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[TOOLS AND APPLICATIONS OF CRISPR-CAS IN PRO- AND EUKARYOTES]

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-Cas systems are an acquired and adaptive immune system of archaea and bacteria. By using a small guide RNA (sgRNA) and a single protein or a cascade of proteins they are able to target and degrade exogenous DNA or RNA. This bacterial defence system since has been studied extensively, which has lead to several key discoveries enabling its use as a programmable molecular tool. CRISPR-Cas systems allow scientists to perform insertions, deletions and translocations with high target specificity. With modified Cas proteins, several more tools have been developed that allow control of gene expression or visualization of chromosomes. In this review the possible tools and applications of CRISPR-Cas in both prokaryotes and eukaryotes are shown and discussed.

Introduction

Since the discovery of the Clustered regularly interspaced short palindromic repeats(CRISPR)-Cas9 system in 2005, research has grown rapidly and it has become a new topic of study for molecular biology (Ledford, 2016; Sander & Joung, 2014). By the end of 2014 some 600 research papers had been published that mentioned CRISPR-Cass9 (Ledford, 2015). Over that short period of time CRISPR-Cas9 already had an enormous impact on molecular biology and especially the biomedical sciences (Hammond et al., 2016; Maxmen, 2015). In this review I will discuss the mechanism of the CRISPR-Cas9 system and its potential in prokaryotes and eukaryotes.

Mechanism

CRISPR-Cas9 is a mechanism that acts as an acquired immune system, and has been found to be present in 47% and 86% of bacteria and archaea respectively (Grissa, Vergnaud, & Pourcel, 2007). It does so by degrading foreign DNA that has been inserted by bacteriophages or DNA obtained from other sources such as bacterial conjugation or natural transformation (Boyaval, Moineau, Romero, & Horvath, 2007; Marraffini & Sontheimer, 2008, 2011). CRISPR-Cas systems can be ordered into two classes, five types and several subtypes(Figure 1) (Haft, Selengut, Mongodin, & Nelson, 2005; Makarova et al., 2015). The difference between the CRISPR-Cas classes is the amount of components used during interference. Class 1 uses a cascade of proteins to bind the CRISPR targeting RNA (crRNA), find and cleave the target (Carte, Wang, Li, Terns, & Terns, 2008; Charpentier, Richter, van der Oost, & White, 2015; Haft et al., 2005; Haurwitz, Jinek, Wiedenheft, Zhou, & Doudna, 2010; Makarova et al., 2015). Whereas class 2 uses one multifunctional protein (Biolabs, 2007; Makarova et al., 2015; Sander & Joung, 2014). Moreover, many bacteria and archaea have multiple Cas loci with different types of Cas systems. Therefore it is thought that CRISPR-Cas systems are able to share components (Makarova et al., 2015).

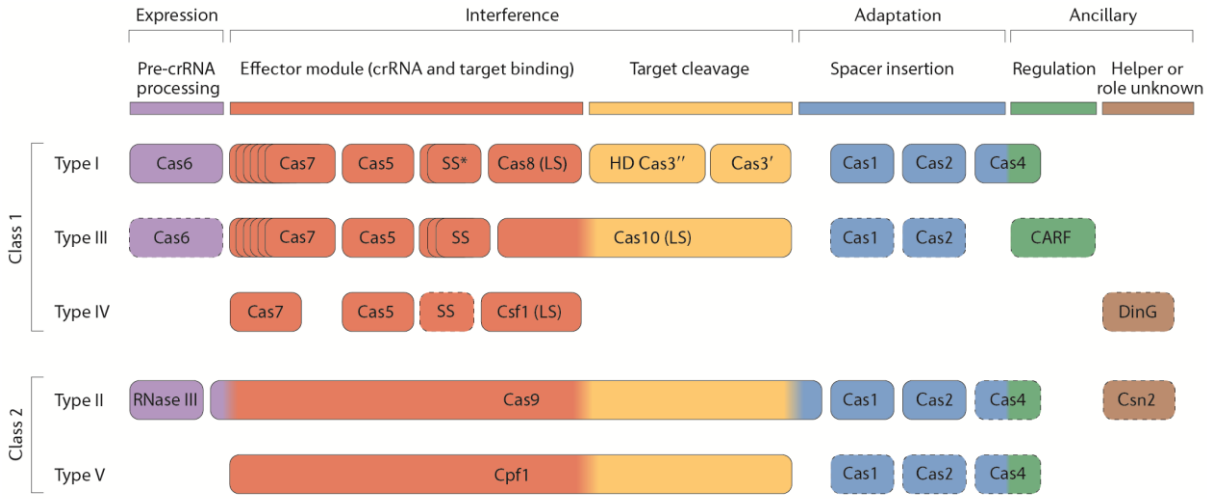

Figure 1 Functional classification of Cas proteins. Dispensable components are indicated by dashed outline. Image from (Makarova et al., 2015)

From the Cas protein family, the Cas-1 and -2 proteins are present in almost every CRISPR-Cas system (Makarova et al., 2015; van der Oost, Jore, Westra, Lundgren, & Brouns, 2009; Yosef, Goren, & Qimron, 2012). These proteins are involved in the acquisition of the spacers, a template to recognize foreign DNA. Spacers are inserted in between palindromic repeats in an array (CRISPR array)(Babu et

al., 2011; Nam et al., 2012; Nuñez, Harrington, Kranzusch, Engelman, & Doudna, 2015; Nuñez, Lee, Engelman, & Doudna, 2015). In *E. coli,* Cas-1 and -2 proteins form a hexameric complex of two Cas1 dimers and one Cas2 dimer (Amitai & Sorek, 2016; Yosef et al., 2012). This complex binds invading pieces of double and single stranded DNA after which the protospacer is inserted into the CRISPR array (Figure 2)(Amitai & Sorek, 2016; Babu et al., 2011). In type II CRISPR-Cas systems the acquisition process has found to be random. Type I and type II CRISPR-Cas systems select the foreign DNA based on a short conserved sequence called the protospacer adjacent motif (PAM) (Horvath et al., 2008; Mojica, Díez-Villaseñor, García-Martínez, & Almendros, 2009). The PAM sequence varies per species, subtype and even per Cas loci (Díez-Villaseñor, Guzmán, Almendros, García-Martínez, & Mojica, 2013; Horvath et al., 2008; Mojica et al., 2009). The protospacers is excised three to five base pairs from the PAM resulting in spacers with similar lengths (Díez-Villaseñor et al., 2013; Erdmann & Garrett, 2012). The excision leaves a nucleophilic alcohol group at the 3' end (Amitai & Sorek, 2016; Nuñez, Harrington, et al., 2015). Aided by the Cas-1 and -2, the protospacers are guided to the correct location and inserted in-between the repeats forming an array of spacers called the CRISPR array (Amitai & Sorek, 2016; Díez-Villaseñor et al., 2013; Erdmann & Garrett, 2012). A CRISPR array always starts with the genes of the respective Cas family, followed by a leader sequence and an initial repeat (Figure 2) (Amitai & Sorek, 2016; Haft et al., 2005; Nuñez, Lee, et al., 2015). In most cases, the spacers are inserted closest to the leader with one CRISPR array holding several spacers of the same bacteriophage (Díez-Villaseñor et al., 2013; Erdmann & Garrett, 2012). However, it has been found that in some cases the spacers are inserted randomly into the CRISPR array (Erdmann & Garrett, 2012). The CRISPR array RNA gets transcribed as a whole after which it is cleaved so that each separate piece consists out of a spacer and a repeat (crRNA)(Carte et al., 2008; Gesner, Schellenberg, Garside, George, & Macmillan, 2011; Haurwitz et al., 2010; Niewoehner, Jinek, & Doudna, 2014). Depending on the CRISPR-Cas system the pre-crRNA gets cleaved by different proteins. (Carte et al., 2008; Charpentier et al., 2015; Gesner et al., 2011). Type II CRISPR-Cas systems requires a small complementary RNA strand to bind to the crRNA to make it functional (Gasiunas, Barrangou, Horvath, & Siksnys, 2012), this small RNA is known as trans-activating crRNA (tracrRNA) (Figure 2) (Dugar et al., 2013; Gasiunas et al., 2012). The protein Cas9 is the core part of type II systems is a single multifunctional protein. Upon binding of the tracrRNA-crRNA, or small guide RNA (sgRNA), RNase III cleaves the 3' end side after which the 5' end of the sgRNA is also cleaved (Charpentier et al., 2015; Gesner et al., 2011). This results in a Cas9 protein with bound sgRNA that can find the target sequence and cleave the DNA (Figure 2)(Gasiunas et al., 2012). Cas9 contains two active sites, an HNH domain and RuvC, which bears similarities to RNase H domains (Gasiunas et al., 2012; Nishimasu et al., 2014). These two sites cleave the DNA resulting in a double stranded blunt end break (Gasiunas et al., 2012). The final step is reparation of the DNA either via Non-Homologous End Joining (NHEJ) or Homolog Directed Repair (HDR)(Bayat, Omidi, Rajabibazl, Sabri, & Rahimpour, 2016; Li & Heyer, 2008). The CRISPR-Cas systems allow for the bacteria to protect itself against invading and unwanted DNA (Boyaval et al., 2007; Marraffini & Sontheimer, 2011). However, by doing so it also created an incredibly efficient all-in-one molecular tool to alter genomic sequences, thus making it an interesting subject for further investigations (Hammond et al., 2016; Sander & Joung, 2014; Wiedenheft, Sternberg, & Doudna, 2012).

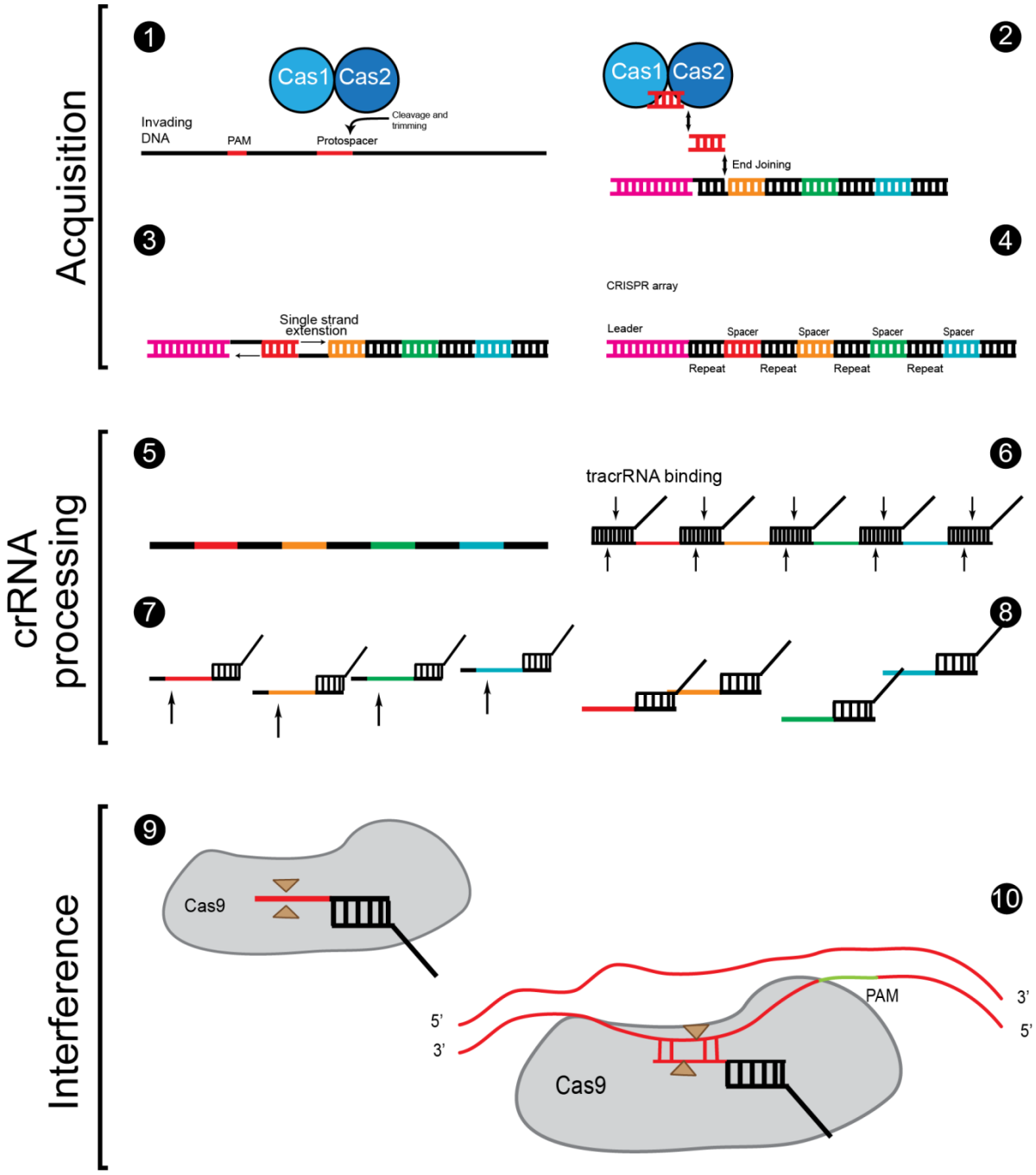

Figure 2 Stages of Type II CRISPR-Cas system. (1): Invading DNA gets bound by Cas1-2 complex. Complex recognizes PAM sequence and cleaves protospacer. (2): Cas1-2 complex brings protospacer to CRISPR array. Protospacer is inserted via end joining. (3): array is extended to complete the single strands. (4): CRISPR array with new spacer. (5): CRISPR array is transcribed. (6): tracrRNA binds to repeats and is bound by Cas9 and respectively cleaved by RNAse III. (7): crRNA is trimmed at the 5' end. (8): sgRNA is now functional and bound to Cas9. (9): Cas9 has bound the functional sgRNA and will look for a complementary match of the spacer. (10): when complementary sequence has been found Cas9 will cleave DNA after which it can be repaired by NHEJ or HDR. Image adapted from (Wikipedia, 2017)

Existing tools

Several techniques to help modify the genome already existed before CRISPR-Cas. Those that can make a knock-out or a knock-in of a gene and obtain similar effects of that as CRISPR-Cas systems. The most used tool to induce new genetic information in an organism is the transformation or transfection of plasmids. Plasmids are circular strands of DNA that replicate independently from the host's chromosomal DNA (Addgene, 2014; Glick, Pasternak, & Patten, 2010). Although most common

in prokaryotes, plasmids are also frequently used in eukaryotes (Addgene, 2014; Glick et al., 2010). Over the years various plasmids have been developed for both pro- and eukaryotes and can be ordered online (Addgene, 2014). These plasmids are often optimized for transient expression of protein or studying of pathways via overexpression (Addgene, 2014). Moreover, there is a plethora of techniques to modify and customize plasmids *ex vivo*, such as TOPO (Patel, 2009), GATEWAY (Katzen, 2007), Gibson assembly (Thomas, Maynard, & Gill, 2015), Golden gate (Engler, Kandzia, & Marillonnet, 2008) and many more (Cohen, 2013; Hunt, 2005; Marsischky & LaBaer, 2004). Although arduous in eukaryotes, plasmids have the added benefit that they are generally easily introduced in organisms through conventional transfection and transformation (Johnston, Martin, Fichant, Polard, & Claverys, 2014; Kim & Eberwine, 2010).

The core of most genetic engineering mechanisms and a common tool is recombineering (Sharan, Thomason, Kuznetsov, & Court, 2009; Thomason, Sawitzke, Li, Costantino, & Court, 2014) or Gene Targeting (Capecchi, 1989), which relies on homologous recombination (HR). The key components of HR are regions of homology in the vector or amplicon that are complementary to a part in the host genome. After introduction of the exogenous DNA the host can, by chance, replace a part of its genome that is homologous to the complementary strands of the vector or amplicon. This reaction results in an insertion or deletion of a part of the genome (Capecchi, 1989; Copeland, Jenkins, & Court, 2001; Heyer, Ehmsen, & Liu, 2010; San Filippo, Sung, & Klein, 2008; Sharan et al., 2009; Thomason et al., 2014; Vrančić, Gregorić, Radičević, & Gjuračić, 2008). The size of the homologous region is dependent on the host for highest efficiency and accuracy. In *E.coli* the homologous part can to be 40 to 50 base pairs (Sharan et al., 2009; Thomason et al., 2014) whereas in higher eukaryotes this becomes kilo base pairs (Capecchi, 1989; Copeland et al., 2001; Vrančić et al., 2008). Although one of the most used mechanisms, HR remains very inefficient. To further optimize HR efficiency , damage such as double or single strand DNA breaks can be induced on the genome that incites the HDR or NHEJ repair pathways, increasing the chances of uptake in the genome (Heyer et al., 2010; San Filippo et al., 2008).

Viral transfection is the most efficient ways to introduce foreign DNA into the host genome, as the viral proteins have evolved to hijack its host. To some extent, bacteriophage λ helped molecular biology take off when it was found and studied as it has adapted system that help integrate its genes into the host genome (Murray & Gann, 2007; Salmond & Fineran, 2015). Bacteriophage λ injects its viral genome into the host, after which it either starts the lytic cycle which ends in cell death or the lysogenic cycle which results in the insertion of its genome in the host (Groth & Calos, 2004; Murray & Gann, 2007; Salmond & Fineran, 2015). Bacteriophage λ uses an integrase and a specific small recognition region, called the att site, to store the viral genome at a specific genetic position in its host *E.coli* (Groth & Calos, 2004). For prokaryotes however, many techniques are available which are quicker and more efficient. In eukaryotes, various viral vectors can be used, depending on the host and goal of the experiment. Retroviruses which insert the DNA in the host genome, very similarly to bacteriophage lambda, are used most often (Nayerossadat, Maedeh, & Ali, 2012; Zhang & Godbey, 2006). Contrary to bacteriophage λ, retroviruses don't have a specific insertion site, however they do use an att sequence and integrases to help insert DNA into the genome (Nayerossadat et al., 2012; Zhang & Godbey, 2006). In eukaryotes, viral vectors remain the most efficient way of genomic engineering (Zhang & Godbey, 2006). However, working with viral agents will always require a lot of preparation and risk, as an error can result in a new and possibly more virulent virus (Murray & Gann, 2007; Zhang & Godbey, 2006).

Recombinases are enzymes of which the function is to insert of a strand of DNA (Turan & Bode, 2011). Moreover, many of these recombinases originate from viruses (Datsenko & Wanner, 2000; Murray & Gann, 2007). Recombinases can be divided into the Tyr- and Ser-recombinases. Where the difference lies in the reversibility of the process (Turan & Bode, 2011). Some of the most used recombinase systems are the Cre-LoxP and the Flp-FRT systems from the Tyr family. The Cre-LoxP and the Flp-FRT recombinase systems are tools that give more temporal and spatial control over genetic modification (Edwards, Young, & Deiters, 2009; Nagy, 2000; Sauer, 2002; van Duyne, 2014). Cre is an enzyme that binds DNA at the loxP site forming a dimer, after which it can form a tetramer with another bound Cre dimer (Nagy, 2000; Sauer, 2002; van Duyne, 2014). This tetramer creates a Holliday-junction resulting in a recombination of DNA. Depending on the orientation and location of the LoxP insertions, deletions, inversions or translocations can be made (Nagy, 2000; Sauer, 2002; Schlake & Bode, 1994; van Duyne, 2014). Spatial and temporal control can be obtained by creating hosts with tissue and cofactor or temperature sensitive promoters (Nagy, 2000; Sauer, 2002; van Duyne, 2014). Although this technique is very reliable, the drawback is that a host strain has to be made beforehand with the Cre recombinase and the specific loxP sites in place, making it a potentially laborious tool (Nagy, 2000; Sauer, 2002; Schlake & Bode, 1994; van Duyne, 2014). Moreover, in prokaryotes it is said that it can destabilize genomic DNA due to native recognition sites, therefore making it a technique that is used rarely in prokaryotes (Campo, Ritzenthaler, Bourgeois, & Leenhouts, 2002; Lambert, Bongers, & Kleerebezem, 2007).

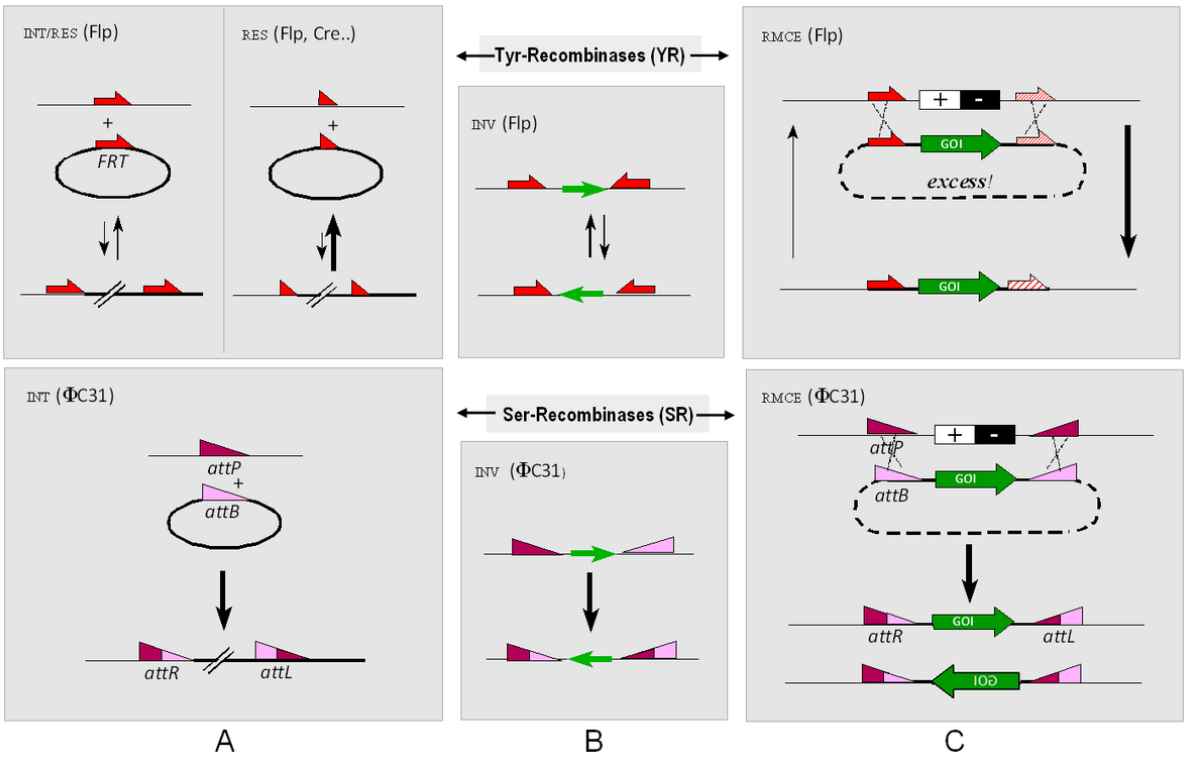

Figure 3 Examples of the tyr and ser recombinase mechanisms,A: INTegration/RESolution B; INVersion and C; Recombinase Mediated Cassette Exchange (RMCE). Depending on the orientation of recognition sites three different mechanisms of action can be executed. Tyr recombinases have reversible reactions as the sites remain intact, whereas ser recombinases alter the site upon reaction. Image adapted from (Turan & Bode, 2011).

RNA interference (RNAi) is a way to exert control of gene regulation in eukaryotes. Micro RNA (miRNA) is expressed in long double stranded RNA. The dsRNA is bound by the RNA Induced Silencing Complex (RISC) and cleaves it into small strands between 20 and 30 nucleotides long(Wilson & Doudna, 2013). The miRNA is then bound by an Argounaute protein which aids it in find its complementary strand (Wilson & Doudna, 2013). When the Argounaute with miRNA binds to mRNA it initiates degradation or represses ribosome binding leading to the silencing of protein translation (Wilson & Doudna, 2013). By inserting exogenous strands of RNA called short interfering RNA (siRNA) the same process can be initiated and targeted gene silencing will occur (Wilson & Doudna, 2013). This is a powerful method of regulation in eukaryotes, prokaryotes however, do not have the tools to process mi- and si-RNA and thus is unusable (Wilson & Doudna, 2013). It has been suggested that besides an adaptive immune system, CRISPR-Cas systems are orthologous to the RNAi mechanism (de Souza, 2012).

The predecessors the CRISPR-Cas systems are Zinc Finger Nucleases (ZFN) and Transcription Activator-Like Effector Nucleases (TALEN)(Gaj, Gersbach, & Barbas, 2013). The ZFN's are combined Cys2His2 Zinc Fingers (ZF) that have DNA binding capabilities (Peisach & Peisach, 2001). Each ZF has binding specificity of 3-4 base pairs, Therefore when combining the ZF's various sequences can be obtained (Peisach & Peisach, 2001). TALEN's have a DNA binding region that exists out of 17 nearly identical 34-amino acid repeats. These repeats are all specific for one nucleotide due to a hyper variable region (Boch, 2011; Joung & Sander, 2013). Therefore, these repeats can be shuffled around to create new TALEN's that are sequence specific (Boch, 2011; Joung & Sander, 2013; Kusano et al., 2016). The TALE is then fused to the FokI nuclease to create a sequence specific nuclease protein (Boch, 2011; Joung & Sander, 2013; Kusano et al., 2016). These two techniques are very similar to the CRISPR-Cas systems. FN's require a lot of protein engineering and optimization of binding whereas TALEN's mutations need to be made in the protein to obtain the right sequence specificity (Boch, 2011; Gaj et al., 2013; Kusano et al., 2016; Peisach & Peisach, 2001). However, many groups and companies have taken up these tools and premade proteins are readily available (Gaj et al., 2013).

Technique	Prokaryotes	Eukaryotes	Description
Recombineering / Gene Targeting	✓	✓	By either constructing a vector with target gene through endonucleases and ligases or amplify it by PCR. Vector or amplicon contain homologous regions that match the hosts genome. Vector and amplicon are inserted via e.g. electroporation, heat shock, microinjection. After which homologous recombination occurs and the host is selected for inserted trait (Capecchi, 1989; Copeland et al., 2001; Sharan et al., 2009; Thomason et al., 2014; Vrančić et al., 2008).
Viral Transfection	✓	✓	By using bacteriophages or retroviruses DNA can be inserted into the genome via the viral mechanisms. Depending on the host and goal different viruses can be used to obtain either transient or permanent modification (Zhang & Godbey, 2006).
Cre-Lox / Flp-FRT	✓	✓	Recombination events occur through the recombinases. Recognition and binding of their respective sites is done after which, depending on the orientation and the amount of binding sites, insertion, deletion, inversion or translocation of DNA can be performed(Nagy, 2000; Sauer, 2002; Schlake & Bode, 1994; van Duyne, 2014).
RNAi		✓	Exogenous dsRNA is cleaved by the RNA-induced silencing complex (RISC) and bound by an Argonaute protein. The siRNA can bind to its complementary part on which the Argonaute protein degrades the mRNA, silencing translation (Wilson & Doudna, 2013).
ZNF/TALEN	✓	✓	ZFN's or TALEN's are able to recognize DNA sequences. By altering the combination of ZNF's or redesigning the TALEN's DNA binding site, sequence specificity can be obtained. An attached nuclease is then able to create dsDNA breaks (Gaj et al., 2013; Joung & Sander, 2013; Peisach & Peisach, 2001).

Table 1 already existing techniques described and their availability in prokaryotes and eukaryotes.

CRISPR-Cas for eukaryotes

Several molecular tools already have been developed based on the CRISPR-Cas systems (Biolabs, 2007; Ledford, 2016; Sander & Joung, 2014). Target genome editing, expression, methylation or mapping can be achieved with the CRISPR-Cas9 system (Hsu, Lander, & Zhang, 2014; Sander & Joung, 2014). It has been shown that HDR and NHEJ are powerful mechanisms to edit genomes (Bibikova et al., 2001; Bibikova, Golic, Golic, & Carroll, 2002). With the CRISPR-Cas9 system and a homologous template recombination can occur through the HDR pathway (Cong et al., 2013; Hsu et al., 2014; Mali, Yang, et al., 2013). Without a homologous template to aid the HDR pathway insertions and deletions can take place trough the NHEJ pathway resulting in codon shifts (Bibikova et al., 2002; Hsu et al., 2014). However, the pathways to fix double strand DNA breaks are not present in prokaryotes, making this form of genome editing a method hard to use (Cui & Bikard, 2016; Li & Heyer, 2008). Recently, a group found another Class two Cas protein, Cpf1, that creates a small overhang during cleaving (Zetsche et al., 2015). This allows for better control of orientation during insertion through NHEJ or HDR.

One of the largest problems in genome editing is target specificity, as off-target mutations can lead to disastrous results. It has been found that Cas9 allows for mismatches and can thus cleave DNA on off-target sites (Fu et al., 2013; Hsu et al., 2013). Cas9 proteins allow most mismatches in the 5'-end of the sgRNA. Therefore it was suggested to shorten or truncate the sgRNA, although counter intuitively, it lead to increased target specificity (Fu, Sander, Reyon, Cascio, & Joung, 2014). Some of these problems can be avoided by carefully designing the sgRNA, and several programs have been developed to help find binding sites for Cas proteins and their off-target sites (Bae, Park, & Kim, 2014; Hsu et al., 2013; Kleinstiver et al., 2015). To increase Specificity of Cas9 was altered to have only one functional cleavage site, termed Cas9 nickase, so two Cas9 nickases are required to create a dsDNA break (Gasiunas et al., 2012; Jinek et al., 2012; Shen et al., 2014). Not only will this lead to less severe damage on off target catalysis but also increase specificity due to the requirement of two sgRNAs (Gasiunas et al., 2012; Jinek et al., 2012; Shen et al., 2014).

Cas9 has been further engineered to obtain other functions as well. The main key to that was turning off both active sites created a non-catalytic or dead Cas9 (dCas9) protein that binds DNA but does not cleave (Qi et al., 2013). This was first used as a way to repress expression levels by letting several Cas9 proteins bind on a gene promoter (Qi et al., 2013). Moreover, Cas9 transcription factor complexes have been made where the added transcription factor can ensue control over gene expression (Chavez et al., 2015; Konermann et al., 2014). Another way to control expression is to degrade the mRNA, which is also a possibility with a type III Cas system (Hale et al., 2009) or change methylation of the genome with a Cas9-protein complex (Liu et al., 2016). To study the effect of mutations in vivo, like cancer, a CRISPR-Cas system has been developed that is inducible with the chemical compound doxycycline (Dow et al., 2015). Therefore allowing a spatial and temporal control of mutagenesis *in vivo* (Dow et al., 2015). Finally a Cas9 has been fused to GFP allowing for genomic loci visualizations in real time (Chen et al., 2013; Ma et al., 2015).

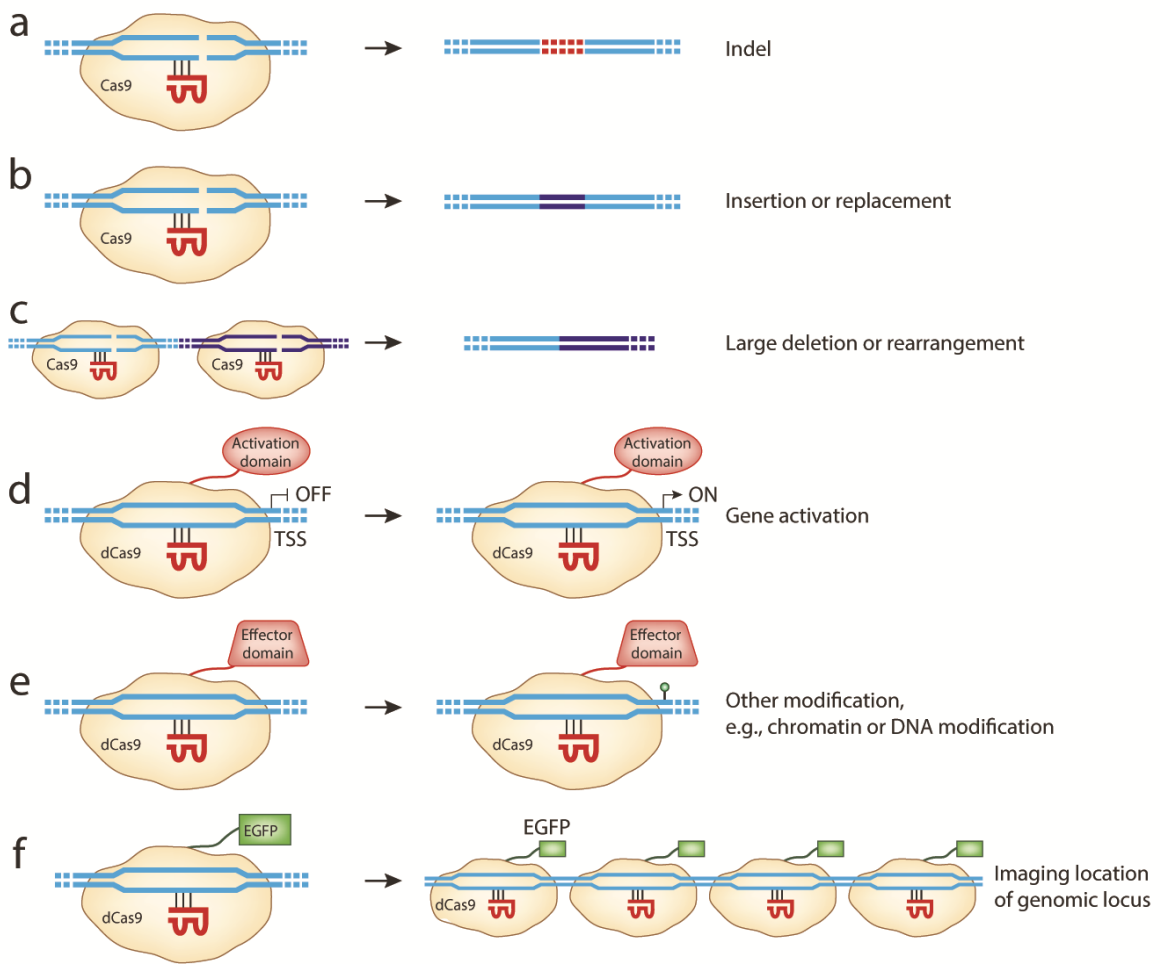

Figure 4 Examples application for CRISPR-cas9 systems. (a) Cas9 cleaves dsDNA. Repair by NHEJ results in indel. (b) with a homologous template present insertion or replacement can occur through repair by HDR. (c) by using two Cas9 proteins large parts of the DNA can be deleted or rearranged. (d) a non catalytic Cas9-transcription factor complex excess control over gene expression. (e) a non catalytic Cas9-effector can change epigenetic modification of the genome. (f) non catalytic Cas9-EGFP fusion allows dynamic visualization of the genome in vivo. Image from (Sander & Joung, 2014).

The most important part of CRISPR-Cas9 is the fact that it allows for multiplexing. Targeting multiple sites in one go. Creating a CRISPR array with several targets of interest Cas9 will bind all of them allowing for easy multiplex genome engineering (Cong et al., 2013). Although the largest problem being target specificity another limitation can be the PAM. Cas9 requires a PAM to bind target DNA, therefore not allowing of binding everywhere in the genome. The most commonly used Cas9, from *Streptococcus pyrogens,* has a PAM of 5'-NGG-3',this short motif occurs approximately every 8 bp on the human genome (Cong et al., 2013; Díez-Villaseñor et al., 2013; Horvath et al., 2008; Hsu et al., 2014; Mojica et al., 2009). However, it has been shown that the PAM specificity can be altered (Kleinstiver et al., 2015). Moreover, as more Cas9 homolog's get characterized a variety of PAMs will be available (Díez-Villaseñor et al., 2013; Mojica et al., 2009).

CRISPR-Cas systems are a great an easy way for genome engineering. The two major improvements with that the CRISPR-Cas tool provide is the ease of design. Firstly, with already premade Cas9 vectors, only the small guide RNA has to be made by the user. Secondly, the ability of simultaneous modification only by the addition of multiple guide RNA's gives it incredible power as a genetic engineering tool (Cong et al., 2013).Various tools have been developed and vectors are readily available. Additionally CRISPR-Cas doesn't have to be transfected as a plasmid. Genetic modification

can be obtained by injecting purified Cas9 protein together with sgRNA (Bayat et al., 2016; Capecchi, 1989; Kingston, R. E., Chen, C. A., Rose, 2003; Shen et al., 2014; Turan & Bode, 2011). When applied with viral agents or other carriers CRISPR-Cas9 might open ways to more efficient gene therapy (Fogleman, Santana, Bishop, Miller, & Capco, 2016; Konermann et al., 2014; X. Wang, Huang, Fang, Zhang, & Wang, 2016) Although more research and optimization has to be done to make it a more efficient system. In the world of eukaryotes, CRISPR-Cas has been accepted rapidly and is already frequently used. The question remains, is it an addition to the already developed molecular toolset available in prokaryotes?

CRISPR-Cas for prokaryotes?

For eukaryotes CRISPR-Cas is the designer's tool of the future, allowing ease of design similarly to that of prokaryotic. The number of articles using CRISPR-Cas in prokaryotes was far less than that for eukaryotes. Although CRISPR-Cas genome editing has already been used several times in bacteria with industrial relevance (Cobb, Wang, & Zhao, 2014; Oh & Van Pijkeren, 2014; Y. Wang et al., 2015). The main application of CRISPR-Cas9 is genome editing through dsDNA breaks, which bacteria have a hard time repairing (Li & Heyer, 2008; Murray & Gann, 2007). It has been reported that high recombination rates can be achieved by mutating the PAM sequence with the homology regions (Jiang, Bikard, Cox, Zhang, & Marraffini, 2013). Resulting in the inability of recurring recognition by Cas9, at the same time all the unsuccessful recombination's will be cleaned up by the lethality of dsDNA breaks (Jiang et al., 2013). The Cas9 nickases which only make a single strand DNA breaks have shown to achieve higher recombination rates (Xu et al., 2015). Possibly due to increase of time to achieve recombination or it could be driving HR via strand invasion (Cong et al., 2013; Mali, Aach, et al., 2013). Some bacteria contain simple NHEJ pathways. It has been found that by co-expressing these protein with Cas9 led to generation of indels with high efficiency (Tong, Charusanti, Zhang, Weber, & Lee, 2015).

Genome editing in prokaryotes with CRISPR-Cas9 still requires more improvement before it can be used. However, the catalytically dead Cas9 (dCas9) shows more potential. Genetic expression can be repressed by targeting dCas9 at the promoter (Bikard et al., 2013; Qi et al., 2013). Moreover, it has also been shown to up regulate gene expression by Cas9 fusion to a subunit of RNA polymerase (Bikard et al., 2013; Qi et al., 2013). CRISPR-Cas systems also allow for the generation of complex genetic circuits dCas9 targeting promoters with specific sgRNAs (Kiani et al., 2014; Nissim, Perli, Fridkin, Perez-Pinera, & Lu, 2014). Gene silencing has also been shown by using endogenous type I systems where Cas3, which digests the DNA, has been knocked out (Luo, Mullis, Leenay, & Beisel, 2015; Rath, Amlinger, Hoekzema, Devulapally, & Lundgren, 2015). Type III systems that digest mRNA have also been shown to be programmable to target specific RNAs, allowing for RNAi in prokaryotes (Zebec, Manica, Zhang, White, & Schleper, 2014). Moreover, data indicates that Cas9 might also have inherent RNA targeting properties (Sampson, Saroj, Llewellyn, Tzeng, & Weiss, 2013).

Maybe one of the most promising tools the CRISPR-Cas systems provide is a specific antimicrobial function (Gomaa et al., 2014). By targeting specific genes that only some strains have the lethality of Cas9 dsDNA break can be used. A bacteriophage vector holding the Cas9, tracrRNA and CRISPR array already has been designed and used to kill an antibiotic *Staphylococcus aureus* strain (Bikard et al., 2014; Citorik, Mimee, & Lu, 2014). Another phage was used to kill enterohaemorrhagic *E.coli* (EHEC) in moth larvae which increased survival. A more subtle approach is to target antibiotic resistance

plasmids, which returns the effectiveness of the antibiotic and any chance of regaining the plasmid through bacterial conjugation (Bikard et al., 2013; Citorik et al., 2014; Cong et al., 2013).

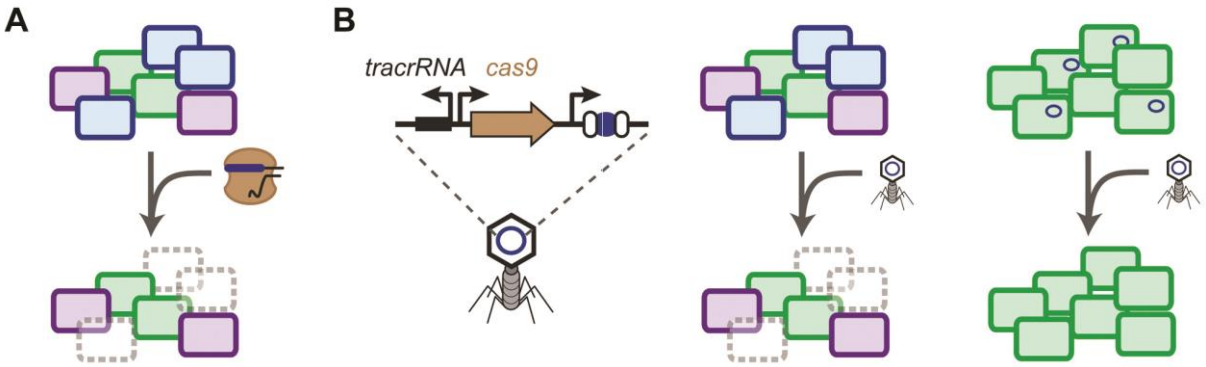

Figure 5 Guided antimicriobial function of CRISPR-Cas9. A: CRISPR-Cas9 can be introduced to a mixture of species. With the right sgRNA only the specific species will be killed. B: By using a viral vector to introduce the CRISPR-Cas9 system it can be used similarly to antibiotics. sgRNA can also be a target to antibiotic plasmid destroying obtained antibiotic resistance. Image taken from (Luo, Leenay, & Beisel, 2016).

In eukaryotes dCas9 fused to GFP has been used to image chromosomal structures, this has not been reported yet in prokaryotes. Therefore, this could aid the understanding of chromosomal dynamics in prokaryotes (Anton, Bultmann, Leonhardt, & Markaki, 2014; Chen et al., 2013). Currently, only the interference end of the CRISPR-Cas systems are utilized for techniques and applications. It has been suggested that the almost universal Cas1 and Cas2 proteins that play a role in spacer acquisition might be able to be manipulated for targeted gene insertion (Luo et al., 2016).

Lastly, the characterization of the CRISPR-Cas systems will help increase the recombination efficiency with older techniques, as CRISPR-Cas was evolved to interfere with exogenous DNA integration (Marraffini & Sontheimer, 2008; Nuñez, Harrington, et al., 2015).

Conclusion

The already established techniques are powerful tools in genome editing and they will be used in the future. With the addition of the CRISPR-Cas systems the possibilities become even greater. The versatility of the Cas proteins allow for simple solutions to difficult problems. Moreover, the ease of use and design has already made it a popular tool. Although there is plenty of room for improvement, mainly in target specificity, CRISPR-Cas systems show incredible potential. CRISPR-Cas systems might play a significant role in the future medicine in the form of gene therapy. Additionally, CRISPR-Cas systems will likely be employed in the field of biotechnology and possibly also in other microbiology sub-disciplines. Undoubtedly, the CRISPR-Cas systems will play an impactful role in the future of molecular biology.

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