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Genetic tools for investigating Mucorales fungal pathogenesis

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summary

Purpose of review—Mucormycosis is an emerging opportunistic fungal infection whose causative agents are found within the Mucorales family. A recent increase in immunocompromised cohorts with solid organ transplants, diabetes mellitus, and other medical conditions have resulted in increased fungal infections including mucormycosis. Our current knowledge about Mucoralean fungi is in its infancy compared to other fungal pathogens, which may be due to lack of robust genetic tools for Mucorales. In this review we summarize recent advances in genetic tools to study the two most prevalent and genetically amenable Mucoralean fungi, *Mucor circinelloides* and *Rhizopus delemar.*

Recent findings—There have been advances made in the study of Mucorales family genetics. These findings include the construction of recyclable markers to manipulate the genome, as well as silencing vectors, and the adaptation of the CRISPR/Cas9 gene editing system.

Summary—We present how these genetic methods have been applied to understand basic biology, morphogenesis, pathogenesis, and host-pathogen interactions in the two Mucoralean fungi, *M. circinelloides* and *R. delemar.* With these advances in Mucorales the opportunity to further understand the pathogenesis of these organisms is opened.

Keywords

mucormycosis; *Mucor*; *Rhizopus*; recyclable marker; CRISPR/Cas9 in fungi

Introduction

Mucormycosis is a fungal infection characterized by rapid progression, and is caused by fungi belonging to the Mucorales family. Mucormycosis has been observed sporadically in the past; however, a continual increase has been observed [1–4]. Mucorales are ubiquitous in nature [5]. The lifestyle of Mucorales can span from being saprobic to being opportunistic pathogens in both humans and animals [5]. A recent increase in fungal infections, including mucormycosis, can be attributed to an influx of susceptible cohorts with solid organ

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Conflict of Interest

The authors declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent

This article does not contain any studies with human or animal subjects performed by any of the authors

transplants, diabetes mellitus, and other medical conditions that cause impaired immunity [6–8]. Phagocytic cells may play crucial roles to control Mucorales infections because approximately 15% of the patients with severe neutropenia are likely to develop mucormycosis due to defects in phagocytic cell functions [3, 9]. Wüster et al. characterized the response of human mononuclear cells to various Mucorales, in which both inactivated germ tubes and spores significantly stimulated the secretion of proinflammatory cytokines TNF-α and IL1ß [10]. They also observed an upregulation of co-stimulatory molecules on monocyte derived dendritic cells along with T- helper cell activation [10]. Another study found that Toll-like receptor 2 and NF-kB pathway- related genes are induced in human polymorphonuclear neutrophils in response to *Rhizopus* [11]. The causative agents for mucormycosis include *Rhizopus* spp., *Mucor* spp., *Rhizomucor* spp., *Lichtheimia* spp., *Cunninghamella* spp., and others [12, 13, 3]. Mucormycosis presents itself with a mortality rate of ~50% in all cases and over 90% mortality in disseminated cases [7, 14, 15]. Patients with mucormycosis often suffer from permanent disfiguration as a result of surgical debridement of affected tissues followed by antifungal drug treatments [6, 7, 16].

Two pathogenic Mucorales species, *Mucor circinelloides* and *Rhizopus delemar* (previously known as *R. oryzae,* respectively denoted as "*Mucor*" and "*Rhizopus*") are genetically amenable among other human pathogenic Mucorales fungi. These pathogenic Mucorales species typically grow as hyphae, but exhibit dimorphism as they are able to grow as both hyphae and yeast. This dimorphism was found to be regulated by the calcineurin gene [17, 18]. The ability to express dimorphism was also found to have a role in virulence of the Mucorales species [18]. Due to Mucorales naturally growing as moulds, its predominant growth form is that of filamentous hyphae. In a laboratory setting Mucorales can be grown on a variety of media, such as Yeast Peptone Glucose (YPG) or Minimal Media with Casamino acids (MMC) [19]. Mucorales have been observed to grow best at 26°C with plenty of light present [19, 1]. Under these conditions the Mucorales will utilize light to activate carotenoid biosynthesis and asexual sporangiospore production [20]. *Mucor* and *Rhizopus* are the most studied Mucorales models today. They are used to study and understand pathogenesis and host-pathogen interactions in mucormycosis. However, the current study of these fungal organisms presents itself with a series of challenges. The genetics of these organisms are far from being extensively understood. A whole-genome duplication occurred early in the Mucormycotina lineage and the duplication of genes may have provided new proteins which expand the sensory and signaling pathways [21]. Mucorales are known to be haploid and present zygotic meiosis when sexually reproducing [22]. Interestingly, Mucorales genomes encode genes that have been known to be metazoan specific, such as GTPases Rab32, the Ras-like GTPase Ral, and their possible positive regulators [23]. The lack of scientific attention this organism receives, and the resulting lack of available genetic tools, could be the key factors as to why it is understudied when compared to other fungal organisms. Nonetheless, the interest in *Mucor* and *Rhizopus* as subjects of further study has increased. This review discusses how genetic analysis has been achieved in Mucorales and provides updates on the newly developed genetic systems, which can enhance our understanding of mucormycosis.

Gene Knock-out in *Mucor* and *Rhizopus*

Mucoralean fungi are understudied due to the challenges faced by researchers. When compared to other pathogenic fungi, Mucorales research is still in its primordial stages. The two genetically amenable Mucorales, *Mucor* and *Rhizopus,* express a greater than usual drug resistance. The idea of traditional genetic manipulation involving drugs as dominant selective markers therefore is not attainable, which significantly limits our ability of genetic manipulation [24]. Auxotrophic markers therefore have been selected to replace a gene in *Mucor* and *Rhizopus.* In *Mucor,* there are two main auxotrophic markers, *leuA* and *pyrG,* used for genetic manipulation. The *leuA* gene is an ortholog of the *Saccharomyces cerevisiae LEU1* gene, which encodes an isopropylmalate isomerase that is required for the leucine biosynthesis pathway [25]. The *pyrG* gene is an ortholog of the S. *cerevisiae URA3* gene, which encodes an orotidine- 5'-phosphate (OMP) decarboxylase that participates in de novo biosynthesis of pyrimidines [24, 18]. In *Rhizopus,* the *pyrF* gene has been used as a selection marker; *pyrF* is an ortholog of the *URA5* gene that encodes an orotate phosphoribosylatransferase, which is also an enzyme required for the biosynthesis of pyrimidines [26]. These *leuA* and *pyr* genes in *Mucor* and *Rhizopus* have been used to construct a gene deletion allele.

Gene knock in *Mucor* and *Rhizopus* has also incorporated conventional gene deletion construction (Fig 1). For example, the approximately 1-kb DNA fragments upstream and downstream of a target gene were put together at the 5' or 3' end of the marker gene, in this case *pyrG.* Overlapping PCR or cloning methods have been used to generate the deletion construct. The deletion construct is then delivered into the fungal cells via protoplasting transformation and/or electroporation, or via biolistic transformations for homologous gene replacement [24, 19]. The juxtaposed ~1-kb fragments of the target gene then enhance the homologous recombination between the gene of interest and the marker.

This conventional gene deletion method advances our understanding of Mucorales. The light sensing mechanisms in *Mucor* have been elucidated by gene deletion of the related white color genes [27, 28, 24]. These gene knockout methods have also demonstrated the roles of the *sexM* gene in sexual development, those of *rdrp* genes, argnaute genes, dicer genes in gene silencing, and those of calcineurin genes in dimorphic transition and virulence [29, 18, 17]. Additionally, the roles of ADP-ribosylation factors and protein kinase A in morphogenesis were elucidated by related gene deletions [30, 31].

In *Rhizopus, a* similar approach to generate a deletion construct, followed by transformation to generate a deletion allele, is employed [32]. For example, Ibrahim et al. elucidated the function of the high affinity iron permease gene *(FRT1)* in iron uptake and virulence via this conventional gene knockout approach [33].

Recyclable marker system for genetic manipulation of *Mucor*

The genomes of *Mucor and Rhizopus* underwent duplications and have multiple copies of genes in signaling pathways [34, 23]. For example, in *Mucor* there are three gene copies for the calcineurin A catalytic subunit, two copies for the immunophilin cyclophilin A, and ten copies for the protein kinase A regulatory subunit ([18] and Garcia and Lee unpublished

data). Thus, studying a signaling pathway in *Mucor* is difficult, given the few auxotropic markers available for gene deletion. Garcia et al developed a recyclable marker to achieve serial gene deletions by using the *pyrG* gene in *Mucor* (Fig 1). In similar fashion to the *URA* blast system in *Candida albicans,* in which a 200-bp tandem repeat was flanked around the *URA3* gene [35], the *pyrG* gene is flanked on either side with a 237-bp repeat, resulting in the *pyrG-dpl237* marker [24]. The presence of tandem repeats of 237-bp around the *pyrG* gene facilitates the excision of the *pyrG* marker after target gene deletion, thus making it recyclable and possible to achieve a series of gene deletions in a single organism [24].

Selection for mutants that have excised the *pyrG* marker can be achieved by using the 5-fluoroorotic acid (5-FOA), which is decarboxylated by the *pyrG* gene product resulting in the production of the toxic compound 5-florouracil (Fig 1) [24]. Thus, mutants that lack the wild-type *pyrG* gene can survive on the media containing 5-FOA and uracil/uridine. With this method it is possible to selectively grow any mutants that have excised the *pyrG* marker. With the marker excised, the mutants generated from the first transformation can be used for a second gene deletion.

There have been mutants of *Mucor* developed utilizing this recyclable *pyrG* marker system. One example would be how Garcia et al generated double deletion mutants with a yeast-locked and albino colony phenotype [24, 18]. *Mucor* is a dimorphic fungus that can transition between yeast and hyphal forms, and this dimorphism is regulated by the calcineurin pathway [18]. There are three genes encoding calcineurin A catalytic subunits *(cnaA, cnaB,* and *cnaC)* and one gene encoding calcineurin B regulatory subunit *(cnbR)* [18]. The disruption of the *cnbR* gene in *Mucor* resulted in mutants that exclusively grow as multi-budded yeast [18]. *Mucor* produces carotene that results in a brownish colony phenotype, and carotene production in *Mucor* is governed by *car* genes. With the recyclable *pyrG-dpl237* marker, Garcia et al deleted the *carRP* gene that encodes for a protein with dual enzyme activities (phytoene synthase and lycopene cyclase), both of which are involved in carotenoid production [27]. The deletion of the *carRP* gene generated mutants with a white colony phenotype due to a lack of carotene accumulation. The *pyrG* marker was then excised in the presence of 5-FOA. Eventually the *cnbR* gene in the albino *carRP* mutants was replaced with the *pyrG* marker to generate double mutants that only grow as white yeast. This experiment provides a proof of concept for the use of a recyclable marker system that can be in Mucorales. A recyclable genetic marker is yet to be tested in *Rhizopus.* It is apparent that development of a recyclable *pyrF* marker for *Rhizopus* will further enhance our ability to perform genetic analysis.

Gene Silencing as a genetic tool in *Mucor* and *Rhizopus*

Mucor has been found to have a conserved RNAi pathway that allows posttranscriptional gene regulation [36–39]. The endogenous short RNAs (esRNAs) generated by the cleavage of double stranded RNA (dsRNA) regulates expression of endogenous genes by degrading their corresponding mRNAs in *Mucor* [40]. Transformation of self-replicative plasmids or vectors expressing inverted repeat transgenes resulted in the identification of two different classes of small antisense RNAs linked with silencing [41, 42]. The long antisense RNAs (25nt) are only produced or detected very early in the vegetative growth stage, whereas the

short antisense RNAs (21nt) are only detected in the spore RNA (late stage). Two distinct RNA polymerases are involved in the silencing process. The first polymerase (RdRP1) initiates silencing by producing antisense RNA transcripts from the target gene, and the second polymerase (RdRP2) amplifies the silencing signal by producing new dsRNA molecules from target mRNA template [43]. A study in which silencing was triggered by a hairpin RNA shows that a single dicer gene *(dcl-2)* is essential for gene silencing, and vegetative development [37]. The *dcl-2* product cleaves the dsRNA molecules into the two classes of siRNAs which are then loaded onto a single *Argonaute* gene *(ago-1)* in order to mediate exogenous and endogenous gene silencing [39]. It is important to note that the RdRP genes are also involved in dicer independent degradation of endogenous mRNA (non-canonical pathway) [44].

Calo et al. have shown that *Mucor* regulates its epigenetic, RNAi mediated, post-transcriptional silencing of the *fkbA* gene to confer resistance to FK506 [45]. A mRNA profiling study by Nicolas et al. further confirmed that RNAi machinery controls responses to specific environmental signals [46]. The gene silencing machinery has not only been extensively studied but is also utilized as a molecular tool to perform genetic manipulation in this fungus.

Trieu et al. have developed a silencing vector pMAT1700 to generate a whole-genome silencing library [47]. The vector can also be modified to silence any target gene, and hence this is an important tool for functional genomic screenings. This self-replicative vector comprises of a *pyrG* selection marker and a silencing cassette. The silencing cassette has two convergent promoters (*Pgpdh1* and *Pzrt1*), between which a multiple cloning site (MCS) is flanked to generate dsRNA that can trigger the gene silencing mechanism in *Mucor* (Fig 2). The *carB* gene, which is another *car* gene responsible for carotene production, is also present between the two promoters to function as a silencing reporter. A vector with target DNA can be transformed into *Mucor* by electroporation, and successful silencing of the cloned genomic fragment will result in easily detectable white colonies due to the *carB* gene being silenced simultaneously with the target gene. The authors have used this approach to identify two novel virulence factors - Phospholipase D and Myosin 5. Deletion of these genes resulted in reduced virulence in the two heterologous hosts *Galleria mellonella* (insect) and *Mus musculus* (murine). Thus, gene silencing can be used as a tool for screening and identification of unique phenotypes or any other characteristics that are different from the wild-type strain, and then the functional validation can be performed by generating knockdown strains.

A gene silencing system is also used as a molecular tool for genetic manipulation in *Rhizopus.* A small interfering RNA (siRNA) mediated knockdown of lactate dehydrogenase A resulted in 85.7% reduction in lactic acid but a 15.4% increase in ethanol yield [48]. In patients with chronic kidney failure, *Rhizopus* utilizes iron from ferrioxamine to boost its growth. Liu et al. have identified two surface proteins/receptors (Fob 1 and Fob 2) in *Rhizopus* that bind to ferrioxamine and facilitate iron uptake [49]. When RNAi attenuated the expression of Fob 1 and Fob 2, there was a decrease in iron uptake by the fungus.

Recently, it was discovered that a glucose-related protein (GRP78) was acting as an endothelial cell receptor to which Mucorales could bind during host cell invasion [50]. It was then concluded that a spore coating protein homolog (CotH) was the ligand for GRP78. This study resulted in a CotH3, CotH2 *Rhizopus* mutant (RNAi::*CotH2,3)* [50]. In a separate study, the RNAi silencing pathway present in *Rhizopus* was exploited to down-regulate two genes involved in the uptake of iron from ferrioxamine [49]. The exploitation of the RNAi silencing pathway presented by *Rhizopus* is a solid method for genetic manipulation of this organism. Together the data suggest that RNAi is an important tool for genetic manipulation in *Rhizopus.*

The main advantage of using silencing over gene knockout is that it allows one to study essential genes in Mucorales. Gene silencing can also be used to study closely related genes with shared sequence similarity such as the *CotH* family. The gene silencing system compensates for the lack of markers in Mucorales.

CRISPR-Cas9 in *Mucor* and *Rhizopus*

Clustered Regularly Interspaced Short Palindrome Repeats (CRISPR) systems are DNA sequences in bacteria that confer protection against bacteriophages [51]. The Type II CRISPR- Cas system from *Streptococcus pyogenes* has been extensively studied, and CRISPR- associated protein 9 (Cas9) was determined to be essential for all stages of S. *pyogenes* immunity [52]. This CRISPR/Cas9 system has been successfully modified to perform gene editing in embryos [47], plants [53], and microbes [54–57]. CRISPR/Cas9 system comprises of two essential components, which are a single guide RNA (sgRNA) and a Cas9 nickase [58]. The sgRNA is obtained by combining CRISPR RNA (crRNA; complementary to a specific part of the target gene) and trans-activating crRNA (tracrRNA). The sgRNA directs the Cas9 nuclease to induce a double-stranded break in the 20bp target DNA with a protospacer adjacent motif (PAM; Fig 3). Studies have further shown that chemical modifications at selected sites of the sgRNA could increase the specificity of the Cas9 enzyme and minimize off-target effects [59].

Low gene editing frequencies pose a significant problem for genetic manipulation in filamentous fungi. CRISPR-Cas9 has been applied to overcome this problem in several fungi, such as *Aspergillus spp.*[60, 61, 56], *Tichoderma reseei* [62], *Neurospora crassa* [63], and others. Very recently CRISPR-Cas9 started gaining attention in genetic manipulation of mucormycosis causing pathogens.

In *Rhizopus,* a plasmid-based strategy was implemented to assemble all the required elements into one vector to disrupt a target gene [64]. A biolistic delivery system was used to transform the vector into the M16 strain (*pyrF-*). The transformation resulted in five stable transformants, and partial deletion of the target gene was confirmed by Southern blotting.

On the contrary, a plasmid-free system without *in vitro* RNP formation was used to perform gene editing in *Mucor* [65]. A polyethylene glycol (PEG)-mediated transformation method [66] was adapted to introduce gRNA and Cas9 enzyme with or without donor DNA. This CRISPR-Cas9 system was optimized by first disrupting the *carB* gene which encodes for a carotenogenic enzyme phytoene dehydrogenase [67]. Interestingly, when non-homologous

end joining (NHEJ) mediated mutagenesis was adapted (no donor DNA) to disrupt the *carB* gene, it resulted in disruption of neighboring the *carRP* gene as well. However, introduction of donor DNA resulted in the stable integration of the deletion cassette at the target site via homologous recombination. The authors were also able to disrupt HMG-CoA reductase genes using a similar strategy. Together, the data suggest that homology-directed repair combined with CRISPR- Cas9 is more suitable for genetic manipulation in *Mucor.* This powerful tool can be used to disrupt multiple genes in order to elucidate new mechanisms which contribute to the pathogenicity of this organism.

Conclusions

Fungi belonging to the Mucorales family have proven to play many roles in industry, research, and the medical field. The interest in further study these fungi has increased, but unfortunately the tools to genetically manipulate these fungi are limited. Nonetheless, progress towards overcoming the challenges faced by scientists studying these organisms is being made. Although we are far from fully understanding the Mucorales family, we have presented many methods by which scientists are pioneering new tools for investigating *Mucor.* By having the ability to genetically manipulate *Mucor* and *Rhizopus,* we may be able to find novel methods of treatment for mucormycosis, further manipulate the organism for better biofuel production, and possibly establish a new fungal model organism.

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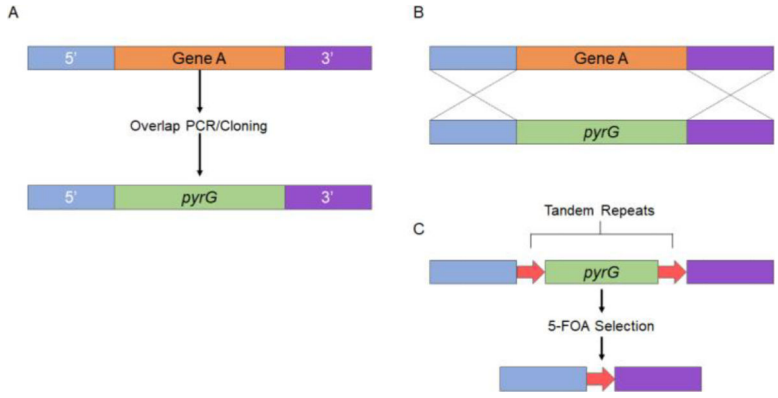

Figure 1.

Gene deletion in *Mucor* and *Rhizopus.* (A) ~1 kb of the 5' and 3' end of the target gene is incorporated on their respective sides to the *pyrG* marker gene. This is accomplished by overlapping PCR or cloning methods. (B) The deletion construct is delivered into the fungal cells via protoplasting transformation and/or electroporation, or via biolistic transformations for homologous gene replacement. (C) To construct a recyclable marker the *pyrG* gene is flanked on either side with a 237-bp repeat, resulting in the *pyrG-dpl237* marker. The tandem repeats of 237-bp around the *pyrG* gene facilitates the excision of the *pyrG* marker after target gene deletion.

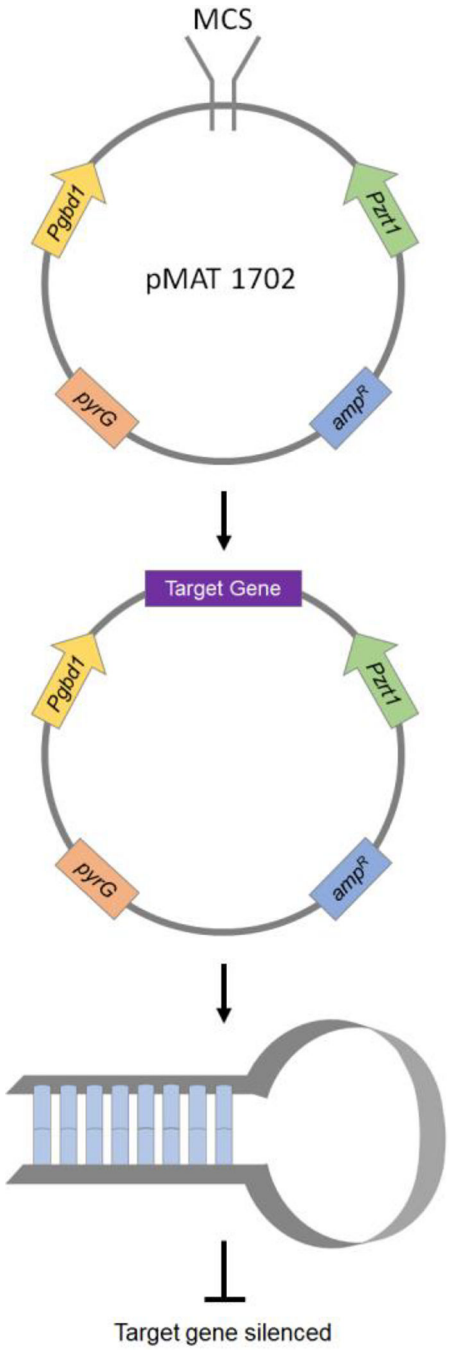

Figure 2.

Gene silencing in *Mucor* and *Rhizopus.* Two convergent promoters flank a multiple cloning site which can be digested, and a target gene can be placed. The Amp and pyrG act as selectable markers, and the presence of carB gene adjacent to the target gene allows for selection of white colonies post-transformation. The dsRNA resulting from this self-replicative vector can silence the target gene.

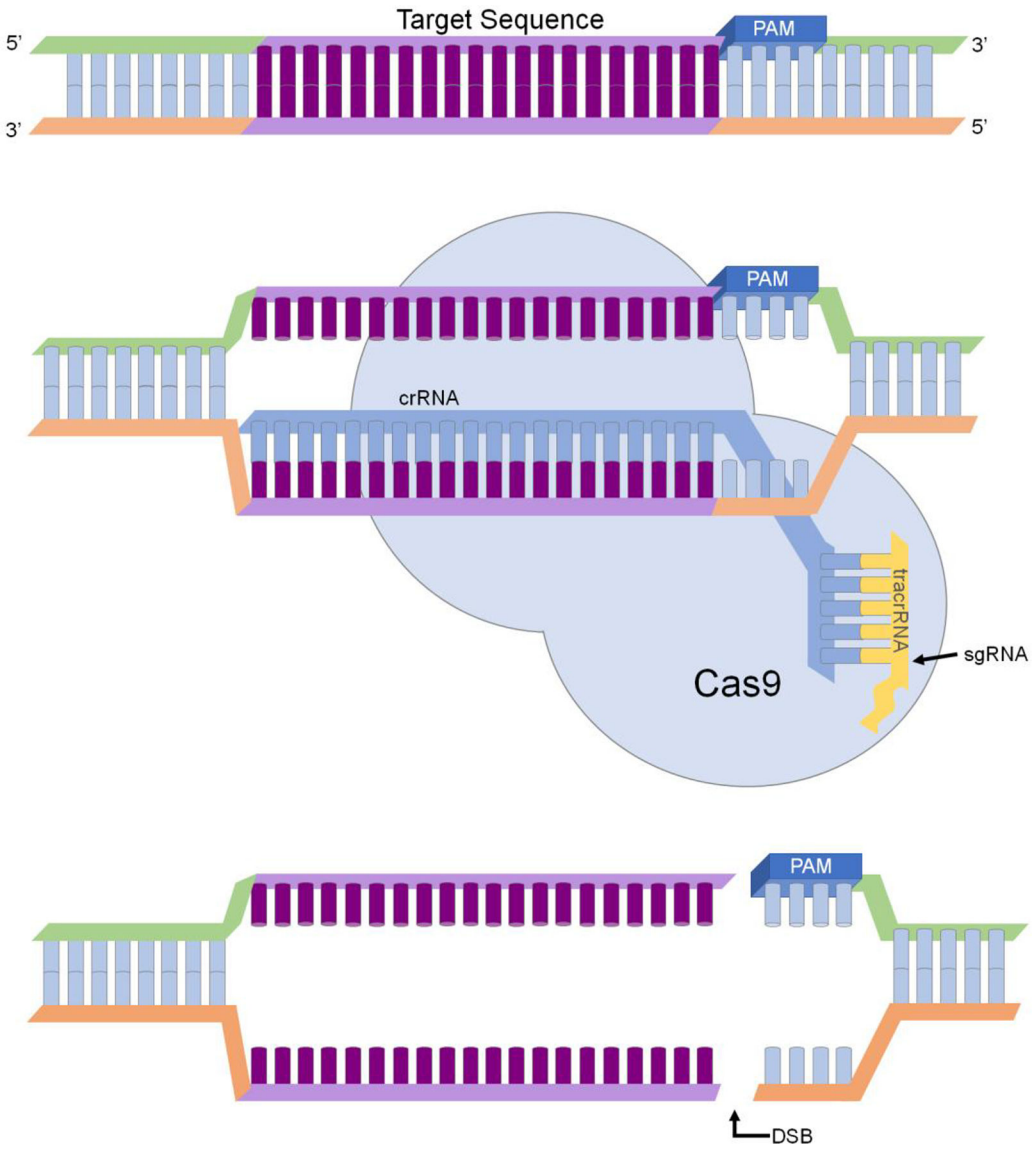

Figure 3.

Cas9 induced double-strand break in a target gene. The sgRNA comprises of tracrRNA and crRNA. The crRNA is specific to the target DNA, and the Cas9 enzyme creates a double-strand break upstream of the PAM motif.