Cas9–crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria

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AUTHOR SUMMARY

Bacterial viruses (bacteriophages) pose a ubiquitous and deadly threat to bacterial populations. To survive in hostile environments, bacteria have developed a multitude of antiviral defense systems (1). Clustered regularly interspaced short palindromic repeats (CRISPR), together with CRISPR-associated genes (*cas*), constitute an adaptive microbial immune system that provides acquired resistance against viruses and plasmids. CRISPR systems are categorized into three main types (2). In type I and type III CRISPR systems, nucleoprotein complexes involved in CRISPR RNA (crRNA)-mediated silencing of foreign nucleic acids comprise large multisubunit aggregates. Here, we showed that in type II systems the silencing complex consists of a single protein (Cas9) that binds to crRNA to mediate sequence-specific cleavage of invasive dsDNA.

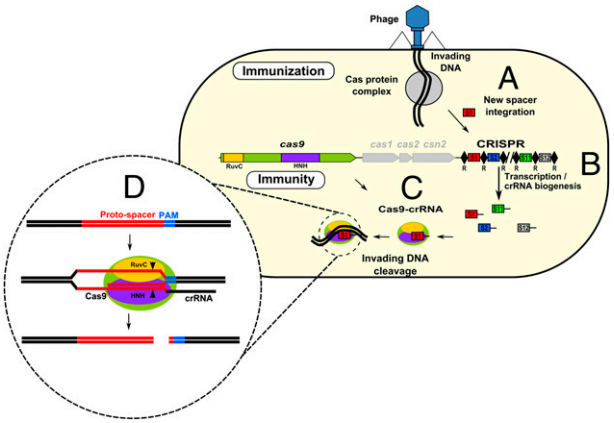

Fig. P1. The Cas9–crRNA complex generates two distinct DNA nicks on opposing dsDNA strands that match the loaded small, interfering crRNA sequence. (*A*) After phage DNA entry into the cell, a piece of the invading DNA is inserted as a spacer into the CRISPR locus. (*B*) The CRISPR repeat-spacer array is transcribed and processed into short crRNAs. (*C*) The crRNA forms a ribonucleoprotein complex with Cas9, which recognizes invading DNA homologous to the crRNA sequence and mediates interference. (*D*) Invading DNA cleavage by Cas9–crRNA. In the presence of Mg^{2+} ions, the signature Cas9 protein nicks each DNA strand 3 nt upstream of the PAM sequence to generate blunt DNA ends through RuvC- and HNH-like active sites that act on separate DNA strands.

The CRISPR/Cas system hijacks short fragments of invasive DNA called "spacers" and subsequently uses them as templates to generate specific small RNA molecules (Fig. P1). These RNAs combine with Cas proteins to form effector complexes that trigger degradation of foreign nucleic acid, thereby preventing proliferation and propagation of invasive genetic elements. CRISPR/Cas systems are categorized into three main types, which differ in the structural organization and function(s) of nucleoprotein complexes (2). In a typical type I system (as exemplified by *Escherichia coli*), crRNAs are incorporated into a multisubunit ribonucleoprotein (RNP) complex called "Cascade" (for "CRISPR-associated complex for antiviral defense"), which binds to the target DNA and triggers degradation by an accessory Cas3 protein (3). In type III systems (as exemplified by *Sulfolobus solfataricus* and *Pyrococcus furiosus*), the Cas RAMP module and crRNA complex recognize and cleave synthetic RNA in vitro (4, 5).

Type II CRISPR/Cas systems typically consist of only four Cas genes; however, their mechanism for DNA interference remains to be established. Here, we elucidated the molecular basis for the RNP complex involved in DNA silencing for type II CRISPR/Cas systems and characterized the mechanism of DNA cleavage. Specifically, we showed that in the CRISPR3 system of *Streptococcus thermophilus* (a model and active type II CRISPR/Cas system), Cas9 associates with crRNA to form an effector complex that specifically cleaves matching target dsDNA (Fig. P1), in sharp to the effector complexes for type I and type III systems, which are multisubunit ribonucleoprotein complexes.

We isolated the Cas9–crRNA complex and demonstrated that in vitro it generates a double-strand break at specific sites in target DNA molecules that are complementary to crRNA sequences and bear a short protospacer-adjacent motif (PAM) in the direct vicinity of the matching sequence. We showed that DNA cleavage is executed by two distinct active sites (RuvC and HNH) within Cas9 to generate site-specific nicks on opposite DNA strands. Sequence specificity of the Cas9–crRNA complex is dictated by the 42-nt crRNA, which includes a 20-nt fragment complementary to the protospacer sequence in the target DNA. Our data demonstrate that the Cas9–crRNA complex functions as an RNA-guided endonuclease with sequence-specific target site recognition and cleavage through two distinct strand nicks (Fig. P1).

Our present findings establish a molecular basis for CRISPR-mediated immunity in bacteria, specifically for type II systems, which rely solely on the signature Cas9 protein. Further, the simple modular organization of the Cas9–crRNA complex, in which specificity for DNA targets is encoded by a small crRNA and the cleavage machinery consists of a single, multidomain Cas protein, provides a versatile platform for the engineering of universal RNA-guided DNA endonucleases. Indeed, by altering the RNA sequence within the Cas9–crRNA complex, pro-

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

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grammable endonucleases can be designed for in vitro and in vivo applications, and we provide a proof of concept for this application. These findings pave the way for the development of molecular tools for RNA-directed DNA surgery. Specifically, this endonuclease system can be reprogrammed readily using customized RNAs to cleave dsDNA specifically, thus expanding the genome-editing enzyme repertoire beyond zinc finger nucleases and transcription activator-like effector nucleases.

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