Supporting Information

Gasiunas et al. 10.1073/pnas.1208507109

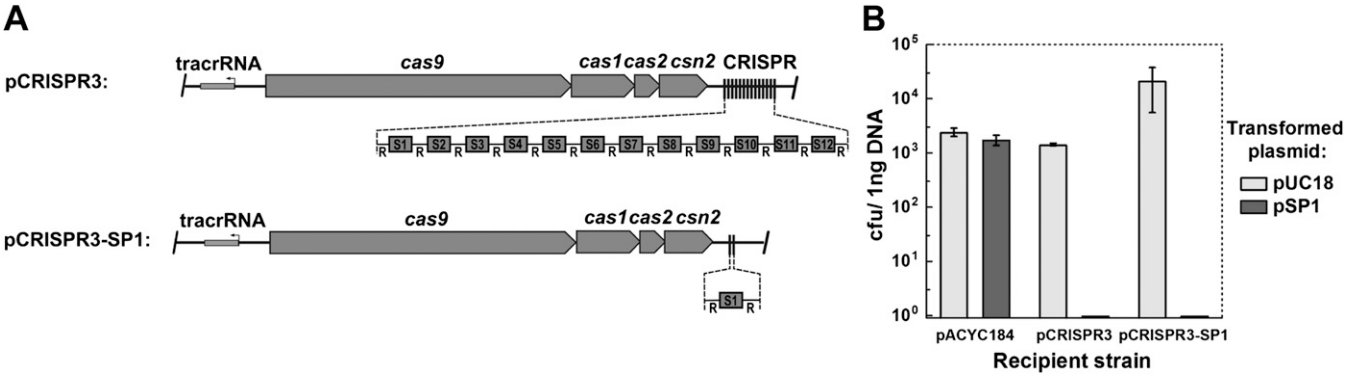


Fig. S1. Immunity against plasmid transformation in *Escherichia coli* cells provided by the *Streptococcus thermophilus* CRISPR3/ CRISPR-associated (Cas) system. (*A*) Schematic representation of the clustered, regularly interspaced, short palindromic repeats (CRISPR)/Cas locus of the recombinant plasmid pCRISPR3 carrying an indigenous 12–spacer-repeat array of the *S. thermophilus* CRISPR3/Cas system (*Upper*) and an engineered pCRISPR3-SP1 plasmid carrying one spacer-repeat unit (*Lower*). (*B*) Interference with plasmid transformation by the *S. thermophilus* CRISPR3/Cas system in *Escherichia coli* cells. *E. coli* RR1 recipient strains carrying plasmids pACYC184, pCRISPR3, or pCRISPR3-SP1 were transformed with plasmid pSP1 carrying protospacers and the protospacer adjacent motif (PAM) or pUC18 (1). Transformation efficiency is expressed as cfu/ng plasmid DNA (mean ± SD).

1. Sapranauskas R, et al. (2011) The Streptococcus thermophilus CRISPR/Cas system provides immunity in Escherichia coli. *Nucleic Acids Res* 39:9275–9282.

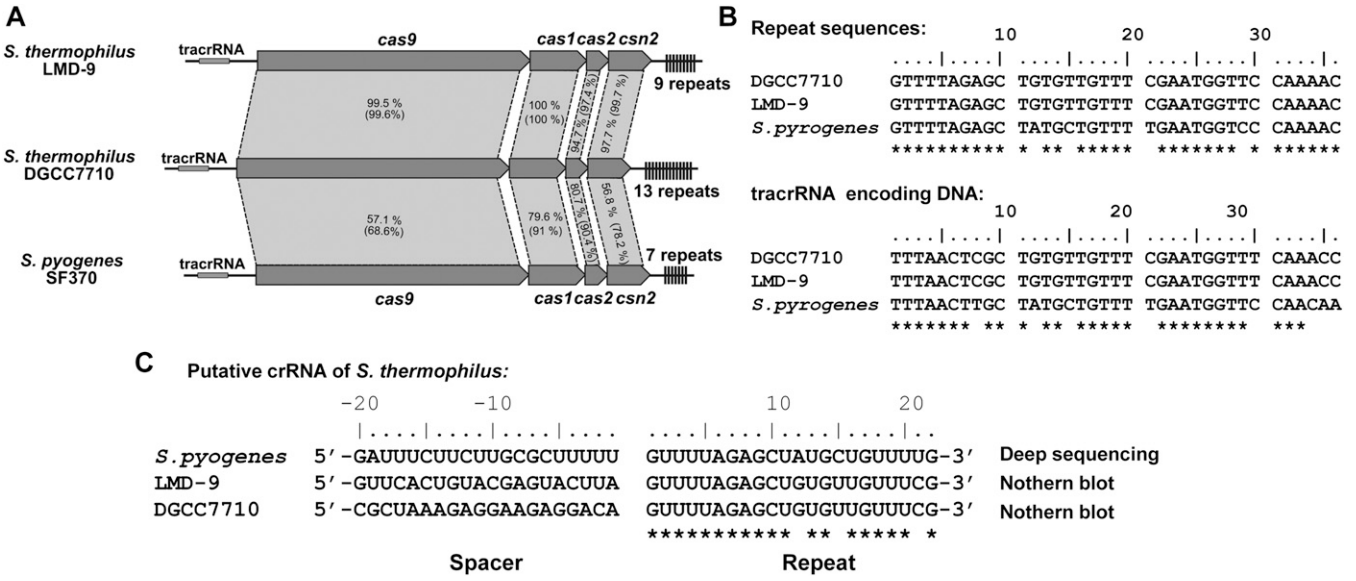


Fig. S2. Comparison of type IIA CRISPR/Cas systems from *Streptococcus thermophilus* strains DGCC7710 and LMD-9 and *Streptococcus pyogenes* strain SF370. (*A*) Schematic organization of the CRISPR/Cas systems. Nucleotide sequences corresponding to the transencoded small RNA (tracrRNA) required for the crRNA maturation in *S. pyogenes* (1) are present in the *S. thermophilus* LMD-9 and DGCC7710 strains. Percentages of identical and similar (in parenthesis) residues among the corresponding protein sequences (demarcated by dashed lines) are shown. (*B*) Alignment of the conserved repeat sequences and tracrRNA. Corresponding sequences from DGCC7710 and LMD-9 are identical. Nucleotide positions that are identical in all three strains are labeled by an asterisk below the aligned sequences. (*C*) Comparison of crRNA sequences. The sequence and length of *S. pyogenes* crRNA was determined by deep-sequencing analysis (1). The approximate lengths of CRISPR RNA (crRNA) from *S. thermophilus* LMD-9 (1) and DGCC7710 (this work) strains were determined by the Northern blot analysis.

1. Deltcheva E, et al. (2011) CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. *Nature* 471:602–607.

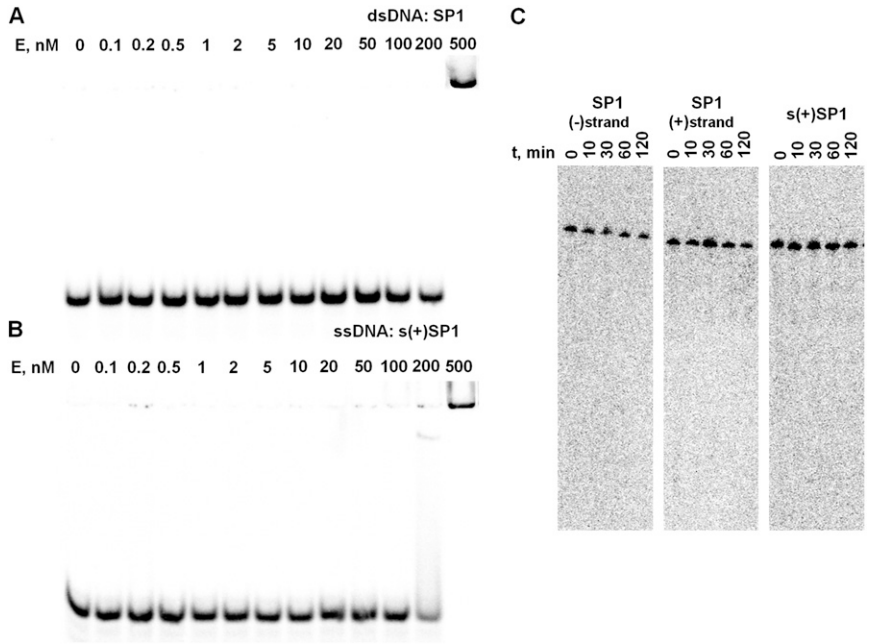


Fig. S3. DNA binding and cleavage analysis of the C-terminal His_6-tagged Cas9 protein variant (Cas9-Chis) lacking crRNA. EMSA of Cas9-Chis protein binding to (*A*) the double-stranded SP1 oligoduplex and (*B*) the single-stranded s(+)SP1 oligonucleotide. EMSA experiments were performed in the binding buffer [40 mM Tris·acetate (pH 8.3), 0.1 EDTA, 0.1 mg/mL BSA, 10% (vol/vol) glycerol] at 25 °C. The reactions contained 0.5 nM of the ^{33}P-labeled oligoduplex and the protein at the concentrations indicated above each lane. (*C*) Oligonucleotide cleavage assay. Cas9-Chis protein (5 nM) was incubated in the reaction buffer [10 mM Tris·HCl (pH 7.5), 10 mM NaCl, 10 mM $MgCl_2$, 0.1 mg/mL BSA] at 37 °C with 1 nM oligonucleotide. The SP1 oligoduplex was labeled with ^{33}P at the 5′ end of the (+) or (–) strand. Single-stranded oligonucleotide s(+)SP1 was labeled with ^{33}P at the 5′ end.

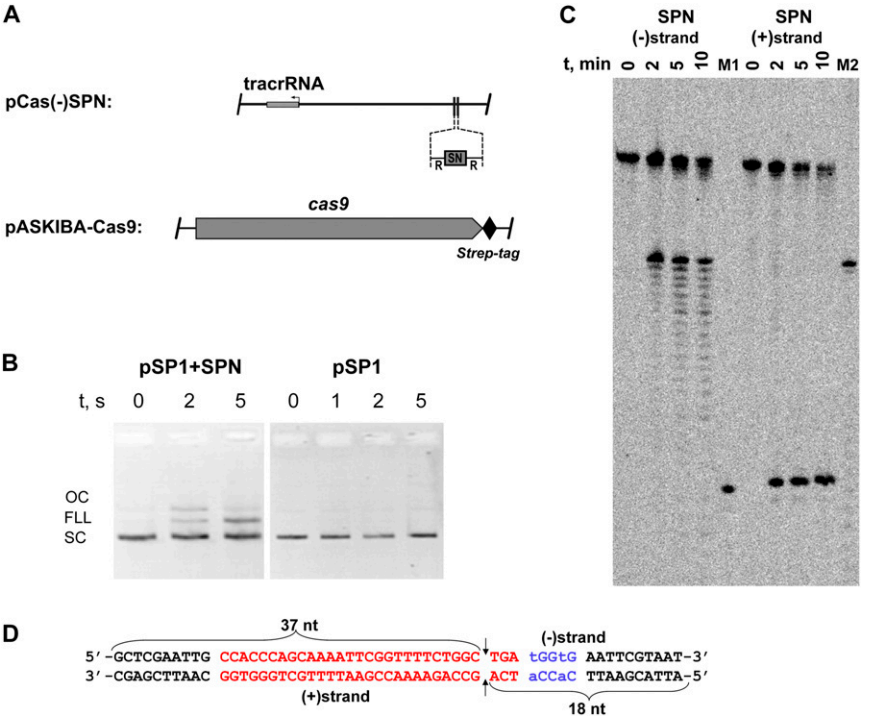


Fig. S4. Reprogramming the Cas9–crRNA complex. (*A*) Schematic representation of heterologous loci in two plasmids used for reprogramming the Cas9–crRNA complex. pCas(–)SPN was constructed from the pCas9(–) plasmid by inserting a spacer sequence, SN (5′-CC ACC CAG CAA AAT TCG GTT TTC TGG CTG-3′), and inactivating the Cas9 gene as described in ref. 1. (*B*) Agarose gel analysis of plasmid DNA cleavage products. pSP1 and pSP1+SPN (pSP1 plasmid with inserted SN protospacer and a PAM over the AatII site) were incubated at 2.5-nM concentration with 2 nM of the Cas9–crRNA complex in the reaction buffer [10 mM Tris·HCl (pH 7.5), 10 mM NaCl, 10 mM $MgCl_2$, 0.1 mg/mL BSA] at 37 °C for various time intervals, and reaction products were analyzed in the agarose gel. FLL, full-length linear DNA cut at both strands; OC, open circular DNA nicked at one of DNA strands; SC, supercoiled plasmid DNA. (*C*) Oligoduplex SP1 cleavage. Cas9–crRNA complex (2.5 nM) and 1 nM SPN oligoduplex (Table S1) labeled with ^{33}P at the 5′ end of either the (+) or the (–)strand were incubated in the reaction buffer [10 mM Tris·HCl (pH 7.5), 10 mM NaCl, 10 mM $MgCl_2$, 0.1 mg/mL BSA] at 37 °C. M1, 18-nt length marker. Lanes M1 and M2 contain chemically synthesized 5′-end, ^{33}P-labeled, 18-nt and 37-nt oligodeoxynucleotides corresponding to the cleavage products of the (+) and (–) DNA strands, respectively. (*D*) Schematic representation of the SPN oligoduplex substrate and cleavage products. The SPN (spacerSN) oligoduplex (Table S1) contains the inserted protospacer (red letters) and PAM (blue letters). Cleavage positions are designated by arrows.

1. Sapranauskas R, et al. (2011) The Streptococcus thermophilus CRISPR/Cas system provides immunity in Escherichia coli. *Nucleic Acids Res* 39:9275–9282.

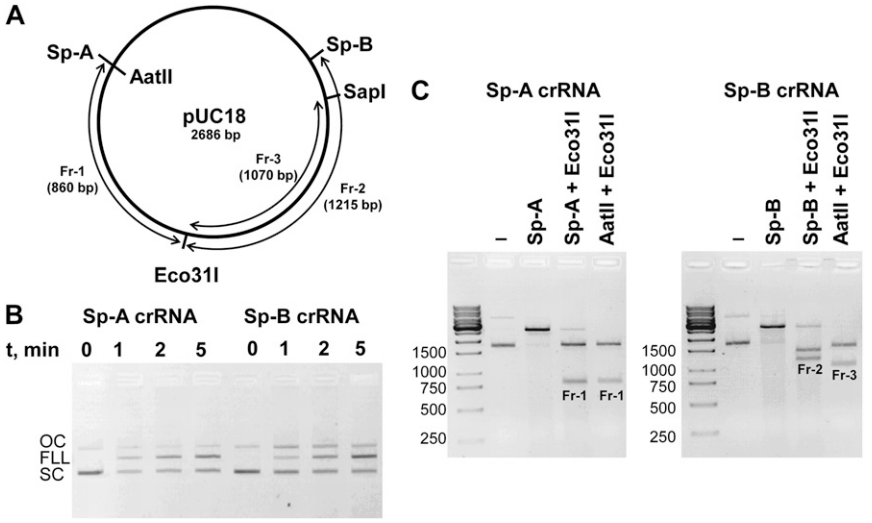


Fig. S5. pUC18 cleavage by reprogrammed Cas9–crRNA complexes. (*A*) Schematic representation of target sites and length of cleavage fragments. The Sp-A sequence overlaps the AatII site. The Sp-B sequence is located 145 bp away from the SapI site. The pCas(–)SP-A and pCas(–)SP-B plasmids encoding the corresponding crRNAs were constructed by inserting spacer repeat unit-encoding oligoduplexes containing spacer sequences (5′-TTAATGTCATGATAA-TAATGGTTTCTTAGACGTC-3′ and 5′-ACGAGCCGGAAGCATAAAGTGTAAAGCCTG-3′, respectively) into the pCas(–)-R plasmid as described in ref. 1. (*B*) Agarose gel analysis of pUC18 cleavage products. pUC18 was incubated with 2.5-nM concentration with 2 nM of complex containing Sp-A or Sp-S crRNA in the reaction buffer [10 mM Tris·HCl (pH 7.5), 10 mM NaCl, 10 mM $MgCl_2$, 0.1 mg/mL BSA] at 37 °C for various time intervals, and reaction products were analyzed in the agarose gel. FLL, full-length linear DNA cut at both strands; OC, open circular DNA nicked at one of DNA strands; SC, supercoiled plasmid DNA. (*C*) pUC18 cleavage by the combined action of the Cas9–crRNA complex and a restriction enzyme. pUC18 (2.5nM) was incubated with 5 nM of the Cas9–crRNA complex for 15 min at 37 °C (Sp-A and Sp-B lanes). Reactions were stopped by heating at 65 °C for 5 min. The Eco31I restriction enzyme was added to the mix and incubated at 37 °C for 15 min to yield fragments Fr-1 (Sp-A + Eco31I) and Fr-2 (Sp-B + Eco31I). The DNA fragments obtained by cleaving pUC18 plasmid with AatII + Eco31I (Fr-1) and AatII + Eco31I (Fr-3) restriction enzymes were used as size markers. Traces of linear pUC18 DNA plasmid in lanes Sp-A + Eco31I and Sp-B + Eco31I are the results of incomplete Cas9–crRNA cleavage.

1. Sapranauskas R, et al. (2011) The Streptococcus thermophilus CRISPR/Cas system provides immunity in Escherichia coli. *Nucleic Acids Res* 39:9275–9282.

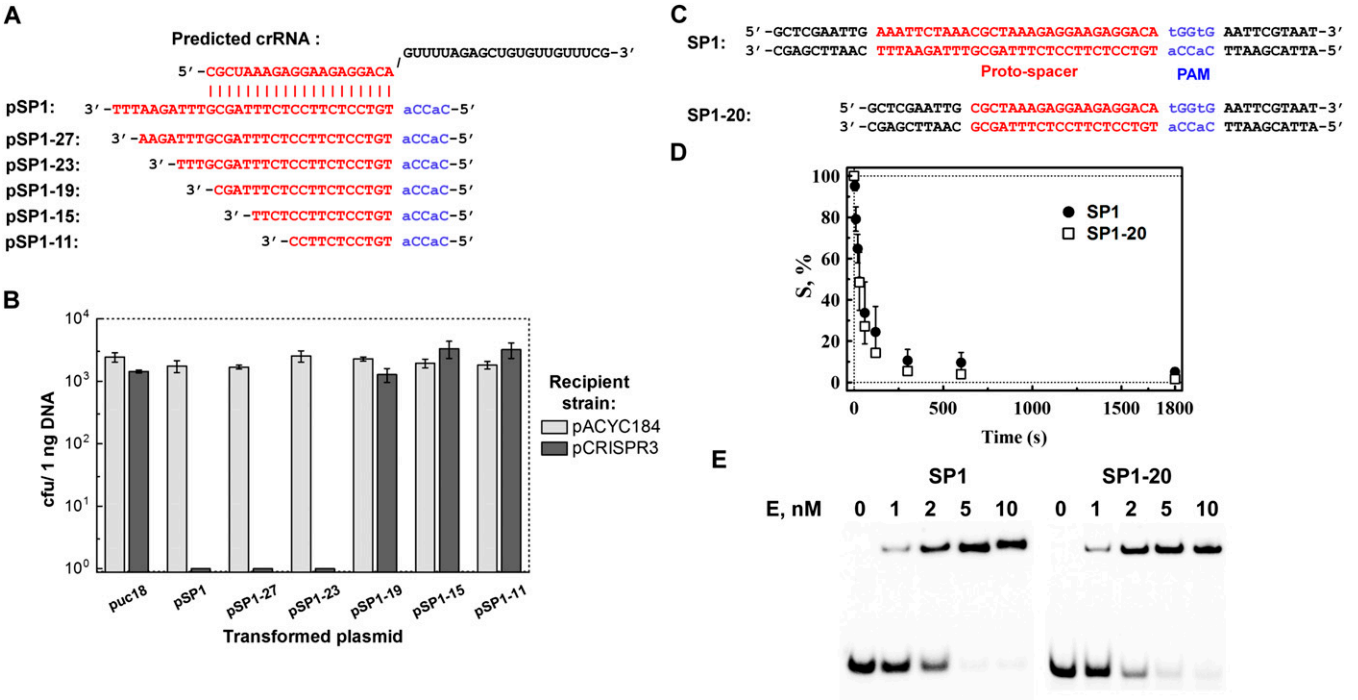


Fig. S6. Impact of spacer length on CRISPR-encoded immunity. (*A*) Schematic representation of shortened versions of protospacers inserted in the transformed plasmids. (*B*) Effect of protospacer length on the plasmid transformation efficiency. Transformation efficiency is expressed as cfu/ng plasmid DNA (mean ± SD). (*C*). Schematic representation of oligoduplexes used in the in vitro cleavage and binding experiments. (*D*) Time courses of the cleavage of 30-bp oligoduplex (full-length protospacer SP1, filled circles) and the 20-bp oligoduplex (truncated protospacer SP1-20, open squares) by the Cas9–crRNA complex. (*E*) EMSA of SP1 and SP1-20 oligoduplex binding by the Cas9–crRNA complex.

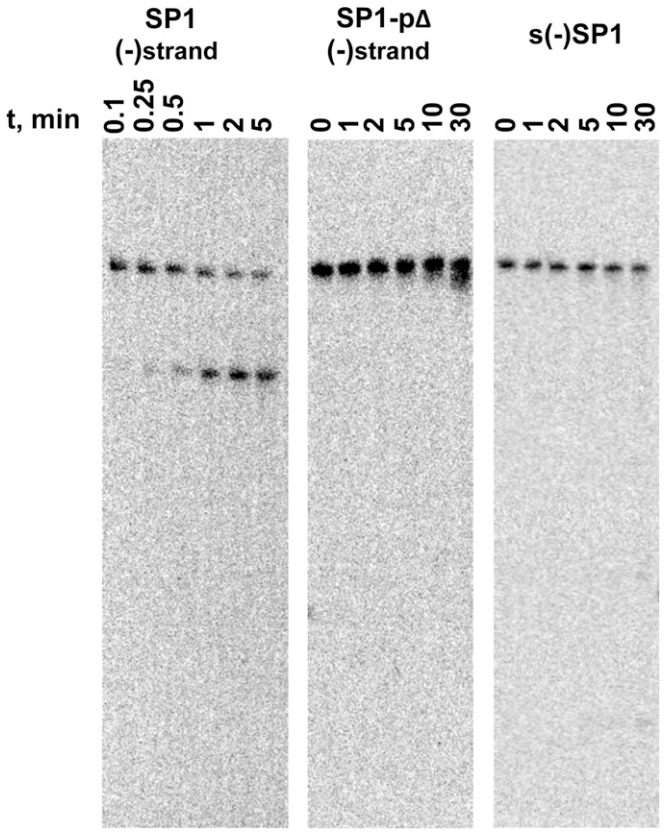


Fig. S7. (–)Strand cleavage in oligoduplexes containing or lacking a PAM (SP1 and SP1-pΔ, respectively) and in single-stranded form [s(–)SP1]. Cas9–crRNA complex (2.5 nM) was incubated in the reaction buffer [10 mM Tris·HCl (pH 7.5), 10 mM NaCl, 10 mM $MgCl_2$, 0.1 mg/mL BSA] at 37 °C in the presence of 1 nM of respective oligonucleotide.

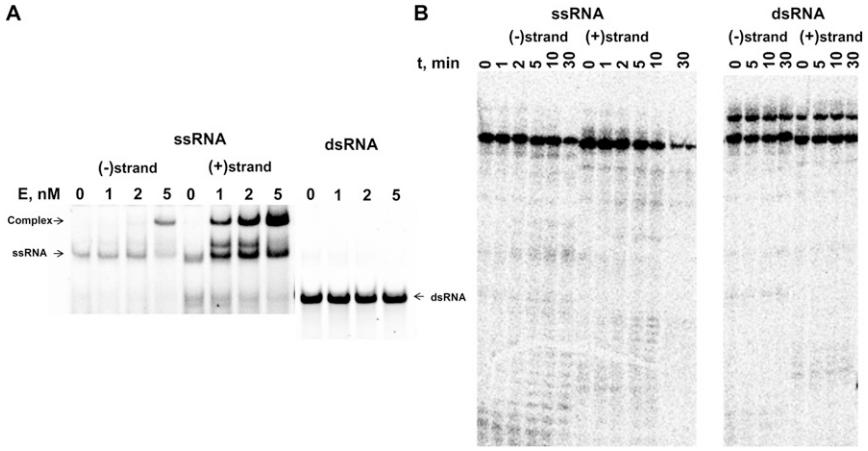


Fig. S8. RNA binding and cleavage analysis of the Cas9–crRNA complex. (*A*) EMSA of Cas9–crRNA complex binding to the 84-nt RNA fragment containing protospacer-1, a PAM, and 24-nt flanking sequences on both sides. (*Left*) RNA (–)strand. (*Center*) RNA (+)strand. (*Right*) dsRNA. RNA fragments used for analysis were generated by in vitro transcription (TranscriptAid T7 High Yield Transcription Kit; Fermentas) from PCR fragments with the T7 promoter inserted at the front end of the RNA-coding sequence. PCR fragments coding (+) and (–) RNA strands were obtained from pSP1 plasmid (1) with the primer pairs 5′-TAATACGACTCACTATA**G**GGTACCGAGCTCGAATTG-3′/5′-GGGAAACAGCTATGACCATGATTACGAATTC-3′ and 5′-GGGTACCGAGCTCGAATTGAAATTCTAAACG-3′/5′-TAATACGACTCACTATA**G**GGAAACAGCTATGACCATGATTACG-3′, respectively. (The T7 RNA polymerase promoter is underlined, and the transcription start is in bold.) The reactions contained 1 nM of the ^{33}P-labeled RNA fragment and the protein at the concentrations indicated above each lane. After 15 min at room temperature, the samples were subjected to PAGE for 2 h and analyzed as described in *Materials and Methods*. (*B*) RNA cleavage assay. Cas9–crRNA complex (2.5 nM) was incubated in the reaction buffer [10 mM Tris·HCl (pH 7.5), 10 mM NaCl, 10 mM $MgCl_2$, 0.1 mg/mL BSA] at 37 °C in the presence of 1 nM (+) and (–) ssRNA strands (*Left*) or dsRNA labeled on the (+) or (–) strand (*Right*). Reaction products were analyzed on denaturing polyacrylamide gel.

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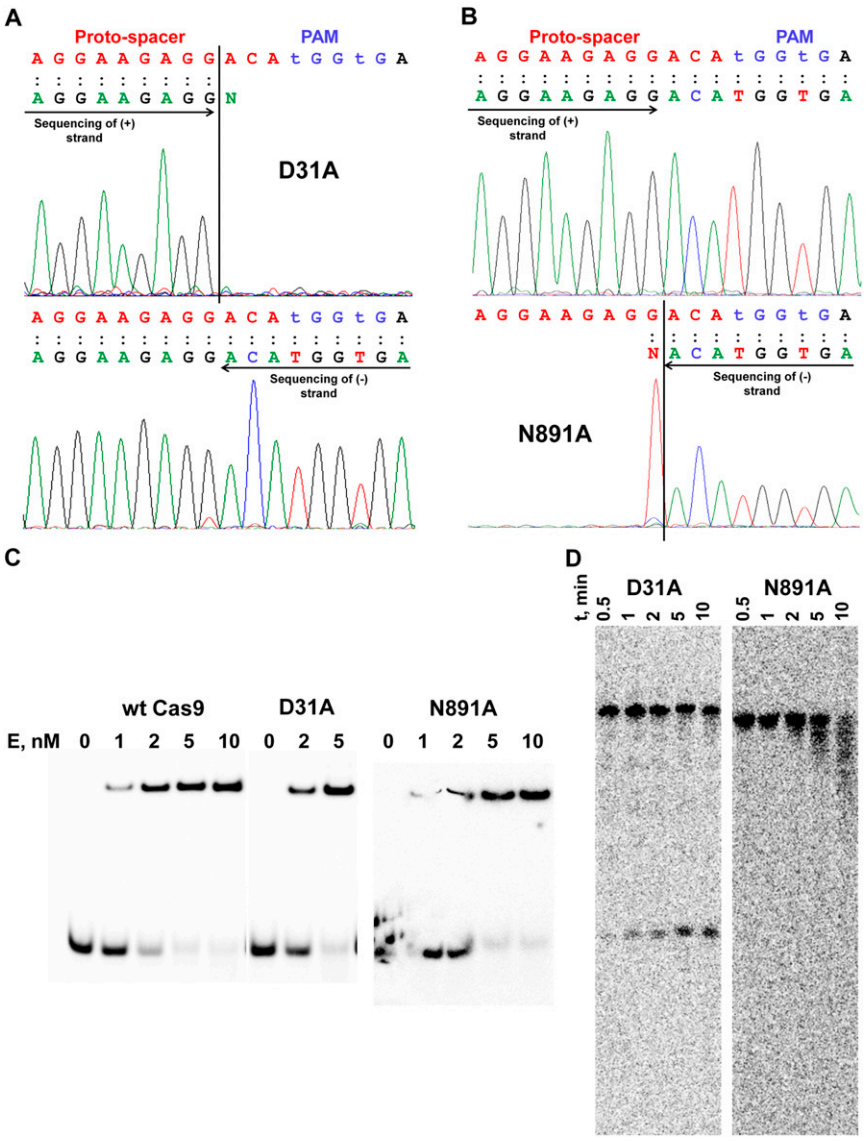


Fig. S9. Properties of Cas9 active-site mutant crRNA complexes. (*A*) Direct sequencing of reaction products obtained with the Cas9 mutant D31A (RuvC-like active-site motif). (*B*) Direct sequencing of reaction products obtained with the Cas9 N891A mutant (HNH-like active-site motif). (*C*) SP1 oligoduplex binding by WT Cas9–crRNA and active-site mutant complexes. (*D*) Cleavage of the (+)SP1 strand by Cas9–crRNA mutant complexes.

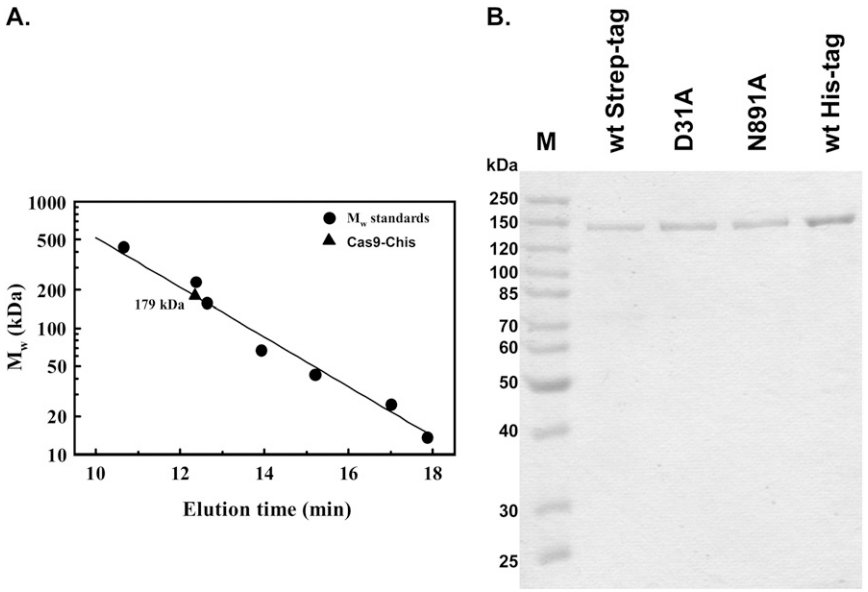


Fig. S10. (*A*) Molecular mass of the WT Cas9-Chis protein. Gel filtration experiments were carried out at room temperature using a Superdex 200 10/300 GL column (GE Healthcare) preequilibrated with 10 mM sodium phosphate (pH 7.4) buffer containing 500 mM sodium chloride. The apparent molecular mass of Cas9 (black triangle) was calculated by interpolation from the standard curve obtained using a set of proteins of known molecular mass (black circles) (Bio-Rad Gel Filtration Standards). (*B*) SDS/PAGE of the purified Cas9 proteins and mutant variants. M, protein molecular mass marker "PageRuler Unstained Protein Ladder" (Fermentas). The numbers on the left indicate markers' size in kDa. Fifty nanograms of His-tagged and 30 ng of Strep-tagged Cas9 proteins were loaded on the gel.

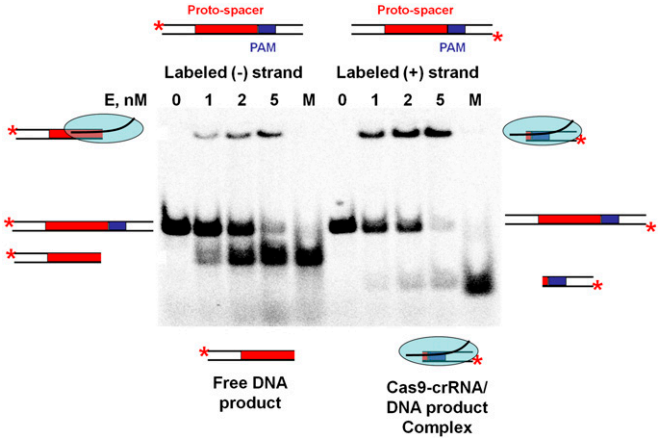


Fig. S11. Native electrophoresis of Cas9–crRNA and cleavage products. The protein at the concentrations indicated above each lane was incubated in the reaction buffer [10 mM Tris·HCl (pH 7.5), 10 mM NaCl, 10 mM $MgCl_2$, 0.1 mg/mL BSA] at 37 °C for 30 min in the presence of 0.5 nM SP1 oligoduplex. Samples were mixed with loading dye solution [0.01% bromophenol blue and 75 mM EDTA in 50% (vol/vol) glycerol] and analyzed by nondenaturing PAGE. M, melted form of cleavage reactions products. The cartoons on each side of the gel illustrate protein–DNA complexes and DNA corresponding to each band. Cartoons below the gel illustrate the major substrate form after reaction.

Table S1. Oligonucleotide substrates

Oligonucleotide	Sequence	Specification
SP1	5′-GCTCGAATTGAAATTCTAAACGCTAAAGAGGAAGAGGACAT**GG**T**G**AATTCGTAAT-3′ 3′-CGAGCTTAACTTTAAGATTTGCGATTTCTCCTTCTCCTGTA**CC**A**C**TTAAGCATTA-5′	55-bp oligoduplex substrate containing protospacer1 and PAM
SP1-pΔ	5′-GCTCGAATTGAAATTCTAAACGCTAAAGAGGAAGAGGACAAATTCGTAAT-3′ 3′-CGAGCTTAACTTTAAGATTTGCGATTTCTCCTTCTCCTGTTTAAGCATTA-5′	50-bp oligoduplex substrate containing protospacer2
SP2	5′-GCTCGAATTGTACTGCTGTATTAGCTTGGTTGTTGGTTTGT**GG**T**G**AATTCGTAAT-3′ 3′-CGAGCTTAACATGACGACATAATCGAACCAACAACCAAACA**CC**A**C**TTAAGCATTA-5′	50-bp oligoduplex substrate containing protospacer2 and PAM (oligoduplex without protospacer1)
s(+)SP1	5′-ATTACGAATT**C**A**CC**ATGTCCTCTTCCTCTTTAGCGTTTAGAATTTCAATTCGAGC-3′	55-nt ssDNA oligonucleotide substrate, (+) strand of SP1 oligoduplex
s(+) SP1-pΔ	5′-ATTACGAATTTGTCCTCTTCCTCTTTAGCGTTTAGAATTTCAATTCGAGC-3′	50-nt ssDNA oligonucleotide substrate, (+) strand of SP1-pΔ oligoduplex
s(+) SP2	5′-ATTACGAATT**C**A**CC**ACAAACCAACAACCAAGCTAATACAGCAGTACAATTCGAGC-3′	55 nt ssDNA oligonucleotide substrate, (+) strand of SP2 oligoduplex
s(–)SP1	5′-GCTCGAATTGAAATTCTAAACGCTAAAGAGGAAGAGGACAT**GG**T**G**AATTCGTAAT-3′	55-nt ssDNA oligonucleotide substrate, (–) strand of SP1 oligoduplex
SP1-20	5′-GCTCGAATTGCGCTAAAGAGGAAGAGGACAT**GG**T**G**AATTCGTAAT-3′ 3′-CGAGCTTAACGCGATTTCTCCTTCTCCTGTA**CC**A**C**TTAAGCATTA-5′	45-nt oligoduplex substrate containing 20 nt of protospacer1 and PAM
SPN	5′-GCTCGAATTGCCACCCAGCAAAATTCGGTTTTCTGGCTGAT**GG**T**G**AATTCGTAAT-3′ 3′-CGAGCTTAACGGTGGGTCGTTTTAAGCCAAAAGACCGACTA**CC**A**C**TTAAGCATTA-5′	55-bp oligoduplex substrate containing protospacerN and PAM

Protospacer sequence is underlined. PAM is in bold.