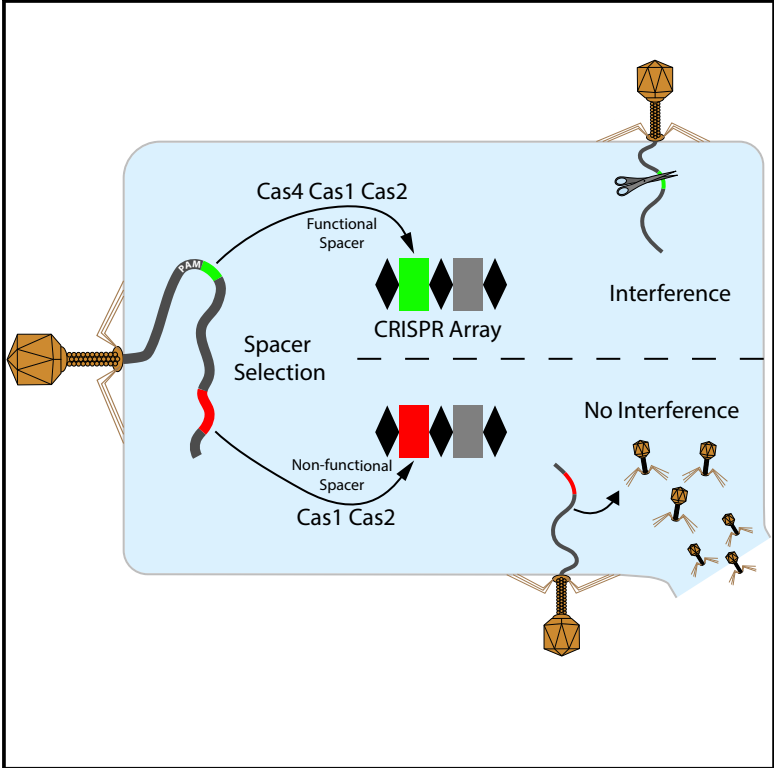


## Cas4 Facilitates PAM-Compatible Spacer Selection during CRISPR Adaptation

### Graphical Abstract



### Authors

Sebastian N. Kieper,  
 Cristóbal Almendros, Juliane Behler, ...,  
 Jochem N.A. Vink, Wolfgang R. Hess,  
 Stan J.J. Brouns

### Correspondence

stanbrouns@gmail.com

### In Brief

Kieper et al. demonstrate that the ubiquitous protein Cas4 assists Cas1 and Cas2 in the selection of new CRISPR spacers with a PAM licensing efficient CRISPR interference.

### Highlights

- Cas4 facilitates the integration of PAM-compatible spacers
- Spacer length variation is dictated by Cas1-2
- Cas4 shortens spacer length
- Cas4-selected PAMs license type I-D CRISPR interference



# Cas4 Facilitates PAM-Compatible Spacer Selection during CRISPR Adaptation

Sebastian N. Kieper,<sup>1,5</sup> Cristóbal Almendros,<sup>1,5</sup> Juliane Behler,<sup>2</sup> Rebecca E. McKenzie,<sup>1</sup> Franklin L. Nobrega,<sup>1</sup> Anna C. Haagsma,<sup>1</sup> Jochem N.A. Vink,<sup>1</sup> Wolfgang R. Hess,<sup>2,3</sup> and Stan J.J. Brouns<sup>1,4,6,\*</sup>

<sup>1</sup>Kavli Institute of Nanoscience, Department of Bionanoscience, Delft University of Technology, Van der Maasweg 9, 2629 HZ Delft, the Netherlands

<sup>2</sup>Genetics and Experimental Bioinformatics, Faculty of Biology, University of Freiburg, Schänzlestraße 1, 79104 Freiburg, Germany

<sup>3</sup>Freiburg Institute for Advanced Studies, University of Freiburg, Albertstr. 19, 79104 Freiburg, Germany

<sup>4</sup>Laboratory of Microbiology, Wageningen University, Stippeneng 4, 6708 WE Wageningen, the Netherlands

<sup>5</sup>These authors contributed equally

<sup>6</sup>Lead Contact

\*Correspondence: [stanbrouns@gmail.com](mailto:stanbrouns@gmail.com)

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## SUMMARY

CRISPR-Cas systems adapt their immunological memory against their invaders by integrating short DNA fragments into clustered regularly interspaced short palindromic repeat (CRISPR) loci. While Cas1 and Cas2 make up the core machinery of the CRISPR integration process, various class I and II CRISPR-Cas systems encode Cas4 proteins for which the role is unknown. Here, we introduced the CRISPR adaptation genes *cas1*, *cas2*, and *cas4* from the type I-D CRISPR-Cas system of *Synechocystis* sp. 6803 into *Escherichia coli* and observed that *cas4* is strictly required for the selection of targets with protospacer adjacent motifs (PAMs) conferring I-D CRISPR interference in the native host *Synechocystis*. We propose a model in which Cas4 assists the CRISPR adaptation complex Cas1-2 by providing DNA substrates tailored for the correct PAM. Introducing functional spacers that target DNA sequences with the correct PAM is key to successful CRISPR interference, providing a better chance of surviving infection by mobile genetic elements.

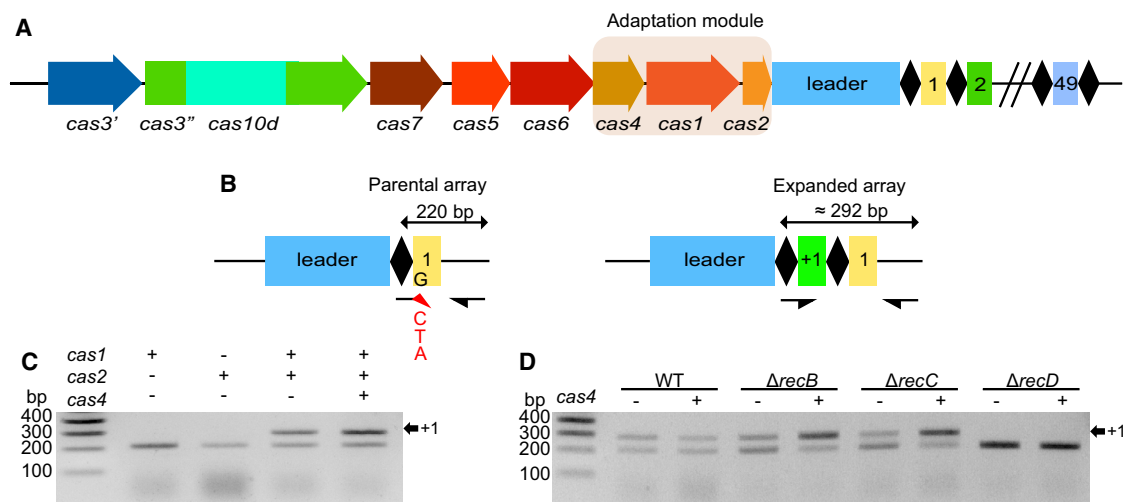
## INTRODUCTION

Microbes require updating their adaptive immune repertoire to keep up with ever-changing mobile genetic elements (MGEs) such as bacteriophages and conjugative plasmids. The prokaryotic CRISPR-Cas system is an adaptive immune system that uses clustered regularly interspaced short palindromic repeats (CRISPRs) and their associated proteins (Cas) (Jansen et al., 2002; Mojica et al., 2005; Barrangou et al., 2007). In a process termed CRISPR adaptation, microbes integrate short sequences from MGEs into their CRISPR array (Amitai and Sorek, 2016; Sternberg et al., 2016; Jackson et al., 2017). This array then becomes a source for small RNAs (i.e., CRISPR RNA [crRNA]) that guide Cas nuclease complexes to their target (van der Oost et al., 2014; Marraffini, 2015; Mohanraju et al., 2016).

CRISPR-Cas systems are grouped into two major classes that each hold several types and multiple subtypes, and remarkably, all contain *cas1* and *cas2* genes (Koonin et al., 2017). In the type I-E system of *Escherichia coli*, *cas1* and *cas2* are necessary and sufficient to mediate expansion of the CRISPR array (Yosef et al., 2012). However, next to *cas1* and *cas2*, a number of other *cas* genes in class I and II systems have been directly linked to the spacer integration process, suggesting that CRISPR adaptation across different systems has different requirements (Koonin et al., 2017). Type II-B (Cas9), type V (Cas12a), and most type I (I-A, I-B, I-C, I-D, and I-U) CRISPR-Cas systems contain *cas4* genes in conserved gene clusters with *cas1* and *cas2* genes, while in some systems, *cas4* is fused with *cas1* (I-B, I-U, and V-B) (Hudaiberdiev et al., 2017). Deletion of *cas4* from the I-A type abrogated CRISPR adaptation in a *Sulfolobus islandicus* strain overexpressing *csa3*, a regulator of *cas* gene expression (Liu et al., 2017), while deletion of *cas4* in type I-B revealed that *cas4* is essential for CRISPR adaptation against HHPV-2 in *Haloarcula hispanica* (Li et al., 2014). Additionally, interaction between the Cas1/2 fusion protein, Csa1, and Cas4 of the archaeal type I-A system was found *in vitro* (Plagens et al., 2012). These findings suggest a strong functional association of Cas4 and the Cas1 and Cas2 adaptation proteins. Despite the conservation of the *cas4* gene among these highly diverse CRISPR-Cas systems, a functional role for Cas4 has not been shown *in vivo*. Early biochemical studies have found different Cas4 proteins as monomers, dimers, and decamers and containing either [2Fe-2S] or [4Fe-4S] iron-sulfur clusters (Zhang et al., 2012; Lemak et al., 2013, 2014). Furthermore, Cas4 proteins were shown to be active nucleases with catalytic domains belonging to the PD-DEXK phosphodiesterase superfamily (Hudaiberdiev et al., 2017). It was suggested that the observed catalytic activities play a role in either the generation or the processing of spacer precursors; i.e., DNA substrates that are used by Cas1 and Cas2 to form spacers (Zhang et al., 2012; Lemak et al., 2013; Lemak et al., 2014). Recently, Rollie et al. showed *in vitro* that Cas4 cleaves 3' overhangs of prespacer substrates containing protospacer adjacent motifs (PAMs) (Rollie et al., 2018).

Obtaining new spacers that target an invading DNA sequence with a correct PAM is central to the success of CRISPR





**Figure 1. Genetic Requirements of Type I-D CRISPR Adaptation**

(A) Overview of the type I-D CRISPR-Cas locus as found on the *Synechocystis* 6803 pSYS A megaplasmid. The putative adaptation module consisting of *cas4-1-2* is highlighted in light brown. Downstream of *cas2* is the leader sequence in blue, followed by the I-D array consisting of 37-bp repeats interspaced by 49 spacers with a statistical mode length of 35 bp (Figure S2C).

(B) Degenerate primer PCR used for the detection of spacer acquisition (Heler et al., 2015). The 3' end of the forward primer mix mismatches the first 5' nucleotide of spacer 1 (indicated in red). Spacer integration restores complementarity allowing for efficient amplification. For more sensitive detection, the amplicon of expanded arrays was extracted and subjected to a second round of PCR (see Figure S1A).

(C) Co-expression of *cas1* and *cas2* is necessary and sufficient for the integration of new spacers.

(D) Assessing spacer integration in WT *E. coli* K12 and different *recBCD* mutant backgrounds in the presence or absence of *cas4*. The presence of *cas4* enhances spacer integration in the  $\Delta recB$  and  $\Delta recC$  genotypes, while spacer integration is below the detection limit of this PCR (described in B) in the  $\Delta recD$  mutant regardless of the presence of *cas4*.

adaptation. The PAM is a short sequence motif (Deveau et al., 2008; Mojica et al., 2009; Shah et al., 2013) that is required for crRNA-effector complexes such as Cascade, Cas9, and Cas12a to find their target DNA and avoid targeting host CRISPR arrays (Mohanraju et al., 2016). Only when a new spacer has been selected from a target adjacent to a PAM can CRISPR interference efficiently take place. In type II systems, Cas9 assists Cas1-2 to select PAM-compliant spacers (Heler et al., 2015), but it remains unknown what other factors also contribute to PAM selection.

Here, we have determined the biological role of Cas4 by employing *in vivo* spacer acquisition assays in a heterologous *E. coli* host. We show that the type I-D adaptation proteins Cas1 and Cas2 from the cyanobacterium *Synechocystis* sp. 6803 are necessary and sufficient to integrate spacers into the CRISPR array. However, providing *cas4* results in a significant enrichment of new spacers with PAM motifs that support CRISPR interference in the type I-D CRISPR-Cas system of the native host *Synechocystis*. Altogether, our results demonstrate that Cas4 enhances functional memory formation, which increases the chance of surviving infections by MGEs.

## RESULTS

### The Cas1-Cas2 Complex Integrates Spacers Independently of Cas4

To determine the minimal requirements for spacer acquisition in the type I-D system, we cloned *cas4*, *cas1*, and *cas2* genes from *Synechocystis* (Figure 1A) into T7-based expression vectors.

A minimal CRISPR array with one repeat was obtained by cloning the full-leader sequence followed by the first repeat and the leader proximal spacer (Sp1) of the type I-D system into the pACYC-Duet1 vector system. The resulting plasmids were co-transformed into a wild-type (WT) *E. coli* K12 strain (BW25113) devoid of T7 RNA polymerase. This setup ensured constitutive and low expression levels of the adaptation genes from *Synechocystis*. We first tested the ability of the cells to integrate new spacers into the minimized CRISPR array either in the presence or absence of the *cas4-1-2* genes. With a sensitive PCR approach (Figure 1B), spacer acquisition was readily detectable in the presence of *cas1-2* regardless of the presence of the *cas4* gene (Figure 1C). Further, deletion of either *cas1* or *cas2* abolished spacer integration, indicating that the combination of *cas1* and *cas2* is necessary and sufficient to mediate the integration of new spacers (Figure 1C). The detection of expanded CRISPR arrays in *E. coli* K12 demonstrates that spacers were acquired even though *E. coli* is not the natural host of the type I-D system. Consequently, the type I-D adaptation module does not rely on any specific host factors only present in *Synechocystis*.

### Cas4 Enhances Spacer Acquisition in the Absence of the RecBCD Complex

Next, we were interested in knowing whether the observed integration was dependent on the presence of host factors in our heterologous expression system. Since the *E. coli* strain used is not the natural host of the type I-D system, CRISPR adaptation by I-D Cas1-2 does not rely on cyanobacterial factors that are

only present in *Synechocystis*. For the type I-E system of *E. coli*, a role for the RecBCD complex has been proposed in generating spacer precursors during double-stranded DNA break repair at stalled replication forks (Ivančić-Baće et al., 2015; Levy et al., 2015). The *Synechocystis* genome contains cyanobacterial orthologs of *E. coli* RecB and RecD, but RecC appears to be absent (Cassier-Chauvat et al., 2016). Hence, we sought to assess spacer integration in *E. coli*  $\Delta recB$ ,  $\Delta recC$ , and  $\Delta recD$  mutant backgrounds from the KEIO collection (Baba et al., 2006). While we observed no difference in spacer acquisition frequencies for pCas1-2 in *recB* and *recC* deletion mutants, integration of spacers in the *recD* mutant was greatly reduced (Figure 1D) but could still be detected with the sensitive spacer detection approach (Figure S1B). Interestingly, when we supplied *cas4* in the *recB* and *recC* deletion backgrounds, we observed a relative increase of array expansion (Figure 1D). The results demonstrate that Cas1-2 is the core requirement for type I-D adaptation as has been found for type I-E (Nuñez et al., 2014; Yosef et al., 2012). The presence of Cas4 seems to facilitate uptake of spacers in the absence of RecB or RecC, which is consistent with competing pathways for the generation of spacer precursors.

### Cas4 Influences Spacer Length

To understand the nature and origin of newly acquired spacers in the presence or absence of *cas4*, we subjected amplicons of expanded I-D arrays to next-generation sequencing. Analysis of novel spacers in the absence of *cas4* revealed that spacers of 36 bp length were incorporated most frequently (Figure 2A). This length deviates by one nucleotide from the spacer length found in the native CRISPR array of *Synechocystis* in which the statistical mode of spacer length is 35 bp (Figure S2C). Interestingly, when *cas4* was supplied to the system, the mode of spacer length was restored to 35 bp. To assess if Cas4 activity was responsible for the change in spacer length, we created an active site mutant in the RecB-domain by substituting a divalent metal-ion binding aspartic acid for alanine (i.e., D76 corresponding to D99 in Sso0001) (Zhang et al., 2012). When this mutant was introduced in strains containing pCas1-2, the same spacer length mode was observed as when *cas4* was absent, showing that the catalytic activity of Cas4 influences spacer length. Furthermore, it suggests that Cas4 is involved in processing spacer precursors (i.e., pre-spacers) before they are integrated into the CRISPR array.

### New Spacers Are Mostly Genome Derived

Next, we mapped the unique spacer sequences to the *E. coli* BW25113 genome as well as to the plasmids harbored by the cells. Approximately 60% of the spacers that were acquired in the absence of the *cas4* mapped to the genome (Figure 2B). We observed increased numbers of spacers targeting the *lacI* gene, which is present both on the plasmid and the genome. Spacers were also preferentially acquired from the chromosomal replication terminus *terC* (Figure S3). The enrichment of spacers at the replication terminus is similar to what has been observed previously for type I-E (Levy et al., 2015) and suggests that the I-D Cas1-2 adaptation complex can use DNA degradation products from RecBCD as substrates for new spacers. When we supplied WT or mutant *cas4*, spacer acquisition from the genome further

increased to 85% and 90%, respectively. However, the preferential uptake of spacers from *terC* was lost (Figure S3). We observed no orientation bias of the newly integrated spacers for either strand of the genome (Table S3). Although *E. coli* is not the native host of the I-D CRISPR system, the results are consistent with the notion that the adaptation proteins of I-D use pre-spacer substrates from abundant DNA sources in the cell (in this case, the genome).

### Cas4 Facilitates Selection of Spacers with a Specific PAM

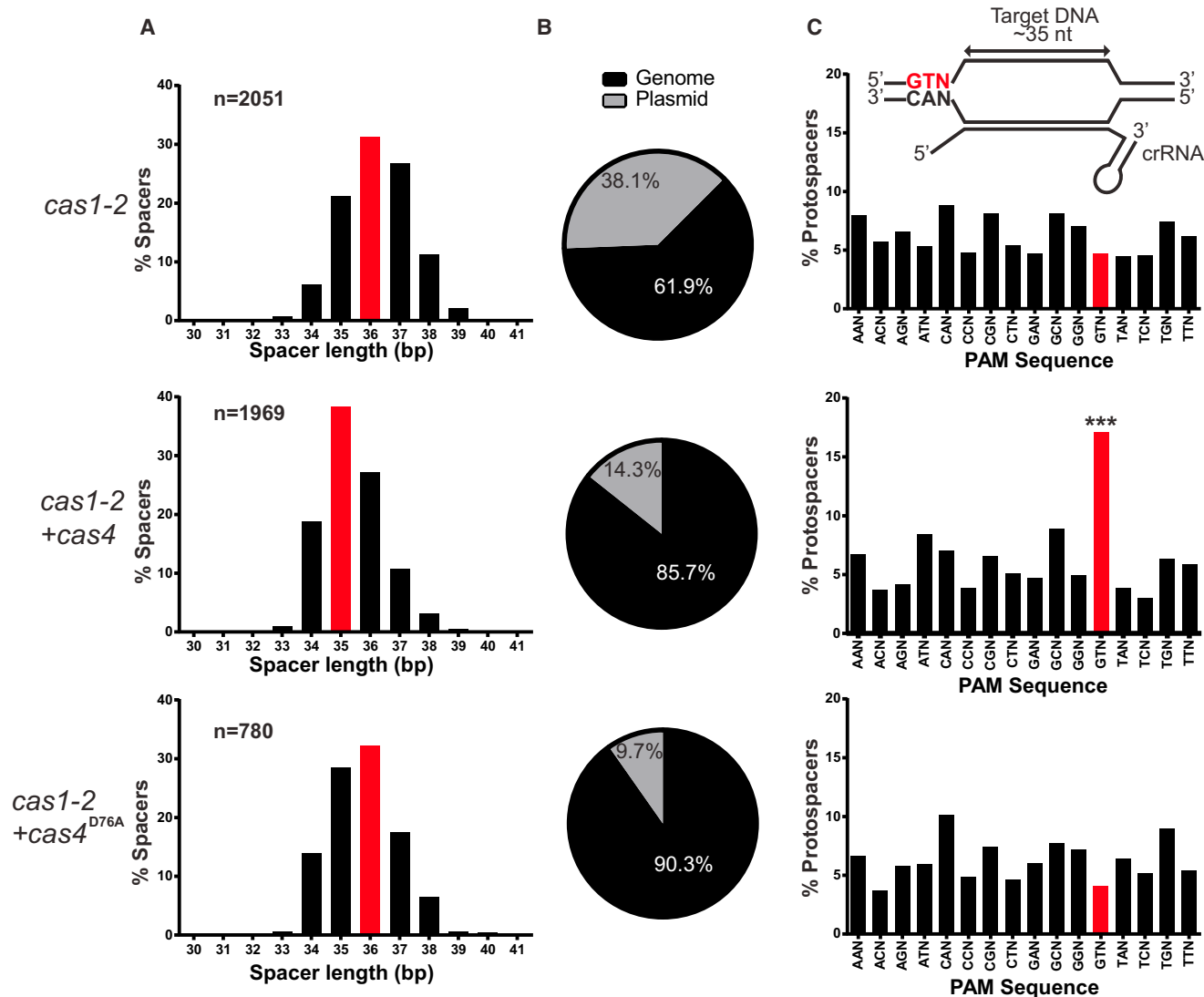
In order to determine which PAMs had been selected during spacer acquisition, we mapped the unique spacers to their targets and retrieved their flanking sequences. This revealed that in the absence of *cas4* no particular sequence motifs were enriched in the flanking regions of the target. Interestingly, when we analyzed upstream flanking sequences of targets from spacers acquired in the presence of *cas4*, we observed that spacers with GTN PAMs were significantly enriched (Figure 2C). This GTN PAM matched the previously predicted PAM for I-D systems (Shah et al., 2013). When we introduced *cas4*<sup>D76A</sup>, the enrichment of GTN PAMs was no longer observed, indicating that the metal-ion coordinating residue, which is likely important for catalytic activity of Cas4, is also essential for PAM selection. Cas1-2 alone displays no inherent PAM selection preference. In order to assess whether inactivation of the *recB* gene would reduce background levels of spacers derived from RecBCD products, we subjected the expanded arrays from the *recB* mutant to high-throughput sequencing. Although this genetic background did not abolish background spacer integration, the presence of *cas4* further increased GTN-PAM-compliant spacers (Figures S2A and S2B; Table S3).

### GTN Is a Functional PAM in the Native Type I-D Host *Synechocystis*

To test whether the GTN PAM enriched in the presence of *cas4* licenses CRISPR interference in *Synechocystis*, we performed interference assays using a conjugative plasmid containing a protospacer matching spacer 1 of the type I-D array. The protospacer was flanked by one of the four GTN PAMs (GTA, GTC, GTG, or GTT) and carried gentamicin resistance for selection. Compared to a non-target control plasmid, we observed a dramatic reduction in the numbers of transconjugants with each of the four possible GTN PAMs (i.e., no transconjugants for GTC, GTG, and GTT and 1 for the GTA PAM) (Figure 3). In contrast, plasmids containing protospacers flanked by AGC PAMs resulted in the same conjugation efficiency as found for the non-target control. We conclude that the type I-D system in *Synechocystis* is active and provides efficient CRISPR interference with GTN PAMs.

## DISCUSSION

Microbes face a number of challenges when they update their CRISPR memory. First, how can they select new spacers from invading elements while preventing sampling from their own genome? Second, how can they maintain a balance between spacer uptake and turnover? Third, how can they select spacers that give functional CRISPR interference? Here, we addressed the last question and show that the highly ubiquitous Cas4 protein



**Figure 2. Analysis of Spacers Acquired by Type I-D CRISPR Adaptation**

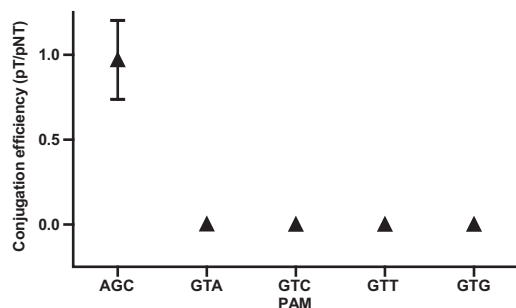
(A) Spacer length distribution in cells harboring different combinations of *cas* genes. The variation in spacer size does not depend on the presence of *cas4* but is solely dependent on the Cas1-2 adaptation complex. The presence of *cas4* restores the statistical mode of spacer length as found in the native I-D array (35 bp; Figure S2C). Statistical mode of spacer length is indicated with red bars.

(B) Origin of newly acquired spacers. The type I-D adaptation proteins acquire mostly from genomic DNA.

(C) Percentage of protospacers with different PAMs. The presence of *cas4* significantly increases the incorporation of spacers that match protospacers with the consensus GTN PAM, while no significant enrichment of PAMs is observed for spacers acquired by Cas1-2 alone or in conjunction with the Cas4<sup>D76A</sup> mutant. n, number of analyzed spacer sequences; significance level  $\alpha = 0.001$ .

present in type I, II, and V systems helps to integrate spacers targeting DNA sequences with PAMs that support CRISPR interference. Because crRNA-effector complexes such as Cascade, Cas9, and Cas12a are critically reliant on PAMs to find their target DNA and to avoid host CRISPR arrays, the selection of PAM-compliant spacers enhances the success rate of CRISPR interference and promotes clearance of invader DNA from cells (Mohanraju et al., 2016). Apart from influencing the PAM, we found that the statistical mode of spacer length was shifted by 1 nt to shorter spacers, suggesting a role for Cas4 in processing spacer substrates before or during integration. The variable spacer size

itself is dictated only by Cas1-2 from type I-D. While structural constraints of the Cas1-2 complex from type I-E, and presumably also of the Cas1-2/3 complex from type I-F, act like a molecular ruler that predetermines a fixed spacer length of predominantly 32 nt (Nuñez et al., 2015; Wang et al., 2015; Fagerlund et al., 2017; Rollins et al., 2017), the integration complex of the type I-D system likely displays plasticity and enables incorporation of spacers that vary in size by 5 or 6 nucleotides. This spacer size variation is observed not only in type I-D but also in type I-B (Li et al., 2017) and many CRISPR systems containing *cas4* genes. Our data are consistent with a model in which the nuclease



**Figure 3. Conjugation Efficiency of Target Plasmids Carrying either AGC-Protospacer1 or GTN-Protospacer1 in *Synechocystis* 6803**

The AGC PAM does not license interference, while plasmids containing the observed GTN PAM are efficiently cleared. Data points represent mean  $\pm$  SE (n = 3).

activities of Cas4 tailor prespacer substrates for the Cas1-2 adaptation machinery during the integration of new spacers.

The *cas4* gene has long been implicated in CRISPR adaptation. Many *cas4* genes have been found adjacent to *cas1* and *cas2*, and in some cases, fusions between *cas4* and *cas1* have been observed (Hudaiberdiev et al., 2017; Koonin et al., 2017). The *cas4* gene was shown to be essential for CRISPR adaptation in the type I-B system of *Haloarcula* (Li et al., 2014). Interestingly, a *Campylobacter* bacteriophage containing a *cas4* gene was reported to promote acquisition of self-targeting spacers in the *Campylobacter* type II-C system (Hooton and Connerton, 2015), which is in line with our finding that Cas4 promotes the integration of spacer from abundant DNA populations in the cell. The Cas4 protein has been observed in a complex with a Cas1-Cas2 fusion protein and Csa1 in the *Sulfolobus* type I-A system and this complex was coined Cascis after CRISPR-associated complex for integration of spacers (Plagens et al., 2012). The Cas4 protein of the *Bacillus halodurans* type I-C system forms a tight heterohexameric complex with Cas1 consisting of two Cas1 dimers and two Cas4 subunits (Lee et al., 2018). Although different catalytic activities have been assigned (Zhang et al., 2012; Lemak et al., 2013, 2014; Hudaiberdiev et al., 2017), the biological role of Cas4 has remained elusive. Only recently it was shown that Cas4 nuclease activity participates in PAM-dependent cleavage of 3' overhangs of prespacers (Rollie et al., 2018). Furthermore, Lee et al. demonstrate that this PAM-dependent cleavage of prespacers ensures that only functional spacers are integrated into the CRISPR array (Lee et al., 2018). This sequence specific cleavage is in line with the findings presented in this study in which Cas4-derived spacers are shorter and enriched in functional GTN PAMs.

While Cas4 may aid the generation of spacers with the correct PAM in a number of CRISPR-Cas systems, some other Cas proteins have been found to influence PAM selection as well. The crRNA-guided effector complex Cas9 present in type II-A systems is required for spacer acquisition (Wei et al., 2015) and helps to select new spacers with a correct PAM (Heler et al., 2015). Next to Cas1-2, the integration of new spacers in type II-A requires the toroidal DNA binding protein Csn2, a protein known to interact with Cas1 (Heler et al., 2015; Ka et al., 2016).

Other ways to improve taking up spacers with the correct PAM include primed CRISPR adaptation (Datsenko et al., 2012; Swarts et al., 2012), which appears to be a general feature of type I systems only (Li et al., 2014; Rao et al., 2016; Staals et al., 2016; van Houte et al., 2016). In contrast to naive spacer acquisition, primed CRISPR adaptation uses preexisting spacer matches to trigger updates of the CRISPR memory against that target. Apart from Cas1-2, the priming process requires the presence of a crRNA-effector complex (e.g., Cascade) and the DNA nuclease Cas3. It seems that the frequency of acquiring functional spacers is much higher during priming than during naive spacer acquisition (Jackson et al., 2017). This can be partly explained by considering that functional spacers confer a selective advantage to the host when the interference machinery is present. On the molecular level, the increased frequency of functional spacers during priming may be explained by the observation that the Cas3 nuclease cleaves target DNA in a PAM-compatible manner to fuel the Cas1-2 adaptation machinery with suitable DNA substrates for integration (Künne et al., 2016).

Taken together, a picture has emerged that it is important for microbes to acquire functional instead of randomly selected spacers in their CRISPR arrays and that there is a variety of ways in which CRISPR systems can accomplish this. The conserved component Cas4, which is present in about half of all CRISPR subtypes, appears to be a Cas protein dedicated to the task of facilitating the integration of functional spacers during CRISPR adaptation.

## EXPERIMENTAL PROCEDURES

### Bacterial Strains and Growth Conditions

*E. coli* strains DH5 $\alpha$ , BW25113 (WT), JW2788 (BW25113  $\Delta$ recB), JW2790 (BW25113  $\Delta$ recC), and JW2787 (BW25113  $\Delta$ recD) were grown in lysogeny broth (LB) at 37°C and continuous shaking at 180 rpm or grown on LB agar plates (LBA) containing 1.5% (wt/vol) agar. *Synechocystis* 6803 was cultivated as described previously (Scholz et al., 2013). When required, the media were supplemented with 100  $\mu$ g/mL ampicillin, 50  $\mu$ g/mL spectinomycin, 25  $\mu$ g/mL chloramphenicol, and 7.5  $\mu$ g/mL gentamicin (see Table S1 for plasmids and corresponding selection markers).

### Plasmid Construction and Transformation

Plasmids used in this study are listed in Table S1. All cloning steps were performed in *E. coli* DH5 $\alpha$ . Primers described in Table S2 were used for PCR amplification of the type I-D CRISPR-Cas locus (*cas4*, *cas1*, *cas2*, and leader-repeat-spacer1) from *Synechocystis* cell material using the Q5 high-fidelity Polymerase (New England Biolabs). PCR amplicons were subsequently cloned into Berkeley MacroLab ligation-independent cloning (LIC) vectors (<http://qb3.berkeley.edu/macrolab/addgene-plasmids/>) using either LIC or into the pACYCDuet-1 vector system (Novagen, EMD Millipore) using conventional restriction-ligation cloning. The *cas4*<sup>D76A</sup> mutant (Zhang et al., 2012) was obtained using a PCR-based mutagenesis using primers listed in Table S2. The conjugative plasmid pVZ322 used in the interference study was obtained by fusing the 5' PAM (GTA, GTT, GTG, GTC, and AGC)-protospacer1 3' sequence in-frame with a gentamicin resistance cassette upstream of its stop codon using inverse PCR using primers listed in Table S2. The gentamicin resistance cassette with and without the PAM-protospacer sequence (pT and pNT, respectively) was then assembled with the linearized pVZ322 backbone. All plasmids were verified by Sanger sequencing (Macrogen Europe, Amsterdam, the Netherlands; GATC Biotech, Konstanz, Germany). Bacterial transformations were either carried out by electroporation (2.5 kV, 25 mF, 200 V) using a ECM 630 electroporator (BTX Harvard Apparatus) or using chemically competent cells prepared according to manufacturer's manual (Mix&Go,

Zymo research). Electrocompetent cells were prepared following a protocol adapted from [Gonzales et al. \(2013\)](#). Transformants were selected on LBA supplemented with appropriate antibiotics.

### In Vivo Spacer Acquisition Assay

*E. coli* BW25113 and *E. coli* mutant strains JW2788 (BW25113  $\Delta recB$ ), JW2790 (BW25113  $\Delta recC$ ), and JW2787 (BW25113  $\Delta recD$ ) were transformed with pCas1-2, pCRISPR, and pCas4, pCas4<sup>D76A</sup>, or the pEmp control plasmid (Table S1). Cultures were inoculated from single colonies and passaged once after 24 hr of growth at 37°C and continuous shaking at 180 rpm. 200  $\mu$ L of cells was harvested by centrifugation and resuspended in 50  $\mu$ L MilliQ water. Subsequently, 2  $\mu$ L cell suspension was subjected to spacer detection PCR using a forward primer annealing in the 3' end of the CRISPR repeat of pCRISPR but mismatching the first nucleotide of spacer 1 (degenerated primer mix; [Heler et al., 2015](#)) and a reverse primer annealing in the vector backbone (Table S2). When higher sensitivity was required, amplicons of expanded pCRISPR arrays were separated from parental pCRISPR array amplicons using the BluePippin automated agarose-electrophoresis system (3% agarose gel cassette, SageScience). The extracted expanded CRISPR array amplicons were then subjected to an additional PCR reaction using the same degenerated primer mix but a different reverse primer matching spacer 1.

### Next-Generation Sequencing and Statistical Analysis

After validation of PCR amplicons by gel electrophoresis and clean up with the GeneJET PCR Purification kit (Thermo Fisher Scientific), the samples were analyzed using Invitrogen Qubit fluorometric quantification. Samples were prepared for sequencing with the Nextera XT DNA Library Preparation Kit (Illumina) and each library individually barcoded with the Nextera XT Index Kit v2 SetA (Illumina). Libraries were pooled equally and spiked with ~5% of the PhiX control library (Illumina) to artificially increase the genetic diversity before sequencing on a Nano flowcell (2  $\times$  250 base paired-end) with an Illumina MiSeq. Image analysis, base calling, de-multiplexing, and data quality assessments were performed on the MiSeq instrument. FASTAQ files generated by the MiSeq were analyzed by pairing and merging the reads using Geneious 9.0.5 and subsequently extracting newly acquired spacers by identifying the 3' end of the degenerate primer and the 5' end of the single repeat present in the parental pCRISPR. Unique spacer sequences were mapped to the chromosome and the replicons carried by the corresponding strains with the BLAST-function of Geneious 9.0.5.

### Statistical Tests

To infer the likelihood of finding a certain distribution of PAMs we used a binomial test, where we estimated the likelihood of the observed frequency of each PAM in the case of a randomly distributed PAM-pool (likelihood per PAM: 1/16). As we performed the test multiple times on the same dataset, we used a Bonferroni correction to decrease the probability of a type I error. Spacer size preference was tested by using a bootstrapping resampling method with replacement. 10,000 bootstrap resamples were generated from each observed dataset (each of similar size to the observed dataset). The statistical mode of a certain spacer size within these resamples represented the likelihood of observing this mode in the observed dataset.

### Synechocystis Interference Assay

*Synechocystis* 6803 contains on its megaplasmid pSYSa a type I-D and two type III CRISPR-Cas systems (III-D and III-B) ([Scholz et al., 2013](#)). *Synechocystis* I-D interference assays were performed as described previously ([Behler et al., 2018](#)) with a *Synechocystis* 6803 derivative strain with 16 instead of 49 spacers (spacers 1–14 and 48–49 retained) in its I-D CRISPR array ([Trautmann et al., 2012](#)). Conjugation assays were performed using the self-replicating conjugative vector pVZ322 and the gentamicin resistance cassette for selection. Target plasmids with a number of different PAMs were constructed containing the target of spacer 1 of the I-D CRISPR array. Plasmids were conjugated into *Synechocystis* by triparental mating as described previously ([Scholz et al., 2013](#)). Briefly, overnight cultures of the helper strain *E. coli* J53/RP4 and the donor strain *E. coli* DH5 $\alpha$  with the plasmid of interest were diluted and incubated for 2.5 h at 37°C with shaking at 180 rpm. For

each conjugation, an optical density 600 (OD<sub>600</sub>) of 7.0 of the plasmid-bearing and helper cultures were harvested, resuspended in LB and combined. The mixed culture was incubated for 1 hr at 30°C without shaking. In parallel, a *Synechocystis* culture with an OD<sub>750</sub> of 1.0 was harvested and combined with the mixed culture of the plasmid-bearing and helper culture. The pellet was resuspended and placed on a sterile filter. After overnight incubation at 30°C, the filter was rinsed and 30  $\mu$ L of the resulting cell suspension was plated on BG11 agar plates containing 7.5  $\mu$ g/mL gentamicin. Transconjugants were counted after further incubation at 30°C for 2 weeks. Mean values of conjugation efficiency and corresponding standard errors were calculated by dividing the number of transconjugants obtained with the target plasmids (pT) by the number of transconjugants obtained with the non-target control plasmid (pNT). Experiments were performed in biological triplicates and in parallel with the control plasmid.

### DATA AND SOFTWARE AVAILABILITY

The accession number for the sequencing data reported in this paper is European Nucleotide Archive: PRJEB25213.

### SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and three tables and can be found with this article online at <https://doi.org/10.1016/j.celrep.2018.02.103>.

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### AUTHOR CONTRIBUTIONS

S.N.K., C.A., and S.J.J.B. designed research. S.N.K., C.A., J.B., R.E.M., F.L.N., A.C.H., and J.N.A.V. performed research. S.N.K., C.A., J.B., R.E.M., F.L.N., J.N.A.V., W.R.H., and S.J.J.B. analyzed data. S.N.K., C.A., and S.J.J.B. wrote the paper with input from all authors.

### DECLARATION OF INTERESTS

The authors declare no competing interests.

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## **Supplemental Information**

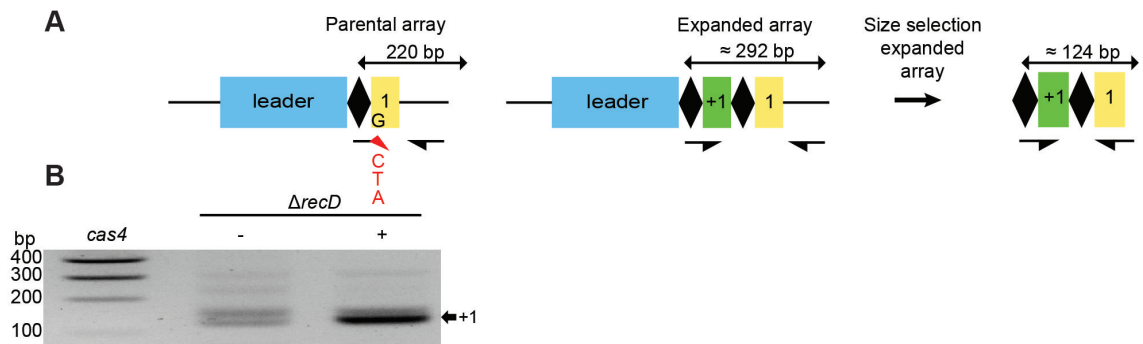
### **Cas4 Facilitates PAM-Compatible Spacer**

#### **Selection during CRISPR Adaptation**

**Sebastian N. Kieper, Cristóbal Almendros, Juliane Behler, Rebecca E. McKenzie, Franklin L. Nobrega, Anna C. Haagsma, Jochem N.A. Vink, Wolfgang R. Hess, and Stan J.J. Brouns**

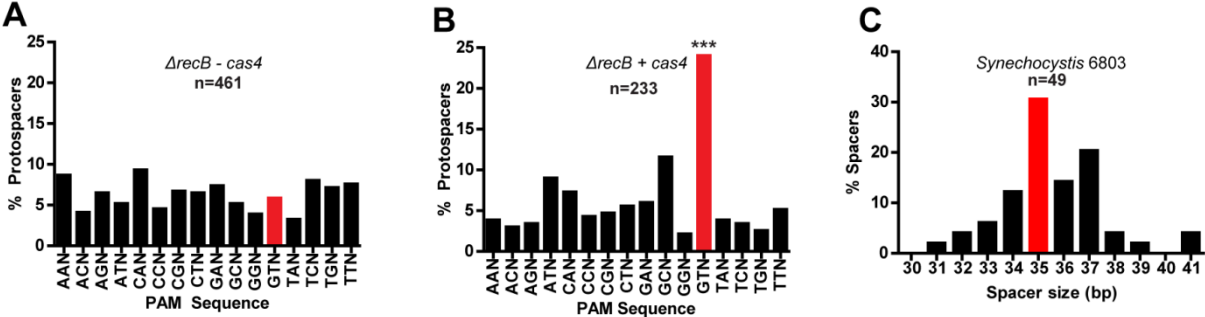
## Supplemental figures

### Figure S1. High sensitivity spacer detection PCR, Related to Figure 1 D



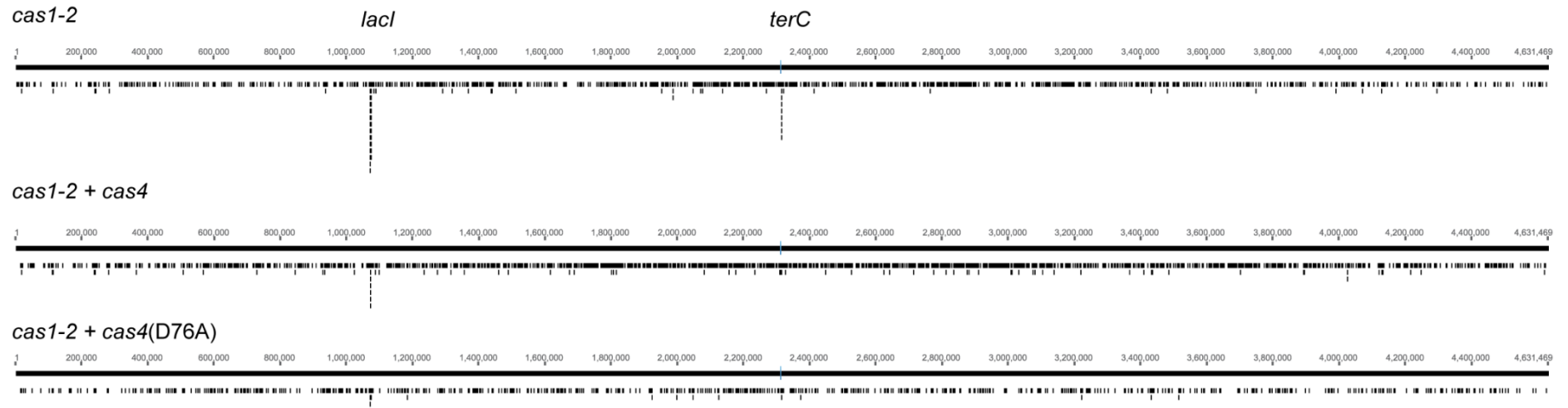
**Figure S1 A.** Following the first round of amplification, the hypothetical amplicon of expanded arrays of ~292 bp (not visible on gel in **Fig. 1D**) is size selected using the BluePippin system (3% agarose cassette, SageScience). The extracted band is then subjected to a second PCR using the same forward degenerate primer mix (Heler et al. 2015) with a reverse primer annealing in spacer1 of the parental array. **B** By applying the PCR approach described in **A** spacer integration is observed in the *E. coli* K12  $\Delta recD$  background in the presence or absence of *cas4*. Band intensity is not a quantitative measure for integration efficiency, but rather a binary result (yes/no), because PCR product input resulting from automatized size selection cannot be normalized. The amplicon corresponding to expanded arrays is indicated with a black arrow.

**Figure S2. PAM and size distribution of new spacers, Related to Figure 2.**



**Figure S2, A** Percentage of protospacers adjacent to indicated PAM matched by spacers acquired in **A** *ΔrecB* without *cas4* and **B** with *cas4*. **C** Spacer size distribution in the native type I-D host *Synechocystis*. n= number of analyzed spacers, significance level  $\alpha = 0.001$ .

**Figure S3. Genomic spacer mapping, Related to Figure 2.**



**Figure S3.** Origin of spacers acquired from the WT *E. coli* K12 genome. In the absence of *cas4* the Cas1-2 integration complex acquires spacers with preference for the *lacI* sequence and *terC* site. The preferential uptake of spacers derived from the *terC* site is lost when supplying Cas4 WT or Cas4<sup>D76A</sup>.

## Supplementary Tables

**Table S1. Plasmids used in this study, Related to Figure 1, 2, 3.**

Name in this study	Name	Insert	Vector	Resistance	Source
pCas2	pTU084	Synechocystis PCC6803 Type I-D <i>cas2</i> (deltaCas1)	pET-T7	Amp	This study
pCas1	pTU085	Synechocystis PCC6803 Type I-D <i>cas1</i> (deltaCas2)	pET-T7	Amp	This study
pCas4 <sup>D76A</sup>	pTU086	Synechocystis PCC6803 Type I-D <i>cas4</i> (D76A)	pET-T7	Spec	This study
pCas4	pTU130	Synechocystis PCC6803 Type I-D <i>cas4</i>	pET-T7	Spec	This study
pCRISPR	pTU134	Synechocystis PCC6803 Type I-D Leader-R-S1	pACYCDuet1	Cm	This study
pCas1-2	pTU70	Synechocystis PCC6803 Type I-D <i>cas1-cas2</i>	pET-T7	Amp	This study
pEmp	pTU116	NA	pET-T7	Spec	Addgene Plasmid #48329
pVZ322	pNT	NA	pVZ322	Gent	This study
pVZ322 pT-GTA	pT-GTA	GTA-protospacer1-GentR	pVZ322	Gent	This study
pVZ322 pT-GTT	pT-GTT	GTT-protospacer1-GentR	pVZ322	Gent	This study
pVZ322 pT-GTC	pT-GTC	GTC-protospacer1-GentR	pVZ322	Gent	This study
pVZ322 pT-GTG	pT-GTG	GTG-protospacer1-GentR	pVZ322	Gent	This study

**Table S2. Oligonucleotides used in this study, Related to Figure 1, 2, 3.**

Name	Sequence	Description
BG7615	TTATGGAGTTGGGATCTTATTAGATAATAAT ACTACCAGGTTTTCTGGTTG	<i>cas2</i> rv
BG8223	TACTTCCAATCCAATGCAATGGATGATTATT TACCTTTAGC	<i>cas4</i> fw
BG8224	TTATCCACTTCCAATGTTATTATTTAGTAAGT TTTTTTAATTCTTTCGG	<i>cas4</i> rv
BN015	CGTCCATGGGAAGTCATTCTTCAAATTTTGG C	leader fw
BN016	TACAAGCTTAGGCATTGAAAGCGACC	Sp1 Rv (for degenerate PCR)
BN114	TTTAAGAAGGAGATATAGATCATGTCTACAC TTTACTTGACTIONCAACC	<i>cas1</i> fw
BN135	GCTGCAGTGGGAAGAAAGTG	Type I-D <i>cas4</i> mutagenesis D76A Fw
BN136	AATAATCCCTTTAACTTTTAGGCG	Type I-D <i>cas4</i> mutagenesis D76A Rv
BN143	GCGATCGGGACTGAAACT	Degenerated Fw1
BN144	GCGATCGGGACTGAAACA	Degenerated Fw2
BN145	GCGATCGGGACTGAAACC	Degenerated Fw3
BN156	AGGCATTGAAAGCGACC	Degenerate PCR Rv (internal. Sp1)
BN172	AGATCTGCCATATGTATATCTCCTTC	pAcyc backbone Rv (for degenerate PCR)
BN212	GATCTATATCTCCTTCTTAAAGTTAAAC	<i>cas1</i> deletion Rv
BN213	CAGTTATCAGTTGTGTTTTGAC	<i>cas1</i> deletion Fw
BN214	TTTTTAGTCGTCAAAACACAAC	<i>cas2</i> deletion Rv
BN215	TAATAAGATCCCAACTCCATAAG	<i>cas2</i> deletion Fw
GentaR_pUC 19_fwd	CGGTGATGACGGTGAGATTCCATTTTTACAC TGATGAATGTTCCGTTGCG	Gentamicin resistance cassette with overlaps to pUC19
GentaR_pUC 19_rev	CGCCTTTGAGTGAGCTCCCGGCATTCGCTG CGCT	Gentamicin resistance cassette with overlaps to pUC19
CRISPR1_G TG_S1_fwd	GTGGATTGTTGTGCCCTGGCGGTGCGTTT CAATGCCTTTAACAATTCGTTCAAGCCGAGA TC	GTG PAM motif spacer 1
CRISPR1_G TG_S1_rev	AAGGCATTGAAAGCGACCGCCAGGGGCAC AACAATCCACGGTGGCGGTACTIONTGGGTC	GTG PAM motif spacer 1
CRISPR1_G TA_S1_fwd	GTAGATTGTTGTGCCCTGGCGGTGCGTTT CAATGCCTTTAACAATTCGTTCAAGCCGAGA TC	GTA PAM motif spacer 1
CRISPR1_G TA_S1_rev	AAGGCATTGAAAGCGACCGCCAGGGGCAC AACAATCTACGGTGGCGGTACTIONTGGGTC	GTA PAM motif spacer 1
CRISPR1_G TT_S1_fwd	GTTGATTGTTGTGCCCTGGCGGTGCGTTT CAATGCCTTTAACAATTCGTTCAAGCCGAGA TC	GTT PAM motif spacer 1
CRISPR1_G TT_S1_rev	AAGGCATTGAAAGCGACCGCCAGGGGCAC AACAATCAACGGTGGCGGTACTIONTGGGTC	GTT PAM motif spacer 1
CRISPR1_G TC_S1_fwd	GTGATTGTTGTGCCCTGGCGGTGCGTTT CAATGCCTTTAACAATTCGTTCAAGCCGAGA TC	GTC PAM motif spacer 1
CRISPR1_G TC_S1_rev	AAGGCATTGAAAGCGACCGCCAGGGGCAC AACAATCGACGGTGGCGGTACTIONTGGGTC	GTC PAM motif spacer 1

CRISPR1_A GC_S1_fwd	AGCGATTGTTGTGCCCTGGCGGTCGCTTT CAATGCCTTTAACAAATTCGTTCAAGCCGAGA TC	AGC mock-PAM motif spacer 1
CRISPR1_A GC_S1_rev	AAGGCATTGAAAGCGACCGCCAGGGGCAC AACAAATCGCTGGTGGCGGTACTTGGGTC	AGC mock-PAM motif spacer 1
GentaR_pVZ 322_fwd	TCTGCTCCTGCAGGTCGACTGATTCCATTTT TACTGATGAATGTTCCGTTGCGCTGCC	Gentamicin resistance cassette with overlaps to pVZ322
GentaR_pVZ 322_rev	CCCGGCATTCGCTGCGCTTATGGCAGAGCA	Gentamicin resistance cassette with overlaps to pVZ322



**Table S3. Spacer origin analysis of unique spacers, Related to Figure 2**

Strain	Cas4	Genome		pCas4/pEmp		pCas1-2		pCRISPR		% Protospacers		% Protospacers with GTN PAM
		Fw	Rv	Fw	Rv	Fw	Rv	Fw	Rv	G	P	
WT	+	852	835	89	98	10	11	44	30	85.7	14.3	17.1
WT	-	642	627	262	247	27	26	123	97	61.9	38.1	4.7
WT	D76A	351	353	25	26	2	1	10	12	90.3	9.7	4.1
$\Delta recB$	+	49	51	46	47	4	6	16	14	42.9	57.1	24
$\Delta recB$	-	125	138	51	73	8	13	25	28	57.0	43.0	5.9

**Table S3.** Total number of unique spacers acquired from the *E. coli* K12 genome, the *cas4* expression plasmid and corresponding empty vector control, the *cas1-2* expression plasmid and the minimalized type I-D array plasmid in different strains and either presence or absence of *cas4*. Strand orientation indicated with Forward (Fw) or Reverse (Rv). The two last columns represent the percentage of protospacers that match the genome (G) or plasmids (P) and spacers matching a protospacer with the GTN PAM.

### **Supplemental reference**

Heler, R., Samai, P., Modell, J.W., Weiner, C., Goldberg, G.W., Bikard, D., and Marraffini, L.A. (2015). Cas9 specifies functional viral targets during CRISPR-Cas adaptation. *Nature* 519, 199-202.