Characterization of a novel type III effector provides new insights into the allosteric activation and suppression of the Cas10 DNase

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Abstract

Antiviral defense by type III CRISPR-Cas systems relies on two distinct activities of their effectors: the RNA-activated DNA cleavage and synthesis of cyclic oligoadenylate. Both activities are featured as indiscriminate nucleic acid cleavage and subjected to the spatiotemporal regulation. To yield further insights into the involved mechanisms, we reconstituted LbCsm, a lactobacilli III-A system in *Escherichia coli*. Upon activation by target RNA, this immune system mediates robust DNA degradation but devoids the synthesis of cyclic oligoadenylates. Mutagenesis of the Csm3 and Cas10 conserved residues revealed that Csm3 and multiple structural domains in Cas10 function in the allosteric regulation to yield an active enzyme. Target RNAs carrying various truncations in the 3' anti-tag were designed and tested for their influence on DNA binding and DNA cleavage of LbCsm. Three distinct ternary LbCsm complexes were identified. In particular, binding of target RNAs carrying a single nucleotide in the 3' anti-tag to LbCsm yielded an active LbCsm DNase regardless whether the nucleotide shows a mismatch, as in the cognate target RNA (CTR), or a match in the noncognate target RNAs (NTR), to the 5' tag of crRNA. In addition, further increasing the number of 3' anti-tag in CTR facilitated the substrate binding and enhanced the substrate degradation whereas doing the same as in NTR gradually decreased the substrate binding and eventually shut off the DNA cleavage by the enzyme. Together, these results provide the mechanistic insights into the allosteric activation and repression of LbCsm enzymes.

Introduction

CRISPR-Cas (clustered regularly interspaced short palindromic repeats, CRISPR-associated) systems constitute the adaptive and heritable immune system in bacteria and archaea, which mediates antiviral defense against invasive genetic elements in a small RNA-guided fashion[1-8]. The immune system consists of two parts: CRISPR arrays containing spacers derived from invading nucleic acids, and *cas* gene cassettes coding for enzymes or structural proteins that function in mediating the CRISPR immunity. CRISPR-Cas systems are classified into two broad classes based on the composition of their

effector complexes: Those of Class 1 carry multi-subunit effectors and those of Class 2 possesses a single effector protein, and these antiviral systems are further divided into six main types (types I - VI) with >20 subtypes[9-11].

Type III CRISPR systems are unique because they exhibit both RNA interference and DNA interference *in vivo* to protect their microbial hosts against invading nucleic acids[4,12-23]. Three activities are associated with these Type III immune systems, including target RNA cleavage[18,24-30], target RNA-activated indiscriminate single-stranded (ss) DNA cleavage, a secondary DNase activity[15,19,20,31-37] and synthesis of cyclic oligoadenylate (cOA), a second messenger that activates RNases of Csm6/Csx1 families, mediating cell dormancy or cell death[38-45]. Activation of the immunity requires mismatches between the 3' anti-tag of a cognate target RNA (CTR) and the 5' tag of crRNA whereas the full match between the 3' anti-tag of non-cognate target RNA (NTR) and the 5' tag of crRNA completely represses the immune response (see reviews[46-49]). Upon activation by the secondary messenger, Csx1/Csm6 RNases exhibit indiscriminate cleavage of viral and cellular RNAs, leading to cell dormancy or cell death to curb virus infection[38-43,50,51]. It is further believed that the type III CRISPR DNase eventually clears up remaining invading nucleic acids[52], whereas the cOA secondary messenger is to be removed by ring nucleases[53], allowing cells recover from the Type III immune response and restore the growth.

Structure of the *Streptococcus thermophilus* III-A (StCsm) effector complex has recently been resolved. This includes that of the StCsm binary complex and those of the StCsm^{CTR} and StCsm^{NTR} ternary effector complexes. The two ternary complexes have undergone major conformational change, relative to the binary one, but the active StCsm^{CTR} complex and the inactive StCsm^{NTR} complex show minimal conformational change except for the fact that the 3' anti-tag of CTR and that of NTR are placed in different channels[54]. Very similar results were obtained from the structural analysis of the *Thermococcus onnurineus* Csm effector complex[55] and the StCsm effector of different composition[56]. In particular, the active site of these Csm DNases exhibits little difference between the NTR-bound effector complex and the corresponding CTR-bound effector complex. As a result, the process of the activation of a binary Csm complex by CTR as well as its inhibition by NTR remains elusive.

To gain an further insight into the molecular mechanisms of the allosteric regulation of type III-A effector complexes, we characterized a Csm present in *Lactobacillus delbrueckii* subsp. *bulgaricus* (LbCsm). Its effector complex was reconstituted by expression in, and purified from, *Escherichia coli*. Investigation of the interactions between purified effector complexes and their target RNAs unravels an active ternary LbCsm complexes with CTR or NTR carrying a single nucleotide at their 3' anti-tag regions, and these results provide a novel insight for allosteric activation and repression of the LbCsm DNase.

Results

L. delbrueckii subsp. *bulgaricus* encodes a novel III-A CRISPR-Cas system defective in cOA synthesis

The *L. delbrueckii* subsp. *bulgaricus* strain carries a type III-A CRISPR-Cas system (LbCsm) including a CRISPR array of 16 spacers (Fig. 1A), in addition to a type II CRISPR-Cas9 system in another chromosome location (GenBank: CP016393.1). This LbCsm system was chosen for characterization since phylogenetic analyses of a selected set of Cas10 proteins revealed that its Cas10, the *L. delbrueckii* subsp. *bulgarius* Csm1, is distantly related to the Cas10 proteins that have been studied

thus far (Fig. S1).

We chose to reconstitute the LbCsm effector in *E. coli* since recombinant protein purification procedure has not been established for this lactobacillus yet. Three different *E. coli* vectors, i.e. p15AIE, pUCE and pET30a, were employed to clone all components of the immune system, including the complete set of III-A *cas* genes, a His-tag version of *csm2* and a synthetic CRISPR array (Fig. S2). Expression of these plasmid-borne genes in the same cell yielded all this III-A Cas proteins, a His-tagged LbCsm2 protein and crRNAs (Fig. 1B), which were allowed to assemble into recombinant LbCsm ribonucleoprotein complexes in *E. coli*. The resulting effector complexes were then purified in two-step purification, the nickel-His tag affinity chromatography and the size exclusion chromatography (SEC). A single protein peak appeared in 10-12 ml in the SEC purification (Fig. 1C), indicative of copurification of large complexes. Analysis of these SEC samples by SDS-PAGE showed that each SEC sample contained 5 protein bands corresponding to the predicted sizes of Csm1, Csm2, Csm3, Csm4, and Csm5, respectively (Fig. 1D). The RNA component was extracted from the effector complexes using the Trizol agent. Denaturing PAGE analysis of extracted RNAs by radio-labelling and northern blot analysis of these RNAs by the radio-labeled DNA probe revealed 3 major RNA components of ~76, 38 and 32 nt. The largest species represented the complete unit of S1 crRNA carrying the 5'-repeat handle+S1 spacer+3'-repeat handle, representing the Cas6-cleaved crRNA product of a single spacer, whereas the two smaller RNA species were matured crRNAs (Fig. 1E and Fig. S3). Together, these results indicated that LbCsm ribonucleoprotein complexes were reconstituted in *E. coli*. Since F5-F7 fractions mainly contained the LbCsm complex of 32 nt crRNA (Fig. 1E), the smallest ribonucleoprotein effector complex, they were pooled together, annotated as LbCsm and characterized.

To test if the *E. coli*-expressed effector could be active in RNA cleavage, LbCsm was mixed with four different radio-labeled RNAs individually, i.e. (a) a non-homologous RNA, S10, (b) the protospacer target RNA (PTR), S1-40, lacking any 3' anti-tag, (c) the cognate target RNA (CTR), S1-46, exhibiting mismatches between its 6 nt 3' anti-tag and the 5' tag of the corresponding crRNA, and (d) the noncognate target RNA (NTR), S1-48, possessing the fully complementary sequence between its 3' anti-tag and the 8 nt 5' tag of the crRNA (Fig. 2A). These reaction mixtures were incubated at 37°C for 10 min and analyzed by denaturing PAGE. We found that, while the non-homologous RNA was not a substrate of LbCsm (Fig. S4A), the effector cleaved all three homologous target RNAs in 6-nt periodicity with similar efficiencies. These results indicated that LbCsm possesses the backbone RNA cleavage activity and 3' anti-tag sequences on target RNAs do not influence the RNA cleavage (Fig. 2B). Then, all three target RNAs were tested for their capability of mediating RNA-activated DNA degradation to S10-60, a single-stranded non-homologous DNA substrate (Table S1). As shown in Fig. 2C, only CTR activated the LbCsm DNase, indicating that mismatches between the 3' anti-tag of the target RNA and the corresponding 5' tag of crRNA are essential for the activation. These results are in good agreement with those reported for other characterized type III CRISPR-Cas systems[15,17,19,20,31,34,35].

To date, all studied type III effectors are capable of producing cOAs, a secondary messenger that activates CRISPR-associated Rossmann fold (CARF) domain RNases of the Csm6/Csx1 family for general degradation of cellular RNAs[38-42]. To investigate if LbCsm could also do that, the effector was mixed with ATP and CTR in the presence of Mg^{++}, and incubated for 2 h. Samples were analyzed by denaturing PAGE. As shown in Fig. 2D, whereas the *Sulfolobus islandicus* III-B Cmr-α (a positive

reference[38]) consumed almost all 100 μM ATP for cOA synthesis, but LbCsm did not produce any detectable cOA in the same reaction setup. We repeated the experiments several times and also tested with a number of different metal ions, but constantly failed to detect any cOA in the ATP reaction (Fig. S4F).

We noticed that LbCsm1 carries a QGDD motif in Palm 2, the active site for cOA synthesis, which differs from the GGDD consensus (Fig. S5A). To test if the occurrence of glutamine (Q597) residue in the motif could be responsible for cyclase inactivation in LbCsm, a Q597G substitution mutant was constructed to restore the common GGDD motif in LbCsm1, denoted Csm1^{Q597G}. Characterization of the resultant mutated effector revealed that the GGDD-restored LbCsm still appeared to be inactive in cOA synthesis (Fig. S4F). To this end, the LbCsm cyclase inactivation could more likely be resulted from evolution rather than any spontaneous mutation at the Cas10 cyclase domain.

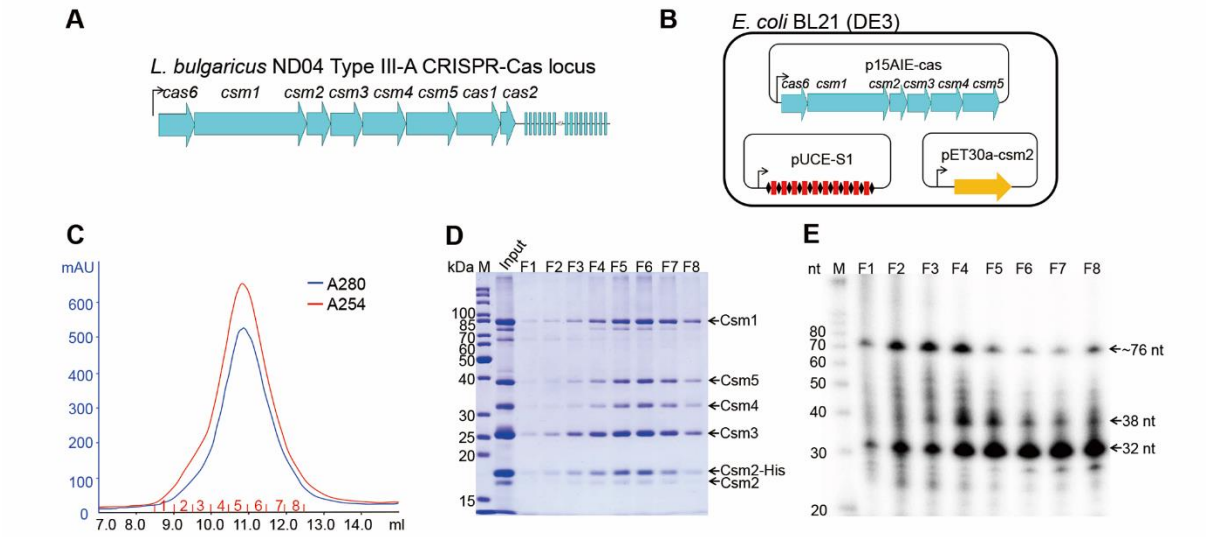

Fig. 1 Cloning, expression and purification of the *L. delbrueckii* subsp. *bulgaricus* Csm complex in *E. coli*. (A) Schematic of the LbCsm system. *Lbcsm* genes and the adjacent CRISPR assay are indicated with filled large arrows and small rectangles, respectively. Line with an arrowhead denotes the promoter of the *csm* gene cassette and the direction of transcription. (B) Strategy for reconstitution of the LbCsm effector in *E. coli*. *Lbcsm* genes (*cas6+csm1-5* genes) were cloned into p15AIE, yielding p15AIE-cas (Fig. S2). A CRISPR array carrying 10 copies of S1 spacer was generated and inserted into pUCE, giving pUCE-S1 (Fig. S2). *Lbcsm2* was cloned into pET30a, giving pET30a-Csm2 that yields the His-tagged Csm2 upon plasmid-born gene expression in the cell. All three plasmids were introduced into *E. coli* BL21(DE3) by electroporation. (C) UV spectrum of SEC purification of LbCsm effector complex. *E. coli* cell extracts were employed for Nickel-His tag affinity purification of LbCsm2 by which LbCsm effector complexes were copurified. The resulting protein samples were further purified by SEC. Blue: UV absorbance at 280 nm; red: UV absorbance at 254 nm. (D) SDS-PAGE analysis of SEC samples collected in the peak region. M: protein mass marker; Input: proteins purified by nickel Csm2-His affinity chromatography. (E) Denaturing gel electrophoresis of 5'-labeled crRNAs. RNAs were extracted from the SEC-purified LbCsm samples. M: RNA size ladder.

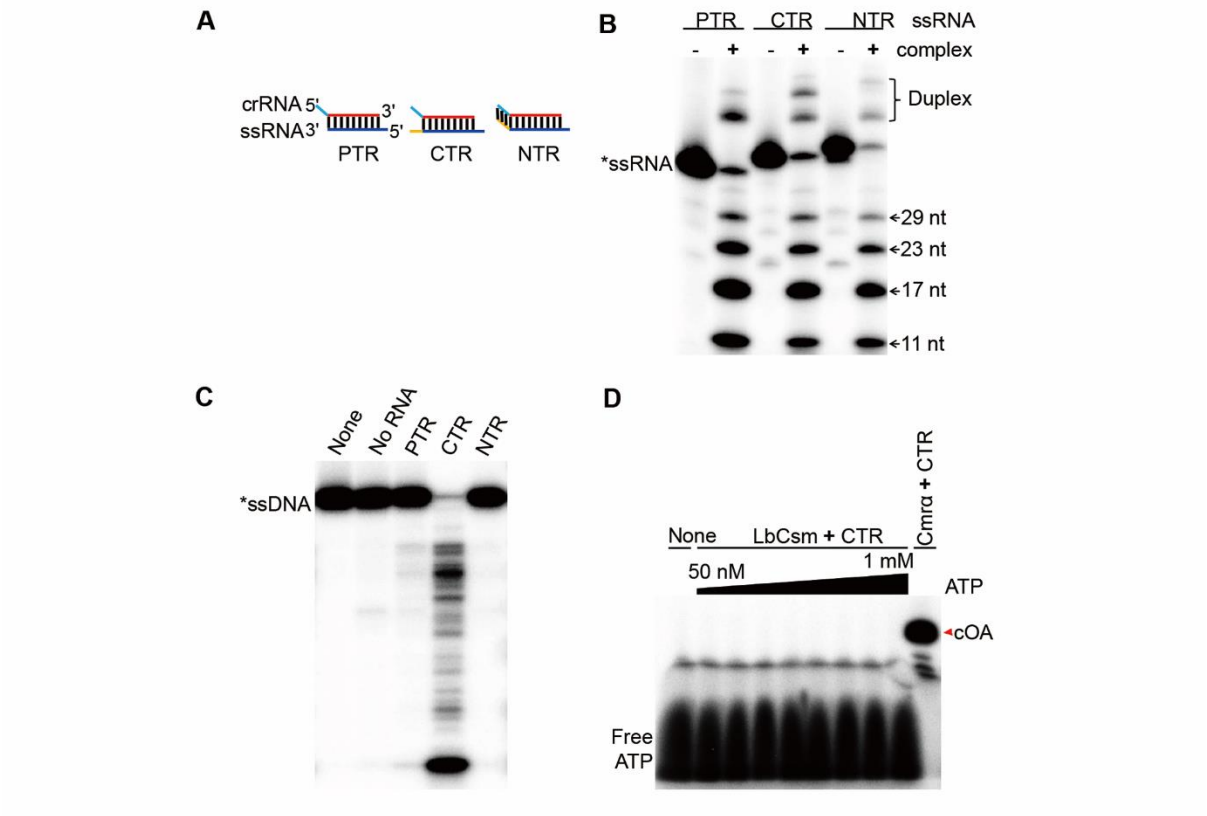

Fig. 2 Biochemical characterization of the LbCsm effector complex. (A) Schematic of three different homologous target RNAs: CTR, cognate target RNA carrying 6-nt 3' anti-tag with mismatch to the 5' tag of the corresponding crRNA; NTR, noncognate target RNA containing 8-nt 3' anti-tag that is complementary to the 5' tag of the corresponding crRNA, and PTR, 40 nt protospacer target RNA completely lacking 3' anti-tag. (B) Analysis of target RNA cleavage by LbCsm. Different target RNAs (50 nM) were individually mixed with 50 nM LbCsm and incubated for 10 min. The resulting samples were analyzed by denaturing PAGE. (C) Analysis of RNA-activated ssDNA cleavage by LbCsm. 50 nM S10-60 ssDNA substrate was mixed with 50 nM LbCsm and 500 nM of each of the target RNA and incubated for 10 min. Samples were analyzed by denaturing PAGE. (D) Analysis of cOA synthesis by LbCsm. ~2 nM [α-^{32}P]-ATP was mixed with a range of cold ATP (48 nM – 1 mM) and incubated with 50 nM LbCsm in the presence of 500 nM CTR for 120 min, the *S. islandicus* Cmr-α complex was used as the positive reference.

LbCsm system mediates anti-plasmid interference in *E. coli*

To investigate if the LbCsm system could mediate DNA interference *in vivo*, three test plasmids were constructed using pBad, an *E. coli* vector exhibiting arabinose-inducible expression. These included pBad-eGFP (pBad-G), a reference plasmid and two protospacer-carrying plasmids: one containing the CTR-S1 protospacer-GFP fusion gene (pBad-CTR), and the other possessing the NTR-S1 protospacer-eGFP (pBad-NTR) (Fig. 3A). Meanwhile, *E. coli* strain for the *in vivo* assay was generated by introduction of the expression plasmid p15AIE-Cas-S1 into *E. coli* BL21 (DE3) by electroporation. The rationale of the experiment is that plasmid-borne gene expression from p15AIE-Cas-S1 would yield binary LbCsm effector complexes in the host cells, which would mediate anti-plasmid activity if a

cognate target RNA was to be expressed from a test plasmid. In addition, since the expression of CTR-S1 or NTR-S1 target RNA is controlled by the Bad promoter that confers very stringent arabinose-inducible expression, target RNAs would only be synthesized in L-arabinose media but not in glucose media. Therefore, examination of colony formation efficiency of each plasmid on both arabinose plates and glucose media would reveal *in vivo* transcription-dependent anti-plasmid interference of the immune systems to be tested (Fig. 3B).

All test plasmids were introduced into the genetic host by transformation, and transformed *E. coli* cells were plated on 6 different medium plates containing 0.5% glucose or arabinose of different contents (0.01-0.08%). Following transformation data were obtained: (a) pBad-G and pBad-NTR produced very similar numbers of transformants on all 6 growth media, (b) pBad-CTR gave very similar transformation efficiency data on plates containing glucose or very low contents of L-arabinose (0.01 or 0.02%), (c) increasing the L-arabinose content in the medium to 0.04% and 0.06%, yielded ca. 10 and 100-fold decrease in transformation efficiency, and (d) the presence of 0.08% inducer completely abolished colony formation by pBad-CTR-containing cells (Fig. 3C). These results indicated, while NTR can effectively turn off the LbCsm immunity, CTR is capable of conferring plasmid clearance to the LbCsm system albeit it only possesses the RNA-activated ssDNA cleavage activity, suggesting LbCsm represents the novel type III CRISPR-Cas system, which exhibits the robust RNA activated DNase activity to resist the invasive plasmid.

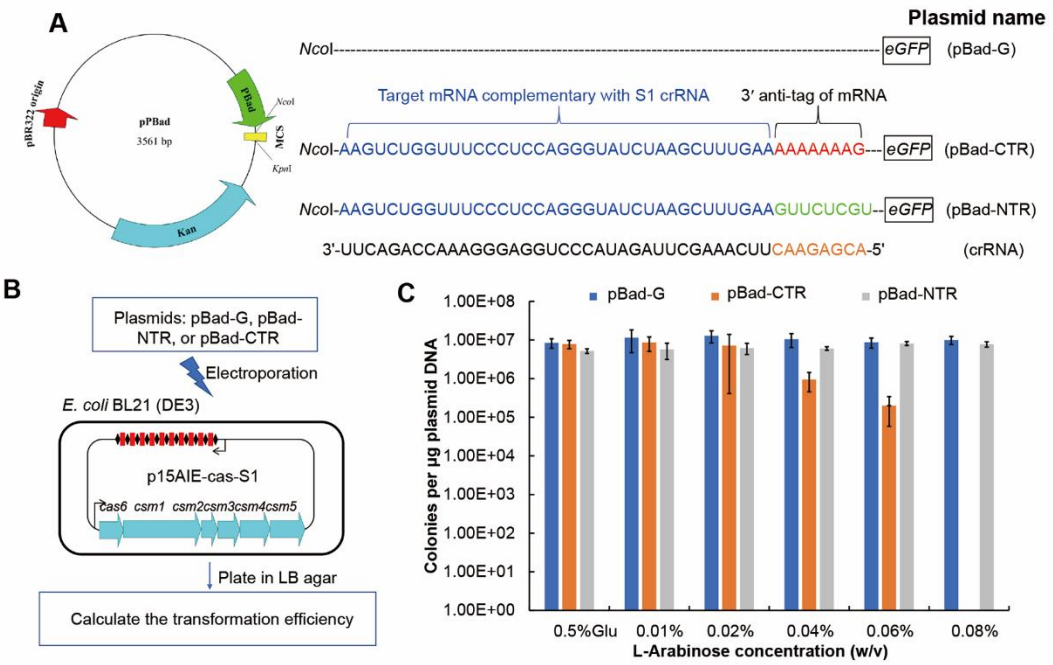

Fig. 3 Anti-plasmid activity of the LbCsm system. (A) Schematic of the test plasmids pBad-G, pBad-CTR and pBad-NTR. Gene expression from these plasmids is under the control of a L-arabinose-inducible Bad promoter. Therefore, mRNAs carrying CTR or NTR are expressed from the corresponding plasmids (pBad-CTR or pBad-NTR) in the presence of L-arabinose but their expression is completely repressed in glucose media. (B) Schematic of the interference plasmid assay for determination of the LbCsm anti-plasmid activity. (C) Transformation efficiency data of the three different plasmids obtained from different growth media.

Multiple Cas10 domains contribute to the LbCsm DNase activity

The largest subunit Cas10 in type III-A effector complexes contains 4 conserved domains, including a HD domain, two Palm domains (Palm1 and Palm2), a Linker and a D4 structural region (Fig. 4A). Biochemical and structural analyses of different type III CRISPR-Cas systems have revealed that the HD domain is responsible for ssDNA cleavage[15,19,20,31,34,35], Palm1 and Palm2 domains function in cOA synthesis[38-41,43] whereas the Linker domain plays a regulatory role in both target RNA-activated DNA cleavage and cOA synthesis[54]. However, it remained to investigate whether a concerted action of different Cas10 domains would be required to yield Csm DNase with optimal activity. The robustness of the LbCsm DNase rendered it a good system for this characterization.

Nine conserved amino acids present in the HD, Linker, and Palm2 domains of LbCsm1 were chosen for substitution mutagenesis, giving 6 single domain mutants. These included Csm1^{dHD}, a HD domain mutant carrying H15A and D16A double substitution, 3 Linker domain mutants, i.e. Csm1^{LinE} (E415A substitution), Csm1^{LinEC} (E415A and C416A substitutions), and Csm1^{LinCC} (C416A and C419A mutations), and 2 Palm2 domain mutants, Csm1^{P2DxD} (D541A and D543A substitutions) and Csm1^{P2DD} (D599A and D600A mutations). Then, some mutated motifs were combined, yielding double domain mutations including two HD/Linker mutants (Csm1^{dHD_LinEC} and Csm1^{dHD_LinCC}), two HD/Palm2 mutants (Csm1^{dHD_P2DxD} and Csm1^{dHD_P2DD}) and Csm1^{LinE_P2DxD}, a Linker and a Palm2 mutant (Table S2).

Each mutated *csm1* gene was used to replace the wild-type *csm1* on p15AIE-cas, and the resulting plasmids were into *E. coli* to express LbCsm effectors carrying each of the mutated LbCsm1 protein. These mutated LbCsm effector complexes were obtained as described for the wild-type LbCsm effector. The protein components of these effector complexes were checked by SDS-PAGE, and this showed that they all contained 5 different Csm subunits as for the wild-type LbCsm complex (Fig. S5B), indicating that none of these Csm1 mutations affected the effector assembly. These effector complexes were then tested for target RNA cleavage using S1-46, the CTR, as the substrate and they all exhibited the backbone cleavage (Fig. S5C).

Next, RNA-activated DNase activity was examined for each mutated complex in reactions containing 50 nM effector, 50 nM ssDNA and 500 nM CTR. For single domain mutants, we found that all mutations (except for Csm1^{P2DD}) strongly impaired the ssDNase activity, including Csm1^{dHD}, Csm1^{LinE}, Csm1^{LinEC} Csm1^{LinCC} and Csm1^{P2DxD} (Fig. 4B). These results indicated that, in additional to the HD domain, the putative cleavage site, three other Csm1 motifs, i.e. E415 and C416 C419 in the Linker region and DxD in the Palm2 domain are also important for the LbCsm DNase. Analysis of the double motif mutants further showed, while combined mutations of Csm1^{dHD_LinEC}, Csm1^{dHD_LinCC} and Csm1^{dHD_P2DD} possessed similar DNase activities relative to those of single motif mutants, those of Csm1^{dHD_P2DxD} and Csm1^{LinE_P2DxD} completely abolished the LbCsm DNase cleavage (Fig. 4B). To this end, we concluded that three Cas10 motifs, including HD, E415, C416 and C419 (Zinc finger) in the Linker as well as D451and D453 (DxD) in Plam2, play critical roles in the LbCsm DNA cleavage.

To yield a further insight into the LbCsm DNA cleavage, all these Csm1-mutated effector complexes were tested for substrate binding in which 25, 50 or 100 nM effector complex was mixed with 500 nM CTR and 5 nM radio-labeled S1-60 ssDNA. After incubation for 3 min, the formation of LbCsm-ssDNA complexes was checked by nondenaturing PAGE. As shown in Fig. S5E, the substrate affinity of these effector complexes fell into three different categories. First, wild-type (WT) and LbCsm_Csm1^{P2DD} showed a strong binding (100% and ~80%), which is consistent with their unimpaired DNase activity;

second, LbCsm_Csm1^{dHD} and LbCsm_Csm1^{P2DxD} retained ~30% of the binding capacity of the wild-type LbCsm effector, whereas the last group included those with mutations in Linker, only exhibiting 5-20% of substrate binding. Taken together, these data indicated that the HD motif, the Zinc finger and the DxD motif function in facilitating substrate binding of the LbCsm effector complex.

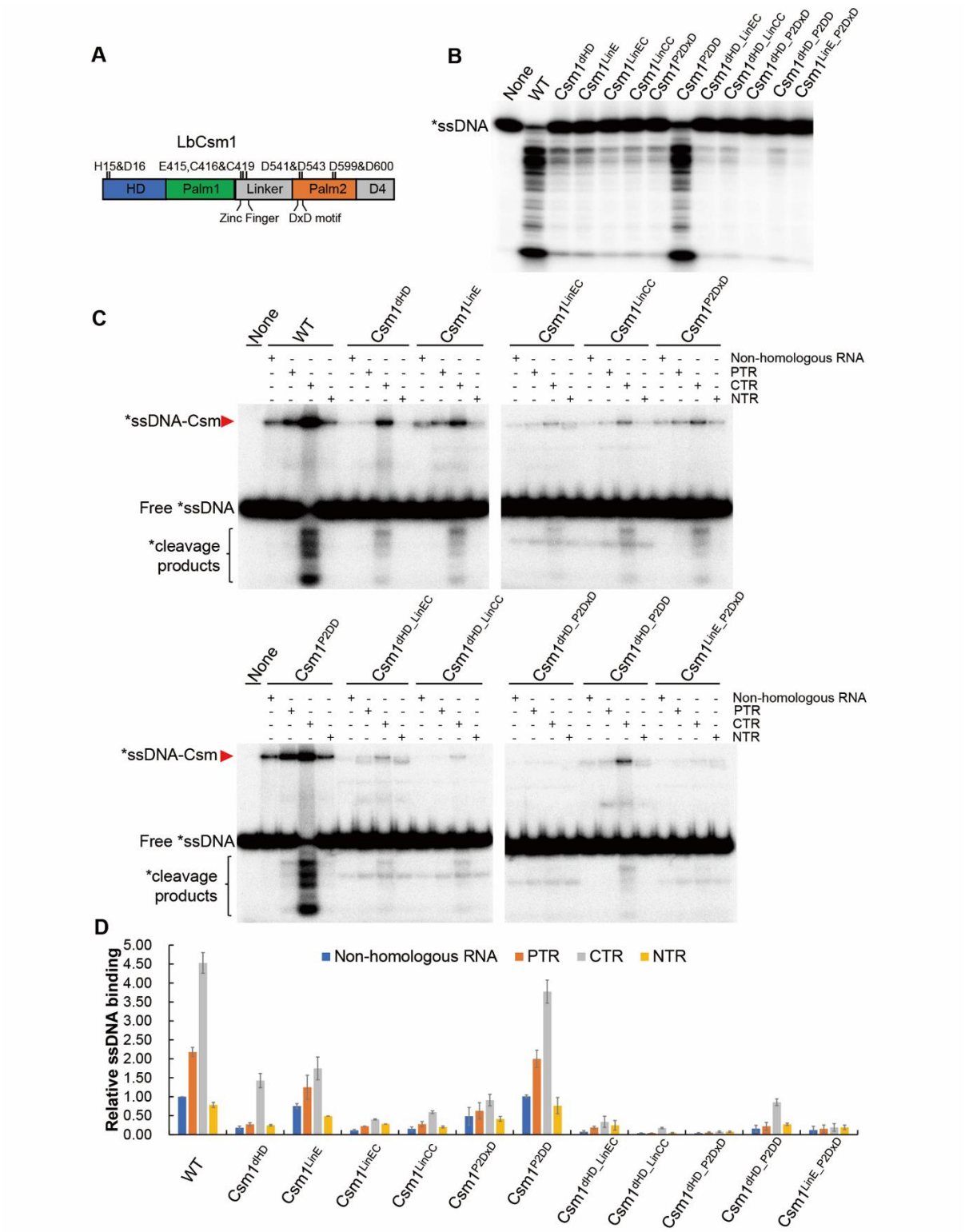

Fig. 4 Effect of LbCsm1 mutations on ssDNA binding and cleavage by the LbCsm effector

complex. (A) Domain architecture of the LbCsm1 protein. HD represents the HD-type nuclease domain; Palm 1 and Palm 2 denote the two cyclase domains; Linker is a domain that adjoins the Palm1 and Palm2 domains, consisting 4 cysteine residues, D4 is located in the C-terminus rich in α-helices. Amino acid residues selected for alanine substitution mutagenesis are indicated with their names and positions. (B) RNA-activated ssDNA cleavage by effectors carrying one of the constructed LbCsm1 mutants. 50 nM S10-60 ssDNA substrates were mixed with 50 nM mutated LbCsm carrying each of LbCsm1 mutant proteins and 500 nM CTR, and incubated for 10 min. Samples were analyzed by denaturing PAGE. (C) ssDNA binding by effectors carrying each of the constructed LbCsm1 mutants. 5 nM labeled S10-60 ssDNA were incubated with 100 nM of LbCsm effectors indicated in each experiment. 400 nM of non-homologous RNA (S10 RNA) or in the presence of 500 nM of one of the target RNAs, PTR or CTR or NTR for 3 min. Samples were analyzed by non-denaturing PAGE. Red arrowheads indicate the Csm-ssDNA complex. (D) Relative ssDNA binding between the wild-type LbCsm effector and its LbCsm1 mutated derivatives. The relative ssDNA binding activities were estimated by image quantification of the non-denaturing PAGE in (C) by the accessory analysis tool in Typhoon FLA 7000, the ssDNA activity of LbCsm in non-homologous RNA was used as the standard and set up as 1. Results shown are average of three independent assays, bars represent the mean standard deviation (± SD).

LbCsm1 Linker domain and Palm2 DxD motif function in the allosteric control of the LbCsm DNase

The establishment of ssDNA binding assay with LbCsm prompted us to investigate how target RNAs could regulate the LbCsm DNase. For this purpose, 400 nM, S10 RNA (non-homologous RNA) and 500 nM of PTR, NTR or CTR was mixed with 100 nM LbCsm and 5 nM of labeled S1-60 DNA individually. After incubation at 37 °C for 3 min, samples were analyzed by non-denaturing PAGE. We found that, while the WT LbCsm effector showed little DNA-binding activity in the presence of non-homologous RNA or NTR, PTR greatly enhanced the DNA binding, and that activity was further facilitated by CTR for ca. 4-fold (Fig. 4C & 4D), indicating that CTR induces allosteric regulation on substrate binding of LbCsm. These results also indicated that the CTR-mediated allosteric regulation of LbCsm DNase involves a protospacer region-induced minimal regulation, in which protospacer region-bound facilities the ssDNA substrate binding but the complexes remain inactive, and CTR-dependent activation of the LbCsm DNase. Therefore, 3' anti-tag of both CTR and NTR regulate LbCsm DNA binding.

Analyses of all above Csm1-mutated effector complexes revealed that, while five mutated effectors (LbCsm_Csm1^{P2DD}, LbCsm_Csm1^{dHD}, LbCsm_Csm1^{LinCC}, LbCsm_Csm1^{dHD_LinCC}, and LbCsm_Csm1^{dHD_P2DD}) exhibited a pattern of target RNA activation that is very similar to that of the wild-type LbCsm, those carrying E415A and/or alanine substitutions in Palm2 DxD did not show such a significant CTR-enhanced ssDNA substrate binding; their CTR and PTR ternary complexes basically remained inactive (Fig. 4C & 4D). These results indicated that among the three motifs essential for the LbCsm DNase, HD is not involved in CTR-induced allosteric regulation of LbCsm whereas both E415 and P2-DxD are essential for the allosteric regulation.

Examination of ssDNA cleavage products in Fig. 4C further revealed that target RNA-independent DNA cleavage was observed for mutated effectors carrying LinEC, LinCC, dHD_LinEC or dHD_LinCC mutations on LbCsm1. Common for these mutants was a mutation in the CC motif of the Zinc finger within the Linker domain, and these results indicated that the Zinc finger motif is essential for preventing

target RNA-dependent activation of the LbCsm DNase. To this end, the Zinc finger motif could function as another layer of LbCsm DNase regulation by influencing substrate binding.

Taken together, our results unraveled three distinctive functions for the Cas10 protein: (a) consistent with the results obtained with other type III effectors, the HD motif of LbCsm1 hosts the catalytic site of the DNase; (b) E415 and P2-DxD are involved in mediating allosteric regulation of the ternary effector to yield active enzyme.

Identification of Csm3 amino acids involved in the regulation of the LbCsm DNase

The ssDNA substrate binding assay showed that PTR-bound ternary LbCsm showed a higher substrate binding compared with that in the presence of non-homologous RNA (Fig. 4C), indicating that the protospacer region-bound drives certain regulation of LbCsm complex. To yield an further insight into the mechanism, some Csm3 variants were constructed and the mutated effector complexes were purified and characterized *in vitro*.

Alignment of a selected set of Csm3 proteins revealed a number of conserved amino acids among which may have a structural function or form the intermolecular contacts with crRNA or target RNA (Fig. S6). There are three conserved residues: H20, D34 and D106 do not a structural function or crRNA and target RNA interaction (LbCsm3 number). D34 was the predicted active site of the LbCsm RNase whereas functions of the remaining two amino acids were uncertain. To study that, we constructed several LbCsm3 mutants, including alanine substitution of H20, D34, or D106 as well as double substitutions (H20/D34 and D34/D106). LbCsm effector complexes carrying each of these Csm3 mutations (designated $Csm3^{H20A}$, $Csm3^{D34A}$, $Csm3^{D106A}$, $Csm3^{H20/D34-A}$ and $Csm3^{D34/D106-A}$) were purified from *E. coli* and characterized. Target RNA cleavage assay showed that, while neither $Csm3^{H20A}$ nor $Csm3^{D106A}$ substitutions influenced the target RNA cleavage, $Csm3^{D34A}$ substitution greatly impaired the RNA cleavage of the effector (Fig. 5A). These results confirmed that the D34 is the active site of target RNA cleavage of the LbCsm system as demonstrated for the conserved aspartic acid residue in all other known type III CRISPR-Cas systems. Analysis of RNA-activated ssDNA cleavage showed that, the LbCsm complex containing the $Csm3^{D34A}$ substitution exhibited higher ssDNase activity relative to WT complex, DNase activity was strongly impaired in the remaining 4 mutated Csm3 complexes (Fig. 5B).

To test their spatiotemporal regulation, these Csm3 mutated complexes were incubated with the ssDNA substrate in the presence of different RNAs. We found that, except for $Csm3^{H20A}$ substitution exhibiting an impaired ssDNA binding for all tested RNAs, $Csm3^{D34A}$ exhibited a higher ssDNA substrate binding enhancement in presence of PTR vs. non-homologous RNA than WT complex (ca. 3-fold vs. 2-fold, respectively), which might due to the reduced target RNA cleavage allows a longer persistence of the PTR-bound ternary LbCsm complex. However, $Csm3^{D106A}$ substitution and the double substitutions $Csm3^{H20/D34-A}$ and $Csm3^{D34/D106-A}$ did not show such a significant PTR-induced ssDNA substrate binding enhancement relative to WT complex (Fig. 5C & 5D). Together, These results confirmed that the target RNA-mediated regulation of LbCsm complex involves a protospacer region-induced regulation, consistent with the results obtained from structural analysis with other Csm or Cmr complexes[54,55,57]. These results also indicated that two Csm3 residues, H20 and D106, are involved in mediating regulation of the ternary LbCsm complex to yield active enzyme.

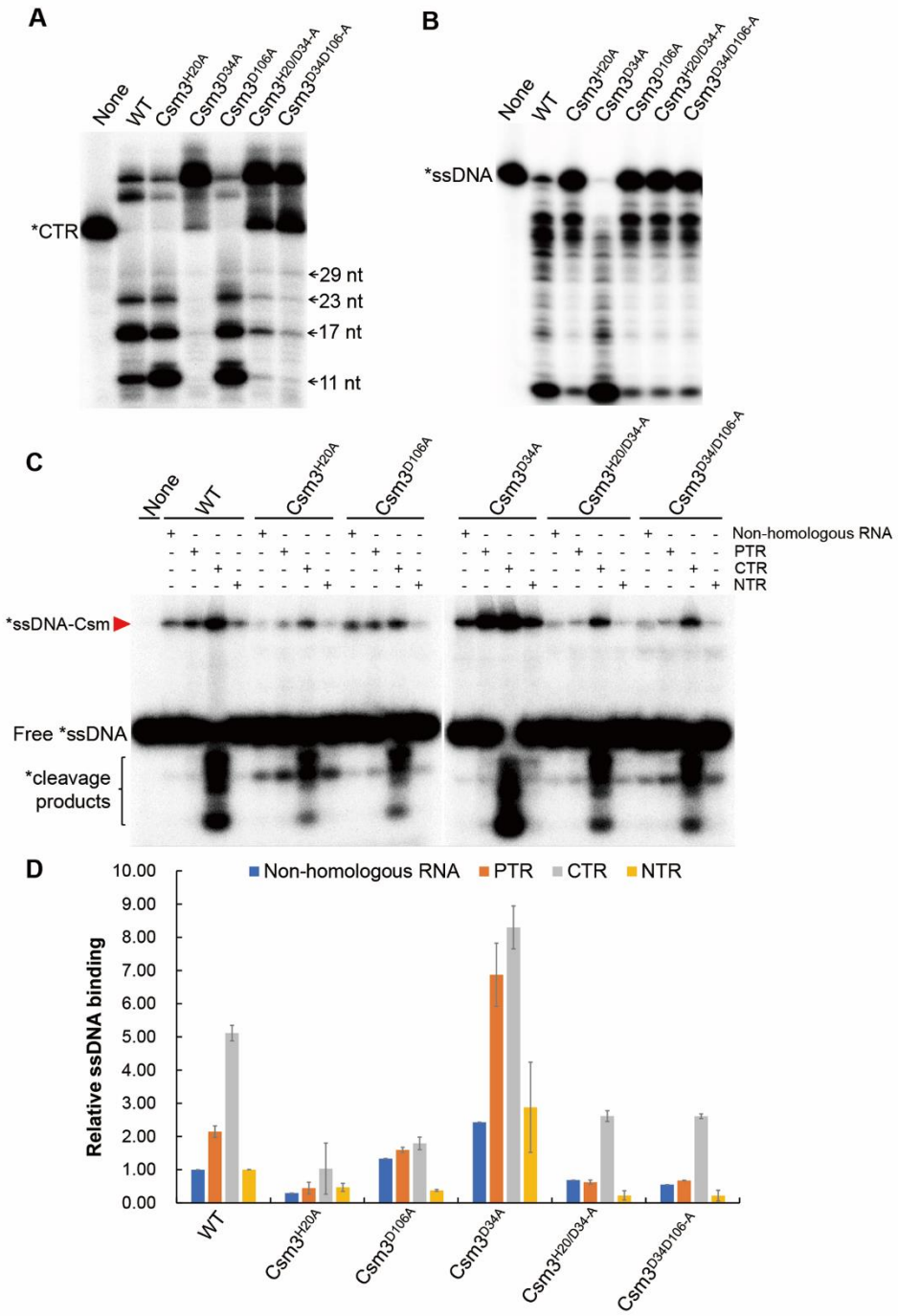

Fig. 5 Effect of LbCsm3 mutations on the ssDNA cleavage and binding of LbCsm. (A) Target RNA cleavage of LbCsm3 mutated derivatives. 50 nM of S1-46 RNA were incubated with 50 nM of LbCsm or the indicated mutant derivatives for 10 min and the samples were analyzed by denaturing PAGE. (B) RNA-activated ssDNA cleavage by effectors carrying one of the constructed LbCsm3 mutants. Reaction conditions were the same as in Fig. 4B. (C) ssDNA binding by effectors carrying each of the constructed LbCsm3 mutants. Reaction conditions were the same as in Fig. 4C. (D) Relative ssDNA binding between the wild-type LbCsm effector and its LbCsm3 mutated derivatives. The ssDNA activity of LbCsm in non-homologous RNA was used as the standard and set up as 1. Results shown are average of three independent assays, bars represent the mean standard deviation (± SD).

Identification of different target RNA-LbCsm ternary complexes with distinctive substrate binding and DNA cleavage

To investigate how target RNA could activate the LbCsm DNase, 3'-truncation variants of the cognate target RNA were generated, carrying +1, +2, +3, +4, +5, +6 nt of the 3' anti-tag region of CTR (annotated as CTR^{+1} to CTR^{+6}) as well as the full length CTR (CTR^{Full}) (Table S1). Each target RNA was tested for its capability to facilitate DNA binding and DNA cleavage by LbCsm. As shown in Fig. 6A, while the binary LbCsm showed little DNA binding, PTR, the target RNA lacking any 3' anti-tag sequence, greatly facilitated the substrate binding of the LbCsm complex (ca. 55% of CTR-LbCsm) but it failed to mediate DNA cleavage, suggesting that this complex (LbCsm-PTR) could represent the one step in the allosteric regulation of LbCsm. Next, when CTR1, a target RNA that extended the PTR by a nucleotide at the 3' anti-tag position, formed a ternary effector complex with LbCsm, the resulting effector not only showed further increased substrate binding activity (ca. 90% of the full activity) but also activated for the DNA cleavage (ca. 35% of full activity). This suggested that a single nucleotide at the 3' anti-tag of CTR is flipped by the LbCsm to convert the inactive ternary LbCsm complex to an active one. Finally, DNA cleavage activity by the LbCsm effector peaked with CTR^{+4} that carries 4 nt 3' anti-tag of CTR, which could have completed the allosteric regulation of the immune system (Fig. 6A).

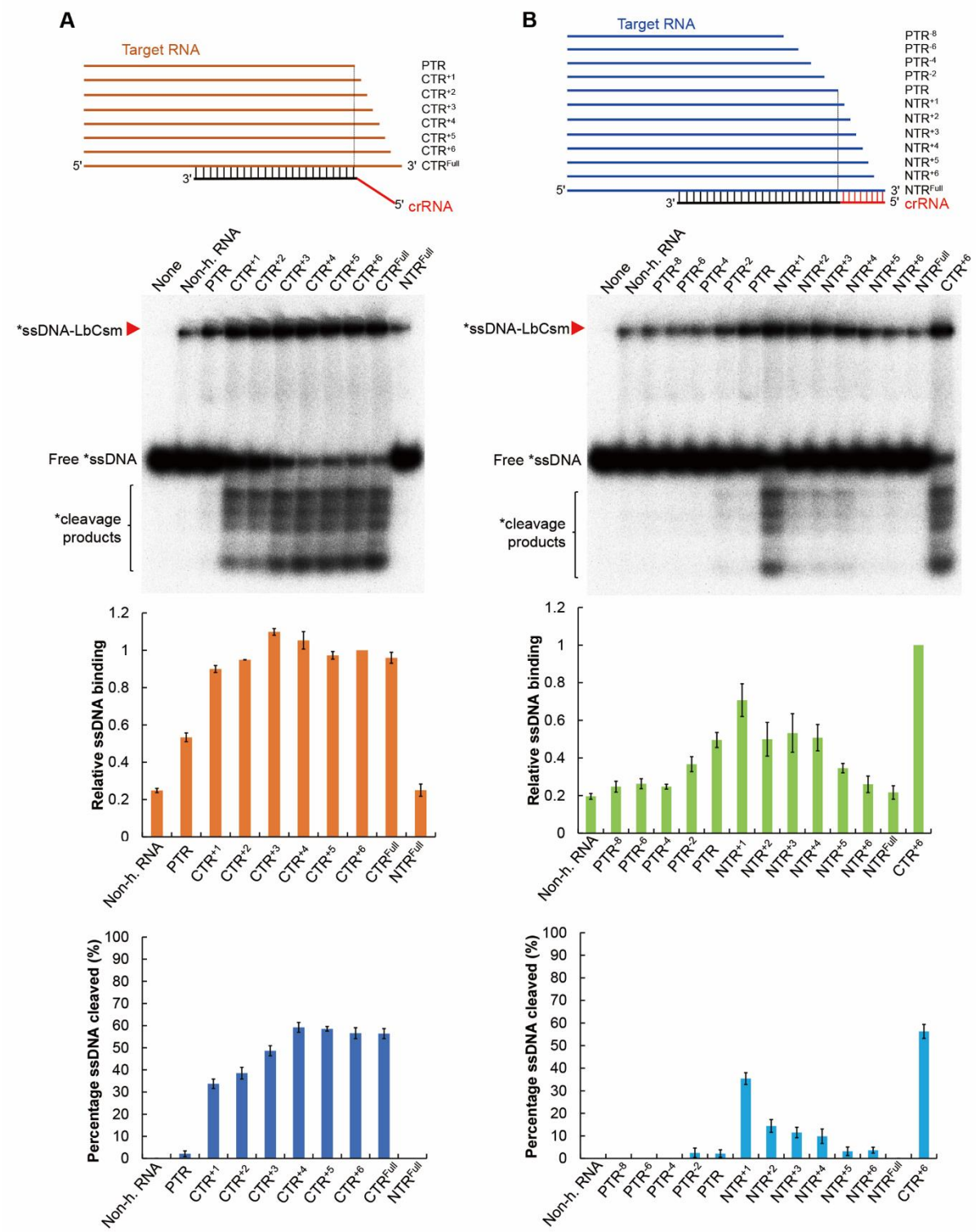

Fig. 6 Target RNA-directed allosteric regulation of LbCsm involves activation and deactivation mechanisms. (A) CTR activates the LbCsm DNase. (B) NTR mediates autoimmunity avoidance by deactivation. Reactions were set up with 5 nM S10-60 ssDNA, 100 nM of LbCsm and 400 nM non-homologous RNA (non-h. RNA) or 500 nM target RNA. After addition of one of target RNAs, the mixture was incubated at 37 °C for 3 min. Samples were then analyzed by non-denaturing PAGE (in this page) or denaturing PAGE (Fig. S7). Red arrowheads indicate the LbCsm-ssDNA complex. Relative ssDNA binding and percentage ssDNA cleaved of LbCsm facilitated by each of these target RNAs were

estimated by image quantification of bands on non-denaturing PAGE and denaturing PAGE, using the accessory analysis tool equipped with a Typhoon FLA 7000. For the quantification of the substrate binding, the amount of ssDNA-LbCsm-CTR^{+6} complex is arbitrarily defined as 1. Results of average of three independent assays are shown with bars representing the standard deviation (± SD).

NTR inhibits the LbCsm DNase by preventing substrate binding

The same approach was then employed to investigate how NTR could inhibit the LbCsm DNase. Truncated derivatives of NTR were generated (i.e. NTR^{+1} through NTR^{+6}) as for the CTR derivatives, and in addition, a few PTR truncations (PTR^{-8} to PTR^{-2}) were also made (Table S1). These target RNAs were tested for their capability of facilitating substrate binding and cleavage as above described. We found that PTR and its truncated derivatives (PTR^{-4} to PTR^{-8}) showed marginal differences both in ssDNA binding and in ssDNA cleavage, suggesting that these ternary complexes could belong to the same category, equivalent to the stage of the binary LbCsm effector (Fig. 6B). While PTR^{-2} and PTR showed similar effects on ssDNA binding, NTR^{+1}, however, mediated a major stimulation to the LbCsm DNA binding (ca. 70% of the full activity), and it also facilitated the DNA cleavage (ca. 35% of full activity), and in fact, the stimulating effect by NTR^{+1} is comparable to that by CTR^{+1} (Fig. 6). These results not only implied the first nucleotide at the 3' anti-tag of NTR can still be flipped by the complex to allow activation of the HD nuclease domain, but also indicated that the process of repression by NTR shares some common activation with the CTR-dependent activation process of LbCsm DNase. Thereafter, extension of the 3' anti-tag of NTR greatly reduces the DNA binding and gradually decreases DNA cleavage (Fig. 6B), suggesting that the interaction between 3' anti-tag of NTR and the 5' tag of crRNA could have deactivated the enzyme by restricting the accessibility of the ssDNA substrate to the active site.

Discussion

Type III CRISPR-Cas systems characterized thus far show three different interference activities: target RNA cleavage, RNA-activated indiscriminate DNA cleavage and cOA synthesis among which the latter two activities are responsible for the DNA interference by these systems whereas target RNA cleavage plays a regulatory role (see reviews[46,47,49]). Here we report a type III-A subtype CRISPR-Cas system that shows robust RNA-activated ssDNA cleavage, but devoiding of cOA synthesis. This is consistent with the fact that the genome of the *L. delbrueckii* subsp. *bulgaricus* genome does not code for any detectable Csm6 homologs. Interestingly, we find that a plasmid-borne LbCsm system is sufficient to mediate interference plasmid clearance in *E. coli*. This suggests that the LbCsm system probably only utilizes the RNA-activated ssDNase to mediate the antiviral immunity in the original host. We have also explored the simplicity of the LbCsm antiviral mechanism for investigating mechanisms of the DNA cleavage by type III CRISPR-Cas systems and our research has yielded mechanistic insights into the target RNA-mediated activation and repression of the LbCsm DNase.

Structures of several type III effector complexes have been solved[54-58] and these analyses have revealed that formation of target RNA-Csm ternary effectors involves a minimal conformational change that is very similar between the CTR ternary effectors and the corresponding NTR ternary ones although only the former has been activated for DNA cleavage[54-56]. As a result, there is no major structural difference at the catalytic site of active versus inactive Csm DNases, and the only difference is the 3'

anti-tag sequences of the two different types of target RNAs are individually placed in different channels in their structures[54]. Furthermore, in the structure of the PTR-StCsm complex, major conformational change also occurs although the target RNA lacking any 3' anti-tag. Since structure of a DNA substrate-bound Csm effector complex has not been resolved, how these effectors interact with their DNA substrates remains to unknown. For this reason, the only biochemical criterion to distinguish different effector complexes is to analyze their DNA cleavage activity. In this work, we have established a ssDNA binding assay for LbCsm, and our detailed analysis of substrate binding for this immune system has revealed that different target RNAs are capable of facilitating differential substrate binding and DNA cleavage to the LbCsm effector. Thus, it is very interesting to study how allosteric regulation differentially influences the two activities of the LbCsm DNase.

Structural analysis of StCsm by You et al.[54] has indicated that the Cas10 Linker domain functions in mediating conformational change. Here, we find that that both the DxD motif in Palm 2 and the E415 residue in the Linker domain play an essential role in mediating the allosteric regulation of the LbCsm complex *in vitro*. To date, detailed functions of the Cas10 linker E415 and P2 DxD motifs have not been studied for other type III CRISPR-Cas systems although all three amino acids are well conserved in Cas10 proteins (Fig. S5A). Therefore, it is of a great interest to investigate if their functions revealed for LbCsm represent a general mechanism for all type III immune systems.

In this paper, we have presented the detailed biochemical analysis of substrate binding by LbCsm effector complex, and our analysis shows that the Cas10 Zinc finger modulates the enzyme substrate binding since E415A substitution yielded an enzyme with greatly reduced DNA binding and inactive in DNA cleavage (Fig. 4). As E415 is probably part of the Zinc finger motif, this suggests that the Cas10 Zinc finger domain can have dual function in this immune system, namely both as an executor to the allosteric regulation and as a regulator to substrate binding[46,59]. The other study on substrate binding was conducted by single-molecule fluorescence microscopy analysis of *Staphylococcus epidermidis* Csm by Wang et al[60]. They find that Cas10 subunit is locked in a static configuration upon NTR binding in which the DNA binding pocket of the effector appears to be in a closed form, inaccessible to substrate. However, upon CTR binding, Cas10 exhibits a larger conformational space in the active site[60]. Together with our biochemical data, it is plausible to predict that there exists a substrate-binding pocket in the DNase of type III effector complexes although this structural domain remains to be illustrated by their structural analysis.

The possibility of characterization of effector complexes for both substrate binding and DNA cleavage allows us to identify target RNA-LbCsm effector complexes exhibiting differential activities, and we propose that these effector complexes probably represent intermediates for structural analysis to study the molecular mechanisms of the CTR activation or NTR repression of the LbCsm system (Fig. 7). These include: (a) The LbCsm-PTR complex showing an elevated level of substrate binding but inactive in catalysis, (b) the LbCsm-CTR^{+1} and LbCsm-NTR^{+1} exhibiting most of substrate binding and catalysis activities, representing another type of intermediates. There are two apparent features with the two effectors: they differ from LbCsm-PTR only by the first nucleotide present in the 3' anti-tag of target RNAs, and the nucleotide in CTR is different from that in NTR. Thus, this nucleotide could functions as a trigger to induce the major allostery in LbCsm ternary to yield an active effector complex and the interaction between the effector with the nucleotide should not be specific, (c) LbCsm-CTR^{+4} represents another effector complex that should have adopted the same conformation since it exhibits the maximal

substrate binding and catalysis, and finally (d) and LbCsm-NTR^{Full} represents the inactive conformation of the effector completely deactivate the LbCsm complex, ensuring autoimmunity avoidance of the LbCsm system (Fig. 7). These LbCsm effector complexes provide good model for structural analysis to reveal the molecular mechanisms of activation and repression of Csm DNases.

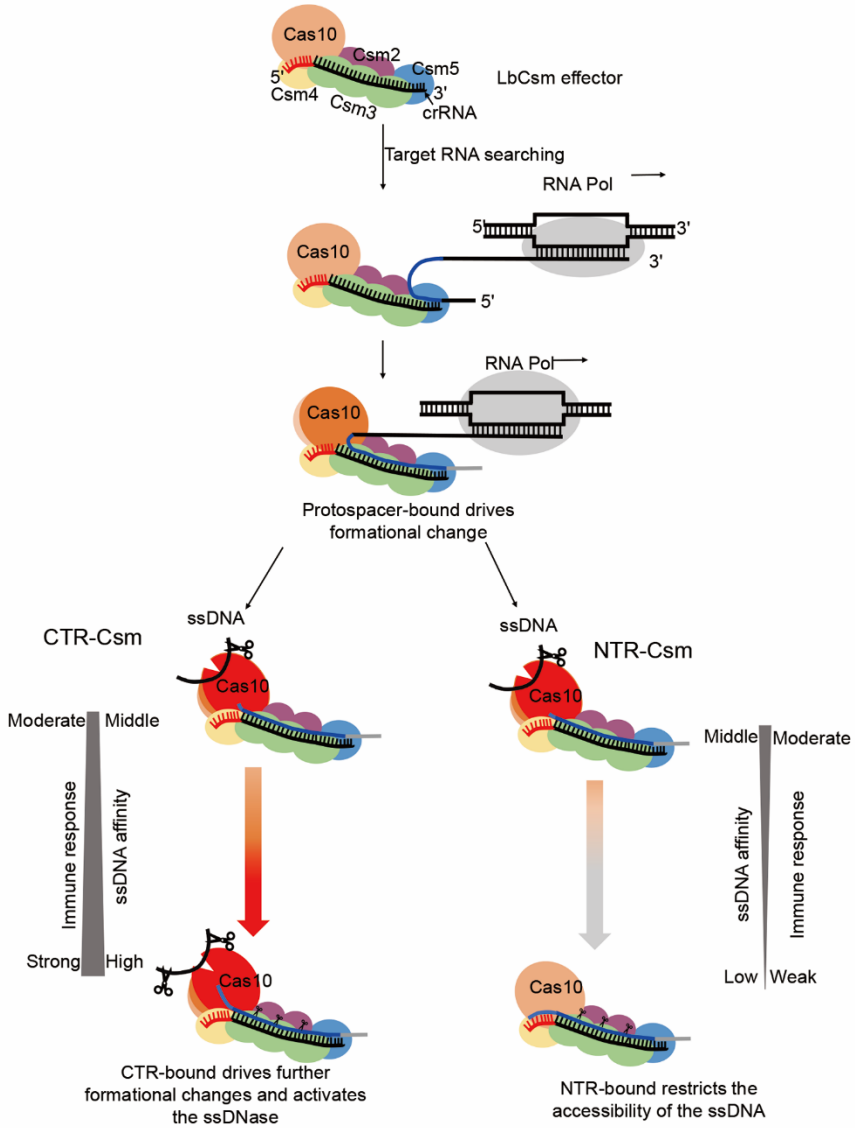

Fig. 7 Model of allosteric activation and repression of the LbCsm DNase. The previous works have proposed that the initial recognition of nascent transcript at the 5' end of target RNA for type III complex, since both of Csm5 subunit in Csm complex and Cmr1 subunit in Cmr complex are crucial for target RNA binding[36,37,66]. These suggested that the binary LbCsm effector complex interacts with target transcript initially at the 5' end of target RNA and further via sequence complementarity between the protospacer and the corresponding crRNA, leading to the formation of a ternary effector complex with a major formational change. Addition of a single nucleotide at the 3'-end of protospacer RNA results in an important allosteric change in the LbCsm DNase, giving an active enzyme. CTR-bound LbCsm exhibits the full level of substrate binding and DNA cleavage whereas NTR-bound LbCsm closes the substrate-binding pocket, which deactivates the DNase. Finally, multiple Csm3 subunits cleave the

target transcripts, and release of target RNA cleavage products restores the binary conformation, completing the spatiotemporal regulation of LbCsm systems.

Materials and Methods

Bacterial strains and growth conditions

L. delbrueckii subsp. *bulgaricus* ND04 (GenBank: CP016393.1) was grown in MRS broth (Oxoid, UK) at 37°C without shaking. *E. coli* JM109 and BL21(DE3) were propagated in Luria-Bertani (LB) medium at 37°C with 200 rpm/min shaking. If applicable, antibiotics were added as the following: ampicillin (100 μg/ml, sigma), kanamycin (25 μg/ml, sigma) and chloramphenicol (10 μg/ml, sigma).

Construction of different vectors and plasmids

To construct a p15A replicon-based expression vector, the origin fragment was obtained from pTRKH2 [61], ampicillin resistance gene (Amp) was derived from pUC19 (New England Biolabs) whereas gene expression cassette were amplified from and pET30a (Novagen) by PCR, using three sets of primers (p15A-F and-R, Amp-F and -R, as well as LacI-F and T7-R) listed in Table S1. Ligation of these DNA fragments generated the expression vector p15AIE (Fig. S2A). The same strategy was employed to construct pUCE in which the origin was amplified from pUC19, chloramphenicol resistance gene was obtained from pCI372 whereas the expression cassette, from pET30a, using the primer sets of pUC, Cm and T7, respectively (Table S1). Ligation of these DNA fragments gave pUCE (Fig. S2B).

The vector for invader plasmid assay was constructed in two steps. First, fragment 1 containing the origin of pBR322 and kanamycin resistance gene (Kan) was obtained from pET30a by PCR (pBR-Kan primers); second, DNA fragments carrying an arabinose-inducible Bad promoter, multiple cloning sites (MCS) and transcriptional terminator (T) were generated by PCR from *E. coli* BL21(DE3) genome and plasmid pELX1 [62], using the primer sets of PBad and MCS-T listed in Table S1, respectively, and they were then fused together by splicing overlapping extension PCR (SOE-PCR) [63]; third, ligation of the fragment 1 and the SOE DNA fragment yielded pBad vector. Then, three DNA fragments carrying *eGFP* gene were amplified from pEGFP-N1 [64] by PCR using GFR-F/GFR-R, CTR-GFR-F/GFR-R and NTR-GFR-F/GFR-R, and insertion of each fragment into pBad individually gave pBad-G, pBad-CTR and pPBad-NTR. All primers employed in this work were listed in Table S1.

Chromosomal DNA was extracted from cells of *L. delbrueckii* subsp. *bulgaricus* ND04 using OMEGA Genomic DNA Purification Kit (OMEGA Bio-tek). DNA fragment covering the cas6-cas10-csm2-csm3-csm4-csm5 gene cassette was amplified by PCR with the primers *Sal*I-*Cas6*-F and *Not*I-*Csm5*-R using ND04 genome DNA as template. The PCR product inserted into p15AIE via *Sal*I and *Not*I, yielding p15AIE-Cas. *csm2* gene was amplified from ND04 genome using primers Csm2-F and Csm2-R. PCR product was then digested with *Nde*I and *Xho*I and cloned into pET30a expression vectors, giving pET30a-Csm2. To construct a CRISPR array plasmid, fusion PCR amplification was performed using three primers Re-S1-F, S1-R1, Re-S1-R to generate the multiple copies of 36 nt length repeats interspaced by multiple S1 spacer (40 nt) of identical sequence, then PCR products of ~1 kb were recovered from an agarose gel using an OMEGA gel-purification kit (OMEGA Bio-tek). The purified DNA fragments were cloned into pJET1.2 (CloneJET PCR Cloning Kit, Thermo Scientific). After confirming the sequence of the synthetic CRISPR array on pJET clones (GATC Bio-tech), the DNA fragment was amplified and cloned into plasmid pUCE at the *Bgl*II site, yielding pUCE-S1 carried 10 identical spacers

(S1) in the CRISPR array. Finally, plasmid p15AIE-Cas-S1 was constructed by insertion of the CRISPR array into p15AIE-Cas at the *Nhe*I site.

Purification of LbCsm effector complexes from *E. coli*

Three plasmids (p15AIE-Cas, pUCE-S1 and pET30a-Csm2) were introduced into *E. coli* BL21 (DE3) by electroporation, yielding a *E. coli* strain containing all three plasmids. This bacterial strain was employed as host to overexpress the LbCsm system. The strain was cultured 200 ml LB medium containing ampicillin, kanamycin, chloramphenicol (at 37°C, 200 rpm) to the mid-log phase (OD_{600} was 0.8), then IPTG was added to 0.3 mM and the culture was further cultured at 25°C for 16 h. Cells were harvested by centrifugation at 5,000 rpm for 5 min, and cell pellets were resuspended in 20 ml buffer A [20 mM Tris-HCl (pH 8.5), 0.25 M NaCl, 20 mM imidazole and 10% glycerol], yielding cell suspension that was treated with a French press for cell lysis at 4°C. Cell debris was then removed from treated cell suspension by centrifugation at 10,000 rpm for 30 min at 4°C. The Csm complex was captured on the HiTrap affinity column (GE Healthcare) by LbCsm2 copurification and eluted with buffer B [20 mM Tris-HCl (pH 8.5), 0.25 M NaCl, 200 mM imidazole and 10% glycerol]. The resulting LbCsm effector complex preparation was further purified by size exclusion chromatography (SEC) with Superdex 200 (GE Healthcare) using the chromatography buffer [20 mM Tris-HCl (pH 8.5), 0.25 M NaCl and 5% glycerol]. SEC fraction samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and those containing the complete set and high quality of Csm complex components were pooled together and used for further analysis. Csm complex concentration was measured according to the Bradford method using the protein assay kit (Thermo Scientific) with bovine serum albumin as the standard.

Extraction and analysis of crRNA

The purified LbCsm complex (100 μl) was first mixed with l00 μl Trizol agent (Sigma), and then 200 μl chloroform:isoamylalcohol (24:1, v/v) was added. After vortex for 30 s, the mixture was centrifuged at 12,000 rpm for 10 min at 4 °C. The upper phase was transferred into a new tube and reextracted with 200 μl chloroform:isoamylalcohol. crRNA in the upper phase was precipitated with one volume of isopropanol and washed twice with 1 ml of 70% ice-cold ethanol. The pellet was air-dried for 30 min at the room temperature and dissolved in 20 μl DEPC-H_2O. Ten nanograms of crRNA was 5'-labeled with [γ-^{32}P]-ATP (PerkinElmer) using T4 polynucleotide kinase (New England Biolabs) and separated on a 12% denaturing polyacrylamide gel. The labeled crRNAs were identified by exposing the gel to a phosphor screen (GE Healthcare) and scanned with a Typhoon FLA 7000 (GE Healthcare). For northern blotting of crRNA, 100 ng of unlabeled crRNA was mixed with equal volume of 2 × RNA loading dye (New England Biolabs) and fractionated in the 12% denaturing polyacrylamide gel. Northern blotting analysis was conducted as described previously [65], using radiolabeled RNA S1-40 (Table S1).

Labeling of DNA and RNA Substrates

All DNA, S10 (nonhomologous RNA), S1-40 (PTR), S1-46 (CTR) and S1-48 (NTR) oligonucleotides were purchased from IDT, other RNA oligonucleotides were generated by *in vitro* transcription using TranscriptAid T7 High Yield Transcription Kit (Thermo Scientific) (Table S1). DNA and RNA oligonucleotides to be used as substrate for cleavage and binding assays were purified by recovering

the corresponding bands from either a native polyacrylamide gel (for double-stranded DNA) or from denaturing polyacrylamide gel (for ssDNA or RNA) after electrophoresis. ssDNA and RNA substrates were 5' labeled with [γ-^{32}P]-ATP and T4 polynucleotide kinase followed by denaturing gel purification. Double strand DNA, bubble DNA and R-loop DNA was generated as described previously [31].

Cleavage assay

Nucleic acid cleavage assays were conducted in 10 μl of reaction containing the indicated amount of effector complex and substrates in the cleavage buffer (50 mM Tris-Cl (pH 6.8), 10 mM $MgCl_2$, 50 mM KCl, 0.1 mg/ml BSA]. In DNA cleavage assay, 500 nM (unless otherwise indicated) unlabeled RNA was supplemented to activate DNA cleavage activity. Samples were incubated at 37°C and stopped for indicated time periods and the reaction was stopped by addition of 2 × RNA loading dye (New England Biolabs). For electrophoresis, samples were heated for 3 min at 95°C and analyzed on an 18% polyacrylamide denaturing gel. RNA ladders were generated by Decade™ Marker RNA (Ambion) following the instructions and labeled by [γ-^{32}P]-ATP with T4 polynucleotide kinase. Results were recorded by phosphor imaging.

Determination of cyclic oligoadenylates synthesis activity

Each reaction mixture contained 50 nM Csm complex, 500 nM unlabeled S1-46 RNA (CTR), ~2 nM [α-^{32}P]-ATP (PerkinElmer) and an indicated content of ATP in 50 mM Tris-Cl (pH 6.8) and 0.1 mg/ml BSA supplementing with ions. Reactions were incubated at 37°C for 120 min and 2 × RNA loading dye was added at the indicated time points to stop the reaction. Samples were kept on ice until use. For electrophoresis, samples were treated at 95°C for 3 min and analyzed by 24% denaturing PAGE. Gels were analyzed by phosphor imaging.

Electrophoretic Mobility Shift Assay

ssDNA binding assay was performed by incubating different amounts of Csm complex (specified in each experiment) with 5 nM ^{32}P-5'-labeled S10-60 ssDNA in the cleavage buffer. All reactions were incubated at 37°C for the indicated time periods. Then, the same volume of 2 × native loading buffer [0.1% bromophenol blue, 15% sucrose, w/v] was added and the samples were immediately put on ice and kept there until needed for electrophoresis on an 8% nondenaturing polyacrylamide gel. Electrophoresis was carried out at 4°C using 40 mM Tris, 20 mM acetic acid (pH 8.4 at 25°C) as the running buffer. Gels were analyzed by phosphor imaging.

Relative ssDNA binding and cleavage activities of LbCsm facilitated by each of these target RNAs were estimated by image quantification of bands on non-denaturing PAGE and denaturing PAGE, respectively, using the accessory analysis tool equipped with a Typhoon FLA 7000. Results of average of three independent assays are shown with bars representing the mean standard deviation (± SD).

Mutagenesis of LbCsm1 and LbCsm3

Lbcsm1 mutants were generated using the splicing overlapping extension PCR protocol previously reported [63]. In brief, several mutations were designed in the internal partial overlapping primers, initial PCRs were preformed using the external primer and their corresponding internal primers to generate overlapping gene segments, then, the two PCR products were fused together by overlapping extension

PCR. The resulting fragments were digested with restriction endonuclease (DNA fragment containing LbCsm1 H15D16A mutation was cleaved with *Sal*I and *Sac*I, those carrying LbCsm1 E415A, E415C416A, C416C419A, D541D543A, D599D600A or Q597G were digested with *Stu*I and *Sac*I. LbCsm3 H20A, D34A and D106A was obtained via *Stu*I and *Kpn*I. After purification, these DNA fragments were inserted into the plasmid p15AIE-Cas at the corresponding restriction sites, yielding the plasmids carrying each designed *Lbcsm1* mutation. All mutations were verified by DNA sequencing (GATC Biotech).

Plasmid interference assay

Plasmid interference assays were performed as previously described [51]. Briefly, an *E. coli* BL21(DE3) strain carrying p15AIE-Cas-S1 (80 μl) was transformed with 100 ng of one of the following plasmids, pBad-G, pBad-CTR or pBad-NTR. Electroporation was performed in a 1-mm cuvette (Bio-Rad, USA), with the setting of 1,600 V, 200 Ω and 25 μF, using a Gene Pulser II Electroporation System (Bio-Rad). Then, 920 μl of SOC medium was immediately added to electroporated cells and incubated with shaking (200 rpm) at 37°C for 60 min. A series of dilutions were then made for each transformation, and 100 μl of each dilution was plated onto LB agar plates containing 0.05 mM IPTG, Ampicillin, Kanamycin and various concentrations of L-arabinose. Plates were incubated overnight at 37°C and the transformation efficiency was calculated. Transformation experiments were conducted for three independent times.

Acknowledgments

We thank colleagues in the Archaea Centre for stimulating discussions and Dr. Søren M. Madsen (Bioneer A/S, Hørsholm, Denmark) for a kind gift of pTRKH2 and pCI372 plasmids. This work has been supported by grants from Danish Council for Independent Research (DFF-4181-00274), National Science Foundation of China (31771380) and the National Transgenic Science and Technology Program (2019ZX08010-003) to Q.S. and grants from Shandong University.

Author contributions

H.Z. provided the *L. delbrueckii* subsp. *bulgaricus* strain; J.L., and Q.S. designed experiments; J.L. and M.F. performed experiments; J.L., M.F. and Q.S. analyzed data; J.L. and Q.S. wrote the paper. All authors read and approved the final manuscript.

Conflict of interests

The authors declare no competing interests.

Additional information

Supplemental Information includes Supplemental figures and Supplemental tables can be found with this article online.

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