Chapter 4
Potential for CRISPR Genetic Engineering to Increase Xenobiotic Degradation Capacities in Model Fungi

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4.1 Xenobiotic Compounds

The Industrial Revolution marked a major turning point in human history, producing changes in economy, politics, society, and especially the environment. Since the 1980s, there has been a shift in attention to recognize the increasing presence of stable and often toxic, anthropogenic compounds. These synthetically derived compounds are called "xenobiotics" and are chemically distinct from molecules found in nature derived from biological and abiotic process.

The classification of xenobiotic compounds can be made according to (i) the chemical structure (amine, aromatic, halogenated hydrocarbon; metals; etc.), (ii) the industrial origin (agricultural chemistry, textile processing industry, leather

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R. Prasad, E. Aranda (eds.), *Approaches in Bioremediation*, Nanotechnology in the Life Sciences, https://doi.org/10.1007/978-3-030-02369-0_4

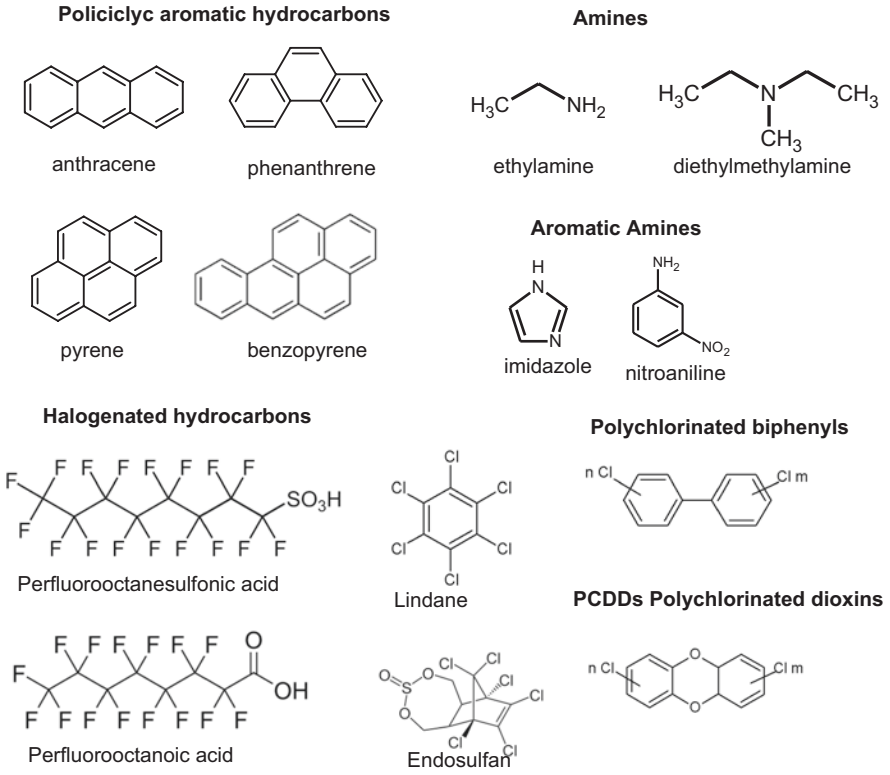

Fig. 4.1 Some of the most representative groups of xenobiotic compounds found in nature, according to their chemical structure. Polycyclic aromatic hydrocarbons, amines, aromatic amines, halogenated hydrocarbon polychlorinated biphenyl, polychlorinated dioxin

industry, personal domestic care, pulp and paper industry, etc.), (iii) the function/use (absorbents, adhesives, coloring agents, cosmetics, flame retardants, fuel additives, pharmaceutical active compounds, etc.), or (iv) the regulation (priority or emerging pollutants) (Iovdijová and Bencko 2010) (Fig. 4.1).

Xenobiotic compounds are often found in sources of chemical waste, for example, excessive use of chemical fertilizers in agriculture contributes to refuse pollution. In addition, surface runoff, transport emission, heating, urban wastes, and natural disaster increase the presence of these substances in the environment. Industry is the main source responsible for large-scale contamination. The fate of these compounds is determined by the metabolism of the microorganism in addition to abiotic processes, such as photooxidation.

Though microorganisms tend to funnel xenobiotic compounds into the natural metabolic pathways based on certain structures (aromatic) and substituents (halogen, nitro groups, etc.), xenobiotics are less susceptible to degradation, and consequently they tend to persist in the environment (Knackmuss 1996). The accumulation of xenobiotic compounds in the environment is problematic, specifically as it relates to bioaccumulation in living organisms.

Bioaccumulation in living organisms can upset every level of the biosphere, from changes in the diversity of microbial communities to endocrine disruption in fish. In addition, bioaccumulation is not only problematic for the natural environment. Through consumption of food products and freshwater from contaminated sites, long-term problems in human health result (Baun et al. 2004).

Public concern about the widespread presence and the effect of these substances in the environment was first incited by the 1962 publication of Rachel Carson's *Silent Spring*.

The first experiments of toxic mineralization began in the 1950s, and until today, researchers continue searching for solutions to reduce health hazards and pollution within the discipline of environmental biotechnology (Wittich and González 2016; Connell 2018).

4.2 Environmental Biotechnology Using Fungi

Bioremediation exploits organisms in contaminated settings to transform, neutralize, or remove toxic compounds. Species of bacteria, archaea, fungi, nematodes, plants, and even insects have demonstrated the ability to remediate contamination. Processes such as immobilization or adsorption are considered useful tools for bioremediation yet may still result in accumulation of the compounds in question. The most desirable effect in bioremediation events is the avoidance of the accumulation of xenobiotic compounds in the environment. Biomineralization takes place through catabolic reactions and the use of this energy for anabolisms to break down the toxic xenobiotics. In some cases, biomineralization can occur using only the contaminant. In other cases, the contaminant is not available for use as the sole source of carbon and energy. In these cases, the pollutant can still be transformed by the induction of specific enzymes in response to the presence of other utilizable compounds in a process known as cometabolism. Both bioremediation processes occur in nature and in engineered systems alike, though it remains unclear whether pathways are predominantly metabolic or cometabolic (Tran et al. 2013).

For bacteria, which can more quickly adapt to the presence of xenobiotics in the environment, xenobiotics can be more readily applied as source of carbon and energy in catabolic pathways. This adaptation is generally attributed to spontaneously arising mutants that allow the use of these compounds as a carbon and/or energy source. Genetic sequences of benefit can potentially circulate through bacterial communities through donation and further horizontal gene transference mechanisms in the form of genomic island, islets, and profages (Perna et al. 2001). Horizontal gene transfer has, for example, allowed for the insertion of the TN4371 biphenyl transposon from *R. oxalatica*, which circulated genes for biphenyl degradation (Toussaint et al. 2003).

In fungi, these kinds of adaptive events are more complex, due to the eukaryotic cell type characterized by the presence of a true membrane-bound nucleus and the compartmentalization functions. DNA is blocked in chromosomes. Beneficial mutations can be transferred to the progeny when mutations are produced within the cell. In addition, if a mutation partially affects an intron region, this can be removed

during RNA splicing during maturation of the RNA and may result in the loss of the mutation in the next generation.

Regardless, fungi have special features that make them suitable microorganisms for bioremediation processes (reviewed by Harms et al. 2011). They can spatially grow through several hectares by forming hyphae, do not require continuous water phases for dispersion, and allow the transport and growth of other microorganism such as bacteria (reviewed by Harms et al. 2011). Fungi can attenuate pollutant concentrations by physically adsorbing different contaminants through the presence of a thick cell wall composed of polymers such as cellulose and chitin. On the other hand, fungi are heterotrophic microorganisms that produce a set of enzymes involved in the decomposition of the organic matter for further mineralization to CO_2 and H_2O, thereby contributing to global carbon cycling. Enzymes that degrade, modify, or create glycosidic bonds are classified according to the carbohydrate-active enzyme (CAZYme; http://www.cazy.org/) database. Among these enzymes, auxiliary enzymes, including the lignin-modifying enzymes (LME), have been well studied in the transformation of xenobiotic compounds (Jiang et al. 2014). LMEs can transform aromatic substrates in cometabolic processes, due to the characteristically lenient substrate specificity. From that point, transformation of the target substances can result in less toxic or nontoxic compounds, which can then be further metabolized by the same fungi or by the microbial community. Lignin-modifying enzymes include laccases, tyrosinases, lignin peroxidases, manganese peroxidases, versatile peroxidases, *Coprinopsis cinerea* peroxidases, and others (Table 4.1). These enzymes are oxidoreductases; thus the capability to transform a specific compound depends on the redox potential of the enzymes as well as the ionization potential of the target compound.

Several studies concerning the fungal metabolism of xenobiotic compounds have shown the importance of the participation of the intracellular enzymatic system during aerobic xenobiotic transformation (Cerniglia 1997; Marco-Urrea et al. 2015; Aranda 2016; Olicón-Hernández et al. 2017). This intracellular system is comprised of a multifamily of enzymes widely distributed in the fungal kingdom and is involved in the detoxification systems. The xenobiotic degradation process involves the internalization of the compounds and the preliminary attack by cytochrome P450 (CYP) enzymes, followed by further transformations by epoxide hydrolases. This transformation produces hydroxylated metabolites that can lead to the ring cleave and mineralization through the β-ketoadipate pathway (Fuchs et al. 2011). In phase II of the fungal xenobiotic metabolism, miscellaneous transferase enzymes can include functional groups to the xenobiotic compounds, leading to the formation of more soluble compounds (Cerniglia and Sutherland 2010).

This intracellular enzymatic system is not well-known and may be more diverse than initially thought, as recent studies reveal the high diversity in the CYP family (Morel et al. 2013). In addition, the possibility of an alternative anaerobic pathway in fungi remains understudied. Thus, genetic engineering experiments with these fungal microorganisms can present an important tool for the future of the bioremediation.

Table 4.1 Main fungal enzymes involved in the metabolism of xenobiotic

Enzyme	Known gene examples	Regulation of fungal production	Bibliography
Caldariomyces fumago haem chloroperoxidase (CPO)	*Htp1– htp8 Caldariomyces fumago*	Production in complex liquid media	Kellner et al. (2016)
Cytochrome P450 monooxygenases	*CYP52X2*, *CYP53A1*, *CYP53A11*, *CYP53A24*	Differential transcriptional regulation in response to xenobiotic compounds and carbon sources	Huarte-Bonnet et al. (2018)
Dye decolorizing peroxidases (DyP)	*AauDyP (Auricularia auricula-judae)*, *MepDyP (Exidia glandulosa)*	Fungal production in phenolic complex liquid media	Liers et al. (2013)
Laccases	*lacc1 (pox4)*; *lacc2 (Lpoxa3) Pleurotus ostreatus*	Differential transcriptional regulation in response to Cu, Mn, Cd, Ag, various organic compounds, N, and C	Pezzella et al. (2013)
Lignin peroxidases (LiP)	*Pclip1 Phanerochaete chrysosporium*		Holzbaur and Tien (1988)
Mn peroxidases (MnP)	Mnp1, mnp, mnp6, mnp7, mnp8 (*Pleurotus ostreatus*)	Differential transcriptional regulation in response to Mn	Knop et al. (2014)
Quinone reductases	qr1 (*Gloeophyllum trabeum*); qr (*Phanerochaete chrysosporium*)	Differential transcriptional regulation in response to heat shock and chemicals	Jensen et al. (2001)
Unspecific peroxygenase (UPO)	*UPO1 Agrocybe aegerita*	Fungal production in soya media	Pecyna et al. (2009))
Versatile peroxidases (VP)	vpl2 (*Pleurotus eryngii*); vp (*Bjerkandera adusta*); mnp2, mnp4, mnp5 (*Pleurotus ostreatus*)		Ruiz-Dueñas et al. (2008), Martínez (2002), and Knop et al. (2014)

Adapted from Harms et al. (2011)

4.3 The Relevance of Gene Databases in Fungal Engineering

Recent advancements in biotechnology have made new opportunities for research in genetic engineering with respect to biodegradation. The idea of engineering a microbial metabolism to break down toxic compounds in soil and water is an appealing possibility for remediating heavily polluted areas. The application of fungi as tools in bioremediation can present many advantages over manual removal of toxic chemicals in terms of cost and time efficiency. Strains of fungi isolated from heavily contaminated soils have already proved to play a role in the degradation

of xenobiotics such as polycyclic aromatic hydrocarbons (PAHs) and polychlorinated hydrocarbons and polychlorinated biphenyls (PCBs) (Potin et al. 2004; Mineki et al. 2015).

To exploit this element of fungal metabolism that results in PAH and PCB degradation requires a greater depth of understanding the genetics at play in the system. Identifying and targeting the genes which control xenobiotic metabolism in fungi can allow researchers to better attribute elements of the xenobiotic degradation mechanism to specific enzymatic activity. With this kind of control, it would be possible to fine-tune the process, upregulating the production of enzymes which have proven directly responsible for xenobiotic degradation.

Metabolic engineering in fungi has already been applied in the model filamentous fungi, *Aspergillus niger*. Generally, the organism is engineered with the intent of producing industrially relevant proteins or chemicals (Poulsen et al. 2005; Schmid et al. 2009; Kuivanen et al. 2015).

Sequenced and annotated genomes are immensely useful, if not required for more nuanced genetic and metabolic engineering (Albertsen et al. 2006; Ghosal et al. 2016). Specialized processes in fungal biodegradation tend to be less genetically conserved than other more basal functions of cellular metabolism (Wisecaver et al. 2014; Wakai et al. 2017). Pinpointing exact sequence data encoding for enzymatic elements of the fungal xenobiotic metabolism is not yet a possibility. Regardless, there is an increasing push for fully or partially sequenced fungal genomes, which may aid in the efforts of geneticists interested in fungal xenobiotic degradation mechanisms (Grigoriev et al. 2014). Fungal genetics are becoming increasingly available in the NCBI BLAST search base, as well as in specific gene libraries.

The Aspergillus Genome Database (AspGD.org) is one example of a library specific to the genus *Aspergillus* and focuses on the genetics of four popular species in academic and industrial research. The four species of fungi featured in the AspGD includes *A. niger*, *A. nidulans*, *A. oryzae*, and *A. fumigatus*. AspGD offers alignment tools, primer design, and links to relevant literature, among other features. The specificity of the AspGD is useful and convenient in its concentrated focus and may offer more carefully curated genomes. However, while the data bank is still available online, some aspects of the sequence data may be dated. The AspGD catalogue has been inactive since 2015; as such, users would be wise to cross-check information from the AspGD with other fungal genetic libraries (Descorps-Declère et al. 2008).

One such library of fungal genetics is the FungiDB (FungiDB.org), a subset of the EuPathDB, which focuses mainly on eukaryote pathogens. FungiDB has combined datasets from a variety of online sources to integrate nearly 100 fungal genomes on one platform. In addition to a gene catalogue, the FungiDB also hosts functional data (proteomics, transcriptomics) and offers tools for gene ontology searches (Basenko et al. 2018). Ontology is further emphasized by the Comparative Fungal Genomics Platform (CFGP). The CFGP offers a localized workspace with features for comparing and organizing genetic and functional data, facilitating trend analysis. For example, the proteomics tool dubbed InterProScan offers protein prediction from sequence data. Additionally, and perhaps more useful for gene

inference and manipulation, the CFGP offers multiple sequence alignment by ClustalW, to locate sequence similarities across species. The CFGP library is linked to the NCBI BLAST search base, and its BLASTMatrix feature runs multiple, simultaneous BLAST searches from CFGP datasets. All data mining can be organized and saved to the workspace, making the CFGP a convenient package for layered studies in genomics and functional genetics (Park et al. 2007).

There has been a significant push for data in fungal genetics of late. In efforts with the US Department of Energy Joint Genome Institute, the MycoCosm fungal genetics portal launched a scheme to sequence the genomes of 1000 species of fungi. To help close gaps in knowledge of fungal genetics, users could input their own data or nominate species for genome sequencing (https://genome.jgi.doe.gov/programs/fungi/1000fungalgenomes.jsf) (Grigoriev et al. 2014). This push for sequenced fungal genomes proves fungal genetics to be an emerging and important field with respect to bioengineering. The massive data input in this pursuit may still present challenges in the parsing of these genomes. Most sequenced fungal genes, even in popular model species, remain uncharacterized. While it is relatively straightforward to directly compare DNA and RNA sequence reads, for the information to be more readily accessible, researchers must prioritize processing sequence data to identify protein-coding genes, promotor regions, intron/exon boundaries, etc. through vigorous annotation (Descorps-Declère et al. 2008; Cerqueira et al. 2013; Schmidt-Dannert 2014; de Vries et al. 2017).

4.4 Earlier Biotechnologies for Gene Manipulation

Sequenced and annotated genomic data from native fungi with capacity for biodegradation is imperative to the study of microbial metabolism for the goal of understanding and designing species with a competitive advantage for xenobiotic degradation in situ, particularly when the method of gene engineering is highly specific (Ghosal et al. 2016; Thion et al. 2012). Gene manipulation can generally be classified into four different categories, from lowest to highest specificity: mutagenesis (random or site-directed), recombinase-based editing, posttranscriptional gene silencing by RNA interference (RNAi), and endonuclease-based editing.

Traditionally chemical and physical mutagens have been used in fungi to induce base substitutions, UV light and monofunctional alkylating agents (4-nitroquinoline 1-oxide, 4NQO; N-methyl-N′-nitro-N-nitrosoguanidine, MNNG; or ethyl methanesulfonate, EMS) or deletions, ionizing radiation, and bifunctional alkylating agents (diepoxybutane, DEB, or diepoxyoctane, DEO) (reviewed in Talbot 2001).

Ectopic integration of exogenous DNA is frequent in fungi and has proven useful to control the expression of genes for large-scale industrial production processes. In addition, this methodology has allowed the identification of gene function and drug discovery (Li et al. 2017). Since the 1980s, ectopic DNA integration has been used to induce random or quasi-random insertional mutagenesis based on (1) *Agrobacterium tumefaciens*, as in *Aspergillus* (Wang et al. 2014), *Colletotrichum* (Cai et al. 2013) and *Verticillium* (Santhanam 2012), (2) restriction enzymes

(restriction enzyme-mediated integration, REMI mutagenesis) (Riggle and Kumamoto 1998; Wang et al. 2007), (3) electroporation (Chakraborty 2015), and also, but less common, (4) particle bombardment (Herzog et al. 1996; Barcellos et al. 1998), or (5) transposon-based DNA delivery (Dufresne and Daboussi 2010).

More precise gene manipulation, gene editing, involves applied biotechnology techniques used to modify the genomes of target organisms by knocking out or replacing specific genes. Gene-editing events are carried out by means of insertions, deletions, or substitutions of DNA sequences and have shown to produce modified organisms with more desirable traits for more than two decades. Unlike transgenesis, gene editing allows changes in the DNA sequence without adding exogenous genes from other organisms. Gene editing makes genetic modification a faster, cheaper, and more accurate method than classical genetics approach (i.e., artificial selection, breeding rare individuals that have desirable phenotypes caused by mutations).

Site-directed mutagenesis and recombinase-mediated gene editing take advantage of the natural ability of the cell to both integrate exogenous DNA and to apply the endogenous recombination system by DNA repair mechanisms. Site-directed mutagenesis uses PCR amplification methods and homologous recombination (HR) to replace/modify the sequence of a specific gene with a copy containing the desired mutation (Stuckey and Storici 2013). In recombinase-mediated editing, site-specific recombinases (i.e., Cre/loxP and Flp/FRT) can knockout or knock-in genes by recognizing 30–40 nucleotide sequences, inducing DNA exchange and enhancing the frequency of HR (Aguiar et al. 2014). In most fungal systems, site-specific recombination frequency is low (<1%) posing a problem for the production of knockout strains (Nakayashiki and Nguyen 2008; Stuckey and Storici 2013).

As an alternative to recombination-based methods, posttranscriptional gene silencing by RNA interference (RNAi) is being successfully used for the manipulation of fungal gene expression (Salame et al. 2011). The expression of one or several genes can be reduced or completely silenced (transcript level can be close to zero in some transformants (Kück and Hoff 2010)) inserting double-stranded RNAs that trigger the degradation of mRNAs. This is particularly useful when the target sequence belongs to a multi-copy gene family or the deletion of the gene is lethal.

The recent development of cost-effective methods for high-throughput DNA sequencing and genome annotation has triggered the rapid development of gene-editing tools based on the action of endonucleases. The capacity of endonucleases to produce double-stranded breaks (DSBs) and break repairs in vivo through DNA damage response pathways has been taken as an opportunity for genetic engineers to edit genomes more precisely by inserting changes at targeted loci. Breaks repaired by nonhomologous end joining (NHEJ) often yield insertions or deletions which can then cause frameshift mutations to knockout gene function. By contrast, breaks repaired by homology-directed repair (HDR) result in a precisely targeted sequence replacement. As such, a template DNA needs to be provided for HDR. These mechanisms for repair include not only single nucleotide substitutions but also single or multiple transgene insertion.

Endonuclease-based editing generally consists of a bipartite system integrated by a customizable DNA-binding domain to recognize virtually any nucleotide sequence in addition to an endonuclease, which uses the recognition site to specifically produce DSBs. Programmable, site-specific nucleases, the so-called molecular scissors, were chosen as Method of the Year in 2011 for being the most promising tool "to knock out or knock in genes, to make allelic mutants, to change gene-regulatory control and to add reporters or epitope tags, all in the endogenous genomic context" (Nature Methods 2012). There are four main classes of endonuclease-mediated tools used for genome editing: meganucleases (MNs), zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats and associated proteins (CRISPR/Cas) (Abdallah et al. 2015).

MNs or homing nucleases were discovered in the 1990s. Since then, hundreds of naturally occurring meganucleases have been described in many different organisms. Although their function is still unclear, MNs are known to recognize and cleave 12–40 bp DNA sequences (most likely unique or nearly so). I-SceI and I-CreI are the most commonly used MNs for genome engineering (Muñoz et al. 2011; Silva et al. 2011). Engineering MNs to modify their recognition site, when possible, is costly and time-consuming compared to other endonuclease-mediated systems.

Synthetic zinc finger proteins have been engineered for nearly two decades, combining different zinc finger domains able to recognize and bind 9–20 bp sequences. Each zinc finger consists of approximately 30 amino acids that can recognize 3 bp motifs. Pairs of zinc finger domains are generally designed and combined to dimeric nuclease FokI to specifically produce DSBs (Davies et al. 2017).

More recently, TALEN, a system of naturally occurring DNA binding proteins derived from transcription activator-like effectors from the plant pathogen *Xanthomonas* sp., has been applied as a technique for creating targeted genetic modifications. TALEN domains are built by tandem conserved repeat modules of 34 amino acids. Each amino acid recognizes a single base pair, but the ones located at positions 12 and 13 are highly variable (repeat variable di-residue, RVD) and crucial for the recognition of a specific nucleotide. Also, gene targeting requires that binding sites have a thymine base in the 5′ end. TALEN domains can be designed to recognize 33–35 bp sequences (reviewed in Abdallah et al. 2015). TALENs form dimers separated by 10–30 bp regions where dimeric FokI can produce DSBs (Guha and Edgell 2017). In comparison with ZFNs, TALEN engineering is simpler, presents higher rates of cleavage activity, and, apart from nucleases, can be combined to numerous effector domains as site-specific recombinases and transcriptional activators (Joung and Sander 2013; Gaj et al. 2013). However, some authors have suggested that hybrid ZFN and TALEN nucleases can increase specificity in multiple cell types (Yan et al. 2013).

By far, the RNA-guided nuclease system of CRISPR/Cas is the method that has shaken the field of gene editing. In 1987 researchers characterized a prokaryotic system for viral defense in *Escherichia coli*, later named for the idiosyncratic region of DNA characterized by clustered regularly interspaced short palindromic repeats (CRISPR). When first described, it was difficult to foresee the impact CRISPR has

in modern genetics (Ishino et al. 1987). The system, with apparent role in DNA repair or gene regulation, was described as a series of short repeats, split up by spacers of similar size (Makarova et al. 2002). Very soon, it was found that spacers are sequences of foreign DNA from phages or plasmids (Mojica et al. 2000). Up to 10% of bacteria and archaea (Burstein et al. 2016) use these spacer sequences to match and recognize invading viral DNA, which is then inactivated by a double-stranded break produced by the CRISPR-associated (Cas) proteins, enzymes with putative nuclease, and helicase domains (Haft et al. 2005). As such, CRISPR is a form of adaptive viral immunity for microorganisms which regularly take in foreign sequences of DNA and RNA (Mojica et al. 2005; Barrangou et al. 2007; Marraffini 2015).

4.5 An Introduction to CRISPR/Cas Theory and Methodology

While conventional gene induction techniques have been successful in fungi, the process remains time- and labor-intensive and is considered to be generally inefficient as a means of genetic engineering (Wang et al. 2015). As indicated in the previous section, recent developments in biotechnology introduced a fine-tuned methodology for accessible genetic engineering by the exploitation of a unique element found in bacterial viral defense, CRISPR (Jinek et al. 2012; Doudna and Charpentier 2014). Now widely available is the CRISPR-associated enzyme, Cas9, which has been co-opted as a specific DNA-cutting machine. Cas9 is an RNA-guided endonuclease essential to the microbial adaptive immune system and is presently utilized as a high-precision gene engineering tool in an increasing range of organisms (Jinek et al. 2012; Ran et al. 2013; https://www.broadinstitute.org/what-broad/areas-focus/project-spotlight/crispr-timeline).

There are three types of Cas proteins (I–III) which can be further subdivided into ten subtypes (Makarova et al. 2011). While type I and III systems are complex and need the intervention of multiple Cas proteins to cleave the targeted DNA, type II system (described in *Streptococcus pyogenes*) uses only Cas9 protein (Haft et al. 2005; Makarova et al. 2011, 2013; Chylinski et al. 2013). CRISPR-Cas9 works generally in three steps (reviewed in Karvelis et al. 2013); a first contact with the foreign DNA produces (1) immunization: spacers are acquired and included in CRISPR locus. The presence of recurring viral DNA into the cell induces (2) expression; CRISPR is transcribed into crRNAs containing an individual spacer followed by a process of maturation mediated by a trans-activating CRISPR RNA (tracrRNA), and (3) interference: Cas protein binds crRNA and produces a DSB break at a site 3 base pairs from the protospacer adjacent motif (PAM) site, a two to six base pair DNA sequence immediately downstream the targeted DNA sequence (Shah et al. 2013) (Fig. 4.2).

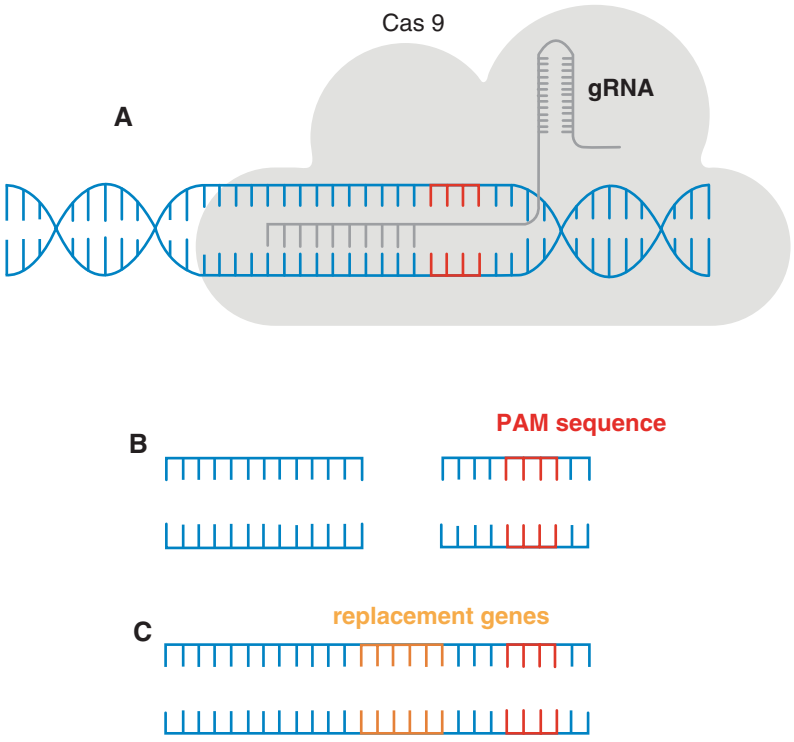

Fig. 4.2 General representation of genome editing CRISPR/Cas9. (**a**) Target DNA to be edited and guide RNA binds. Cas9 enzyme binds and creates double-stranded break in DNA. (**b**) Errors introduced during repairs of gene disrupter. (**c**) Template used during repair and correct sequence restored

For a general CRISPR/Cas9 gene modification, researchers have tuned the Cas9 nuclease to interact with specific sites of host DNA that correspond to a guide RNA. The guide RNA (gRNA) can be essentially any 20-nucleotide sequence flanked by a protospacer adjacent motif (PAM) site, a string of NGG that acts as a signal for Cas9 to make a cut in the gene of interest. When Cas9 binds to the gRNA, the enzyme is then able to traverse the nuclear membrane, localizing to the target site on the genomic DNA which corresponds to the guide. Cas9 then cleaves the DNA at the target site, and the rapid repair mechanism which follows is innately error prone. As such, what results is a precise mutation in the target gene (Rath et al. 2015).

The Cas9 enzyme can also be modified for more complicated engineering purposes. For example, Cas enzymes can be constructed to include fused activators or repressors to respectively up- or downregulate the target gene (Ran et al. 2013; Ceasar et al. 2016; Graham and Root 2015). The efficacy of these modified Cas enzymes and the relatively abundant presence of PAM sites in the genome have made the CRISPR/Cas system something of a watershed for the potential

applicability of biotechnology and genetic engineering. In considering the effectiveness of Cas9, it may be useful in some cases to bear in mind that Cas9 is only one enzyme in a family of Cas proteins. The Cas family is a diverse group which offers four types of CRISPR/Cas systems featuring endonucleases that can be useful in varying degrees to researchers depending on the intent of experimentation. While the type II Cas9 is perhaps the most widely applied variety, the type III Cas10 enzyme, for example, has been shown to target and cut RNA sites as well as DNA sites (Rath et al. 2015).

In fungi, CRISPR/Cas has already proven effective in several species of filamentous fungi (Nødvig et al. 2015; Fuller et al. 2015; Shi et al. 2017; Zheng et al. 2017). Generally, experiments in fungi using CRISPR/Cas systems have been employed to efficiently synthesize chemical products for industry (Kuivanen et al. 2016; Pohl et al. 2016). Though catabolic gene systems in fungi have been of relevance to research in applied bioengineering for some time, in the case of CRISPR/Cas modifications, RNA sequencing events must first be performed due to specificity of the editing system (Fowler and Berka 1991; Descorps-Declère et al. 2008; Kuivanen et al. 2016).

4.6 Potential Experiments and Future Directions for Manipulating Fungal Xenobiotic Metabolism

While many of the CRISPR experiments in fungi have been shown to disrupt genes by insertion/deletion and point mutations, there are several possibilities for experiments to apply a degree of metabolic engineering in the degradation of xenobiotics. For example, using an inactive, enzymatically "dead" Cas nuclease, dCas9, researchers may specifically target genes for visualizations or, as mentioned earlier, regulation through attached biomolecules (Qi et al. 2013).

Through annotated genomics and transcriptomics, researchers can identify not only specific genes responsible for encoding proteins involved in xenobiotic metabolism but also the promoter and enhancer regions. A dCas9 enzyme situated on a promotor region could theoretically inspire an uptick in production of PAH-degrading enzymes at the transcription region. An interesting experiment would be to track if an increase in the rate of, for example, certain cytochrome P450 enzymes, a family known to play a role in PAH degradation, results in faster xenobiotic metabolism (Syed et al. 2013). With so little known about fungal xenometabolism on a genomic scale, a considerable experiment would be a mass silencing of genes thought to be involved in degradation. Each gene pinpointed and silenced, through a static, inactivated dCas9, may produce different insights into the mechanistic aspects and genetic elements of PAH degradation. Silencing by inactivation can be more effective considering mortality of the subject, as edited genes may still be transcribed and translated into dysfunctional protein products, causing potential damage to the cell (Watford and Warrington 2017).

To design modified fungi for in situ xenobiotic bioremediation will depend greatly on such preliminary experiments as mentioned above. Viable mutated fungi have shown a capacity to survive laboratory settings, yet the promise of potential in bioremediation through fungi rests on the ability of modified organisms to compete in and detoxify the chemistry of contaminated sites beyond the realm of the controlled experimental backdrop.

4.7 Conclusion

The production of multifunctional fungal strains able to efficiently degrade xenobiotic compounds can prove fundamental in efforts to rehabilitate contaminated lands. Genetic engineering experiments to specifically upregulate relevant degradative enzymes can offer an avenue of eliminating contamination in polluted areas. There is fundamental groundwork in basic research that must be executed to apply such experiments in the natural environment. Primarily, available and reliable sequence data is the foundation of genetic engineering in fungi. Secondly, access to efficient gene modification techniques, such as the CRISPR/Cas9 system, shows promise for prospective exploration in fungal xenometabolics. The push for fungal genomes marks that investigations in fungal genetics are a growing field, and this basic data collection is crucial to furthering the potential for manipulation and subsequent augmentation of fungal degradation capacities in real-world applications.

As such, there remains a great deal of research and development in the fields of mycology and genetic engineering for these degradation events using modified fungi to take place. Studies concerning the impact of genetically modified microorganisms on microbial communities and the environment should not be overlooked in the process. Regardless, there is great potential for experiments in fungal genetics and genetic engineering as a means of bioremediation and for understand how to better regulate the potential use of microorganisms in natural ecosystems.

Acknowledgment HPS acknowledges the Fulbright Program (PS00247479) for the Open Study/Research Grant. RNP was funded by Ramón y Cajal program (RYC-2011-08653). EA gratefully thanks the Ministry of Economy and Competitiveness (MINECO) and FEDER funds for co-funding the Ramón y Cajal contract (RYC-2013-12481).

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