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dnaK GENE EDITING IN THE *Escherichia coli* GENOME VIA THE CAS9/CRISPR SYSTEM

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ABSTRACT
The Cas9/CRISPR genetic engineering technology is the centerpiece of a robust adaptive bacterial immune system which contains clustered regularly interspaced palindromic repeats. CRISPR-associated protein-9 (Cas9) is part of this immune system that seeks out, cuts, and degrades viral DNA. By taking advantage of the Cas9/CRISPR system, a tool has been developed to delete or insert DNA into target genes in bacterial cells or mammalian cells to obtain cells with desired traits. In this study, we used the Cas9/CRISPR system for engineering the genome of a non-pathogenic, laboratory strain of bacteria, *Escherichia coli*. We introduced a deletion mutation in the dnaK gene of the bacteria which is responsible for DNA replication, thus allowing the bacteria to grow on a dnaK template containing a plate. We investigated the dnaK gene deletion efficiency of the Cas9/CRISPR genome editing system.

KEYWORDS: dnaK, Cas9/CRISPR, *E. coli.*

INTRODUCTION
Despite difficult conditions, such as predatory viruses, bacteria dominate many natural ecosystems, including inhospitable environments. To live, bacteria have devised a number of protection mechanisms, including the recently identified CRISPR system.[1] Clustered regularly interspaced short palindromic repeats, or CRISPR, is an acronym for clustered regularly interspaced short palindromic repeats. CRISPR loci contain short, partially palindromic DNA repeats that occur at regular intervals and form loci that alternate repeated elements (CRISPR repeats) and variable sequences (CRISPR spacers) (Figure 1).[19] Despite the fact that this unusual locus was discovered in 19872, little attention was paid to unusual loci, such as those described in the draft microbial genome.[3] These loci are typically flanked by accompanying CRISPR-associated (cas) genes. Their biological role was difficult until 2005 when three groups identified astronauts with homology to foreign genetic elements, including viruses and plaid. These reports led to the hypothesis that CRISPR may act as an immune system.[7] CRISPR-mediated immunity was quickly established, and subsequent research established that CRISPR-mediated immunity includes sequence-specific, RNA-mediated[9] targeting of mostly DNA[10], but also RNA.[11] Since then, many molecular-based studies have been conducted on the genetics, mechanisms and applications of the Cas9/CRISPR system.

The Cas9/CRISPR system and its components are generally hypervariable and differ significantly in terms of events, genes, sequence, number and size of different genomes. In fact, CRISPR repeats are typically 28 to 37 nucleotides in length and can partially form palindromic structures to form hairpin structures, but can vary widely (23 to 55 nucleotides). Similarly, CRISPR spacers are typically 32 to 38 nucleotides in size, but can vary in size (nucleotides 21-72). Arrays containing up to 588 repeats (in *Haliangium ochraceum*) are described quantitatively, but in most cases, there are less than 50 units. Similarly, up to 19 other loci have been identified in *Methanocalcococcus* and 25 putative CRISPR loci have been proposed in *Methanotorris igneus*, but organisms generally contain 1-2 CRISPR loci.[12] According to the CRISPRdb database, CRISPR is found in almost half of the bacterial genome (1126/2480 or ~45%) and in the majority of archaea (125/150 or ~83%).[12]

The Cas9/CRISPR system was divided into three main types, type I, type II and type III, and 12 subtypes, taking into account the genetic content and structural and functional differences.[13,14] The most important defining traits of Cas/CRISPR types and subtypes are genetically

distinct and highly functional diverse genes and proteins encoded by them that show many of the biochemical functions performed by the different stages of immunity mediated by CRISPR. RNA recognition motifs are common in many Cas proteins, and most Cas protein families have functional domains that interact with nucleic acids such as DNA binding, RNA binding, helicase and nuclease motifs.[13,14,15,16,17] Cas1 and cas2 are common genetic markers for various types and subtypes, while cas3, cas9, and cas10 have been identified as signature genes for type I, type II, and type III, respectively. Type II systems have only been found in bacteria so far, and there is a preference for type I systems in archaea and hyperthermophiles, according to phylogenetic analysis.

The sequence in the exogenous nucleic acid element corresponding to a CRISPR spacer has been defined as a protospacer[18], which is flanked by a system-specific, highly conserved CRISPR motif, subsequently renamed protospacer adjacent motif (PAM) (Figure 2).[19]

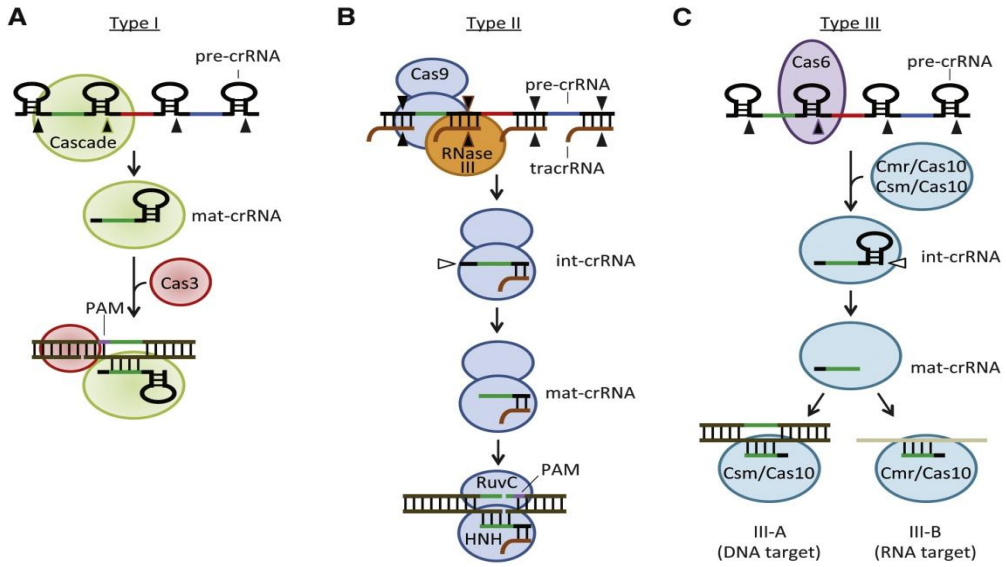

Figure 1: Mechanism of crRNA Biogenesis and Targeting in the Three Types of Cas9/CRISPR Systems Black arrowheads indicate primary processing sites of the crRNA precursor (pre-crRNA) to liberate intermediate crRNAs (int-crRNA). White arrowhead indicates further processing of the int-crRNA to yield mature crRNAs (mat-crRNA). Green line indicates target sequence (same sequence as crRNA spacer). Purple line indicates PAM.[19]

(A) In type I systems, primary processing of the pre-crRNA is achieved by the Cas6 endoribonuclease within the Cascade complex[9] (Brouns et al., 2008). Cleavage occurs at the base of the stem-loop formed by the repeat RNA to release mat-crRNAs. The Cascade recruits the Cas3 nuclease to nick the DNA strand complementary to the proto-spacer, immediately downstream of the region of interaction with the crRNA spacer.[20]

(B) In type II systems, primary processing requires the annealing of the tracrRNA to the repeat sequences of the pre-crRNA and the subsequent cleavage of the dsRNA by the host RNase III.[21] Primary processing occurs in the context of Cas9, and it is followed by the trimming of the 5′ end repeat and spacer sequences of the int-crRNA to yield mat-crRNAs. Target cleavage requires the crRNA, the tracrRNA, and the RuvC and HNH domains of Cas9, each of which cleaves one DNA strand of the protospacer region, 3 nt upstream of the PAM.[22,23]

(C) In type III systems, Cas6 cleaves the pre-crRNA to generate int-crRNAs that are incorporated into a Cmr/Cas10 or Csm/Cas10 complex, where further maturation occurs through the trimming of 3′ end sequences. While genetic evidence indicates that III-A subtypes cleave target DNA.[10,24] sequences[25], biochemical data suggests that subtype III-B cleave RNA molecules.[10]

The recent development of the Cas9 endonuclease for genome editing builds on more than a decade of basic research into the biological function of the mysterious repetitive elements now known as CRISPR (Figure 2).[25] CRISPR loci typically consist of a clustered set of CRISPR-associated (cas) genes and the signature CRISPR array—a series of repeat sequences (direct repeats) interspaced by variable sequences (spacers) corresponding to sequences within foreign genetic elements (protospacers) (Figure 3).[25] Whereas cas genes are translated into proteins, most CRISPR arrays are first transcribed as a single RNA before subsequent processing into shorter CRISPR RNAs (crRNAs), which direct the nucleolytic activity of certain Cas enzymes to degrade target nucleic acids.[25]

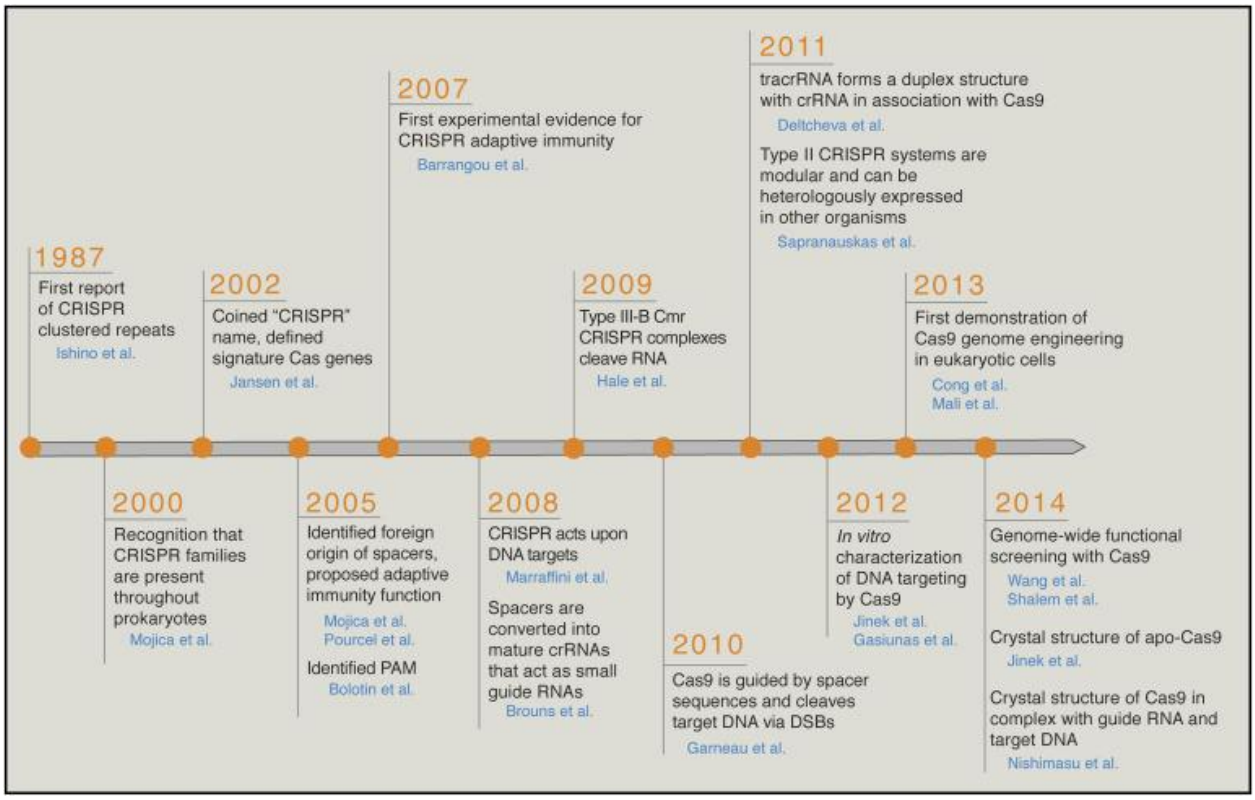

Figure 2: Key Studies Characterizing and Engineering CRISPR Systems.

Major HSPs (DnaK, GroEL) are molecular chaperones that assist in correct folding and assembly of proteins and are involved in diverse cellular processes, including DNA replication, UV mutagenesis, bacterial growth, RNA transcription and flagella synthesis.[26,27]

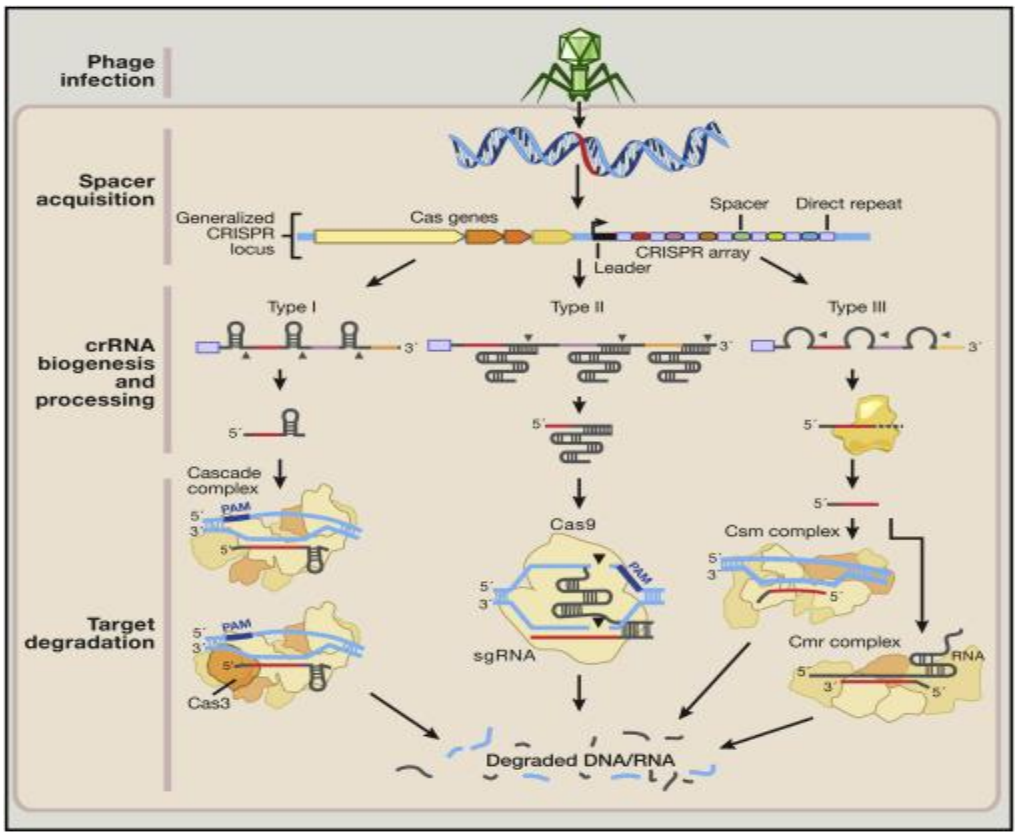

Figure 3: Natural Mechanisms of Microbial CRISPR Systems in Adaptive Immunity.

dnaK encodes a protein that is related to eucaryotic hsp70.[28] DnaK is 50% identical in amino acid sequence to eucaryotic Hsp70. There appear to be no other HSP70-related genes in the *E. coli* genome. DnaK was originally identified as a host gene necessary for lambda DNA replication.[29,30] DnaK appears to interact with the P protein of lambda, as mutations in the lambda phage allow it to grow in a dnaK-host map in the P gene. Biochemical experiments have confirmed and further defined its role in mitochondrial DNA replication. Six proteins are required for the localized unwinding of duplex DNA at the origin of replication, prior to the binding of DnaG primase: two lambda proteins--O and P and four host proteins-- DnaB (a helicase), DnaK, DnaJ and Ssb (single stranded binding protein). A complex of O, P, and DnaB forms at the origin.[31,32,33] The subsequent addition of DnaJ, DnaK, and Ssb proteins plus ATP results in an origin-specific unwinding of the DNA duplex. It is hypothesized that DnaJ and DnaK "loosen" the association between DnaB and P, so that DnaB is able to function as a helicase[33], thus permitting DnaG binding and subsequent DNA synthesis. The dnaK gene was so named because *E. coli* DNA synthesis shuts off when mutant cells are shifted to high temperatures. Until recently, however, there was no direct evidence that DnaK was involved in host DNA replication. Sakakibara[34] isolated a new dnaK allele, dnaKlll, during a screen designed to isolate mutants defective in the initiation of DNA synthesis. Mutations in dnaA (a gene required for DNA initiation) were known to be suppressed in the presence of rnh mutations, which encode RNAase H.[35,36] The suppression is thought to be due to the presence of some latent replication origins, which become active in the absence of RNAase H activity. DNAk111 was isolated as a temperature-sensitive mutant whose defects in DNA synthesis could be relieved upon inactivation of the rnh gene. The DNA mutant is unable to initiate a new round of DNA replication at a high temperature after the termination of the round already in progress. DNA synthesis in both the dnaK111 and dnaA mutants became temperature independent after the reintroduction of a wild-type rnh gene. Unlike DnaA mutants, DnaK111 mutants remain temperature sensitive to growth in the presence of an inactive rnh gene. The inability of DnaK111 to grow at high temperatures even in the absence of Rnh function suggests that this DnaK mutation causes pleiotropic effects. The defect in DNA synthesis can be corrected by inactivating Rnh, but other defects that cause cell death are not. These results imply that DnaK carries out multiple important functions in the cell. Other genetic analyses have shown that DnaK is essential at high temperatures, and perhaps at lower temperatures as well. Cells containing deletions of dnaK can not grow at 42°C.[37] The results at lower temperatures are more equivocal, because it appears that dnaK null mutants quickly acquire secondary mutations that allow more vigorous growth.[37] Original transductants are extremely filamentous; cells containing secondary mutations are less so. Since cells that do not undergo this change are incapable of continued growth at 30°C or grow very poorly, it is suggested that DnaK is necessary for growth at temperatures other than 42°C. In this study, we aimed to investigate Cas9/CRISR gene editing by targeting the dnaK gene.

MATERIALS AND METHODS

Materials

Cas9 Protein

The Cas9 protein is the engine of CRISPR. It binds the tracrRNA part of the gRNA and the gene targeted for editing. If a gene match is found, the Cas9 protein will make a double-stranded cut in the DNA. The cell responds to the cut by trying to repair the DNA damage. Cas9 only cuts, it does not do any actual gene editing. Instead, it tricks the cell into doing it.

guideRNA (gRNA)

The gRNA is a combination of CRISPR RNA (crRNA) and the trans-activating crRNA (tracrRNA) which are connected by a small nucleotide linker (GAA). Some people use the separate tracrRNA and crRNA. We used separate tracrRNA and crRNA. The tracrRNA part of the gRNA binds to the Cas9 protein and to the crRNA. Critically, the crRNA part of the gRNA matches (is complementary to) the DNA in the genome that we want to edit. This crRNA match is how the Cas9 protein recognizes where to cut.

Template DNA

dnaK gene's template DNA sequence was CGTAAACCACGTCATCAAGG. The sequence was 20nts long.

Bacterial Strain

Non-pathogenic E. coli bacteria DH5α.

Plasmids

cas9 Plasmid pBR322*Kan*^{r}
crRNA Plasmid pBR322*amp*^{r}
tracrRNA Plasmid pBR322*strep*^{r}

Media for Bacterial Growth

LB agar media.

Antibiotics

Kanamycin, Streptomycin and Ampicillin.

METHODS

Competent Cell Preparation: 'Competent' means the bacterial cells that can intake foreign DNA. Bacterial cells can be made competent by treating the cells with chemicals polyethylene glycol and calcium chloride. These chemicals increase the permeability of the cell wall and cell membranes, and also neutralize the negative charges of both DNA and cell membrane (thus minimizing repulsion) and hence facilitating the entrance of foreign DNA.

In this study, we used 10 % polyethylene glycol (PEG 3350) and 25mM calcium chloride to make the *E. coli* cells competent. A loop full of bacterial cells grown overnight on LB agar plates were suspended in 100µl of PEG3350 plus calcium chloride solution contained in a microfuge tube and kept on ice. Alternatively, we also used cells from the liquid culture in LB broth for competent cell preparation.

Transformation: To the two competent cells tubes kept on ice, 15µl of *cas9*pBR322*kan*^{r} and crRNAPlasmid pBR322*amp*^{r} and tracrRNAPlasmid pBR322*strep*^{r} mix was added, the tubes were flipped three times to mix the contents and put on ice. 10µl of template DNA was added to one tube and no template was added to other tube, content mixed as before and put on ice. The tubes were incubated on ice for 30 additional minutes.

Heat Shock Step and cell growth: The competent cell mix tubes were then transferred to a water bath set at 42°C and incubated for 30 sec. Then 500µl of LB broth was added to the tubes and incubated at 30°C for 2-4hrs. Then the cells (transformants) were plated on two LB agar plates with Kanamycin and Ampicillin antibiotics to determine whether gene editing has taken place or not.

RESULTS

First We collected two 10µl of *E. coli* bacteria in two microfuge tubes. Then added 25µl of PEG (40%) and 25µl of $CaCl_2$ (400%) into each tube. Finally, we added 5µl of Cas9 (34µg), 5µl of crRNA (20ng/µl), 5µl of tracrRNA (2µM) in each tube, and sufficient template DNA in one tube and in other tube no template DNA. Incubated at 37°C (1hr). Then kept on ice bath for 1 min and for 30sec at 45°C. Poured individual tube mixture on a LB broth plate with Amphicillin (100µg/ml), Streptomycin (100µg/ml), and Kanamycin (100µg/ml). Plates were incubated at 37°C for 24 hours. After 24 hours we found bacterial growth in the plate which contained antibiotic with added cas9, crRNA, tracrRNA and template DNA. No growth was found in the plate where no template DNA was added.

DISCUSSION

As we set out to use the CRISPR-Cas9 technology to edit the genome of *E. coli*, we decided to opt for the development of antibiotic resistance in *E. coli* instead of studying loss of resistance. This is because the selection of development of antibiotic resistance is much easier than selection of loss of antibiotic resistance. Screening of development of antibiotic resistance is done by allowing the bacteria grow on agar plates containing antibiotic whereas selection of loss of antibiotic resistance requires replica plating techniques which is cumbersome and not always conclusive.

We found no growth in our study when we didn't have template DNA. Due to a lack of dnaK template DNA, broken dnaK gene did not able to repair to come back to the wild form. dnaK gene is responsible for DNA replication. For this reason we found no growth in the LB media plate where template dnaK DNA was missing.

CONCLUSION

CRISPR-Cas9 genome editing has revolutionized all areas of biomedical sciences. It brings great promise in the application of this technology both in bacterial and mammalian systems. To fully realize the benefits of this advanced technology, it is imperative that this methodology is established and used in the different Universities and research laboratories of Bangladesh.

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