequent Ni-chelating,

size exclusion, and heparin affinity chromatography steps. SDS–PAGE analysis (Figure 1B) of the isolated complex revealed 5 bands that matched to individual Cas proteins suggesting that CasABCDE proteins assemble into a St-Cascade complex similar to that of *E. coli*. The identity of all Cas proteins in St-Cascade was confirmed by mass spectrometry analysis (Supplementary table S1). The stoichiometry of the protein complex was not directly determined, however the band intensity in the SDS PAGE (Figure 1B) in conjunction with the mass spectrometry analysis of the St-Cascade tryptic digest suggests that the CasC protein, is the most abundant protein present in St-Cascade similar to the Ec-Cascade (Jore et al, 2011). Denaturing PAGE analysis revealed that RNA co-purifies with the St-Cascade complex (Supplementary Figure S1).

Characterization of S. thermophilus CRISPR4-Cas crRNA.

Next we used denaturing RNA chromatography in conjunction with electrospray ionization mass spectrometry (ESI-MS) to characterize the mature crRNAs isolated directly from the St-Cascade complex. Denaturing ion pair reverse phase chromatography was used to purify the crRNA directly from the St-Cascade complex (Dickman & Hornby, 2006; Waghmare et al, 2009). The RNA isolated from this complex consisted of a single mature crRNA with a retention time consistent with an approximate length of 60 nt (Figure 1C). Purified mature crRNA was further analyzed using ESI-MS to obtain the accurate intact mass. A molecular weight of 19,482 Da was obtained (Figure 1D). In addition, ESI MS/MS was used to analyze the oligoribonucleotide fragments generated from RNase T1 and RNase A digestion of the mature crRNA (Supplementary Figure S2 and S3). In conjunction with the intact mass analysis and denaturing PAGE (Supplementary Figure S1), these indicate that processing of St-CRISPR4-Cas crRNAs is similar to that of *E. coli* CRISPR-Cas crRNAs, generating a 61-nt crRNA (consisting of a 7-nt 5' handle, a 33-nt spacer, and a 21-nt 3' handle) with 5'-OH and 3'-P_i (MW 19481.5 Da) (Figure 1E). Further verification of the 3'-P_i termini was obtained upon acid treatment of the crRNA where no change in mass was observed using ESI-MS.

PAM sequence analysis of the S. thermophilus CRISPR4-Cas system.

The PAM located in the vicinity of a proto-spacer is absolutely required for silencing of invading DNA by Type I and Type II CRISPR-Cas systems (Deveau et al, 2008; Horvath et al, 2008; Sapranuskas et al, 2011; Semenova et al, 2011). In the *E. coli* Type I-E system, the PAM corresponds to the 5'-AWG-3' sequence located immediately upstream of a proto-spacer (Mojica et al, 2009) and is essential for Ec-Cascade binding and subsequent DNA interference (Semenova et al, 2011). On the other hand, experimental analysis of CRISPR repeat boundaries in *E. coli* suggests

a dinucleotide 5'-AW-3' as PAM (Goren et al, 2012). To determine the putative PAM sequence of the CRISPR4-Cas system, we analyzed all currently available CRISPR4 spacer sequences found in *S. thermophilus* strains. A CRISPR4 locus is present in DGCC7710 (Horvath & Barrangou, 2010) and 3 other strains from the DuPont culture collection. In DGCC7710, the CRISPR4 locus contains 12 unique spacers, and 26 more unique spacers were identified in the three other CRISPR4-positive strains. Sequence similarity searches, both in public and proprietary sequence databases, showed that most (26 out of 38) of these CRISPR4 spacer sequences have matches (proto-spacers) in *S. thermophilus* phage sequences. Only perfect matches (100% identity over the complete spacer sequence) between spacer and proto-spacer were considered, providing a set of 106 matching sequences. The sequences located immediately upstream and downstream of these proto-spacers were examined for the presence of a possible PAM. After removal of redundant alleles, a Weblogo representation (Crooks et al, 2004) was used to depict sequence conservation over a 15-nt segment of 28 (upstream) and 21 (downstream) unique sequences (Figure 2A). A 2-bp conserved motif 5'-AA-3' could be identified immediately upstream of the proto-spacers.

PAM is required for St-Cascade binding to the proto-spacer.

To determine whether the predicted PAM sequence is important for proto-spacer recognition, we analyzed St-Cascade binding to a set of synthetic 73-bp oligoduplexes containing the spacer-1 sequence and variable nucleotides at positions -2 and -1 (Figure 2B). Oligoduplexes were radiolabeled at the 5' end of either target or non-target strand, and the St-Cascade binding affinity was evaluated by EMSA. Binding analysis revealed that oligoduplexes fall into three categories with regards to St-Cascade binding. Oligoduplexes containing N(-2)A(-1) nucleotides in the predicted PAM display high binding affinity with $K_d \sim 0.2$ nM, oligoduplexes containing N(-2)T(-1) nucleotides show binding with $K_d < 10$ nM, while all other oligoduplexes except A(-2)G(-1) bind with the same affinity as the non-specific oligoduplex containing spacer-3 instead of spacer-1. Thus, these results suggest that a single nucleotide PAM, A or T (W) at the -1 position upstream of the proto-spacer is required for the St-CRISPR4-Cas system. The G and C nucleotides are not tolerated at this position except for the A(-2)G(-1) dinucleotide (Figure 2C, Supplementary Table S2).

To test whether a non-conserved nucleotide at the -3 position in the vicinity of the predicted PAM is important for spacer recognition, we analyzed St-Cascade binding to a set of oligoduplexes containing a conserved A(-2)A(-1) dinucleotide and any nucleotide at the -3 position (SP1-TAA, SP1-AAA, SP1-GAA, SP1-CAA, respectively) (Supplementary Figure S4A). EMSA analysis revealed that St-Cascade bound all oligoduplexes with a variable N(-3) nt with the same affinity

(Supplementary Figure S4B), confirming that the -3 position is not important for St-Cascade binding.

In Type I CRISPR systems, as exemplified by *E.coli* and *P.aeruginosa*, target recognition is governed the crRNA seed sequence located at the 5' end of the spacer region (Semenova et al, 2011; Wiedenheft et al, 2011) and results in formation of an R-loop where the target strand of the proto-spacer is engaged into a heteroduplex, while the non-target strand is displaced as ssDNA. To demonstrate the formation of the R-loop upon St-Cascade binding to a proto-spacer we used the P1 nuclease, which specifically cleaves ssDNA regions (Jore et al, 2011) (Supplementary Figure S5). In the oligoduplexes SP1-AA and SP1-AG that contain correct PAMs, the non-target strand is susceptible to endonuclease P1 cleavage, while the target strand is resistant to P1 nuclease treatment. On the other hand, in the oligoduplex SP1-CC, which lacks a correct PAM, or in the oligoduplex SP3-AA which contains a PAM but lacks a matching proto-spacer sequence, both DNA strands were resistant to nuclease P1 cleavage. Thus, nuclease P1 assay confirms that an R-loop is formed only when both the correct PAM and a matching proto-spacer sequence are present in the oligoduplex.

St-Cascade binding to the proto-spacer triggers St-Cas3 ATPase activity.

St-Cas3 is a metal-dependent nuclease which possesses a single-stranded DNA stimulated ATPase activity coupled to unwinding of DNA/DNA and RNA/DNA duplexes (Sinkunas et al, 2011). St-Cascade complex binding to the proto-spacer creates an R-loop (Figure 2A) where a non-target strand is displaced as ssDNA and may function as a docking site for Cas3. We used a colorimetric assay to monitor St-Cas3 ATPase activity in the presence of St-Cascade and DNA. The St-Cas3 protein was mixed with the St-Cascade complex and pUC19 plasmid variants that either contain or lack proto-spacer-1 in the context of the correct or mutated PAM (Supplementary Table S3), and ATPase reactions were initiated by addition of ATP and Mg²⁺ ions. The assay revealed that ATPase activity of St-Cas3 is triggered only in the presence of the pSP1-AA plasmid, containing a matching proto-spacer-1 and a correct PAM (Figure 3A). Plasmids lacking proto-spacer-1 and PAM or containing a different proto-spacer (pSP3-AA) or incorrect CC PAM (pSP1-CC) did not stimulate St-Cas3 ATPase activity in the presence of St-Cascade complex. No ATPase activity was detected for the St-Cas3 ATPase-deficient mutant D452A with an impaired Walker B motif (Sinkunas et al, 2011). Taken together, these results suggest a link between the ATPase activity and Cas3 docking on ssDNA formed upon Cascade binding to the matching proto-spacer flanked by a correct PAM.

St-Cascade binding to the proto-spacer triggers St-Cas3-mediated plasmid degradation.

Cas3 of *S. thermophilus* is a metal-dependent nuclease that degrades single-stranded DNA using an active site located in the N-terminal HD-domain (Sinkunas et al, 2011). Double-stranded DNA is resistant to St-Cas3 cleavage (Sinkunas et al, 2011). Consistent with published data on the *Thermus thermophilus* Cas3 protein (Mulepati & Bailey, 2011), St-Cas3 degraded single-stranded M13mp18 DNA more rapidly in the presence of Ni²⁺ compared to Mg²⁺ ions (Supplementary Figure S6A). Since the ATPase activity of St-Cas3 is not supported by Ni²⁺ ions (Sinkunas et al, 2011), a mixture of Mg²⁺ and Ni²⁺ ions which supports both ATPase/helicase and nuclease activities of St-Cas3 was used in further experiments (Supplementary Figure S6B).

To determine whether St-Cas3 docking on the ssDNA formed upon St-Cascade binding triggers nuclease activity, we analyzed Cas3-mediated cleavage of plasmid DNA. pSP1-AA plasmid was pre-incubated with St-Cascade and an ATP solution containing Mg²⁺ and Ni²⁺ ions, followed by addition of St-Cas3. Under these conditions the pSP1-AA plasmid was degraded in a St-Cascade and St-Cas3 concentration- and time-dependent manner (Figure 3B, C). On the other hand, the pUC19 plasmid, or plasmids containing a non-matching proto-spacer (pSP3-AA) or a defective PAM (pSP1-CC), were resistant to St-Cas3 cleavage (Figure 3C). ATP hydrolysis was required for pSP1-AA plasmid degradation. In the absence of ATP the supercoiled pSP1-AA plasmid was converted into a nicked form but not degraded (Figure 3B). The identical cleavage pattern was observed for the St-Cas3 ATPase-deficient mutant D452A in the presence of ATP (Figure 3C). In contrast, the D227A replacement in the nuclease active site (Sinkunas et al, 2011) abolished DNA cleavage activity. Taken together, these data suggest that both ATPase/helicase and nuclease activities of St-Cas3 are required for pSP1-AA plasmid degradation in the presence of St-Cascade. It has been recently reported that Ec-Cascade preferentially binds a negatively supercoiled DNA and that a linear or nicked plasmid is not degraded by Ec-Cascade-Cas3 (Westra et al, 2012). To find out whether DNA supercoiling affects DNA cleavage rate by the St-Cascade-Cas3 system, we monitored degradation rates of supercoiled (pSP1-AA) and linearised (pSP1-AA-BamHI) DNA forms by St-Cascade-Cas3 (Supplementary Figure S12). In contrast to the *E.coli* CRISPR system, St-Cascade-Cas3 degraded both supercoiled and linear DNA substrates at similar rates ($k_{SC} = 0.54 \pm 0.06 \text{ min}^{-1}$, $k_{linear} = 0.43 \pm 0.01 \text{ min}^{-1}$, respectively).

PAM is required for St-Cas3-mediated plasmid degradation.

DNA binding studies revealed that St-Cascade binding to a matching proto-spacer sequence requires a correct PAM sequence (Figure 2C). To check whether plasmid DNA cleavage in the *in vitro* reconstituted interference system follows the same dependence on PAM, we engineered

plasmid substrates containing all possible combinations of base pairs at the -2 and -1 positions relative to the proto-spacer (within the predicted PAM), and monitored St-Cas3-mediated cleavage in the presence of St-Cascade and ATP (Supplementary Figure S7). Consistent with previous binding assays, we found that plasmids containing A(-1), T(-1), or A(-2)G(-1) nucleotides upstream of proto-spacer-1 were efficiently degraded, while plasmids with B(-2)G(-1) (where B = T or C or G) or C(-1) sequences were resistant to cleavage. Altogether, DNA binding and cleavage experiments demonstrate that proto-spacer recognition by St-Cascade is PAM dependent, and that subsequent R-loop formation triggers double-stranded DNA degradation by St-Cas3.

St-Cas3 cleaves DNA within the proto-spacer and upstream of the PAM.

To map St-Cas3 cleavage sites, oligoduplexes SP1-AA, SP1-CC, and SP3-AA were ³³P-5'-end-labeled either on the target or non-target strand, and St-Cas3-induced cleavage was assessed on each strand of each duplex in the absence or presence of ATP. In the absence of ATP, only a non-target DNA strand of the SP1-AA oligoduplex is cut in the proto-spacer region, whilst the target DNA strand is resistant to cleavage (Figure 4A and 4B). In contrast, in the presence of ATP, both target and non-target strands of the SP1-AA oligoduplex are cleaved at multiple positions (Figure 4C). The non-target strand is extensively cut within the proto-spacer and upstream of the PAM at the 5'-end proximal region. The target strand is extensively cleaved within the proto-spacer with minor cuts occurring at both the 5'- and 3'-proximal termini (Figure 4D). Consistent with plasmid DNA cleavage data, no St-Cas3-mediated cleavage was observed for the oligoduplex lacking a proto-spacer (SP3-AA) or with a mutated PAM sequence (SP1-CC), neither in the presence or absence of ATP. Furthermore, the nuclease-deficient mutant D227A did not cleave the SP1-AA oligoduplex. In contrast, ATPase-deficient D452A mutant cleaved only the non-target strand, in both the absence and presence of ATP (Figure 4). The cleavage pattern of the SP1-AA oligoduplex explains why the nicked DNA form is a major product during plasmid DNA cleavage by the D452 mutant or WT St-Cas3 in the absence of ATP. Interestingly, almost all cleavage sites are located at the 3'-end of pyrimidine (T and C) bases (Supplementary Figure S8). Preference for pyrimidine bases is also characteristic for the St-Cas3 cleavage of single-stranded oligodeoxynucleotides (Supplementary Figure S9). The non-target strand cleavage at the 5'-end proximal side of the oligoduplex suggests subsequent St-Cas3 translocation in the 3'→5' direction, followed by DNA degradation.

DNA degradation by Cas3 in the St-CRISPR4-Cas system is unidirectional.

To determine whether the St-Cas3-mediated DNA cleavage is directional, we linearized pSP1-AA plasmid using four different restriction endonucleases (XapI, BamHI, PdmI, AlwNI) to generate a set of linear dsDNA molecules of identical length that have a proto-spacer sequence located at different distances with respect to DNA termini (Supplementary Figure S10). In the pSP1-AA-XapI DNA, the proto-spacer is located almost at the 5'-end, while in the pSP1-AA-PdmI DNA it is located ~800 bp away from the 5'-terminus of the non-target strand. In two other linear DNA substrates, pSP1-AA-BamHI and pSP1-AA-AlwNI, the proto-spacer sequence is located at the 3'-end or ~900 bp away from the 3'-end, respectively. Analysis of reaction products resulting from St-Cas3 cleavage in the *in vitro* reconstituted interference system revealed that the linear DNA pSP1-AA-XapI remained nearly intact, while the pSP1-AA-BamHI substrate was degraded in a similar fashion to the circular plasmid DNA. Furthermore, St-Cas3 acting on the pSP1-AA-PdmI and pSP1-AA-AlwNI substrates produced defined ~1.9- and ~0.9-kb products, respectively, while the remaining DNA fragments were degraded into smaller fragments (Figure 5). Interestingly, the reduced mobility of the ~0.9-kb product resulting from the pSP1-AA-AlwNI cleavage (Supplementary Figure S11) suggests that the St-Cascade (St-Cascade-Cas3) complex remains bound to the cleaved DNA. Taken together, these data are consistent with a model in which Cas3 first makes a double-stranded break in a proto-spacer region (or in its immediate vicinity), and subsequently degrades DNA upstream of the proto-spacer in the 3' to 5' direction in respect to the non-target strand. Therefore DNA downstream of the proto-spacer remains intact.

Discussion

CRISPR-Cas systems have been categorized into three main types that differ by the structural organization and function of RNP complexes involved in crRNA-mediated silencing of foreign nucleic acids. In the *E. coli* CRISPR-Cas system, a multisubunit RNP complex called Cascade binds to the target DNA and triggers degradation by an accessory Cas3 protein (Brouns et al, 2008). The CRISPR4-Cas system of *S. thermophilus* DGCC7710 (Horvath & Barrangou, 2010) displays a similar structural organization to that of *E. coli* (Figure 1A). To show that orthologous *S. thermophilus* Cas proteins assemble into a similar St-Cascade complex, we have isolated the St-Cascade and demonstrate that it recruits St-Cas3 to form a functional effector complex which *in vitro* cleaves target DNA. Moreover, we show that mechanistically the process of DNA interference provided by the St-CRISPR4-Cas system can be dissected into target site recognition and cleavage stages, which are executed by St-Cascade and St-Cas3, respectively.

Target DNA recognition by St-Cascade.

St-Cascade isolated from the heterologous *E. coli* host carries a 61-nt crRNA with a 5'-OH and 3'-phosphate, which guides St-Cascade binding to the proto-spacer sequence in the target DNA. In the St-CRISPR4 array 28 nts of repeat sequences are strictly conserved while 29th nt is sometimes degenerated (C or T). Therefore, we postulated that in the St-CRISPR4 array the repeat sequence is comprised of 28 nt and the spacer sequence of 33 nt, respectively. In the orthologous *E. coli* CRISPR/Cas system 29 nt repeat and 32 nt spacer sequences were initially proposed (ref). However, analysis of sequences of newly inserted repeats in an *E. coli* CRISPR array *in vivo* demonstrate that a base previously thought to belong to the repeat is actually derived from a protospacer (Goren et al, 2012; Swarts et al, 2012). Therefore, the conserved repeat sequence (dubbed duplicon by Goren et al.) in the *E. coli* CRISPR array is proposed to be 28 nt and the spacer sequence 33 nt, respectively. The processed Ec-crRNAs (ref) and *St*-crRNA (Figure X) are comprised of 61 nt suggesting that in the precursor crRNA cleavage positions of Cas6e endoribonucleases are conserved and located at the 21st nt within the repeat sequence. In this case, the mature *St*-crRNA will be comprised of 7 nt 5' handle, 33 nt spacer and 21 nt 3' handle.

Similarly to other Type I systems, St-Cascade binding to the oligoduplexes containing a matching proto-spacer sequence requires a PAM sequence located in the vicinity of the proto-spacer. In the *S. thermophilus* CRISPR4 system, the PAM predicted by *in silico* analysis of the matching proto-spacer sequences in *S. thermophilus* phages is a short 2-nt stretch AA located just upstream of the proto-spacer (Figure 2A). Surprisingly, according to EMSA experiments, the PAM required for St-Cascade binding to the proto-spacer sequence is limited to a single A(-1) or T(-1) nucleotide. Nucleotide replacement at the -1 position with a G or C abrogates St-Cascade binding to the proto-spacer, with the only exception of A(-2)G(-1) dinucleotide which still functions as a PAM and promotes St-Cascade binding, albeit less effectively than A(-1) or T(-1) variants. In the presence of the correct PAM, St-Cascade binds to the DNA containing a proto-spacer sequence with a sub-nanomolar K_d . It has been shown that in the *E. coli* CRISPR system the L1 loop of CasA protein in the Ec-Cascade is involved in the PAM recognition (Sashital et al, 2012). Sequence comparison suggests that a similar loop may be present in the CasA of St-Cascade, however we were unable to locate a statistically significant PAM recognition motif in the putative L1 loop of St-CasA protein. We suggest that PAM recognition is a key step which triggers subsequent St-crRNA binding to the matching DNA strand, presumably through the seed sequence in the immediate vicinity of the PAM (Semenova et al, 2011; Wiedenheft et al, 2011). Importantly, plasmid DNA cleavage *in vitro* by the St-Cascade-Cas3 complex shows the same promiscuous PAM pattern identified from the oligoduplex binding studies.

The discrepancy between the predicted and experimentally determined PAM may be due to several reasons. First, because only a small part of available phage sequence space is explored, it is possible that not all PAM variants were identified in the subset of investigated phage genomes. Alternatively, the requirements for the PAM stringency may differ for the spacer acquisition and interference steps (Swarts et al, 2012). To escape CRISPR interference, bacteriophages often mutate PAM or proto-spacer sequences (Deveau et al, 2008), therefore a short and promiscuous PAM like A(-1) or T(-1) identified for the St-CRISPR4-Cas system may be advantageous for the interference. The PAM identified by *in silico* analysis of the matching proto-spacer sequences in *S. thermophilus* phages may reflect the more stringent PAM requirement for the spacer acquisition step putatively executed by Cas1 and Cas2 proteins.

In Type III systems, self versus non-self DNA discrimination, which is essential for CRISPR-mediated immunity, is achieved through base-pairing interactions at -4, -3 and -2 positions upstream of the proto-spacer (Marraffini & Sontheimer, 2010). The complementarity at these positions between the crRNA and the matching bases in the repeat region in the host genome prevents host DNA cleavage. In the St-CRISPR4 repeat sequence, the C nucleotide is conserved in the -1 position and crRNA binding to the complementary spacer sequence will extend the base-pairing into the repeat region. Importantly, the DNA containing the C(-1) base is neither bound nor cleaved by the St-Cascade-Cas complex, suggesting that complementary interactions at the -1 position may be important for self versus non-self DNA discrimination in the St-CRISPR4-Cas system.

DNA cleavage by St-Cas3 in the St-Cascade-target DNA complex.

Nuclease P1 mapping of the St-Cascade-DNA complex suggests the formation of an R-loop where crRNA and the complementary target DNA strand are engaged into a heteroduplex, and the non-target strand is displaced as a single-stranded DNA. The PAM sequence is critical for R-loop formation. Indeed, if G or C nucleotides are present at position -1, no specific St-Cascade binding and concomitant R-loop formation is detected. The R-loop formation is a key pre-requisite for the binding of St-Cas3 protein, which is a single-stranded DNA nuclease that displays an ATPase/helicase activity stimulated by ssDNA (Sinkunas et al, 2011). St-Cas3 alone does not interact with a double-stranded DNA and therefore first requires St-Cascade binding to the dsDNA to generate the R-loop where the non-target strand of the proto-spacer is displaced as ssDNA and serves as a platform for the St-Cas3 loading. Indeed, the St-Cas3 ATPase activity is triggered only when the R-loop is formed by St-Cascade binding. In the absence of ATP, the oligoduplex substrate is only weakly cleaved by St-Cas3 and cleavage is limited to the non-target strand which is displaced as a single-stranded DNA. Consistent with the oligoduplex cleavage pattern without ATP,

plasmid DNA under these conditions is converted into a nicked form. In the presence of ATP the cleavage pattern of both the oligoduplex and plasmid DNA is radically changed. St-Cas3 extensively cuts both DNA strands in the proto-spacer region of the oligoduplex and upstream of the PAM. The plasmid DNA in the presence of ATP is subsequently degraded by St-Cas3. **In the *Ec*-CRISPR/Cas system a role for supercoiling in the cleavage reaction efficiency has been recently demonstrated (Westra et al, 2012). Our data show that degradation rates of supercoiled and linear DNA forms by the St-Cascade/Cas3 are very similar (Figure S12). These results does not exclude the role of supercoiling in the St-Cascade and target DNA interactions but indicate that supercoiling does not limit the cleavage reaction rate.**

Furthermore, DNA degradation by the St-Cas3 nuclease is directional. After cleaving both DNA strands in the proto-spacer region, St-Cas3 further degrades DNA upstream of the proto-spacer in the 3'→5' direction in respect to the non-target strand, while DNA downstream of the proto-spacer remains intact. St-Cas3 cleavage of the non-target strand in the 3'→5' direction generates stretches of single-stranded DNA on the target strand that can serve as loading platform for the same or other St-Cas3 molecules, promoting further degradation.

It is tempting to speculate that the Cas3 cleavage directionality may contribute to the mechanism of adaptive spacer acquisition proposed recently for the *E. coli* K12 CRISPR system (Datsenko et al, 2012). According to this mechanism the DNA strand from which new spacers are selected is largely determined by the priming proto-spacer orientation. We show here that Cascade-crRNA binding to the matching proto-spacer sequence determines which strand will be extruded into the R-loop and subjected to degradation in the 3' to 5' direction. In this way, the unidirectional DNA degradation by Cas3 may contribute to the selection of a specific DNA strand from which new spacers are subsequently acquired (Datsenko et al, 2012).

In summary, we have shown that St-Cascade recruits St-Cas3 to form a functional effector complex which degrades target DNA *in vitro*. This establishes a molecular basis for CRISPR-based immunity in St-CRISPR4-Cas and other Type I systems (Figure 6). St-Cascade guided by the crRNA locates the target DNA site and, if the correct PAM sequence is present, binds to the matching DNA strand, creating an R-loop which serves as a loading site for the St-Cas3. St-Cas3 binding to the ssDNA triggers ATPase/helicase activity which presumably contributes to Cascade remodeling, making both DNA strands in the proto-spacer region available for Cas3 cleavage. After cleaving both DNA strands within the proto-spacer, Cas3 translocates on the non-target strand in the 3' to 5' direction in an ATP-dependent manner and cleaves the translocating strand using its HD-nuclease domain.

Materials and methods

Cloning, expression and purification of proteins

Streptococcus thermophilus DGCC7710 genomic DNA was used as a template for PCR reactions to clone the *casABCDE* gene cassette and *casC* into pCDF-Duet1 and pBAD24-CHis expression vectors, respectively. A CRISPR locus containing six copies of the repeat-spacer-1 unit (6xSP1) of the WT *S. thermophilus* CRISPR4 system was assembled from oligonucleotides and cloned into pACYC-Duet1 vector (Supplementary Table S3). Full sequencing of cloned DNA fragments confirmed perfect matches to the original sequences.

The St-Cascade complex was expressed in *Escherichia coli* BL21 (DE3) grown in LB broth (BD) supplemented with ampicillin (25 µg/ml), chloramphenicol (17 µg/ml) and streptomycin (25 µg/ml). Cells were grown at 37°C to OD_{600nm} of ~0.5 and expression was induced with 0.2 % (w/v) arabinose and 1 mM IPTG for 3 hours. Harvested cells were disrupted by sonication and cell debris removed by centrifugation. The St-Cascade complex was first purified on the Ni²⁺-charged HiTrap column (GE Healthcare) followed by Superdex 200 (HiLoad 16/60; GE Healthcare) and heparin (GE Healthcare) chromatography steps. The St-Cascade complex was stored at +4°C in a buffer containing 20 mM Tris-HCl (pH 8) and 750 mM NaCl. The St-Cascade complex was subsequently analysed by SDS-PAGE and the sequence of the CasABCDE proteins was further confirmed by mass spectrometry of tryptic digests. Cascade complex concentration was estimated by Bradford assay (Fermentas) using BSA as a reference protein. Conversion to molar concentration was performed assuming the St-Cascade stoichiometry CasA₁:B₂:C₆:D₁:E₁:crRNA₁ is analogous to that of the *E. coli* Cascade (Jore et al, 2011).

St-Cas3 and its mutants were produced and purified as previously described (Sinkunas et al, 2011).

Extraction of crRNA

Nucleic acids co-purified with St-Cascade were isolated by phenol:chloroform:isoamylalcohol (PCI) (25:24:1, v/v/v) extraction. Purified nucleic acids were incubated with DNase I (Fermentas) supplemented with 2.5 mM MgCl₂ or RNase A/T1 (Fermentas) for 30 min at 37 °C. Nucleic acids were separated on a denaturing 15% polyacrylamide gel and visualized by CybrGold (Invitrogen) staining.

HPLC purification of crRNA

All samples were analyzed by ion-pair reversed-phased-HPLC on an Agilent 1100 HPLC with UV260nm detector (Agilent) using a DNasep column 50 mm x 4.6 mm I. D. (Transgenomic, San

Jose, CA). The chromatographic analysis was performed using the following buffer conditions: A) 0.1 M triethylammonium acetate (TEAA) (pH 7.0) (Fluka); B) buffer A with 25% LC MS grade acetonitrile (v/v) (Fisher). The crRNA was obtained by injecting purified St-Cascade complex at 75 °C using a linear gradient starting at 15% buffer B and extending to 60% B in 12.5 min, followed by a linear extension to 100% B over 2 min at a flow rate of 1.0 ml/min. Analysis of the 3' terminus was performed by incubating the HPLC-purified crRNA in a final concentration of 0.1 M HCl at 4 °C for 1 hour. The samples were concentrated to 10-20 µl on a vacuum concentrator (Eppendorf) prior to ESI-MS analysis.

ESI-MS analysis of crRNA

Electrospray Ionization Mass spectrometry was performed in negative mode using an UHR TOF mass spectrometer (maXis) (Bruker Daltonics), coupled to an online capillary liquid chromatography system (Ultimate 3000, Dionex, UK). RNA separations were performed using a monolithic (PS-DVB) capillary column (50 mm × 0.2 mm I.D., Dionex, UK). The chromatography was performed using the following buffer conditions: C) 0.4 M 1,1,1,3,3,3,-Hexafluoro-2-propanol (HFIP, Sigma-Aldrich) adjusted with triethylamine (TEA) to pH 7.0 and 0.1 mM TEAA, and D) buffer C with 50% methanol (v/v) (Fisher). RNA analysis was performed at 50 °C with 20% buffer D, extending to 40% D in 5 min followed by a linear extension to 60% D over 8 min at a flow rate of 2 µl/min, 250 ng crRNA was digested with 1U RNase A and RNaseT1 (Applied Biosystems). The reaction was incubated at 37°C for 4 hr. The oligoribonucleotide mixture was separated on a PepMap C-18 RP capillary column (150 mm × 0.3 µm I.D., Dionex, UK) at 50 °C using gradient conditions starting at 20% buffer C and extending to 35% D in 3 mins, followed by a linear extension to 60% D over 40 mins at a flow rate of 2 µl /min. The mass spectrometer was set to select a mass range of 250–2,000 m/z and the capillary voltage was kept at -3,650 V. Oligoribonucleotides with -2 to -4 charge states were selected for tandem mass spectrometry using collision induced dissociation.

Electrophoretic mobility shift assays (EMSA)

Synthetic oligoduplexes (Metabion) 73 bp in length were used in EMSA experiments (Supplementary Table S3). Each oligoduplex contained a 33 bp proto-spacer sequence corresponding to the first spacer (spacer-1) of the *S. thermophilus* CRISPR4 locus and various PAM sequences. In control experiments, 73 bp oligoduplex containing a proto-spacer-3 instead of proto-spacer-1 was used. An oligodeoxynucleotide corresponding to the target strand was labelled at the 5'-end using PNK (Fermentas) and [$\gamma^{33}\text{P}$]ATP (Hartmann Analytic) and an oligoduplex assembled

by mixing the labelled target and unlabeled non-target strands at a molar ratio of 1:1.5, followed by annealing in 2 mM TrisHCl buffer (pH 8). Increasing concentrations of St-Cascade were incubated with 0.1 nM of radioactively labelled oligoduplex in the binding buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.0, 150 mM NaCl, 0.1 mg/ml BSA, 10% glycerol) for 20 min at 37°C. The samples were subjected to electrophoresis in 8% (w/v) polyacrylamide gel, and visualized using a FLA-5100 phosphorimager (Fujilm). The K_d values for St-Cascade-DNA complexes were calculated as previously described (Tamulaitis et al, 2006). K_d values represent the average value of three independent experiments.

P1 nuclease footprinting

Oligoduplexes SP1-AA, SP1-AG, SP1-CC, and SP3-AA were ^{33}P -5'-end-labeled at either the target or non-target strand for probing with P1 nuclease. Labelled oligoduplex at 2 nM concentration was incubated with or without 10 nM of Cascade complex at 37°C for 15 min in 20 μl of buffer containing 10 mM TrisHCl (pH 8), 100 mM NaCl and 0.1 mg/ml BSA. Then, 0.02 U of P1 nuclease (Sigma) in 20 μl of 30 mM sodium acetate buffer (pH 5.3) were added and incubated at 37°C for 10 min. The reactions were stopped by addition of phenol-chloroform followed by sodium acetate/isopropanol precipitation. The cleavage products were separated on a denaturing 20% polyacrylamide gel and visualized by autoradiography. Products of dideoxy sequencing reactions („Cycler Reader DNA Sequencing kit“ (Fermentas)) of TS132 or TS133 oligonucleotides were used as size markers.

ATPase assay

ATPase reactions were conducted at 37°C in the ATPase reaction buffer (10 mM Tris-HCl (pH 7.5 at 25°C), 75 mM NaCl, 40 mM KCl, 7% (v/v) glycerol, 0.1 mg/ml BSA, 1.5 mM MgCl_2 , 2 mM ATP) containing 3 nM supercoiled double-stranded plasmid (Supplementary Table S3), 12 nM of Cascade complex and 300 nM of Cas3 or D452A mutant. Reactions were initiated by adding MgCl_2 and ATP to a mixture of the other reaction components. Malachite green assay kit (BioAssay Systems) was used to measure ATP hydrolysis through the detection of liberated-free phosphate as previously described (Sinkunas et al, 2011).

Nuclease assay

Supercoiled or linearised pUC19 (Fementas) or its derivative plasmids were used as substrates in the DNA cleavage assay. Cleavage reactions were performed at 37°C for indicated time intervals in the Nuclease buffer (10 mM Tris-HCl (pH 7.5), 75 mM NaCl, 40 mM KCl, 7 % (v/v) glycerol, 1.5

mM MgCl₂, 0.1 mM NiCl₂, 2 mM ATP). Supercoiled or linearised plasmid DNA at 5 nM concentration was incubated with 20 nM of Cascade complex and 100 nM of Cas3 or its mutants unless otherwise stated. Reactions were initiated by addition of Cas3 and stopped by mixing with 3× stop solution (67.5 mM EDTA, 27% (v/v) glycerol, 0.3% (w/v) SDS). Reaction products were analysed by 0.8% (w/v) agarose gels electrophoresis and visualised by ethidium bromide staining. To monitor oligoduplex (Supplementary Table S3) cleavage, either the target or non-target strands were ³³P-5'-end-labeled and 2 nM of labelled oligoduplex was incubated with 4 nM of Cascade complex and 100 or 500 nM of Cas3 in the presence or absence of ATP, respectively. The cleavage products were separated on a denaturing 20% polyacrylamide gel and visualized by autoradiography. Products of dideoxy sequencing reactions („Cycler Reader DNA Sequencing kit“ (Fermentas)) of ³³P-5'-end-labeled oligodeoxynucleotides (Supplementary Table S3; section Cleavage markers) were used as size markers.

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Author contribution

T.S., G.G. and V.S. designed research; T.S. performed research; S.P.W. and M.J.D. performed MS analysis; P.H. and R.B. contributed new reagents/analytic tools; T.S., G.G., S.P.W., M.J.D, R.B., P.H., and V.S. analyzed data; and T.S., G.G. and V.S. wrote the paper.

Conflict of interest

P.H. and R.B. are employees of DuPont Nutrition & Health. G.G., V.S., R.B., and P.H. are inventors on patent applications related to CRISPR.

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Figure legends:

Figure 1. *S. thermophilus* CRISPR4-Cas crRNA and Cascade complex. **(A)** Schematic representation of the CRISPR4-Cas locus containing eight *cas* genes and 12 repeat-spacer units (conserved 28-bp palindromic repeats 5'-GTTTTTCCCGCACACGCGGGGGTGATCC-3' are separated from each other by 33-bp spacers of variable sequence). St-Cascade genes homologous to the *E. coli* Cascade are underlined. Genes names according to (Brouns et al, 2008) and (Makarova et al, 2011) are indicated, respectively, above and below corresponding genes. **(B)** Coomassie blue-stained SDS-polyacrylamide gel of St-Cascade complex proteins isolated using the CasC-His₆ protein as bait. **(C)** IP RP HPLC analysis of mature crRNA. **(D)** LC ESI MS analysis of purified *S. thermophilus* crRNA. Inset shows an enhanced view of the 22- charge state. **(E)**. Architecture of crRNA co-purifying with the St-Cascade protein complex.

Figure 2. PAM-dependent St-Cascade binding. **(A)** Predicted PAM for the St-CRISPR4-Cas system. Weblogo representation (Crooks et al, 2004) of 15-nt sequences found immediately upstream (top) and downstream (bottom) of phage proto-spacers that match known CRISPR4 spacers. A conserved, 2-nt PAM (5'-AA-3') is located immediately upstream of the proto-spacers. **(B)** A schematic representation of a putative R-loop structure resulting from the St-Cascade binding to the 73-bp oligodeoxynucleotide. Nucleotides NN at -1 and -2 positions of predicted PAM were varied. In the R-loop structure a target strand bound to the crRNA is engaged into a heteroduplex while the non-target strand is displaced as a single-stranded DNA. **(C)** PAM sequence dependence of a proto-spacer-1 binding by St-Cascade. Bar diagram shows dissociation constant K_d values obtained by EMSA. Error bars represent standard deviations of average K_d value determined in three separate experiments. Oligoduplex containing a non-matching proto-spacer-3 sequence was used as a non-specific DNA control.

Figure 3. ATPase and nuclease activities of St-Cas3 induced by St-Cascade binding to the proto-spacer dsDNA. **(A)** ATP hydrolysis rates. Malachite green assay was used to measure ATP hydrolysis through the detection of free phosphate liberated from ATP. Reaction rate constant k values (min^{-1}) were calculated from linear slopes of time courses of phosphate liberation per St-Cas3 amount added. ATPase reactions were conducted at 37°C in the ATPase reaction buffer supplemented with 3 nM supercoiled plasmids, 12 nM St-Cascade and 300 nM St-Cas3 or the ATPase-deficient mutant D452A. **(B)** dsDNA degradation requires St-Cas3, St-Cascade and ATP.

Nuclease reactions were conducted at 37°C for 10 min in a Nuclease buffer supplemented with 5 nM pSP1-AA and indicated amounts of St-Cas3 and St-Cascade. (C) PAM and a proto-spacer are essential for DNA degradation. Nuclease reactions were conducted at 37°C for indicated time intervals in Nuclease buffer supplemented with 100 nM St-Cas3, 20 nM St-Cascade and 5 nM of respective supercoiled plasmids.

Figure 4. St-Cas3 cleavage of St-Cascade bound to target dsDNA. Oligoduplexes ³³P –labeled in either the non-target or target strand were pre-incubated with St-Cascade without (A) or with (B) ATP and reaction products analysed in denaturing polyacrylamide gels and mapped on the SP1-AA oligoduplex sequence (C, D), respectively. Cleavage reactions were conducted at 37°C in a Nuclease buffer containing 0 mM (A) or 2 mM (B) ATP, 8 nM St-Cascade and 100 nM (B) or 500 nM (A) St-Cas3 or D227A and D452A mutants supplemented with 2 nM SP1-AA, SP1-CC or SP3-AA ³³P-labelled oligoduplexes. Solid lines designate proto-spacer boundaries. Arrows indicate cleavage positions, height of the arrow correlates with a relative amount of cleavage product after 10 min incubation.

Figure 5. St-Cas3-mediated DNA degradation is unidirectional. Four linear 2759 bp DNA fragments pSP1-AA-XapI, pSP1-AA-Pdml, pSP1-AA-BamHI or pSP1-AA-AlwNI that contain a proto-spacer sequence located at different distance in respect to DNA ends were generated by a restriction endonuclease cleavage of pSP1-AA plasmid. Nuclease reactions were initiated by addition of 20 nM St-Cascade and 100 nM St-Cas3 into a Nuclease buffer containing 5 nM of respective DNA. Linear DNA molecules with St-Cas3 and St-Cascade bound to a proto-spacer are schematically depicted at respective gel picture. M – DNA markers to map cleavage products were obtained by cleaving pSP1-AA with Pdml, XapI and AlwNI, XapI restriction endonucleases, respectively.

Figure 6. CRISPR-mediated interference mechanism. Cascade binding to a matching proto-spacer in the presence of the correct PAM generates an R-loop where the crRNA and the complementary target DNA strand are engaged into a heteroduplex, while the non-target strand is displaced as a single-stranded DNA providing a platform for the Cas3 loading (1). ssDNA binding stimulates the Cas3 ATPase activity which may trigger Cascade remodeling making both DNA strands in the proto-spacer region available for the Cas3 cleavage (2). After cleaving both DNA strands at the proto-spacer Cas3 translocates on the non-target strand in the 3' to 5' direction (dashed line) in the ATP-dependent manner and chops the translocating strand using the HD-nuclease domain (3). A

stretch of single-stranded DNA created on the complementary strand may promote binding of another Cas3 molecule (4) followed by concomitant cleavage resulting in the degradation of both strands of invading DNA (5).