Metabolic engineering in bacteria using CRISPR–Cas9 genome editing tool

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ABSTRACT

Today, it is known that most microorganisms can produce important secondary metabolites for humans, most of which can be used as antibiotics, antifungals, anticancer, etc. Most of these secondary metabolites are produced in pathways where other accessory compounds are also synthesized by some active enzymes in this pathway. Therefore, if it is possible to prevent the synthesis of other side products by using genetic engineering or pathway engineering, the production of the main product can be increased. Among the genome editing tools, the CRISPR Cas9 gene editing system has become one of the most powerful tools with high efficiency in removing competing genes that exist in the pathway of secondary metabolite synthesis.

Keywords: Genome editing, CRISPR-Cas9, Metabolic engineering

1. INTRODUCTION

Secondary metabolites are biologically active molecules that have different functions other than the bacteria's immediate needs in their growth or propagation. Some of these compounds have toxic properties against competing species and sometimes help them to cope with difficult conditions. Due to their diverse role and effects, many secondary metabolites have sparked industrial interest[1]. The methods used for genome editing in bacteria have been developed and widely operated by researchers. However, some of these procedures are laborious, inefficient and require wide programing compared to simple gRNA design for CRISPR.

1.2 CRISPR system

Many bacteria and archaea have specific immune systems which protect them against bacteriophage infection and plasmid transfer [2]–[4]. During this immune activity, short fragments of foreign DNA are integrated into the specific part of the host DNA which belongs to clustered regularly interspaced short palindromic repeats (CRISPR) array, therefore, this record of foreign DNA

enables the host to prevent a re-infection by the known invader [5]–[7]. The competition between bacteria and invading phages have driven the appearance of six types of CRISPR-Cas system. CRISPR-Cas system, types I, II and IV are recognized as subunit effector complexes, while types II, V and VI are defined as a single-subunit effector [8], [9]. CRISPR/Cas9, derived from a type II CRISPR system from Streptococcus pyogenes (spCas9), has become one of the genome editing tools with a wide range of applications [10]. S. pyogenes Cas9 (SpyCas9) is a large DNA endonuclease which consists of 1,368 amino acids. The HNH domain cleaves the part of DNA that is complementary with the guide RNA sequence, whereas the RuvC nuclease domain is responsible for cutting the non-target DNA strand [10]–[12].

During the transcription of the CRISPR array, pre-CRISPR RNAs (pre-crRNAs) are created and then by enzymatic processing, the short mature CRISPR RNAs (crRNAs) are constructed [13]. crRNA contains a spacer, a short part of RNA at its 5' which complements with a foreign genetic segment and at its 3' end includes a fragment of the CRISPR repeat sequence. After that, the guide RNA is made by attaching crRNA to tracrRNA (a hairpin RNA transcribed from a repeat region) (figure 1). Another essential component for Cas9 activity is a short-conserved sequence motif (2–5 bp) found in the vicinity of the crRNA-targeted sequence on the invading DNA, known as a protospacer adjacent motif (PAM) which is a 5'NGG3' sequence. Recognition of the PAM site by Cas9 triggers melting double-strand DNA upstream of the PAM site [14]–[18]. Hybridization between crRNA and foreign DNA triggers Cas9 to the cleavage of the target DNA at the complementary sequence [19]–[21]. A remarkable aspect of the CRISPR–Cas system is the possibility to engineer custom crRNAs that guide Cas9 to cleave specific DNA sequences [22]–[24].

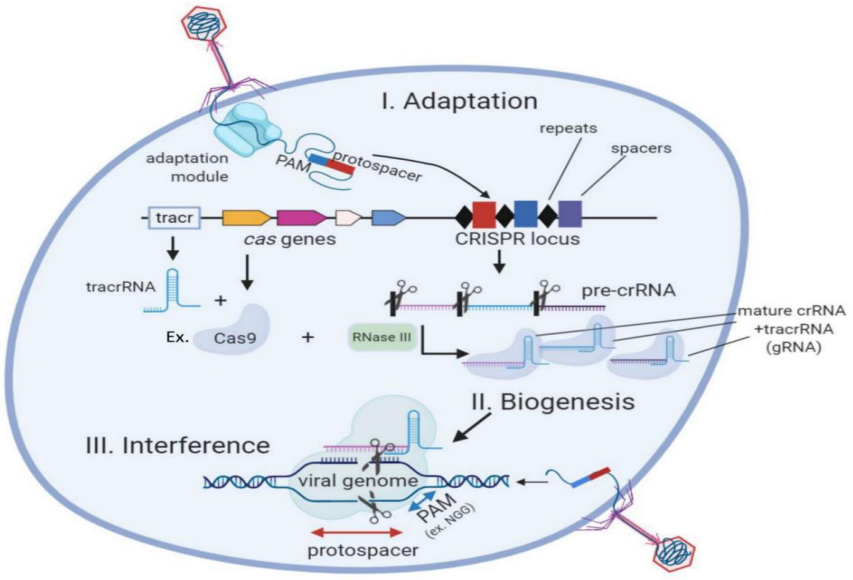

Figure 1: Three stages of the CRISPR system [25]

After the double-strand break (DSB) in the DNA generated by Cas9, the cell has two alternatives to repair its genome: homologous recombination (HR) or non-homologous end joining (NHEJ) (figure 2). These DNA repair strategies are quite mechanistically distinct and generate different repairing DSB repair products [26], [27]. Non-homologous end joining (NHEJ) is an error-prone mechanism that mostly used by higher eucaryotes. It leads to a partial resection of the DNA ends followed by ligation, process by which there are frequently bases lost. DSB repair by NHEJ is not present in most bacteria. DSB by homologous recombination process is suitable for precise genome editing, as it employs donor DNA sequences with homology to both sides of the DSB to supply genetic information to repair the DSB [28]–[30].

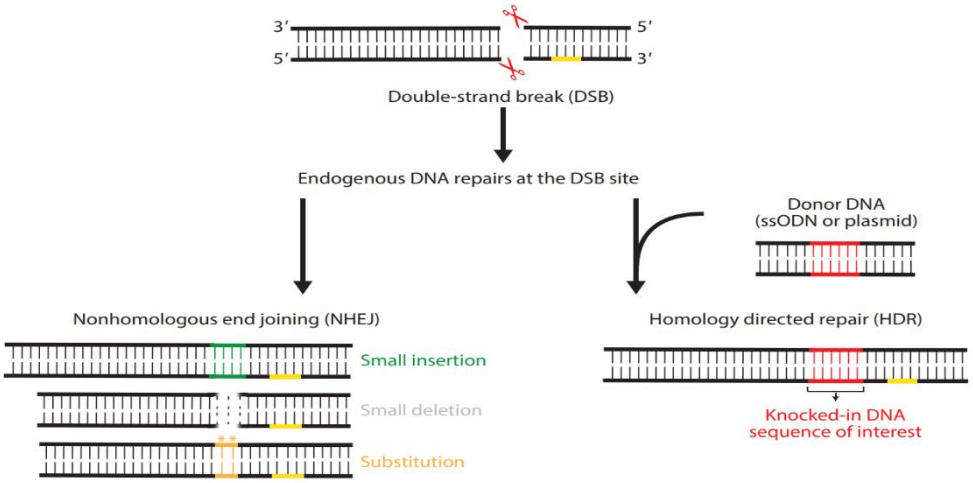

Figure 2: Double strand DNA break repair by NHEJ or HR [14]

Recent advances in using the CRISPR associated Cas system has allowed to utilize this system for precise genome editing and modification in large scale. The advantages of using this system such as high efficiency, low cost, highly customizable and highly versatile genome editing from large genome, have persuaded researchers to apply this system in their laboratories.

For example, CRISPR/Cas9 was used as a genome editing tool in the *Escherichia coli* MG1655 strain. First, the poxb gene was deleted and next, replaced with the *rfp41* gene in the strain. The *E.coli* DH5α was selected as the host for plasmid cloning as well. The results showed that when the homologous arm length is almost 297-298 bp and 101 bp the efficiency of gene editing would be reached 100% and 69.3% respectively [31].

CRISPR/Cas9 was adapted for editing in the *Bacillus subtilis* genome. In this way, the one-plasmid system was used to provide genome editing. The plasmid pJOE8999 is a shuttle vector that encodes a pUC minimal origin of replication for *Escherichia coli*, a temperature-sensitive replication origin of plasmid pE194ts for *B.subtilis*, a kanamycin resistance gene that works in both organisms, a Cas9 encoding gene under the control of the *B. subtilis* mannose-inducible promoter PmanP, and single guide RNA under a strong promoter as well. Utilizing pJOE8999, two deletions were introduced into the *B.subtilis* genome: deletion of the amyE

gene which has 25.1 kb and deletion of a 4.1 kb DNA fragment encoding the pulcherrimin biosynthesis gene. All deletions were confirmed by PCR [28].

CRISPR/Cas9 genome editing tool was also used for genome deletion and insertion in *Clostridium acetobutylicum*. To do this, two cas9-expression plasmids were constructed using the shuttle vector pFW01. In pCas9con, the cas9 gene was placed under the control of a constitutive miniPthl promoter while in pCas9ind it was under an inducible Pcm-tetO2/1 promoter. Then the upp gene, related to phosphoribosyl transferase, was targeted and was successfully removed. This deletion, verified by whole-genome sequencing in the mutant cells, enabled the strain to grow on solid media containing 5-fluorouracil [32].

Staphylococcus aureus is another species that was successfully engineered by CRISPR/Cas9. The pCasSA plasmid constructed by Golden Gate assembly, consists on the rpsL promoter, cas9 gene, single-guide RNA with cap 1A promoter, temperature-sensitive origin (repF), chloramphenicol and kanamycin resistance [33].

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