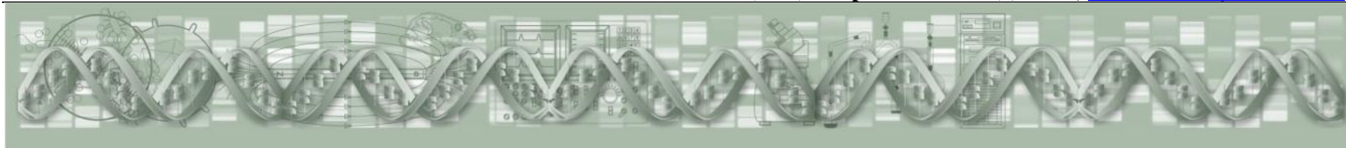

EVOLUTION OF CRISPR SYSTEM AND THEIR APPLICABILITY TO GENOME EDITING IN BACTERIA

IUSTIN-TIBERIUS MUNTEANU [1*]

[1] BioActive Research Group – Biochemistry and Molecular Biology Laboratory, Department of Biology, Alexandru Ioan Cuza University of Iasi, Iasi 700506, Romania
*tibimunteanu897@gmail.com

Abstract

Recombinant DNA and genetic engineering technologies have made it possible to manipulate and carry out important advances in biology. However, designing reliable, safe and accurate system for genome editing in prokaryotes and eukaryotes has been an issue for a long time. Enzymes (such as polymerases, ligases, and restriction endonucleases) and the polymerase chain reaction (PCR) provided ways to isolate genes and gene fragments, as well as to introduce mutations into genes *in vitro*, in cells and in a model organism. Today, the field of biology is undergoing a transformative phase with the advent of easy genome engineering using RNA-programmable CRISPR-Cas9. The CRISPR-Cas9 system is an immune system present in prokaryotes that allows the identification of exogenous DNA or RNA molecules. The Cas9 endonuclease is associated with a guide RNA molecule (gRNA) that allows it to form base pairs with target DNA or RNA sequencing, allowing Cas9 to introduce a site-specific double-strand break. Thanks to this breakthrough in genetic engineering Doudna Jennifer and Charpentier Emmanuelle were awarded the Nobel Prize in Chemistry in 2020 (Doudna and Charpentier., 2014). In what follows I will talk about the applicability of this system to edit bacterial genomes by punctiform, genetic and metabolic modifications.

Keywords: bacterial genome; genetic engineering; CRISPR-Cas9; evolution of the CRISPR system; punctiform (point mutation), genetic and metabolic modifications

TOWARDS AN UNIVERSAL GENE EDITING SYSTEM – CRISPR/Cas9

The CRISPR system (*Clustered Regularly Interspaced Short Palindromic Repeats*) is an adaptative immune system that exists in some microbial species of eubacteria and archaea bacteria and is activated when the cell is attacked by a virus or bacteriophage. This system is based on the recognition and destruction of exogenous DNA or RNA molecules introduced into the host cell. Thus 50% of eubacterial genomes and 87% of archaeal genomes sequenced to date have this defense immunity. In addition to the immune function, this has additional functions: it is involved in replication, adaptation of bacteria to different temperatures or environmental conditions, chromosomal rearrangements, and DNA repair. The CRISPR system was discovered in the genome of *Escherichia coli* in 1987 as a series of palindromic repeats fragments that ranged in length from 29 to 32 nucleotides. Subsequent studies to understand this system allowed the discovery of palindromic sequences that resembled each other in several groups of bacteria (*Mycobacterium tuberculosis*) and archaea (*Haloferax* and *Haloracula* genera). Subsequently, other gene adjacent to those involved in the CRISPR system, including Cas9, were identified, suggesting a functional association. Initially, it was hypothesized that this system might play a role in the replication process and the distribution of cellular DNA between daughter cells. In 2005, the first evidence emerged showing that the CRISPR-Cas system is part of an adaptative prokaryotic immune system – the same short palindromic sequences were identified in the sequence of genome belonging to bacteriophages. In 2007, a series of non-informational zones were discovered in the CRISPR coding *locus* of *Streptococcus thermophilus,* called spacers. In 2008, it was show that mRNA resulting from transcription of specific zones in the CRISPR sequence is matured to from crRNAs guiding the Cas complex in *Escherichia coli* (Rodrigues et al., 2019). The same year, the presence of the CRISPR-Cas system was founded in the pathogen *Staphylococcus epidermidis* (Doudna and Charpentier, 2014). In 2010 it was show that the Cas protein in *Streptococcus thermophilus* can make a single double-stranded break (DSB) in the DNA target molecule, and the next year it was reported that maturation of crRNA requires a small transcoded crRNA (tracrRNA), Cas9 and RNAase III in the *Streptococcus pyogenes* strain. Evidence for the functioning of the CRISPR system in a heterologous system was obtained in 2012 when it was show that the CRISPR-Cas system from *Streptococcus thermophilus* can be transferred into *Escherichia coli* where it provides heterologous immunity against plasmids, DNA and RNA molecules originating from other bacteria or viruses. Also in the same year, various laboratory modifications of the CRISPR-Cas9 system were produced in Streptococcus pyogenes. Those consisted of replacing a tracrRNA and a crRNA with a guide RNA (gRNA) molecule to guide the Cas9 protein to the target and obtain the cleavage. It was only in 2013 that the use of the CRISPR-Cas9 system was first described as a genome editing tool for inserting or correcting certain sequences at specific

Received 2022-07-25; revised 2022-08-22; accepted 2022-08-22; published online 2022-08-23; published 2022-12-23

sites in plant or animal DNA. This method has been extensively used in molecular genetics studies due to its simplicity, as the CRISPR-Cas9 system involves simply targeting the Cas9 endonuclease to the specific target site to be edited using a small gRNA molecule. The final process will allow a permanent modification of the target sequence by introducing a new gene or repairing some damage occurring at a specific site in the DNA molecules. The principles of operation of the CRISPR-Cas9 system, as well as strategies to modify Cas9 enzymes to attenuate non-specific cuts and to activate or inhibit a specific sequence in the genome, are being further investigated (Rodriguez et al., 2019).

Evolution and classification

To understand and classify CRISPR system is quite a difficult task due to the lack of universal Cas genes and their different ways of combining. Therefore, a multiple way of classifying CRISPR system has been developed that considers general aspects (such as gene arrangement and protein structure) for each type and subtype starting from the Cas1 protein (the best-known protein in CRISPR systems). The combined application of these criteria allowed an efficient classification of CRISPR system evolution. The new classification includes 2 classes, 6 types and 33 subtypes of CRISPR systems (Koonin et al., 2017). CRISPR-Cas systems are classified into two main classes and six types according to their mode of action. The first class uses multi-protein complexes for the detection of target sequences, while the second class relies on a single endonuclease and IV are defined s multi-subunit effector complexes, while types II, V and VI are considered as single-subunit effectors. Class 1 system include type I (Cas3), the most common and diversified, type III (Csm and Cmr) is a system present in many species of archaea and type IV very rudimentary and distant from the others. Effector complexes I and III – e.g. Cas5, Cas6 and Cas7 systems – have very elaborate architecture formed from RAMP proteins (Repeated Associated Mysterious Protein). The Cas7 system – having the derivates Csm3, Csm5, Cmr4, Cmr1, Csc2 and Csf2 CRISPR systems – is taken as an example in the exposition of the structure of the RAMP proteins because it is the largest and best-known group of class 1. Class 2 effector modules type II, V and VI have a much more compact and homogeneous organization (having only one protein) than those of class 1. Type II is represented by the Cas9 protein. Type V has been included in the classification scheme but remains currently uncharacterized, only the Cpf1 (derivate from Cas12a), C2c1 and C2c3 endonucleases have been fully elucidated. Only proteins of these types are currently used in genetic engineering, the others having exclusively immune roles. Type VI is represented by the Cas13a and Cas13b proteins capable of recognizing a single-stranded RNA molecules (Hille et al., 2018).

How does it work?

In bacteria and archaea, CRISPR (clustered regularly interspaced short palindromic repeats) and Cas (CRISPR-associated) protein are an adaptive immune system against viruses and other exogenous foreign molecules. Immune adaptation, which is achieved by spacer incorporation, begins with the identification of foreign genetic elements, which are then processed and inserted into the CRISPR array (with random choice). To avoid autoimmunity, it is essential that the adaptive system present in the bacterium can efficiently identify and recognize foreign molecules present in the cytoplasm. This is when the memory of old infections is created and why CRISPR-Cas immunity is an adaptive and heritable system. The CRISPR system (preceded by an AT-rich sequence called the leader region) is comparable as a genetic memory bank for bacteria. Spacer integration takes place in the CRISPR matrix component and thus preserves a chronological memory of previous infections (Hille et al., 2018). The CRISPR-Cas9 (Class 2, type II) complex is one of the most used system for gene editing binds to a tracrRNA (small RNA molecule that is complementary to CRISPR sequence) to form a tracrRNA:crRNA complex (about 100 pb) that will guide the Cas9 protein to the target site. Thus CRISPR-Cas9 achieves sequence-specific cleavage through a simple interaction of the crRNA with the recognition of the target site and the two DNA strands are cleaved by Cas9 protein – an HNH domain will cleave the complementary gRNA strand and RuvC domain will remove the non-primary gRNA strand. Genomic editing can be considered complete once the gRNA has been inserted and fully integrated into the new genome and the DNA molecule has been repaired again. Free phosphodiester ends (DSB - double strand break) within the genome trigger the DNA repair process by joining the adjacent 3' and 5' ends. This is the most delicate time because it can induce deletion or positional changes in different parts of the genome. Once the gRNA has been correctly inserted into the target site, the strands linked by covalent bonds and the DNA molecule repaired, the CRISPR editing process is deactivated. It is an extremely fast and efficient method because a single protein (Cas9) functions as a restriction enzyme (cleaving the DNA molecule at a specific site), as a ligase (attaching phosphodiester chains), as a reverse transcriptase (synthesizing the missing gRNA chain based on complementarity) and as a gyrase (spiralizing the DNA molecule again). The specificity of the CRISPR-Cas9 system increases when, in addition to the complementary gRNA sequences attached to the region of interest, it also requires a PAM (Protospacer Adjacent Motif) sequence that is located immediately after the target sequence. The action of the Cas9 protein is also strictly dependent on this PAM sequence (in the Cas9 system is rich in G) in achieving cleavage of the region of interest in the exogenous DNA molecule. In this way target sites are most frequently located for these short PAM sequences (5-6 nucleotides maximum or rarely 8) which signal the presence of the region of interest where the DNA is to be cleaved and consequently will decrease the possibility of cleavage at inappropriate or non-specific positions. These adjacent sequences will not be cut by CRISPR-Cas9, they will be

left in the genome because they are not part of the complementary gRNA spacer region. They have an exclusionary role only in signaling the sequence region of interest, which is why it is called the adjacent protospacer region (Redman et al., 2016). The structure of the CRISPR-Cas9 complex can be seen in figure 1.

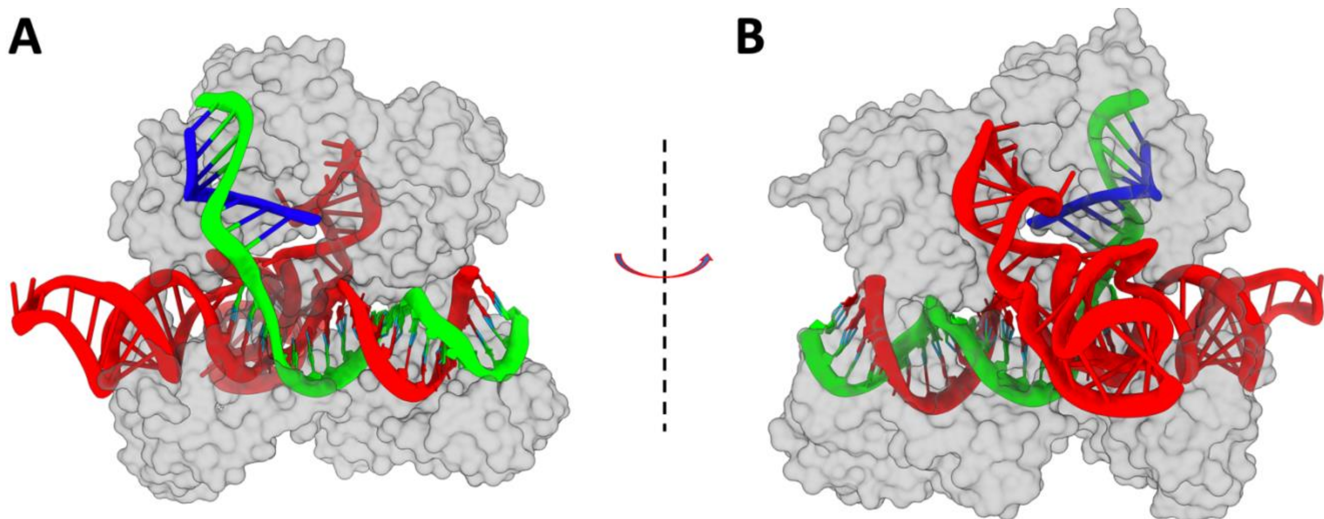

Figure 1. Structure of the CRISPR Cas9 system
A) The complex seen from the front **B)** The complex seen from behind
Gray: Cas9 protein
Red: gRNA
Green: target DNA strand
Blue: complementary Non-target DNA strand

PUNCTIFORME, GENETIC AND METABOLIC MODIFICATIONS

Progress in the application of CRISPR system for base editing

The ability to edit DNA sequences accurately and efficiently in the genome of cells has been a very important goal in science since the first demonstration of cloning using restriction enzymes. Most recently, gRNA-associated CRISPR-Cas system have helped to achieving this goal through their ability to produce a DNA double strand break (DSB) at a precise situs in the genome. One of the challenges of using the CRISPR-Cas9 editing technique is the off-target effect, in which the Cas9 enzyme misacts by cutting genes other than complementary gRNAs. Subsequently, it was realized that specificity for the region of interest is not as simple and rapid as originally assumed. After cutting the complementary sequence, phosphodiester free ends (DSBs) trigger the repair process, which can be either non-homologous and joining (NHEJ) or homology-directed repair (HDR) and is often responsible for insertions, deletions, translocations, and other unwanted internal rearrangements. It has been shown that mismatches of up to one base can be tolerated, but those exceeding 3-5 or more base pairs can cause changes along the DNA strand, leading to other problems in the information chain (Pawluk et al., 2016). To determine the efficiency of the CRISPR system, the target site must be sequenced to confirm whether a point mutation has occurred. We can examine messenger RNA (mRNA) and proteins by quantitative polymerase chain reaction (qPCR) and Western blot analysis, respectively. The performance of genome editing is usually verified by the Nuclease Surveyor assay or using next generation sequencing (NGS), including chromatin immunoprecipitation sequencing (Wu et al., 2018). Alternatively, accurate DNA modification can be achieved by providing a donor DNA sequence that encodes the desired modification to be introduced into the DNA. Homology-directed cellular repair (HDR) results in the incorporation of the sequence from the exogenous DNA template into the DSB. To solve this problem, the catalytically inactive Cas (Casi) protein has been developed, which is also associated with other programmable gRNA binding proteins (base editors) to recognize a target DNA sequence. This system has been programmed to act on single-stranded DNA (ssDNA) but not on double-stranded DNA. When identifying the target sequence on the DNA strand, based on the complementarity between the guide RNA and the target DNA strand results in the displacement of a small

segment of single-stranded DNA generating an 'R-loop' (Rees and Liu, 2018). Subsequently, different types of base editors (now up to the fourth generation) associated with the Cas9 protein capable of acting specifically at the DNA site were developed and refined (Vivien, 2018). Two principal classes of single-base editors have been characterized: the cytosine base editor (CBE) - which allows the conversion of a G-C base into a T-A base pair, and the adenine base editor (ABE) which acts in the opposite way of the CBE editor. CBE and ABE can mediate all four possible transition mutations (C to T, A to G, T to C and G to A). In RNA, the conversion of adenosine to inosine has also been developed using both antisense and gRNA targeting methods from CRISPR-Cas13 (Rees and Liu, 2018). This nucleotide conversion occurs without the need for a double-stranded cut performed by the fully active CRISPR-Cas9 nuclease, without recourse to the DNA repair mechanisms that follow double-strand breaks (without DSB) or using a donor template. Some argue that base editing is an easier way to deliver the editing system into cells, and some evidence suggests that base editing makes fewer unwanted insertions and deletions than classical CRISPR-Cas9 (Vivien, 2018).

An example of this is the application of CRISPR-dCas9 mediated CBE base editing in *Bacillus subtilis 168*. In this case, the CRISPR-dCas9 system (a variant of Cas9 from catalytically deficient *S. pyogenes*) and an activation-induced cytidine deaminase (AID) were used. This method, which has an edit window up to a maximum of 5 nt in positions 17-18, allowed three to four loci to be edited simultaneously (multiplexed editing) with almost 100% efficiency. To test the applicability of this new system, a target plasmid pBAC-dCas9-AID-amyE-18 (a plasmid system expressing both dCas9-AID and sgRNA, but lacking a donor foreign template DNA and without the production of a double-strand break) was constructed and introduced into *B. subtilis* to obtain positive strains. The CRISPR/dCas9-AID system was used here to induce point mutations and inactivate certain genes encoding the amino acids CGA (Arg), CAG/CAA (Gln) on the sense strand of DNA by transforming them into TGA, TAG and TAA codons or by targeting the CCA codon on the antisense strand of a target gene to modify one or both G nucleotides to A within a TGG (Trp) codon. It has been noted through bioinformatics analysis that 569 genes in *B. subtilis 168* cannot be modified by this system, most likely that the underlying problem being in the sequence specificity of PAM. The same system was also applied in *Corynebacterium glutamicum* strain (important for amino acid production) introducing mutations along the DNA strand at positions -16, -19, -20 but with less than 50% efficiency than in *B. subtilis 168*. It is interesting to look for the reason why different changes occur in certain microorganisms when the same editing system is used. The most plausible hypothesis is that the CRISPR-dCas-AID system is toxic to *C. glutamicum* and induces random changes/disruptions along the DNA strand. Various methods are still trying to further refine and improve this type of unique base editing system that may become applicable to more strains in the future (Yu et al., 2020).

Another example of applications of CRISPR-Cas9 system usage is in *Clostridium beijerinckii NCIMB 8052* (biotechnologically important for butanol production). In this study, the CRISPR-Cas9 system was used to select genetically modified strains against unmodified wild-type cells. Plasmids used for either deletion or insertion contain certain key elements, namely: the selection marker erythromycin for selection of *Clostridium* transformants, the CRISPR-Cas9 system and the target DNA to be modified. The mutant is obtained by homologous double cross recombination. The Cas9 system, which is controlled by a lactose-inducible promoter, remains inactive in the initial transformants. Subsequently (after the desired homologous recombination has occurred), Cas9 expression is also induced by the addition of lactose to the culture medium. The gRNA is made to target the DNA sequence that is disrupted during the homologous recombination phase. Hence, such a target sequence exists only in the wild-type cell, but not in the mutant cell. Thus, the Cas9 system can be directed to produce double strand breaks on the chromosome of wild-type cells causing their death. Mutant cells can survive such a process and can thus be easily selected. This method adapted for species belonging to the genus *Clostridium* was effective for any type of single nucleotide modification (SNM) (Zhang et al., 2018). A similar modality was also tested in *E. coli* using CRISPR-Prime Editing (CBE and ABE systems, discussed a little earlier) without inducing double-stranded DSB breaks, proving to be versatile with single nucleotide editing as well. This system can generate substitutions, deletions, insertions and combinations into both plasmids and the *E. coli* chromosome with very high precision. It has been reported that the efficiency of 1-bp deletions is up to 40%. This system has been widely used as a counter-selection tool to remove unchanged cells from a diverse recombinant population. CRISPR Prime Editing is a powerful and versatile addition to the gene editing kit not only for *E. coli* but also for other organisms and can then be enhanced by modifying the specificity of PAM sequences (Tong et al., 2021).

Slowly the CRISPR-Cas9 system is being replaced by the Cas-12a (Cpf1 - intensively applied to species of the genus *Corynebacterium*) system. It is much more efficient, versatile, accurate and does not require an antibiotic resistance marker (so easier for gene manipulation). Such a system has been produced to generate point mutations in the genome and plasmids of *Yersinia pestis* and *Mycobacterium smegmatis* species. Studies have shown that this system is very efficient to insert both large (20 bp) and point fragments into the genome/plasmid of the species of interest (Yan et al., 2017). The CRISPR-Cpf1 system has been found in seven bacterial species such as *Francisella novicida*, *Prevotella disiens*, *Acidaminococcus spp*., *Lachonospiraceae bacterium*, *Candidatus Methanoplasma termitum*, *Moraxella bovoculi* and *Porphyromonas crevioricanis*

(Safari et al. 2019). Like Cas9 (Class 2, type II), the Cpf1 endonuclease (Class 2, type V) can be programmed to target DNA sites of interest by complementing a gRNA molecule. However, Cpf1 possesses several features that distinguish it from Cas9 and could provide for the expansion of punctiform genome editing tools. First, Cpf1 is guided by a single gRNA molecule, whereas Cas9 uses one gRNA and a second trans-acting RNA molecule, thus forming the tracrRNA:crRNA complex. For this reason, the gRNA molecule in Cpf1 averages 42-45 base pairs in length, and the Cas9 system is about 100 base pairs long. Second, Cpf1 recognizes thymine-rich (5'-TTTN-3') PAM sequences, while Cas9 recognizes guanine-rich sequences. Third, after the PAM sequence has been recognized, Cpf1 will perform a cleavage resulting in sticky ends, while Cas9 will cut the DNA molecule generating blunt ends. Therefore, Cas9 will use NHEJ (Non-homologous and joining) to repair the DNA strands that result from cleavage. In the case of Cpf1, the cut is 18-23 base pairs from the PAM sequence, while in Cas9 it is 8-10 base pairs from the adjacent protospacer sequence. Lastly Cpf1 contains the RuvC nuclease domain (initiates cleavage of DNA strands that are not complementary to gRNA) but lacks a second domain, whereas Cas9 uses both the HNH and RuvC endonuclease domains in cleaving the target strand and removing the non-complementary gRNA. Together these observations explain the differences and why the Cas9 system is being replaced by Cpf1 (Yamano et al., 2016) (Vegenas et al., 2019).

Genetic and metabolic modifications

The interest in precisely controlling gene expression in bacteria is attractive for understanding gene function that play a major role in prokaryotic metabolism. Controlling and manipulating genes is a very good way to control the metabolic pathways, function, and interaction between different genes. Moreover, gene expression studies have allowed us to understand the evolution and different phylogenetic relationships between bacteria and to refine genetic engineering to enhance the production of biotechnologically important secondary metabolites. This is extremely useful for precisely controlling the expression of each specific protein. Control of gene expression was first applied in the 1900s to eukaryotes, then in the early 2000s to prokaryotes, and then in the 2010s CRISPR systems began to be tested and used to modify and control genes with very high precision (Vigouroux and Bikard, 2020).

The largest domain where the CRISPR-Cas9 system has been tested is to produce gene and metabolic changes in the bacterial chromosome by introducing, modifying, activating, or inhibiting specific genes. Modifications of 24 - 86 nucleotides have been reported in the *E. coli* genome. Recently, a pRed_Cas9_ΔpoxB300 plasmid capable of inducing modifications of over 300 bp with 100% efficiency has been constructed. Other ΔpoxB100 and ΔpoxB50 plasmids have been developed to test whether they can produce 100 and 50 bp mutations respectively. In this case the efficiency decreased dramatically from 100% to 69.3% and 0% respectively for the plasmid with a 50 bp mutation. This can be explained by the fact that the Cas9 protein is programmed to produce mutations from a few bp to kb. However, to produce mutations of shorter lengths (below 50 bp), the Cpf1 endonuclease must be used, which is much more precise and efficient for short sequences (Zhao et al., 2016). The CRISPR-Cas9 system produces a double strand break (DSB) often causing complete inactivation of genes or random introduction of nucleotides. Two CRISPR systems have recently been developed for metabolic editing in bacteria. The first is catalytically inactive dCas9 - death Cas9 which is obtained by a mutation (Asp10/His840 in Ala) to inactivate the HNH and RuvC domains. Such gRNA-guided binding of dCas9 to a specific DNA locus can inhibit the activity of RNA polymerase (RNAP) to the downstream gene preventing transcription and repress the expression of genes. This system, known as CRISPRi (interference) using the inactive dCas9 protein, is often used to silence genes of interest (Cho et al., 2018). Initially the CRISPRi system was used to reduce the pathogenicity of dangerous bacteria such as *Yersinia*, *Mycobacterium, Klebsiella* and *Vibrio*. Scientists are testing the CRISPRi system as a modern technique to develop a new vaccine method. This method has been applied to *Streptococcus pneumoniae D39* proving effective in silencing murT and gatD genes (responsible for peptidoglycan synthesis) and tarP and tarQ (involved in the polymerization of teichoic acids) (Zhang et al., 2021). An application of the CRISPRi system to inactivate a gene responsible for pathogenicity in *Klebsiella pneumoniae* (recent emergence of drug- and carbapenem-resistant strains) has proven highly effective. The pCasKP-pSGKP plasmid (encoding the CRISPRi system) capable of modifying and silencing genes (Gam, Bet and Exo) conferring drug resistance has been developed (Wang et al., 2018). *Corynebacterium glutamicum* is a bacterium extensively used and tested in molecular biology laboratories for its importance in the production of amino acids, recombinant proteins and secondary metabolites useful in medicine. Different studies have conducted to develop plasmids encoding the CRISPRi system to inactivate genes that are responsible for inhibiting and slowing down the expression of genes important for exploiting biotechnologically useful compounds. In this case, a multiplexed plasmid (containing the dCas9 gene from *Streptococcus pyogenes* and targeting several genes simultaneously) was developed capable of enhancing L-arginine production by silencing genes that slow the expression of argininosuccinate lyase (ArgH), phosphoglucose isomerase (Pgi). ArgH catalyzes the conversion of L-argininosuccinate to L-arginine and fumarate, and Pgi isomerizes fructose-6-phosphate to glucose-6-phosphate in the glycolytic pathway (Gauttam et al., 2019).

One of the most recent applications of the CRISPR/deadCas9 system has been applied to species belonging to the *Arthrobacter* genus (*A. agilis* and *A. bussei*). In this case a plasmid (pCasiART) was developed to silence the gene (CrtB) encoding the

phytoene synthase protein - the first enzyme involved in bacterioruberin biosynthesis. The latter is a rare C50 carotenoid that is synthesized by bacteria as an adaptive response to cold. Thus, the pCasiART plasmid was used to block expression of the enzyme involved in bacterioruberin biosynthesis. The structure of the pCasiART plasmid is as follows: the hdnO promoter of the 6-D-hydroxynicotine oxidase (hdnO) gene in the pAO1 plasmid to express the inactive Cas9 complex (Casi9 - inactive system taken from *Streptococcus pyogenes* with Asp10 and His840 mutation in Ala); BsaI restriction site for Golden Gate assembly; lacZ' (spacer insertion site) gene for β-galactosidase synthesis; KmR marker for kanamycin resistance; ColE1 replication origin (from *E. coli*) and the pCG100 fragment from *Corynebacterium glutamicum* ATCC 13058 required for the recombinant plasmid to replicate in *Arthrobacter* species. To demonstrate and test the efficiency of the pCasiART plasmid, both *Arthrobacter* wilde type (control) and recombinant plasmid cells were grown at different temperatures. At 20°C, species with the pCasiART plasmid had a growth retardation (at the same time the amount of bacterioruberin decreased dramatically - 0.2 μg per 1 g cell dry weight) while at 10°C their growth on culture media was absent. This demonstrates that the CRISPR/dCas9 system to silence and block the transcription of the CrtB gene worked (Flegler and Lipski., 2022).

The second system is called CRISPRa (activated) used in this case to activate gene expression. In this case dCas9 is associated with a transcriptional activating factor that targets the specific gene by activating the RNAP thereby favoring gene expression. The main transactivating factors include: *Herpes simplex* virus viral protein 16 (VP16) or several variants of this protein, such as VP64 and VP160 (Zheng et al., 2019). Numerous examples can be reported recently for metabolic editing by mutations or using the CRISPRi and CRISPRa systems. The ThermodCas9 system (dCas9 system active at 55°C) was applied to *Bacillus smithii* and the classical Cas9 system was applied to *B. licheniformis* for deletion of one gene – BacABC (42.7 kb) with an efficiency of 79% and insertion of one gene with an efficiency of 75.5%. The use of the CRISPRi system has been reported in many other species (biotechnologically important for the production of secondary metabolites, amino acids, toxins – such as botulinum, antibiotics or various biochemicals), for example: *Clostridium beijerinckii, Clostridium acetobutylicum, Clostridium botulinum, Corynebacterium glutamicum, Mycobacterium tuberculosis, Pseudomonas putida, Pseudomonas aeruginosa, Pseudomonas fluorescens, Staphylococcus aureus, Streptomyces coelicolor A3, Streptomyces griseus, Streptomyces hygroscopicus* and *Escherichia coli* (Cho et al., 2018). One of the classic examples in the application of CRISPRi was tested on *E. coli* being the most representative model for testing new CRISPR systems (Fontana et al., 2020). This strain is used for various reasons to produce useful substances such as: biochemicals, biofuels, including fatty acids, amino acids, polyketides, 1,4-butanediol, polyesters, and flavanones (Cho et al., 2018). An interesting complex association between the CRISPRa and CRISPRi systems (namely Inductible Cas-system) has been developed in *Pseudomonas putida KT2440*. It was developed to increase the efficiency of expression of the gene of interest while blocking other genes that interfere with the main biosynthesis pathway for biopterin and mevalonate (very important being biopterin which participates as a cofactor for neurotransmitters like dopamine, norepinephrine, epinephrine and serotonin). This type of combination has been shown to increase the production of these two biotechnologically important compounds by more than 40% (Kiattisewee et al., 2021).

Recent studies on genome editing in bacteria by perfecting endogenous CRISPR systems has been intensively applied to lactic acid bacteria belonging to the genera *Lactobacullus* and *Bifidobacteria.* Endogenous CRISPR-Cas systems can be redesigned to enhance gene expression or provide new properties by improving colonization of the human intestine, or alternatively, they can be modified to enhance probiotic properties or improve therapeutic potential for vaccine delivery or modulation of host immune response. One of the goals of improving *Bifidobacterium* species is to catabolize indigestible oligosaccharides, enhancing their ability to colonize the body and grow on complex carbohydrates in the large intestine (Cantabrana et al., 2017). Various studies attempt to use different multiplexed CRISPR systems (Vo et al., 2021) - thus capable of modifying multiple genes at once, such as the ability to withstand high concentrations of bile salts, tolerate acids, increase specificity and adhesion to substrate pili (increasing colonization capacity), and store major energy via the glycogen synthesis pathway (Cantabrana et al., 2017). Various techniques attempt to modify probiotic gut bacteria as a way of delivering vaccines safely, effectively and without unwanted side effects to both humans and animals. These lactic acid bacteria can also be used to remove proteins that confer resistance to pathogenic species such as *Staphylococcus aureus*. This technique can be used to eliminate both species dangerous to the human body (*Clostridium difficile*) and species responsible for food spoilage (*L. buchneri* - responsible for pickle spoilage) (Cantabrana et al., 2017).

CONCLUSION

The CRISPR system is an adaptive immune complex present in bacteria. This system has been extended and applied in the last decade to both prokaryotes and eukaryotes for genome editing. The versatility, ease and accuracy of this system has led to a single protein (Cas9) being used as a universal genome editing system for various biotechnological, medical, and pharmaceutical purposes. Recently, variants of the classical CRISPR-Cas9 system (Cas9i and Cas9a) have been developed and refined to increase the specificity and accuracy of the Cas9 system. Studies are still under development to correct non-specific

cuts (by modifying PAM sequences) and reduce the toxicity of this system when introduced into other cells (Li and Peng., 2019).

Acknowledgements

This work was supported by a grant from the Romanian Ministry of Education and Research, CNCS - UEFISCDI, project number PN-III-P4-ID-PCE-2020-0656, within PNCDI III.

Thanks to the BioActive Research Group for giving me the opportunity to work in the laboratory of the Faculty of Biology and test the CRISPR system in *Paenarthrobacter nicotinovorans*.

REFERENCES

Cantabrana C., O'Flaherty S., Barrangou R. (2017): CRISPR-based engineering of next-generation lactiv acid bacteria. *Current Opinion in Microbiology*. Vol. 37, pp: 79-87. http://dx.doi.org/10.1016/j.mib.2017.05.015

Cho S., Shin J., Cho B-K. (2018): Applications Of CRISPR/Cas system to bacterial metanolic engineering. *International Journal of Molecular Sciences*. https://doi: 10.3390/ijms19041089

Doudna J.A and Charpentier E. (2014): The new frontier of genome engineering with CRISPR-Cas9. *Science* Vol. 346, Issue 6213. https://doi: 10.1126/science.1258096

Flegler A. and Lipski A. (2022): Engineered CRISPR/Cas9 System for Transcriptional Gene Silencing in Arthrobacter Species Indicates Bacterioruberin is Indispensable for Growth at Low Temperatures**.** *Current Microbiology.* https://doi.org:10.1007/s00284-022-02887-5

Fontana J., Yager D.S., Zalatan J.G., Carothers J.M.V (2020): Challenges and opportunities with CRISPR activation in bacteria for data-driven metabolic engineering. *Current Opinion in Biotechnology.* Vol. 64, pp: 190-198. https://doi.org/10.1016/j.copbio.2020.04.005

Gauttam R., Siebold G.M., Mueller P., Weil T., Weiss T., Handrick R., Eikmanns B.J. (2019): A simple dual-inducible CRISPR interference system for multiple gene targeting in Corynebacterium glutamicum. *Plasmid.* Vol. 103, pp: 25-35. https://doi.org/10.1016/j.plasmid.2019.04.001

Hille F., Richter H., Bratovic M., Ressel M., Charpentier E. (2018): The Biology of CRISPR-Cas: Backward and Forward. *Cell.* https://doi.org/10.1016/j.cell.2017.11.032

Kiattisewee C., Dong C., Fontana J., Sugianto W., Yahya P., Carothets J.M., Zalatan J.G. (2021): Portable bacteria CRISPR transcriptinal activation anables metabolic engineering in Pseudomonas putida. *Metabolic Engineering*. Vol. 66, pp:283-295. https://doi.org/10.1016/j.ymben.2021.04.002

Koonin E., Marakova K., Zhang F. (2017): Diversity, classification and evolution of CRISPR-Cas System. *Current Opinion in Microbiology.* Vol. 37, pp: 67-78. https://doi.org/10.1016/j.mib.2017.05.008

Li Y and Peng N. (2019): Endogenous CRISPR-Cas system-based genome editing and antimicrobials: review and prospects. *Frontiers in Microbiology*. https://doi.org/10.3389/fmicb.2019.02471

Pawluk, A., Amrani, N., Zhang, Y., …, Maxwell, L. (2016): Naturally Occouring Off-Switches for CRISPR-Cas9. *Cell* 167(7), 1829-1838. https://dx.doi.org/10/1016/j.cell.2016.11.017

Redman, M., King, A., Watson, C., King, D. (2016): What is CRISPR-cas9? *Archives of Desease in Childhood Education and practice Edition* 101(4): 213-215. https://doi:10.1136/archdischild-2016-310459

Rees H.A., Liu D.R. (2018): Base editing: Precision chemistry on the genome and transcriptome of living cells. *Nature Review Genetics*. Vol. 19, pp: 770-788. https://doi.org/10.1038/s41576-018-0059-1

Rodriguez D., Solis R., Elizondo E., Barrera-Saldana H. (2019): Genome editing: A perspective of the application of CRISPR-Cas9 to study human diseases (Review*). International Journal of Molecular Medicine* 43(4), pp. 1559-1574. https://doi:10.3892/ijmm.2019.4112

Safari, F., Zare, K., Negahdaripour, M., Mowahed, M. Ghasemi, Y. (2019): CRISPR Cpf1 proteins: structure, function and implications for genome editing. *Cell and Bioscience* 9(36). https://doi.org.10.1186/s13578-019-0298-7

Tong Y., Jorgensen T.S., Whitford C.M., Weber T., Lee S.Y. (2021): A versatile genetic enigeenering toolkit for based on CRISPR-prime editing. *Nature Communications*, article No.5206. https://doi.org/10.1038/s41467-021-25541-3

Vagenas K.G*.,* Jarrczynska Z.D., Strucko T., Mortensen U.H (2019): Cpf1 enables fst and efficient genome editing in Aspergilli. *Fungal Biology and Biotechnology*. https://doi.org/10.1186/s40694-019-0069-6

Vigouroux A. and Bikard D. (2020): CRISPR Tools to control gene expression in bacteria. *Microbiology and Molecular Biology Reviews.* Vol.40, No.2. https://doi.org/10.1128/MMBR.00077-19

Vivien M. (2018): Base editing a CRISPR way. *Nature methods*. Vol. 15, pp: 767-770

Vo P., Ronda C., Klompe S., Chen E., Acree H., Sternberg S. (2021): CRISPR RNA-guide integrases for high-efficiency, multiplexed bacterial genome engineering. *Nature Biotechnology*. https://doi.org/10.1038/s41587-002-00745-y

Wang Y., Wang S., Chen W., Song L., Zhang Y., Shen Z., Yu F., Ji Q. (2018): *CRISPR-Cas9 adn CRISPR assisted cytidine deaminase enable precise and efficient genome editing in Klebsiella pneaumoniae.* Applied and Environmental Microbiology, Vol. 84, Issue 23. https://doi.org/10.1128/AEM.01834-18

Wu W., Yang Y. and Lei H. (2018): Progress in the application of CRISPR: from gene to base editing. *Medicinal Research Reviews*. https://doi.org/10.1002/med.21537

Yamano, T., Nishimasu, H., Zetsche, B., …, Nureki, O. (2016): Crystal Structure of Cpf1 in a complex with Guide RNA and Target DNA. *Cell* 165(4), 949-962. https://dx.doi.ors/10.1016/jcell.2016.04.003
Yan M., **Yan H., Ren G., Zhao J., Guo X., Sun Y.** (2017): CRISPR-Cas12a-Assisted Recombeneering in Bacteria. *Applied and Environmental Microbiology – American Society of Microbiology.* Vol. 83, No. 17. https://doi.org/10.1128/AEM.00947-17
Yu S., **Price M., Wang Y., Li Y., Guo Y., Ni X., Rosser S.J., Bi C., Wang M.** (2020): CRISPR-dCas9 Mediated Cytosine Deaminase base Editing in Bacillus subtilis. *ACS Pubblications – Synthetic biology, American Chemical Society*, pp: 1781-1789. https://doi.org/10.1021/acssynbio.0c00151
Zhang Z., Bonilla P., Seo S., Lu T., Jin Y., Blaschek H., Wang Y. (2018): Bacterial genome editing with CRISPR-Cas9: Taking Clostridium beijerinckii as an example. *Synthetic Biology.* Vol. 1772, Cap. 17, pp: 297-325. https://doi: 10.1007/978-1-4939-7795-6_17
Zhang R., Xu W., Shao S., Wang Q. (2021): Gene silencing through CRISPR interference in bacteria: current advances and future prospects. *Frontiers in Microbiology*. https://doi.org/10.3389/fmicb.2021.635227
Zheng Y., Su T., Qi Q (2019): Microbial CRISPRi and CRISPRa system for metabolic engineering. *Biotechnology and Bioprocess Engineering*. Vol 24, pp: 579-591. https://doi:10.1007/s12257-019-0107-5
Zhao D., Yaun S., Xiong B., Sun H., Ye L., Li J., Zhang X., Bi C. (2016): Development of a fast and easy method for Escherichia coli genome editing with CRISPR-Cas9. *Microbial Cells Factories*. https://doi. 10.1186/s12934-016-0605-5