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Harnessing Type I and Type III CRISPR-Cas systems for genome editing

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ABSTRACT

CRISPR-Cas (clustered regularly interspaced short palindromic repeats-CRISPR-associated) systems are widespread in archaea and bacteria, and research on their molecular mechanisms has led to the development of genome-editing techniques based on a few Type II systems. However, there has not been any report on harnessing a Type I or Type III system for genome editing. Here, a method was developed to repurpose both CRISPR-Cas systems for genetic manipulation in *Sulfolobus islandicus*, a thermophilic archaeon. A novel type of genome-editing plasmid (pGE) was constructed, carrying an artificial mini-CRISPR array and a donor DNA containing a non-target sequence. Transformation of a pGE plasmid would yield two alternative fates to transformed cells: wild-type cells are to be targeted for chromosomal DNA degradation, leading to cell death, whereas those carrying the mutant gene would survive the cell killing and selectively retained as transformants. Using this strategy, different types of mutation were generated, including deletion, insertion and point mutations. We envision this method is readily applicable to different bacteria and archaea that carry an active CRISPR-Cas system of DNA interference provided the protospacer adjacent motif (PAM) of an uncharacterized PAM-dependent CRISPR-Cas system can be predicted by bioinformatic analysis.

INTRODUCTION

Clustered regularly interspaced short palindromic repeats-CRISPR-associated (CRISPR-Cas) systems function in antiviral defense in prokaryotes and are present in about 90% of archaeal and 40% of bacterial genomes (1,2). In the current classification, most known CRISPR-Cas systems are classified into three main groups, i.e. Type I, II and III, each of which is characterized by a signature protein (Cas3, Cas9 or Cas10) (3). The system is adaptive and functions in three functional stages (1,2,4–7). First, a DNA segment (protospacer) is recognized from an invading genetic element and inserted into a CRISPR locus immediately after the leader, becoming the new first spacer of the CRISPR array. Second, the CRISPR array is transcribed from the leader region, forming a long precursor transcript that is processed into mature crRNAs. Finally, crRNAs and Cas proteins form a ribonucleoprotein complex (crRNP) that recognizes invading genetic elements by sequence complementarity between the crRNA and the protospacer and targets their nucleic acids for destruction via DNA or RNA interference. DNA targeting in Type I and Type II systems relies on short conserved sequences defined as protospacer-adjacent motifs (PAMs), which were first identified from bioinformatic analyses (8) and functionally demonstrated in different Type I and Type II CRISPR-Cas systems, and furthermore, DNA interference by all three types of CRISPR-Cas systems requires a seed sequence (1,2,4–7).

Among the three main classes of CRISPR-Cas systems, DNA interference by Type II systems only requires a single Cas protein, namely Cas9. This protein possesses multiple domains and functions together with two small RNAs, a mature crRNA and a trans-acting RNA (tracrRNA) in DNA interference (9–11). Immediately after the discovery, this simplicity has been explored for genome editing in higher eukaryotes at cellular (10) and organismal levels (12), and the past few years have witnessed an explosion of application of this technology in the globe such that CRISPR-Cas9 technology has currently been applied in many different eukaryotes (13–17). Furthermore, CRISPR-Cas-mediated cell killing has been documented for differ-

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ent bacteria (18–21), and explored for selectively eliminating specific bacterial species either by using a genetically engineered Type I or Type II CRISPR-Cas system or by repurposing their endogenous immune systems of bacterial hosts (20,22,23). The immune system is also useful in facilitating generation of genomic island deletion mutants (19,24). However, method has not been reported for repurposing any Type I or Type III CRISPR-Cas system for genome editing.

We were interested in developing CRISPR-based genome editing tools for *Sulfolobus islandicus* to replace the routine genetic manipulation tools recently developed for diverse *Sulfolobus* species, including *S. islandicus, S. acidocaldarius* and *S. solfataricus* (25–29). Studies on multiple CRISPR-Cas systems present in *S. solfataricus* P2 and *S. islandicus* REY15A (30,31) have revealed DNA and RNA targeting activities for *S. solfataricus* (32–35) and for *S. islandicus* (32,36–38). More detailed characterizations of CRISPR-Cas systems in *S. islandicus* REY15A, a genetic model of archaeal research, show that the Type I-A CRISPR-Cas system mediated DNA interference to invading genetic elements by specifically recognizing a PAM located at the 5′-flanking position of the protospacer (32,37). Two PAM sequences, i.e. CCN and TCN, were previously identified by bioinformatic analysis of genomes of *Sulfolobus* hosts and their genetic elements (39), and they were found to function equally well in mediating DNA interference by the Type I-A system (32,40). Furthermore, genetic analysis of Type I-A *cas* genes indicated that depletion of *cas6*, *cas5*, *cas7*, *cas3* or *casHD* by gene deletion inactivated the DNA interference by the I-A system, but deleting *csa5* did not show any influence, indicating only *csa5* is not essential for the immune system (37). This archaeon also encodes two Type III-B CRISPR-Cas systems, also named as Cas RAMP modules (or Cmr systems), one of which is designated as Cmr-α, containing six different Cmr proteins (Cmr-1α through -6α), while the other (Cmr-β) possessing seventh different subunits (41). Six of the seven Cmr-β subunits are homologous to those present in Cmr-α while the seventh is unique. It has been shown that a homologous Cmr-β system in *S. solfataricus* mediates RNA targeting *in vitro* and *in vivo* (35,42), and we show that the Cmr-β system also confers RNA interference in *S. islandicus* (38). Furthermore, Cmr-α is capable of conferring transcription-dependent DNA targeting (36,38). Therefore, both Type I-A and Type III-B Cmr-α show the potential to be employed for testing CRISPR-based genome editing in *S. islandicus* by enabling self-targeting. Here, we tested Type I-A and III-B CRISPR-Cas systems for mediating genome editing and found that both systems greatly facilitate genetic manipulation in this archaeon.

MATERIALS AND METHODS

Strains, growth conditions and transformation of *Sulfolobus*

Genetic hosts and mutants constructed in this work are listed in Table 1. *Sulfolobus* strains were grown at 78°C in a Sucrose-Casamino acids-Vitamin (SCV) medium (0.2% sucrose, 0.2% casamino acids plus 1% vitamin solution) or SCVy (SCV + yeast extract (0.0025%)) (43) and uracil was supplemented to 20 μg/ml if required. *Sulfolobus* competent cells were prepared as previously described (43) and transformed by electroporation. All oligonucleotides (Table 2) were synthesized in Tsingke, Wuhan, China, and restriction enzymes were purchased from ThermoFischer Scientific, Waltham, MA, USA.

Construction of genome-editing plasmids (pGE)

Genome-editing plasmids (pGE) (Table 1) were constructed individually by cloning a single spacer and a mutant allele of the target gene into pSe-Rp, a *Sulfolobus* CRISPR-cloning vector (38). Spacer fragments were generated by annealing of the corresponding complementary oligonucleotides (Table 2) and inserted into pSe-Rp at the BspMI sites, yielding plasmids carrying an artificial mini-CRISPR array. Then, donor DNA fragments containing a mutant allele of each target gene were obtained by splicing and overlap extension PCR (44) using primers listed in Table 2. The PCR products were digested with Sal I and Not I, and purified again. The resulting restriction DNA fragments were inserted into their cognate pAC plasmids at the same sites, giving pGE plasmids listed in Table 1.

Mutant construction and screening of mutated gene alleles by PCR

Each pGE plasmid was introduced into a *S. islandicus* host indicated in each experiment by electroporation. After transformation, pGE plasmids mediated self targeting to the chromosome of wild-type cells and kill them. Only mutants can survive the transformation and form colonies on SCV plates since their chromosomes are devoid of the target sequence. Transformants were screened by PCR amplification of the wild-type target gene and its mutated allele using primers listed in Table 2. The resulting PCR products were analyzed by agarose gel electrophoresis and by DNA sequencing (Tsingke, Wuhan, China).

X-gal assay

β-Glycosidase activity encoded by *lacS* gene was detected in colonies of *Sulfolobus* strains by spraying an X-gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside) solution of 2 mg/ml onto the colonies on plates and incubating for a few hours at 78°C. To detect the enzyme activity in liquid cultures, X-gal was added to 2 mg/ml (final concentration) and incubated in a 78°C incubator for 1 h before observation. Colonies or cultures exhibiting the enzymatic activity show a deep blue color while those that remain colorless are *lacS* mutants.

Western blotting

S. islandicus Cmr-2α-His strain was cultured in SCVy medium. When the absorbance at 600 nm of the culture reached 0.4, cell mass was collected by centrifugation, resuspended in 50 mM phosphate buffer and sonicated. Then, crude protein samples were loaded on 12% SDS-PAGE and fractionated according to their sizes. Fractionated proteins

Table 1. Strains and plasmids used in this work

Strains	Genotype and features	Reference
S. islandicus E233	Δ*pyrEF*	Deng *et al.* (27)
S. islandicus Δcas3	Derived from *S. islandicus* E233S1, carrying deletion of the entire gene of *cas3*	Peng *et al.* (37)
S. islandicus Δcmr-β	Derived from *S. islandicus* E233, carrying deletion of IIIB Cmr-β locus including 7 *cmr*-β genes	This work
S. islandicus ΔlacS	Derived from *S. islandicus* E233, carrying 43 bp deletion in the *lacS* gene	This work
S. islandicus Cmr-2α-His	Derived from *S. islandicus* E233, carrying the *cmr*-2α-His gene	This work
S. islandicus Cmr-2α-HDmut1, 2, 3	Derived from *S. islandicus* Δcmr-β, carrying one or more mutations in the HD domain of *cmr*-2α	This work
pSe-Rp	Contained a DNA fragment of two tandem copies of CRISPR repeat used for construct an artificial mini-CRISPR loci	Peng *et al.* (38)
pAC-lacS1	pSe-Rp carried a CRISPR locus with a spacer matching a protospacer in the *lacS* gene of *S. islandicus*	This work
pGE-lacS1	A genome-editing plasmid derived from pAC-lacS, carrying donor DNA lacking the target site of the plasmid-born CRISPR	This work
pGE-2α-His	A genome editing plasmid, carrying an artificial mini-CRISPR locus with a spacer derived from the stop codon region of *cmr*-2α and a donor DNA containing the coding sequence of the tandem 6 Hisditine residues before the stop codon of the target gene	This work
pGE-2αHD	A genome editing plasmid, carrying an artificial mini-CRISPR with a spacer derived from the coding sequence of the HD domain and a donor DNA for HD domain harboring multiple mutations in the HD domain of the *cmr*-2α gene	This work

Table 2. Oligonucleotides used in this work

Oligonucleotide	Sequence (5′–3′)
LacS-E-SpF	AAAGAGTGTAGTAATTAACACCAATCCAGTCTAACCTACCCCTT
LacS-E-SpR	TAGCAAGGGGTAGGTTAGACTGGATTGGTGTTAATTACTACACT
LacS-E-SOEF	GGGAGGGAGAAGGTTGTGAGAGATGATTTACTGTAGTTAAGAAGACCGAAAAGGGATA
LacS-E-SOER	TATCCCTTTTCGGTCTTCTTAACTACAGTAAATCATCTCTCACAACCTTCTCCCTCCC
LacS-E-SalIF	ACGCGTCGACTAAACATGTACCATTGGCCC
LacS-E-NotIR	ATAAGAATGCGGCCGCCCTTAATGGTCTTATAGGTG
F1	AACTGGCGGTACATAGTGGTA
R1	GGGTAGAAGTGTGTATGAG
F2	GTACAACATTATTCAAGCTC
R2	TTTGATAATCTGCATCATCC
LacS-Seq-F	GTGGCTAAATTAACCGATAC
LacS-Seq-R	GGCATACTATAAGAGGCAAGG
2α-His-SpF	AAAGAATACATGTTTGCTCACCTTAAGTAAGATACT
2α-His-SpR	TAGCAGTATCTTACTTAAGGTGAGCAAACATGTATT
2α-His-SOEF	ATCTTACTTAAGGCATCATCACCATCACCATTGAGCAAACATGTATTTGCTAATAA
2α-His-SOER	ATGGTGATGATGCCTTAAGTAAGATACTGCGTAAATTATACTAAGGAACGTTTCTT
2α-His-SalIF	ACGCGTCGACGGATTTAAGTCATATGCAG
2α-His-NotIR	ATAAGAATGCGGCCGCCTTTTTGGTTTCCCCATTCTAC
F3	GTAAAGTGTAAAGAAGGAAC
R3	ATGGTGATGGTGATGATG
2α-HDmut-SpF	AAAGCGACCCTCCTTGGAAGGCATGGGTAATTACAAGGAATATT
2α-HDmut-SpR	TAGCAATATTCCTTGTAATTACCCATGCCTTCCAAGGAGGGTCG
2α-HDmut-SOEF	CAACCCTCCTTGGGCCGCATGGGTAGCAACAAGGAATATTAGGGAAGGTCAC
2α-HDmut-SOER	CCCATGCGGCCCAAGGAGGGTTGTTAAAATAGGCTATTATTTTCTTATTTAAG
2α-HDmut-SalIF	ACGCGTCGACTAAGGAAAAAGCGATGAGAC
2α-HDmut-NotIR	ATAAGAATGCGGCCGCGTTCCTTCTTTACACTTTAC
2α-Seq-F	CCCAATTATTACAATCCTTC
2α-Seq-R	CACTTGAATACTACCGAACC

1. Restriction sites are underlined whereas 4 nt protruding ends of spacer fragments after primer annealing are shown in bold face.
2. Point mutations in HD domain sequence of *cmr*-2α are highlighted in red.

were transferred onto a nylon membrane using the Semi-Dry Electrophoretic Transfer Cell system (Bio-Rad; Hercules, CA, USA). The membrane was incubated with a hybridization buffer containing an antiserum against His-tag peptide (GenScript, Piscataway, NJ, USA) during which the antiserum bound to His-tagged Cmr-2α protein. The His-tag antiserum was then recognized by a secondary antibody (Goat Anti-Mouse IgG, GenScript) and the protein bands were visualized by chemiluminescent detection using the clarity Western ECL substrate (Bio-Rad; Hercules, CA, USA) and recorded using the MFChemibis 3.2 imaging device (DNR; Jerusalem, Israel).

Co-purification

Cell lysate was prepared from a large culture of *S. islandicus* Cmr-2α-His strain and centrifuged for 30 min at 12 000 rpm. The recombinant protein in the supernatant was purified by Nickel-chelate affinity chromatography (45). Briefly, 500 μl Ni-NTA-agarose (Qiagen, Hilden, Germany) was added to the supernatant and incubated at 4°C by rotating end-over-end for 30 min. Agarose beads were then washed with 10 ml Wash Buffer 1 (50 mM phosphate buffer, pH7.4, 500 mM NaCl, 20 mM Imidizole) and 10 ml Wash Buffer 2 (50 mM phosphate buffer, pH 7.4, 500 mM NaCl, 60 mM Imidizole). Recombinant protein was eluted using the Elution Buffer (50 mM phosphate buffer, pH 7.4, 500 mM NaCl, 200 mM Imidizole).

RESULTS AND DISCUSSION

Strategy for CRISPR-based genome editing in *S. islandicus*

The rationale for repurposing Type I-A and III-B CRISPR-Cas systems for genome editing in *S. islandicus* is to enforce self targeting guided by the crRNA produced from a plasmid-borne CRISPR array, which will selectively kill the wild-type cells by targeting the corresponding protospacer for DNA destruction and selectively retain mutant cells because the mutant chromosome contains a non-target sequence at the corresponding position and is therefore devoid of the self targeting.

The first step of the experiment is to identify a protospacer on the target gene which will be targeted by the CRISPR-Cas immune systems once the corresponding crRNA is provided. For Type I and II CRISPR-Cas systems, a protospacer only serves as a target for DNA destruction when it is immediately flanked by a PAM sequence at a proper orientation, which can either be predicted from bioinformatic analysis (8,39), or experimentally demonstrated (46). Figure 1A illustrates an example of such a target sequence of *Sulfolobus* Type I-A systems, including a protospacer plus a 5′-flanking CCN-PAM motif on the template strand of the *S. islandicus lacS* gene. For Type III CRISPR-Cas systems, their DNA interference is PAM-independent but mismatches between the 5′-repeat handle (5–8 nt sequence) and the sequence at its corresponding position on the DNA target trigger the immunity and the DNA targeting requires transcription at the target site. Therefore, the protospacer of the *lacS* gene shown in Figure 1A also functions as a target for the Cmr-α system. It is important to point out that while DNA targeting by Type I systems can occur on each of the DNA strands, DNA interference by Type III systems only targets the template strand for DNA destruction.

Next, a non-target sequence is to be designed and used to generate a mutant gene allele with which deletion, insertion or gene mutagenesis can be constructed (Figure 1B). Here, it is important to design a donor DNA that does not exhibit any DNA interference activity. This is particularly relevant to *Sulfolobus* CRISPR-Cas systems since they exhibit a great tolerance to mismatches between crRNA and its protospacer (32,34).

Then, a plasmid is to be constructed carrying a mini-CRISPR array with a spacer designed based on the protospacer of the target gene. Once introduced into *Sulfolobus* cells by electroporation, crRNAs expressed from the plasmid will guide the endogenous CRISPR system to initiate DNA interference to the protospacer, and the self-targeting activity would kill cells carrying the wild-type gene, whereas cells that have gained a non-target allele of the gene by recombination will grow and form colonies (Figure 1C).

Proof-of-concept experiment of CRISPR-based genome-editing method with the *lacS* gene

The *lacS* gene of *S. islandicus* was chosen for developing the method since it encodes a β-glycosidase that can be assayed using X-gal; the wild-type enzyme converts the chemical into a strong blue substance (designated as $LacS^+$ phenotype) whereas its mutants fail to do so ($LacS^-$ phenotype). A spacer was designed according to the protospacer shown in Figure 1A, and pAC-lacS, an artificial mini-CRISPR plasmid, was constructed carrying a CRISPR array of a single spacer that could produce the crRNA matching the *lacS* protospacer (Figure 1A). Both pAC-lacS1 and the reference plasmid pSe-Rp were introduced into *S. islandicus* E233 by electroporation. The two plasmids gave ca. 10^4 fold difference in transformation rate (Table 3), suggesting that the CRISPR plasmid mediated cell killing, and this is consistent with the CRISPR-mediated cell killing observed in several bacteria (18–24).

Next, we tested whether genome editing could be done by facilitating homologous recombination with donor DNAs in analogy to genome editing by the well-developed CRISPR-Cas9 technology (13–15,47). Two different donor DNA were prepared including a 58-nt oligonucleotide named LacS-E-SOEF (Table 2) and a double-stranded DNA segment of 985 bp generated with the splicing overlap-extension PCR (SOE-PCR) (44) using the primers listed in Table 2, both of which lacked the target site shown in Figure 2A. We reasoned that mutant *lacS* alleles derived from the recombination between each donor DNA and the wild-type gene would not be targeted by the DNA interference by the CRISPR plasmid.

Each donor DNA was introduced into the archaeon by transformation together with pAC-lacS (co-transformation). The number of colonies obtained from each transformation was comparable to that obtained with transformation of pAC-lacS alone (Table 3). This suggested that these colonies could represent escape mutants in which deletions of spacers in CRISPR arrays or mutations in *cas* genes inactivated DNA interference, as observed in previous invader experiments with *S. islandicus* and *S. solfataricus* (32,36,37). Indeed, X-gal screening revealed that all the colonies were $LacS^+$ and therefore still carried an active *lacS* gene. Since *S. islandicus* is proficient in homologous recombination as demonstrated by conventional genetic manipulations (27,43,48), the failure of recovering the designed mutant hinted that the rate of the CRISPR-mediated genome editing by co-transformation was much lower than the rate of escaping mutations.

To increase the rate of mutant generation in *S. islandicus* cells, a new plasmid vector pGE-lacS1 was constructed, carrying both the mini-CRISPR array and its cognate donor DNA fragment that are exemplified generally in Figure 1C.

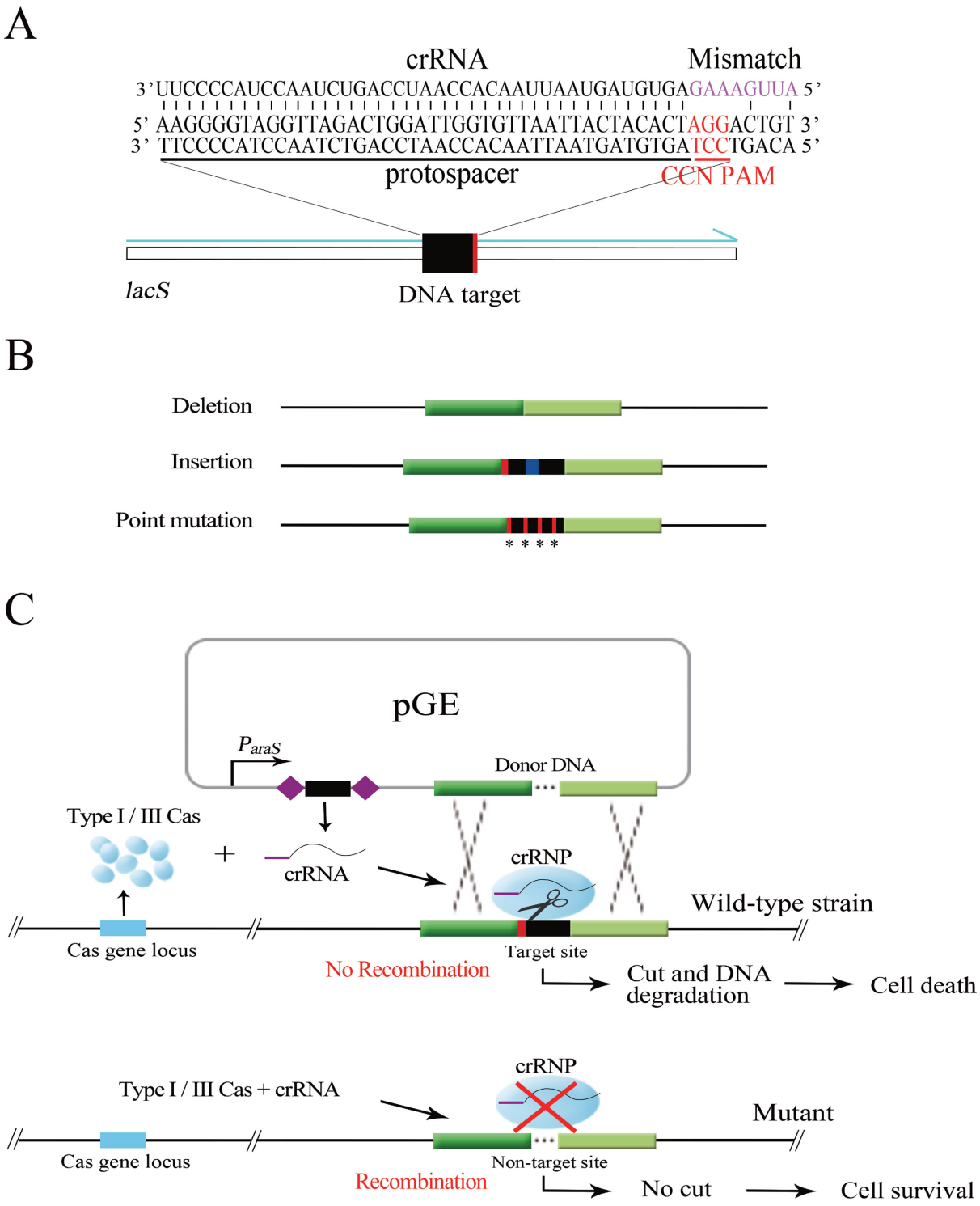

Figure 1. Schematic of the CRISPR-based genome editing in *S. islandicus*. (**A**) An example of a crRNA (top strand) and its corresponding DNA target designed for *lacS* gene editing with data presented in Figure 2. The target sequence is positioned from +933 to +972 relative to the ATG start codon (+1)) in the *lacS* gene, including the protospacer (underlined) and a CCT-PAM (protospacer adjacent motif, shown in red). The presence of the 5′-PAM ensures the Type I-A CRISPR-Cas mediated DNA interference while the mismatch between the 5′-repeat handle and the target sequence (labeled as 'Mismatch') induces DNA interference by the Type III-B Cmr-α system. (**B**) Donor DNAs contain a DNA segment homologous to that flanking the chromosomal target site but it is altered either by Deletion, or Insertion, or Point mutation, such that it is not to be targeted by the endogenous CRISPR systems. (**C**) Two alternative fates for *S. islandicus* cells transformed with a pGE plasmid. pGE carrying an artificial mini-CRISPR locus with a single spacer and a donor DNA fragment. The target site is composed of a protospacer and its adjacent sequence, which is to be recognized by a Type I or Type III-B DNA interference system. If recombination did not occur during transformation, the CRISPR DNA interference selectively targets the wild type gene for degradation, leading to cell death; if recombination yielded the mutant gene on the chromosome, the mutant cell is devoid of the CRISPR immunity, forming colonies on plates.

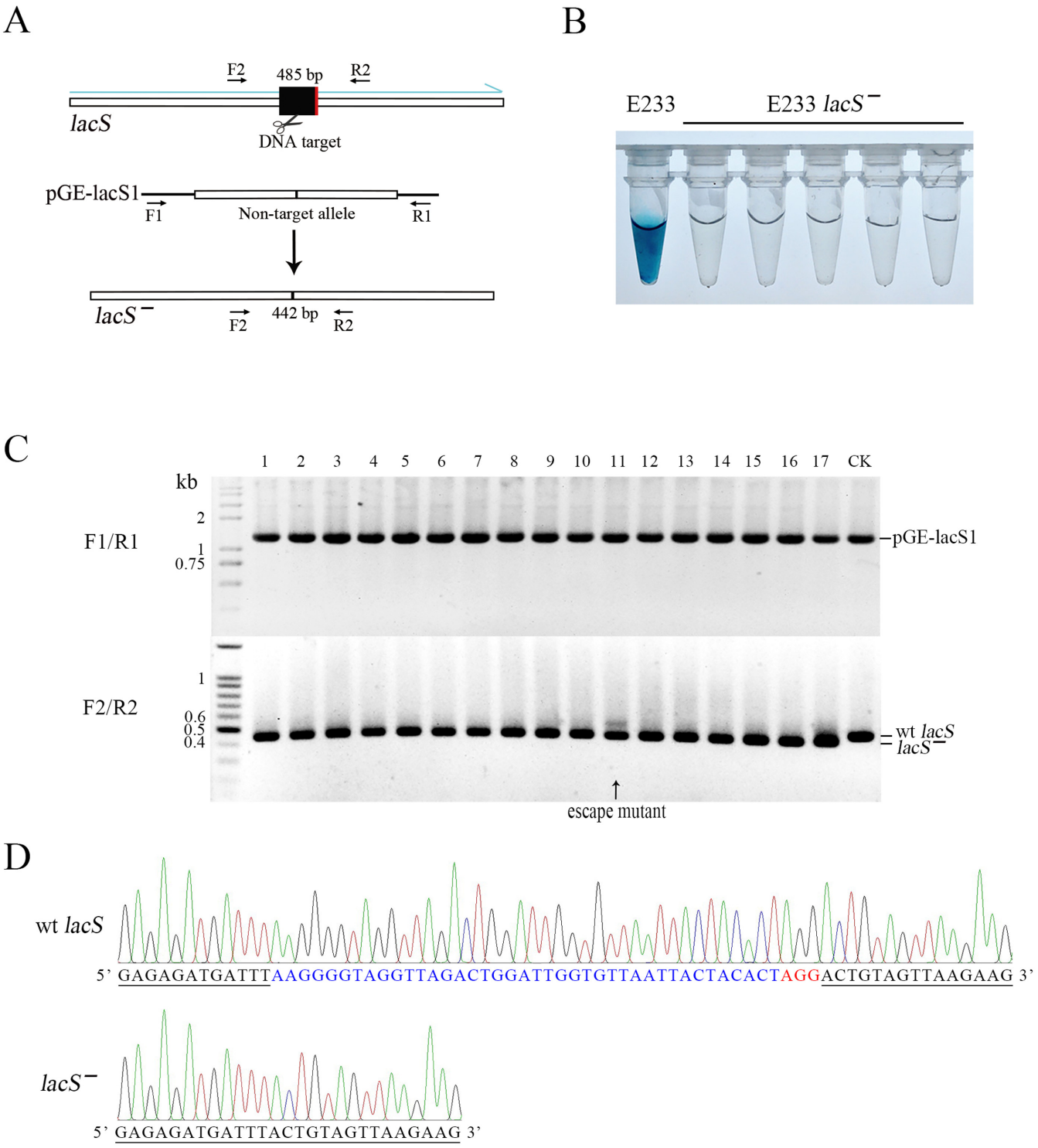

Figure 2. Generating deletion mutants of *lacS* gene by CRISPR-Cas systems in *S. islandicus*. (**A**) Schematic of the mutant identification by PCR. DNA target is detailed in Figure 1A, including a protospacer (underlined) and a CCT-PAM (protospacer adjacent motif, shown in red), and it serves as a target both for the Type I-A and III-B CRISPR-Cas systems. PCR with primers F1/R1 amplifies the donor DNA from pGE-lacS1 whereas PCR with primers F2/R2 yields PCR products both for the wild-type *lacS* gene (wt *lacS*) and for mutant *lacS* lacking the target site (*lacS* $^{-}$). (**B**) Screening of the enzymatic activity of *lacS* in *S. islandicus* strains using X-gal. X-gal was added into *S. islandicus* cultures and incubated at 78°C for 1 h. E233 – *S. islandicus pyrEF* mutant as the genetic host for genome editing. E233*lacS*$^{-}$– Mutants obtained from pGE-lacS1 transformation. (**C**) PCR screen of *lacS* deletion in *S. islandicus* strains. PCR with primers F1/R1 specific for pGE vector amplifies the non-target *lacS* allele (1283 bp) from the plasmid whereas PCR with primers F2/R2 yields 485-bp and 442-bp DNA fragments that are derived from the wild-type *lacS* and the deletion mutant allele, respectively. The double bands indicate an escape mutant (indicated with an arrow). (**D**) Representatives of the chromatographs of the sequencing results of the wild-type *lacS* and its mutant allele *lacS*$^{-}$. The protospacer and the CCT-PAM motif (on the opposite strand) that are highlighted in blue and red, respectively, are present in the wild-type *lacS* but absent from *lacS*$^{-}$.

Table 3. Transformation efficiencies of *S. islandicus* by genome-editing plasmids

Plasmid	Strains	Transformation efficiency [cfu(μg DNA)-1]
pSe-Rp	*S. islandicus* E233	2.6×10^5
pAC-lacS1	*S. islandicus* E233	37
pAC-lacS1 + LacS-E-SOEF	*S. islandicus* E233	14
pAC-lacS1 + 985-bp PCR DNA	*S. islandicus* E233	22
pGE-lacS1	*S. islandicus* E233	5.8×10^2
pGE-2α-His	*S. islandicus* Δcas3	3.1×10^3
pGE-2αHD	*S. islandicus* E233 Δcmr-β	4.3×10^3
pGE-4α$^{W197A\ E199A\ Y201A}$	*S. islandicus* E233 Δcmr-β	4.5×10^2
pGE-1α-del	*S. islandicus* E233 Δcmr-β	7.3×10^2
pGE-1α$^{W58A\ F59A}$	*S. islandicus* E233 Δcmr-β	1.9×10^3
pGE-6α$^{G219A\ G221A}$	*S. islandicus* E233 Δcmr-β	7.1×10^2
pGE-6α$^{L97A\ Y101A}$	*S. islandicus* E233 Δcmr-β	6.3×10^2

Transformation was carried out with each plasmid for at least two independent experiments, and the value represents the average of all transformations.

The plasmid was then introduced into *S. islandicus* E233 by electroporation. Hundreds of colonies were obtained, which is about 15-fold of the transformation rate with pAC-lacS (Table 3). Seventeen of them were picked up and grown in 5 ml SCVy broth. These cultures were incubated with X-gal to detect the enzymatic activity of *lacS* gene. As exemplified in Figure 2B, while the culture of the genetic host showed LacS+, all 5 tested pGE-lacS1 transformants exhibited LacS$^-$. Two sets of primers were then employed to check the plasmid-borne *lacS* fragment and the mutant *lacS* allele in these strains, respectively. As shown in Figure 2C, PCR with F1/R1 primers verified that they all carried pGE-lacS1 whereas PCR with F2/R2 produced a smaller PCR product in all 17 strains, representing the designed *lacS* mutant of the predicted size of 442 bp. Furthermore, a slightly larger PCR product was present in Strain 11 (indicated by an arrow) but absent from all other tested strains, this PCR product is very similar in size to the predicted PCR product of the wild-type gene (485 bp) (Figure 2C). Next, PCR products of the entire *lacS* gene were generated using LacS-Seq-F/LacS-Seq-R primers (Table 2) and sequenced by DNA sequencing. Therefore, only Strain 11 carried the wild-type *lacS* gene while all remaining16 strains harbored the designed *lacS* mutant. Together these results indicated that pGE-lacS1 mediated accurate genome editing in *S. islandicus* at a high efficiency.

Although there are a few reports on CRISPR-mediated genome editing for bacteria in the current literature, only Type II CRISPR-Cas9 systems have been employed for the purpose. In their pioneer research of conducting bacterial genome editing, Jiang *et al*. (2013) employed two different plasmids for genome editing in *E. coli*, one carried the *cas9* and the *tracrRNA* gene of the *Streptococcus pyogenes*, while the other plasmid carry an artificial CRISPR array (21). The CRISPR-Cas system was introduced into the bacterium in the first transformation while the CRISPR array and donor DNA were co-electroporated into the *cas9*-carrying *E. coli* at the second transformation. However, the co-transformation that worked in *E. coli* genome editing failed in *S. islandicus*, which probably reflects that *E. coli* can be transformed by electroporation at a much higher rate ($>10^3$-fold) than *Sulfolobus*. This is also true for *Lactobacillus reuteri* in which a *recT* gene has to be included in order to facilitate the recombination process based on a single stranded donor DNA (49). We constructed genome-editing plasmids carrying both a CRISPR array and a donor DNA fragment and found that they greatly elevates genome-editing efficiencies in *S. islandicus.* Furthermore, successful genome editing was recently reported for using a single plasmid carrying the *cas9* gene, the sgRNA module and homology regions (50–52). Therefore, the strategy of using a single plasmid for genome editing based on endogenous CRISPR-Cas systems should be generally applicable to many other bacteria and archaea as demonstrated in *Sulfolobus*.

When the CRISPR-based genome editing method developed here is compared with the routine genetic manipulation recently developed for *S. islandicus* (26,27), the former method exhibits several advantages over the latter. First, it takes only up to 3 weeks to generate gene mutants by using the CRISPR-Cas genome editing whereas employing the plasmid integration and targeting deletion previously developed in our laboratory requires >6 weeks to accomplish the same task. This is because growing colonies of pGE-lacS1 transformants in the selective broth yields pure cultures of the desired mutant whereas for a routine *Sulfolobus* genetic manipulation, strains need to be purified each time after colonization on plate. Second, preparation of plasmids for CRISPR-Cas genome editing requires a two-step cloning, comparing with three relatively large fragments to be cloned in the routine genetic manipulation. Finally, whereas pGE plasmids are replicative and will be maintained during growth, knockout plasmids are linearized suicide plasmids to be lost during incubation, and this accounts for a much higher transformation rate (>100-fold) with pGE plasmids comparing with that of gene knockout plasmids. Taken together, CRISPR-Cas genome editing developed here has greatly simplified the procedure of genetic manipulation in *S. islandicus*.

In situ gene tagging of *cmr*-2α gene by repurposing a Type III-B system

Next, we employed this approach for generating *S. islandicus* strains to produce an *in situ* tagged protein. We chose to work on Cmr-2α, a Cas10 homolog of the *S. islandicus* Cmr-α system. Cas10 is the type-specific Cas protein for Type III systems (3), which is implicated in DNA interfer-

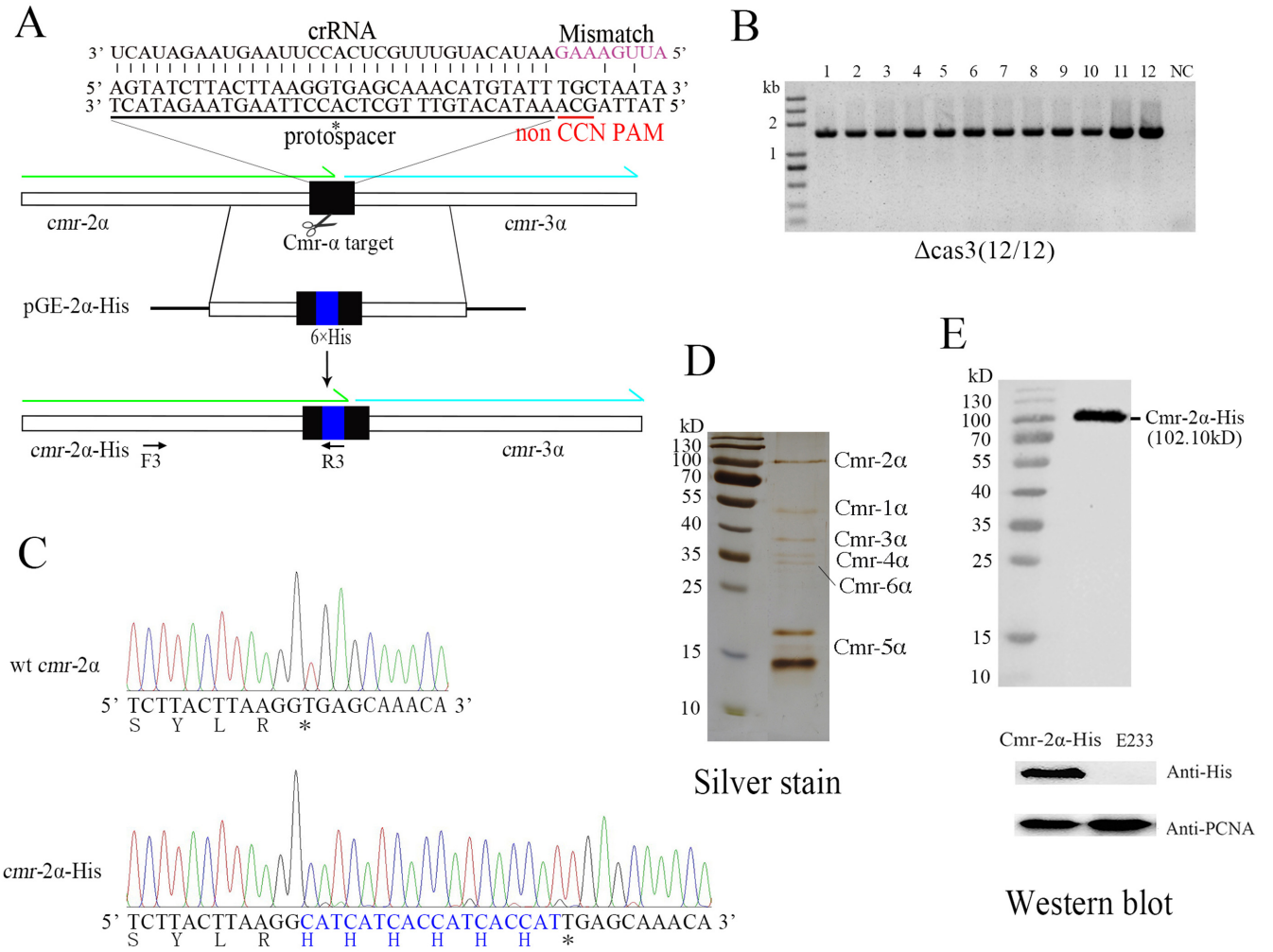

Figure 3. *In situ* gene tagging of *cmr-2α*. (**A**) Schematic of *in situ* gene tagging strategy. The protospacer is underlined in which the TGA stop codon of *cmr*-2α is indicated with an asterisk. Mismatches between the 3-flanking DNA stretch of the protospacer and the 5′ repeat handle of the crRNA facilitate DNA interference to the wild-type cells by Type III-B Cmr-α as experimentally demonstrated previously (36). Insertion of 6x His codons in the middle of the protospacer yields a non-target *cmr*-2α gene. Therefore, cells that carry His-tagged *cmr*-2α gene are devoid of the DNA interference and selectively retained. (**B**) PCR screening of *cmr*-2α-His recombinants generated with *S. islandicus* Δcas3. Primers F3 is specific for genome DNA while R3 contains the 18 nt encoding the 6xHis tag. All 12 tested strains carry the *in situ* tagged gene. (**C**) Representative chromatographs of the sequencing results of the wild-type *cmr*-2α and *cmr*-2α-His recombinants. Precise insertion of His-tag coding sequence (highlighted in blue) in front of the stop codon was observed in all analyzed strains. (**D**) Co-purification of the Cmr-α complex by nickel affinity chromatography purification of His-tagged Cmr-2α. Proteins were stained with Silver staining. (**E**) Western analysis of His-tagged Cmr-2α using antibody specifically recognizes the His-tag peptide. A peptide of the predicted size is hybridized. The DNA replication protein PCNA3 was used as a reference.

ence (53–56). Here, we tested whether Type III Cmr-α DNA interference could be repurposed for the *in situ* gene tagging for the *cmr*-2α gene in this archaeon. A spacer was designed containing the last 19-bp coding sequence of *cmr*-2α, 6-bp intergenic sequence and the first 7-bp of *cmr*-3α, which yields a 6xHis-tagged version of Cmr-2α at the C-terminus. The spacer DNA fragment was generated by annealing of the two oligonucleotides listed in Table 2 and cloned to pSe-Rp, giving pAC-2α-His. Then, a donor DNA (1223 bp) generated by SOE-PCR using oligonucleotides listed in Table 2 was cloned to pAC-2α-His, yielding the genome-editing plasmid pGE-2α-His. To ensure that genome editing is solely based on the Type III-B system, transformation with pGE-2α-His was conducted with *S. islandicus* Δcas3, which is devoid of the Type I-A DNA interference. Thousands of colonies were obtained (Table 3), indicating that the Cmr-α system is very efficient in mediating genome editing.

To reveal the genotype of transformants, their genomic DNAs were used as template for PCR analysis using F3 and R3 (Table 2) to amplify a DNA fragment encompassing the edited genomic region. Twelve colonies were tested and they all yielded a PCR product of the predicted size, which is absent from the original host (Figure 3B), suggesting that all 12 tested strains carried the His-tagged version of *cmr*-2α gene. The authenticity of the His-tagged gene was confirmed for all 12 strains by amplifying a PCR fragment of *cmr*-2α using primers of 2α-Seq-F and 2α-Seq-R (Table 2) for DNA sequencing (Figure 3C). Furthermore, the tagged Cmr-2α protein was purified via nickel affin-

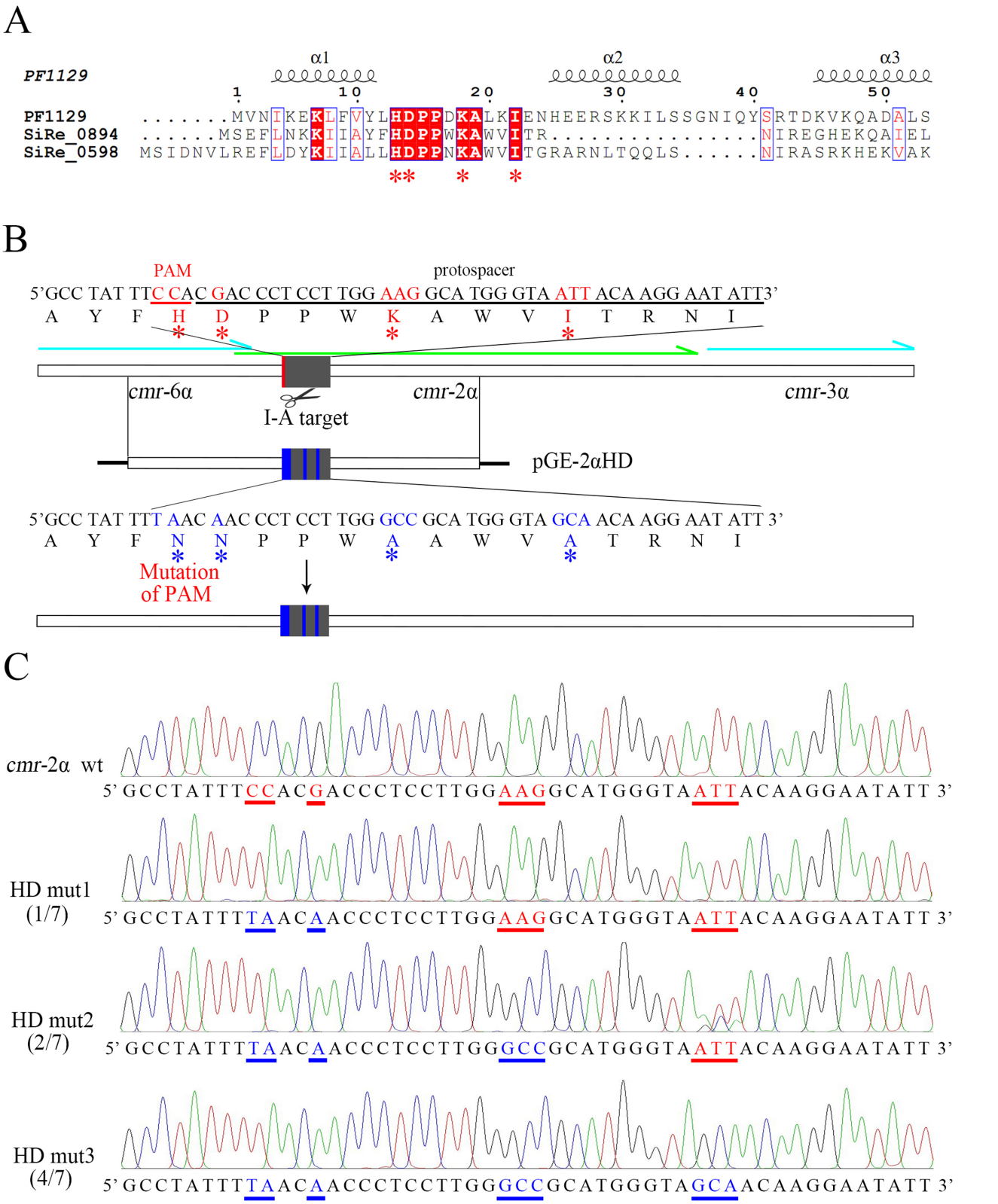

Figure 4. Mutagenesis of *cmr*-2α HD domain. (**A**) A sequence alignment of the N-termini of *S. islandicus* Cmr-2α (SiRe-0894), Cmr-2β (SiRe-0598) and its *Pyrococcus furiosus* homolog PF1129. Four conserved amino acids were chosen for constructing substitution mutations (indicated with red asterisks). (**B**) Schematic of the mutagenic strategy. The I-A target site is underlined and bases highlighted in red are to be mutated whereas bases highlighted in blue represent mutated bases present in DNA donor. The donor DNA containing mutations on the PAM and/or the seed sequence that inactivate the Type I-A DNA interference activity is provided for recombination. (**C**) Chromatographs of the sequencing results for the three types of *cmr*-2α mutant. Sequencing of seven transformants yielded three different types of mutant, carrying either HD mutation, or HD+K mutation or all four designed mutations.

ity purification, which led to the co-purification of a protein complex containing all Cmr-α proteins of the predicted sizes (Figure 3D). In fact, this complex resembles an active Cmr-α complex that was purified by Cmr-6 tagged purification (unpublished data). A few additional proteins of small sizes were co-purified in the Cmr-2α purification (Figure 3D), and these proteins were also present in the Cmr-6α co-purification. But these small proteins are not part of the Cmr-α complex since they have subsequently been removed by gel filtration (data not shown). Furthermore, western blot analysis using a commercial antiserum that specifically recognizes the His tag peptide revealed that a protein band of ca. 100 kDa, which is consistent with the predicted size of *S. islandicus* Cmr-2α (102.1 kDa) (Figure 3E). Together, these results suggest that protein co-purification using *in situ* tagged gene represents a useful method in studying protein-protein interaction *in vivo*.

Generation of substitution mutations by repurposing Type I-A DNA interference

Then, we attempted to construct genes containing point mutations to replace site-directed mutagenesis *in vitro*. Again, the *S. islandicus cmr*-2α gene was used to test *in vivo* mutagenesis of multiple sites in order to identify the active site of DNA cleavage in the Cmr complex. All conserved Cas10 motifs in Cmr-2α were subjected to mutagenesis, and here we started with the HD domain in the N-terminus. Inspection of the coding sequence revealed a CCA-PAM motif overlapping with the HD (histidine and asparatic acid) codons. Thus, the downstream 40 nt was regarded as a protospacer (Figure 4A) with which two oligonucleotides were designed to generate the corresponding spacer DNA fragment that was cloned to pSeRp vector, giving pAC-Sp-cmr-2HD1. Then, a DNA fragment carrying four amino acid substitutions in the HD domain including H14N, D15N, K19A and I23A was obtained by SOE-PCR and cloned into pAC-Sp-cmr-2HD1, yielding pGE-2αHD (Figure 4B). To avoid any possible influence of Cmr-β on the function of cmr-2α, a *S. islandicus* Δcmr-β constructed previously (38) was employed. The genome-editing plasmid was introduced into the host by electroporation, yielding a transformation rate comparable to the above experiments. Analysis of Cmr-2α gene in seven transformants revealed that three different types of *cmr*-2α mutants, among which four of them showed all four mutations, one carried the HD mutation along with K19A whereas the last two only carried the HD mutation (Figure 4C). Notably, recombination occurred for the two sites separated only by 11 bp, suggesting that the persistence of several copies of donor DNA on the plasmid vector (due to the nature of multiple copies of the plasmid (43)) renders it possible to recover a low frequency of homologous recombination. These results also indicated that multiple substitutions are obtainable from the same CRISPR-mediated genetic manipulation.

We did the same for a few other *cmr*-α genes including *cmr*-1α, -4α and -6α and the designed mutants were obtained readily in all experiments with a donor DNA carrying a mutation in its PAM motif (Table 3). However, for a few pGE plasmids in which the PAM is not changed in the donor DNA, transformation rate was seriously impaired, and mutants were only obtained from transformation with some pGE plasmids (unpublished data), suggesting that these donor DNAs was still targeted by the CRISPR immunity albeit at a lesser extent. These results are consistent with the previous finding that DNA targeting by *Sulfolobus* Type I-A tolerate multiple mismatches (32,34). Therefore, it is very important to mutate the PAM motif on any I-A DNA target when generating non-target DNA for gene mutagenesis.

In summary, we show that *S. islandicus* endogenous CRISPR-Cas systems can be repurposed for genetic manipulation including deletion, insertion and site-specific mutagenesis (Figure 1B) and the developed procedures are more straightforward than the classic genetic manipulations. Furthermore, we have successfully conducted genome editing in another *Sulfolobus* strain, i.e. *S. islandicus* HVE10/4 by repurposing its endogenous Type I-A CRISPR-Cas system (unpublished data), which has widely been used as a host for virus study (41) but is constantly refrained from any routine genetic manipulations since electroporation can only yield a low level of transformation to this archaeon. We envision that the CRISPR-Cas genome editing strategy reported here is generally applicable to all archaea and bacteria that encode an active CRISPR-Cas system, representing a simpler CRISPR-based genome editing tool than the existing CRISPR-Cas9 technology for genome editing. In particular, since Type II CRISPR-Cas systems are limited to a few taxa of mesophilic bacteria, their activity needs to be tested vigorously and even improved by mutagenesis before applied in other physiological groups of organisms including thermophilic bacteria and archaea. By contrast, any active endogenous CRISPR-Cas system can readily be applied in genome editing although application of a Type I or Type II CRISPR-Cas system will require to obtain the knowledge of its PAM sequence by bioinformatic analysis as demonstrated previously (8,39).

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