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Genome Editing in Bacteria

(Part 2)

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PREFACE

In the vast landscape of scientific exploration, genetic engineering stands as a beacon illuminating pathways in both basic research and industrial biotechnology. At its heart lie metabolic and genomic manipulations that coax microorganisms to yield invaluable products, sparking innovations that redefine possibilities.

The saga of genetic inquiry into microorganisms hinges upon accessibility to their genomes and the arsenal of molecular tools at our disposal. Early genetic methods for genome editing in bacterial species, rooted in culture and transformation, were painstakingly laborious, often reliant on introducing resistance markers that hindered the pursuit of precise edits such as single amino acid mutations.

Yet, the tide turned with the groundbreaking discovery of CRISPR-Cas technology, unraveling the adaptive immune system of prokaryotes and unfurling vistas of targeted genetic engineering in these organisms. In this tome, we delve into the cutting edge gene editing, exploring diverse strategies employed in prokaryotic genetic manipulation.

This book embarks on a journey that traverses historical perspectives of genome editing, its application in probiotics, and its relevance in agricultural and environmental microbiology. It endeavors to consolidate and update the compendium of knowledge and research in bacterial applications across industries like food and pharmaceuticals, illuminating gene regulation for metabolic engineering through genome editing tools.

Our heartfelt gratitude extends to the esteemed contributing authors who embraced our call to enrich this compendium. Each chapter bears the mark of dedication and expertise, a testament to their profound contributions to bacteriology and molecular biology.

The Bentham Science Group's commitment to publication has facilitated the realization of this comprehensive endeavor, offering a resource intended for researchers, students, teachers, scientists, and enterprising minds intrigued by bacterial metabolic engineering.

In the vast ocean of scientific literature, this book, "Gene Editing in Bacteria," stands as a pioneering compilation, weaving together diverse applications of bacteria across the tapestry of biotechnology.

I dedicate this book to the pioneers of indigenous knowledge in molecular biology and genetic engineering. They not only laid the foundation for an ocean of knowledge but also kindled the flame that propels our relentless pursuit of understanding genome editing techniques in bacteria.

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CHAPTER 1

Advances in Microbial Study for Crop Improvement

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Abstract: Now and in the future, meeting the global demand for healthy food for the ever-increasing population is a crucial challenge. In the last seven decades, agricultural practices have shifted to the use of synthetic fertilizers and pesticides to achieve higher yields. Despite the huge contribution of synthetic fertilizers in agronomy, their adverse effects on the environment, natural microbial habitat, and human health cannot be underrated. Besides, synthetic fertilizers are manufactured from non-renewable sources such as earth mining or rock exploitation. In this context, understanding and exploiting soil microbiota appears promising to enhance crop production without jeopardizing the environment and human health. This chapter reviews the historical as well as current research efforts made in identifying the interaction between soil microbes and root exudates for crop improvement. First, microbial consortium *viz.* bacteria, algae, fungi, and protozoa are briefly discussed. Then, the application of bio-stimulants followed by genome editing of microbes for crop improvement is summarized. Finally, the perspectives and opportunities to produce bioenergy and bio-fertilizers are analyzed.

Keywords: Biofertilizer, Crop improvement, Genetic engineering, Microbial consortium, Rhizosphere.

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INTRODUCTION

The world population is constantly increasing and is projected to be 10 billion by 2050. Barea [1] estimated that by 2050, food demand is supposed to increase by 70% in the agricultural area. Although conventional farming (high-yield varieties, irrigation, synthetic pesticides and fertilizers) has shown an increase in food production by 70% from 1970 to 1995 in developing countries, its adverse effects on the environment, plants, humans, and aquatic ecosystem cannot be overlooked [2, 3]. Therefore, it is time to change our trajectory towards advanced microbial agricultural practices to combat pests and provide natural nutrition resources to plants without compromising the sustainable environment [4]. A microbial consortium is set of microorganisms, including bacteria, *Cyanobacteria*, algae, protozoa, yeast, and fungi, that works synergistically for hydrolyzing biomass, there by increasing soil fertility [5]. Soil bacteria are very important for biogeochemical cycle and agriculture. Plant-soil bacteria interaction plays a key role in determining the plants' health and growth. Usually, such beneficial bacteria are termed plant growth promoting *Rhizobacteria* (PGPR), which colonize in rhizosphere [6]. Species of *Rhizobium* (*Allorhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium*, and *Sinorhizobium*) form symbiotic relationship with legume plants, through flavonoids signals produced by plants. Flavonoids lead to nodule formation by inducing nodulation (nod) genes in Rhizobia [7]. PGPR is being used worldwide to increase crop production [8 - 10]. On the other hand, non-symbiotic PGPR such as *Azospirillum* enhances plant's resistance and ion uptake by producing antibacterial and antifungal compounds, growth regulators and siderophores [11]. Further, *Cyanobacteria* play an important role in raising the oxygen level in the atmosphere and ocean. Oxygenic photosynthesis enabled aquatic and terrestrial environments to undergo diversification and form complex life [12, 13]. *Cyanobacteria Anabaena*, *Calothrix*, *Scytonema*, and *Nostoc* have been widely used in rice cultivation. These *Cyanobacteria* develop specialized cells heterocysts to fix the aerobic nitrogen, particularly when nitrate and ammonia are limited in soil [14]. Recently, a pot experiment study has demonstrated that inoculation of *Nostoc* caused significant increase in root length. However, half dose of recommended chemical fertilizer with *Nostoc* improved the growth and production of rice. Pathum Thani [15]. Rice sheath blight is a serious disease in Asian countries caused by pathogenic fungi *Rhizoctonia solani*. Application of *Nostoc piscinale* (SCAU04) and *Anabaena variabilis* (SCAU26) found to produce bioactive substances to inhibit *R. solani* by 90%, and secrete phytohormones to promote plant growth and development, and induce resistance against disease. Fungi are mostly considered harmful pathogens for both plants and animals, because they produce mycotoxins as secondary metabolites. The major mycotoxins are aflatoxin, ochratoxins, trichothecenes, fumonisins, zearalenone, cyclopiazonic acid, and putulin [16]. In

contrast, *Trichoderma*, *Aspergillus*, and *Clonostachys rosea* are beneficial fungi, found to be very effective against mycotoxin producing *Fusarium* and *Aspergillus* [17, 18]. These fungi have special characteristics such as promoting plant growth, producing antibiotics, and parasitizes other fungi (hyperparasitism) [19]. Seed coating with PGPR, rhizobia, arbuscular mycorrhizal fungi, and Trichoderma resulted in higher yield and resistance against pathogens in several plant species, thus can be used as an ideal biocontrol agent instead of chemical fungicide [20, 21]. In addition to nitrogen fixation, ion uptake, growth promotion, and protection from toxins, microbes are being explored for wastewater treatment, biodiesel production, bioelectricity, and biosensing [22 - 24]. In this regard, *Saccharomyces cerevisiae*, *Pichia stipitis*, and *Kluyveromyces fagilis* have been used extensively for ethanol production [25]. Metabolic engineering of *Clostridium acetobutylicum* enhanced butanol yield of 0.71 mol butanol/mol glucose, which was 245% higher compared to wild-type strains [26]. Some Oleaginous yeasts like *Cryptococcus psychrotolerans* (IITRFD) and *Rhodosporidiobolus fluvialis* (DMKU-SP314) are used for the production of biodiesel [27, 28]. Here, we have diSome Oleaginous yeasts likscussed the current scenario of microbial uses in crop improvement by biochemical and genetic engineering approaches.

MICROBIAL CONSORTIA

Rhizosphere microorganism plays an essential role in sustainable agriculture, influencing natural plant communities' composition and productivity (Fig. **1**). Bacteria, archaea, fungi, algae, viruses, protozoa, oomycetes and microarthropods are the microbial groups residing in the rhizosphere [29]. The leading population of microbes in the rhizosphere is bacteria, trailed by fungi, actinomycetes and other groups. Bacteria, fungi, algae and protozoa coexist in the rhizosphere and exert multiple strategies to utilize minerals and organic wastes. They act as metal sequestering and growth-promoting bioinoculants for plants in metal-stressed soils [29].

Bacteria

Azospirillum, Azotobacter, Bacillus, Enterobacter, Pseudomonas and *Serratia* are successfully used along with *Rhizobium* for microbial consortia for crop improvement [30]. Microbial consortia under extreme environmental conditions enhance crop production. The production of plant growth hormones and vitamins are significantly increased with the application of *Rhizobium* along with *Azotobacter* as consortia [31]. Rhizobium's microbial consortia with *G. intraradices* and *P. striata* show enhanced plant growth in chickpeas root rot along with improved chlorophyll content [32]. Consortia of *Mezorhizobium sp.* and *P.aeruginosa* increased dry weight and nodule formation in chickpeas [33].

CHAPTER 2

Genome Editing Against Bacterial Plant Pathogens

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Abstract: Meeting the crucial demand for sustainable agriculture is an upcoming challenge worldwide, leading to global food security concerns. Approximately 50% of agricultural loss is caused by both biotic and abiotic stresses. As per the estimation of Agrios, 42% of crop loss is characterized by biotic stress alone. Bacteria are the second largest contributor in terms of economic losses caused by various plant diseases. Hence, there is a need to develop elite cultivars in amalgamation with readily available sequenced plant database and progressive genome editing. This has proved to be a groundbreaking/milestone in the field of plant breeding for any desired trait. Until now, many new plant breeding techniques (NPBTs) have been introduced for crop improvement. These techniques include site-specific mutagenesis, cisgenesis, intragenesis, breeding with transgenic inducer lines, *etc*. This book chapter provides a comparative understanding of enrichment in plant genome editing approach about bacterial pathogens aiming for sustainable agriculture development. This chapter also brings a broad aspect of the application, advantages, unsighted aspects of genome editing, and future challenges.

Keywords: Bacterial plant pathogen, NPBTs, Plant genome editing, Sustainable agriculture.

INTRODUCTION

The world's population is rapidly rising and will reach about 9.8 billion by 2050. To fulfill nutrient requirements for the rising population, much more food is needed, but at present, the challenges in the agriculture sector due to various biotic as well as abiotic factors are the biggest concern. Plants are continuously exposed to a large set of pathogens, including bacteria, fungi, oomycetes, and viruses. The world has consistently seen about 20–40% yield loss due to the biotic impacts [1, 2].

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Understanding the molecular mechanism between plants and communities of bacteria, fungi, and other microorganisms has been a significant area of investigation in plant pathology for many years. However, we cannot deny that despite decades of research, we have a very limited understanding of the molecular mechanisms of host-pathogen interactions. The pathogen and the host play an endless arms race game between them. When the plant host and pathogen come in contact, the interaction turns into a fight of recognition and evasion. A multilayer defense system including pathogen-associated molecular patterns (PAMPs)-triggered immunity (PTI) and effector-triggered immunity (ETI) has involved plants in battling against interfering pathogens for survival. The PTI gets activated through the recognition of PAMPs by pattern recognition receptors (PRRs) which results in the production of reactive oxygen species (ROS), callose deposition, and transcriptional reprogramming, which usually prevents the invasion of non-adapted pathogens. In contrary, to modulate host cell physiology, pathogens secrete effectors to interrupt PTI and this results in effector-triggered susceptibility (ETS). In resistant plant varieties, these effectors or byproducts can be recognized by intracellular immune receptors and induce ETI (a robust resistance response). This is usually associated with localized plant cell death leading to pathogen arrest. However, as these pathogens have high evolutionary potential, they can overcome the host's ETI response *via* loss and/or modification of ETI-eliciting effectors as well as meta-effector interactions [3 - 5]. Hence, phytopathogens are difficult to control.

The most effective approaches that control plant disease depend on resistant varieties and agrochemicals. However, as explained earlier, many plant pathogens have high evolutionary potential, novel genotypes no longer sensitive to the resistance gene or the phytosanitary product can rapidly emerge *via* mutation or recombination. Hence, the enhancement of plant resistance plays an important role in adjusting crop production to meet global population increases [6, 7]. The major aim of this chapter is to highlight the applications of genome editing against different bacterial plant pathogens.

EXISTING GENOME EDITING TECHNIQUES AND ADVANCEMENT UNTIL NOW

Over the past few years, new plant breeding techniques have been the most useful approach for developing new crop improvement, including pathogen- resistance [8, 9]. New plant breeding techniques include the usage of Meganucleases (MN), also known as homing endonucleases, zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and newly emerged clustered regularly interspaced short palindrome repeats (CRISPR)/CRISPR-associated protein 9 (Cas9), which have revolutionized targeted modifications of genomes

and have greatly transformed the researches on plants [10]. The requirement of sophisticated protein engineering rendered MN, ZFN, and TALEN techniques less practicable.

Meganucleases

Meganucleases, also called homing endonucleases, have been used for more than 15 years to induce gene targeting. Although they have been rarely used in crop-related gene editing to date, their scope of applications, especially in gene therapy, has further enhanced due to the recent advances in re-engineering meganuclease specificity [11, 12]. They are divided into five families based on the sequence and structure motifs: LAGLIDADG, GIY-YIG, HNH, His-Cys box, and PD-(D/E)XK. Among these families, the LAGLIDADG proteins have been found in all kingdoms of life and are the most well studied [12]. These proteins generally encode within introns or inteins, but freestanding members also exist. They are highly specific endonucleases capable of recognizing and cleaving the exon-exon junction sequence wherein their intron resides (Fig. **1**). Additionally, unlike restriction enzymes, the proteins facilitate lateral mobility of genetic elements within an organism. This process is referred to as "homing" and gives the name to HEs [10].

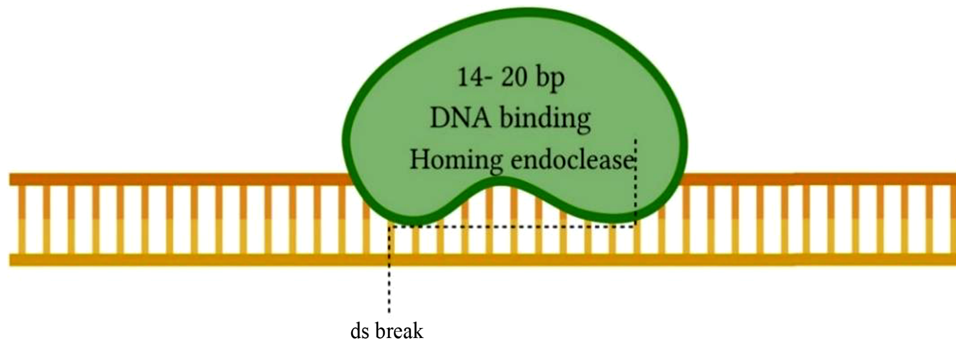

Fig. (1). Schematic representation of Meganuclease components and Meganuclease based genome editing. This figure was created by authors using BioRender.

MN allows insertion, deletion, single-site mutation, and correction in a highly site-specific and controlled fashion. Furthermore, viral vectors are also available for endonuclease delivery as a novel approach to plant engineering; therefore, they are regularly used for applications in medicine, public health, and agronomy. In agriculture, LAGLIDADG Meganucleases I-Crel has been modified for agricultural applications in maize. The endonuclease gene was delivered to immature embryos to generate transgenic plants, with deletions and insertions

CHAPTER 3

CRISPR-Cas for Genome Editing - Molecular Scissors for Combating Pathogens

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Abstract: Clustered Regularly Interspaced Short Palindromic Repeats, abbreviated as CRISPR, is a genome-editing technology that permits the creation of precise knock-out mutants by aiding the modification of gene sequences devoid of the steps involving the insertion of foreign DNA into pathogenic microorganisms. The microorganisms are ubiquitous in nature and harbor in the complex ecosystem of the human being. Cas (acronym for CRISPR-associated) genes are present in many microbial genomes. The variable nature of the microbial genome has been utilized as an integral typing tool in epidemiologic, diagnostic, and evolutionary analyses of the prokaryotic species. The past decade has seen an accumulating growth in the development of gene-editing tools utilizing the CRISPR-Cas system, which essentially is a part of the prokaryotic immune system. The development of these unique gene-editing techniques has empowered researchers to alter and investigate organisms with ease and efficiency as never before. This editing tool can efficiently be programmed and delivered into the bacterial populations to explicitly eliminate members of a targeted micro biome. Manipulation of the gene expression and regulation of the synthesis of metabolites and proteins can be achieved by utilizing an engineered CRISPR-Cas system. Put together, these tools present with the exhilarating opportunity to explore the complex interaction between the individual species of the microbiome and the host organism and thereby reveal novel avenues for the generation of drugs to selectively target the microbiome. CRISPR-Cas technology has been employed to cope with antibiotic resistance in intracellular and extracellular pathogens. The widespread use of antibiotics and the escalation of multidrug-resistant (MDR) bacteria boost the prospect of a post-antibiotic era, which emphasizes the need for novel strategies to target MDR pathogens. The development of permissive synthetic biology techniques offers favorable solutions to carry through safe and efficient antibacterial therapies.

Keywords: CRISPR-Cas, Epigenetic regulation, Genome engineering, Knock-out and -in genes, Synthetic biology.

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INTRODUCTION

The emanation of disease-causing organisms into multidrug-resistant (MDR) variants has metamorphosed into a critical global health quandary. Essentially, the gradual progression of bacteria to MDR strains is an unbridled phenomenon and requires expeditious remedial efforts to curb the longstanding damage. In accordance with the information bulletin on the global prevalence of antimicrobial resistance published by the World Health Organization (WHO) in 2014, an escalation in the frequency of drug-resistant pathogenic bacteria has rendered the treatment of previously easily curable diseases, like pneumonia, urinary infections and circulatory diseases, worldwide. A global action plan has been accredited by the 68th World Health Assembly in 2015 to contrive the perception and assimilation of antimicrobial resistance (AMR) [1]. The aforementioned proposal suggests developing novel drugs, vaccines, diagnostic technologies, and different interventional strategies to ensure continuous management of bacterial diseases. Therefore, there is a pressing need to discern the drug resistance process in MDR bacteria so as to earmark features (like efflux pump for removal of antibiotics, and site mutation at the activity sites) to intercept the pathogenic activity. Furthermore, discovering pioneering and advanced low molecular weight drugs and metabolically designing the synthesis of such small molecule drugs is critical to mitigating the drug resistance in MDR microorganisms.

The emergence of singular synthetic biology (SB) and eukaryotic genome editing/engineering tools impart encouraging diagnostic and management measures to detect and remedy the prevalent refractory bacterial disorders. Major progress in genetic engineering techniques has successfully helped in focusing and editing pathogenic eukaryotic genomes for superior understanding and the extenuation of the drug resistance processes. The implementation of singular genome engineering and SB techniques, like CRISPR-Cas systems, recombination- mediated genetic engineering, and the eukaryotic intercellular signaling mechanisms, for pathogen targeting has been pivotal in the establishment of antibacterial strategies, specifically, the development of vaccines, new antibiotics, phage therapies, and specialized diagnostics.

Innovative genome engineering techniques created as a result of recent developments in SB have made it easier to manipulate microbial genomes for a variety of therapeutic and scientific purposes [2 - 4]. A novel platform is offered by SB to bring together the concepts of basic research and further aid its application in translational research. This kind of approach opens up great potential for providing revolutionary solutions when dealing with infectious agents. SB is a field with great promise due to its inherent nature of combining biological know-how and engineering principles to devise novel, tunable and

modular genetic circuits or products for modulating the existing biological systems. Synthetic biologists have a need and a rising interest in using microbial genome editing technologies to supplement the creation of genetic circuits for specific cellular action or metabolic regulation. Advantageous biological features have been established in the engineered species as a result of a precise modification in the eukaryotic genome. The combination of genome SB and editing tools has also made it possible to use genetically modified bacteria to address a number of significant problems in a variety of fields, from renewable energy to global health. New strategies for preventing bacterial infections have emerged, particularly in light of recent progress in bacterial gene editing techniques that can be directed against a wide variety of bacterial hosts [5]. The generation of new SB tools is proving to be a harbinger for establishing novel strategies to address the imminent peril due to antibiotic resistant bacteria. Implementation of SB in bacteria typically entails the development of brief gene circuits for a chosen pathway or gene function up to the modification of the organism's whole gene pool [6, 7]. Insights into the molecular underpinnings of antibiotic resistance have also been provided by a number of studies utilizing SB. For example, *Escherichia coli* was given a deadly dose of the antibiotic medication triclosan in an effort to identify the potential genes that were involved in resisting triclosan [8]. Identification of the crucial genes in the bacteria (*E. coli*, *Pseudomonas putida*, and *Mycoplasma*) for potential therapeutic use has been possible by synthesizing minimal bacterial genomes using bottom-up and top-down strategies [9]. The potential for growth and the influence of SB in negating the effect of antibiotic resistance are apparent from the aforementioned examples. The application of SB tools and bacterial genome engineering in targeting emergent bacterial pathogens focuses on utilizing SB in introducing pioneering antibacterial therapeutic approaches.

Numerous approaches have been tried to potentially create bacterial gene sets with varying degrees of specificity, efficacy, and application across a wide range of hosts [10]. Bacterial gene editing is more frequently done to introduce mutations into the bacterial genome or to create knock-in and knock-out genes. Although many of these techniques were initially created in *E. coli*, they have recently undergone rapid growth and expansion across a variety of bacterial hosts. It is important that these molecular engineering tools are tractable in various pathogenic bacteria that are paving the avenues for further examination and discernment of these pathogens to impede bacterial infections. The principles and procedures of bacterial genome engineering tools have also been listed in several positive evaluations [11]. Many human diseases are genetically determined; as of now, over 6000 hereditary diseases are known to be accounted for by gene and chromosomal DNA mutations, both by nuclear and mitochondrial [12]. The medicament of hereditary diseases has mostly been symptomatic, until recently

CHAPTER 4

Genome Editing of Plant Growth-Promoting Microbes (PGPM) Towards Developing Smart Bio-Formulations for Sustainable Agriculture: Current Trends and Perspectives

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Abstract: Plant-associated microbes, referred to as plant microbiomes, are an integral part of the plant system. The multifaceted role of plant microbiota in combating both abiotic and biotic stresses is well documented in different crop species. However, understanding the co-evolution of plant growth- promoting microbes (PGPM) and PGP traits at genetic and molecular levels requires robust molecular tools to unravel the functional gene orthologues involved in plant-microbe interaction. The advent of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas9 (CRISPR-associated protein 9) is of paramount importance in deciphering the plant-microbe interaction and addressing the challenges of unraveling endophytic microbes and their benefits thereof. Our knowledge of plant microbiome composition, signaling cues, secondary metabolites, microbial volatiles, and other driving factors in plant microbiome has been enlightened. In recent years, scientists have focused more on below-ground dialogue in recruiting efficient microbiome/engineered rhizosphere. More recently, base editing techniques using endo-nucleolytic ally deactivated dCas9 protein and sgRNAs (CRISPR interference or CRISPRi) have emerged as a useful approach to study the gene functions and have potential merits in exploring plant-microbe interactions and the signaling cues involved. A systemic understanding of the signaling events and the respective metabolic pathways will enable the application of

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genome editing tools to enhance the capacity of microbes to produce more targeted metabolites that will enhance microbial colonization.

Further, it will be exciting to employ CRISPR technologies for editing plant-microbe interactions to discover novel metabolic pathways and their modulation for plant immunity and fitness against abiotic as well as biotic stresses. Such metabolites possess tremendous scope in tailoring newer smart nano-based bio-formulations, besides formulating beneficial microbiomes or cocktails, which is the best alternative for climate resilient farming. The present review sheds light upon the deployment of CRISPR/Cas techniques to comprehend plant-microbe interactions, microbe-mediated abiotic and biotic stress resistance, genes edited for the development of fungal, bacterial, and viral disease resistance, nodulation process, PGP activity, CRISPR interference-based gene repression in the PGPM, metabolic pathway editing and their future implications in sustainable agriculture.

Keywords: CRISPR/Cas, Genome editing, Plant-microbe interaction, Sustainable agriculture.

INTRODUCTION

Plant-microbe interaction plays an integral role in sustaining diverse ecosystem services and sharing common ancestors, however, their survival is interdependent. The term ‘Plant microbiota’ or ‘Plant microbiome [PM] ‘has gained more significance. Microbes reside inside roots and shoots as endophytes, besides occupying the rhizosphere, phyllosphere, and spermosphere [1 - 3]. The unique endobiome of apoplastic fluid [4], guard cell [5], and nodule niche [6] in conferring plant fitness created new vistas in the route map of plant-microbe interaction studies. In general, plant-microbe trade-offs lead to unique partnerships depending on their impact on plant health and fitness, *i.e.*, mutualistic [7], neutral, commensalistic, or harmful [7, 8]. The plant-microbe interactions are bi-directional where microbes derive their nutrients from the host plants and *vice versa*. The plant system produces a nutritionally enriched environment with primary and secondary metabolites (inorganic and organic compounds), which is favorable for diverse microbial colonization. Conversely, the microbiome assists the plant to acclimatize fluctuating environmental conditions. The mechanisms include: promoting plant growth, protection against biotic and abiotic stresses, priming the immune system or induction of defense pathways, mycorrhizal symbiosis, nutrient uptake, and conversion of the unavailable nutrients into plant-accessible forms [9]. More precisely, as a direct mechanism, plant beneficial microbiome enhances plant growth through biological nitrogen fixation, phosphorous uptake, and production of phytohormones, specifically, indole-3-acetic acid (IAA), gibberellic acid (GA) and cytokinins [10 - 12]. As an indirect mechanism, plant-beneficial microbes suppress plant pathogens by producing antimicrobials and promoting induced systemic resistance in plants [13 - 15]. In

contrast, many plant-pathogenic microorganisms cause devastating diseases in various crops. These PM interactions are crucial in sustainable agriculture and the environment, for food security and plant health management [16]. Consequently, profiling plant-associated microbiome [genome assemblies of all microbes] is a dawning concept in the field of molecularplant–microbe interactions. An investigation of the host plant together with the associated microbiome (holobiont) suggests the co-evolution ofplant–microbe, plant–plant, andmicrobe–microbe interactions [17]. Recent studies on plant–microbe interactions detailed Avr protein, computational strategies for protein interactions, molecular diversity, and interactions of virulence genes [18].

Next-generation sequencing [NGS], omics approaches [metagenomics, transcriptomics, proteomics, metabolomics], and other computational tools using system biology approaches shed light on the molecular aspects of plant-microbe interactions governing plant traits. Gene-level understanding of plant traits and associated microbes will be a crucial step towards unraveling microbiomes for sustainable agriculture [19, 20]. Anent to this, modern revolutionary techniques induce precise genetic modifications such as clustered regularly interspaced short palindromic repeats-based genome editing, which is an ideal platform to understand plant-microbe interactions for improving crop productivity and priming resistance [21, 22]. This review envisages the various factors shaping the formation of plant microbiota, and the applications of CRISPR-based tools in the beneficial [symbiotic] or harmful [pathogenic] plant-microbiome interactions for sustainable agricultural practices. The limitations and prospects of genome editing tools to alleviate abiotic and biotic stresses are also discussed.

FACTORS INFLUENCING PLANT-MICROBE INTERACTIONS AND THEIR COMPOSITION

In general, microbial assemblage in plants is determined by assembly rules, where plant-associated factors prefer the growth of a particular set of microbes inhibiting others [9]. Another example is the sequential decrease in microbial diversity from bulk soil to rhizosphere. The rhizosphere and phyllosphere communities vary significantly with plant species [23]. Both the biotic and abiotic factors exert profound effects on plant-microbe interaction, microbial community structure, and composition.

Biotic Factors

Plant factors include geographical location, host genotype, age, root phenomics, root and plant secretomes, and the inherent immune system, of which many reports have confirmed plant genotype as a major intriguing factor governing microbial composition [24]. Plant genotype decides the root metabolome that acts

CHAPTER 5

Applications of Genome Editing in Bioremediation

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Abstract: Excessive utilization of chemicals based substances such as pesticides, pharmaceuticals, fertilizers, inappropriate dumping of industrial materials and local wastes, *etc.,* into the environment is leading towards deliverance of high amounts of contaminants such as chlorinated hydrocarbons, dyes, toxins, petroleum and diesel spills into the soil. The mingling of these materials with soil and water is becoming one of the supreme complications associated with the environment, as these contaminants are a potential menace to human health. Bioremediation is a process that has the ability to destroy harmful contaminants and transform them into less toxic forms using living organisms such as bacteria, fungi, plants, *etc*. It is the most up-to-date nature-friendly approach to lower the extent of pollutants in the environment. With continuous developments in the scientific area, researchers are focussing on improving the process of bioremediation by using genome editing technologies. The gene editing techniques have the potential to significantly improve bioremediation processes such as xenobiotic removal, conversion of toxic compounds to less toxic compounds and pesticide degradation to simple components. The main gene editing techniques, CRISPR-Cas, ZFN and TALEN, have the potential to meet the aforementioned goals. This chapter focuses on the various gene editing tools and different genomic strategies such as gene editing, gene circuit, *etc.,* for the alteration or editing of the genome so that their potential value or applications can be seen in various areas.

Keywords: Bioremediation, Chlorinated, Contaminants, Dumping, Environment, Fertilizers, Hydrocarbons, Industrial, Nature-friendly, Pharmaceuticals, Pesticides, Pollutants, Potential, Spills.

INTRODUCTION

Environment plays a principal role in the well-living of all organisms as it furnishes food, water, shelter and other necessary things to living organisms. The standard of life on earth depends upon the quality of the environment present on earth [1]. In earlier times, all the essential resources were available in sufficient quantity, but nowadays, the excessive use of these resources is leading to their disappearance from nature and is also contaminating the environment. Today, the

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earth is experiencing a lot of changes because of development in almost every field, like agriculture, industry, transport, *etc* [2]. The increase in the standard of living is causing an increase in hazardous human activities which may include the disposal of hazardous waste, no proper sludge treatment, use of pesticides, *etc*. These human activities and accumulation of toxins and other pollutants are contaminating all the essential resources like water, soil, land, *etc.,* and causing a negative impact on the quality of our environment [2]. Some sources of environmental contaminants are shown in Fig. (**1**). There is a need to eliminate these contaminants from the environment to have a sustainable living world. This could be possible with the use of a biological technique called 'Bioremediation' [2].

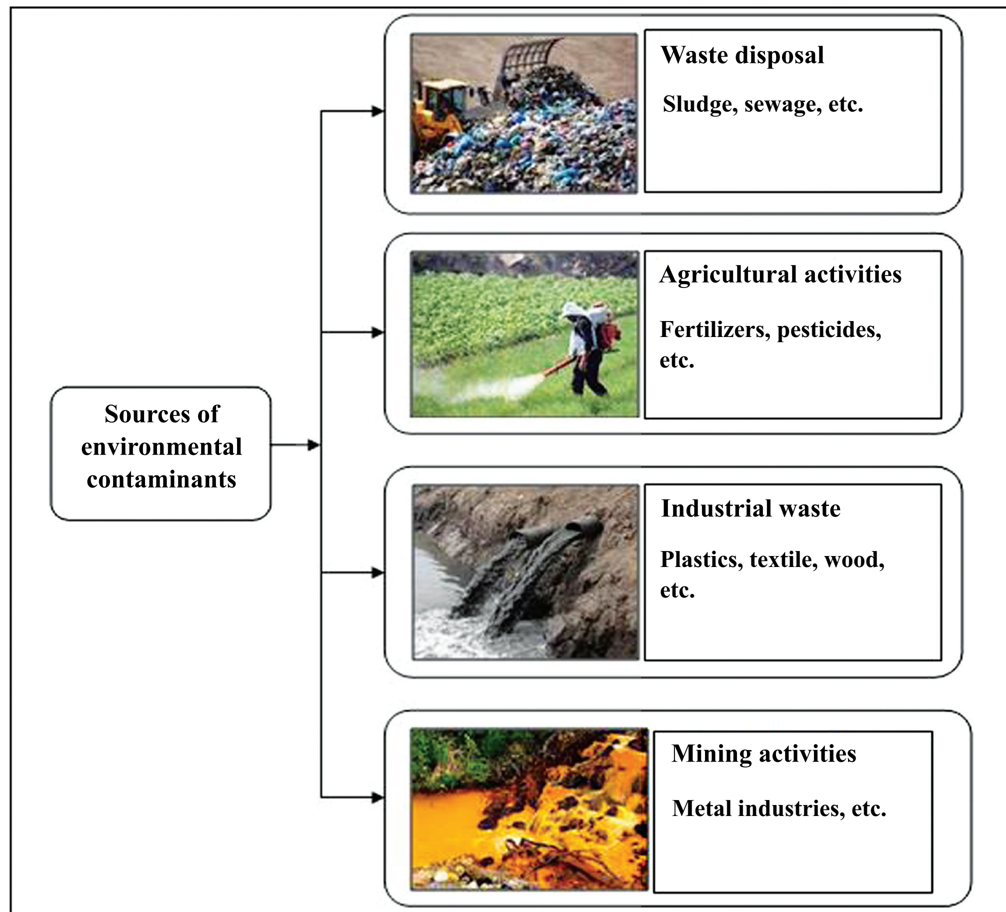

Fig. (1). Some sources of environmental contaminants [3].

Bioremediation is one of the most important practices which involve the use of microorganisms, plants, and fungi to transform hazardous compounds into less-toxic substances under specific conditions. This process is highly involved in the eradication, degradation, immobilization and detoxification of adverse chemical wastes from the environment by the action of microorganisms [4]. Though extremely specialized and diverse microbial groups are present in the environment to remove the harmful pollutants, they perform their task very slowly due to unacceptable environmental conditions, which leads to the accumulation of recalcitrant contaminants such as dichlorodiphenyltrichloroethane (DDT), hexachlorocyclohexane (HCH), *etc.* in the nature [5]. Therefore, to enhance the degradation activities of microbes, there is a need to modify them, which can be achieved by altering their genetic material.

Genomes are highly exposed to errors and changes that arise whenever a cell duplicates its DNA. These errors lead to mutations. When a mutation occurs in a particular gene, it alters its function. Genome editing is a technique of genetic engineering which generally means to change or alter the sequence of DNA or RNA of many organisms, such as microorganisms, plants, animals, *etc.,* by various methods like insertion, deletion, replacement, *etc*. This technology has the ability to treat many genetic disorders which occur due to mutations. The area of genome editing is developing at an immense rate [6]. There are various techniques used in the field of genome editing, which include recombinant DNA technology, Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-Cas9, engineered endonucleases *etc.* These techniques can possibly make the advanced microbes with improved genes of interest that are required for the removal of recalcitrant contaminants from the environment.

BIOREMEDIATION

Bioremediation is a process that depends on some biological processes to degrade, reduce, detoxify, transform or mineralize the congregation of contaminants to a safer state [7]. The process of remediation aided by microbes present at the various polluted frameworks accounts for bioremediation [8, 9]. There are various microorganisms involved in the bioremediation of different harmful contaminants. Some microorganisms are shown in Table **1**.

Table 1. Some microorganisms involved in the bioremediation of contaminants [4].

Microorganism	Contaminants	References
Saccharomyces cerevisiae	Lead, Heavy metals, mercury, nickel, *etc*.	[10]

CHAPTER 6

Genome Editing and Genetically Engineered Bacteria for Bioremediation of Heavy Metals

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Abstract: Genetic engineering involves the manipulation of DNA to either improve, enhance or repair a function by using recombinant DNA technology, which has contributed greatly to the fields of medicine and agriculture. In recent times, the CRISPR-Cas system of gene editing has come to the forefront of genome engineering, transforming disease treatment strategies and the production of modified crops. Industrial activities cause environmental pollution by releasing heavy metal-containing xenobiotic compounds into the environment and affect animal health by causing organ dysfunction and even cancer. Although plants utilize heavy metals from soil in small quantities for their growth, excessive exposure leads to disruption of plant cell machinery and reduces productivity. Similarly, heavy metals degrade soil health by interfering with microbial processes that contribute to soil fertility. Apart from existing methods available for the remediation of contaminated sites, bioremediation is emerging as a potent technique due to its high efficacy, cost-effectiveness and eco-friendly nature. Microbes possess a number of physiological and biochemical properties that have been exploited for the removal and detoxification of metal pollutants. This chapter elaborates on the approaches of gene editing and the development of genetically engineered bacteria to modify the expression of specific genes coding for enzymes that take part in the degradative or detoxification pathway of metals and xenobiotic compounds. It is crucial to address the scope as well as limitations involved in the use of genetically engineered microbes to ensure a safe and cost-effective method for the bioremediation of heavy metal contaminants.

Keywords: Bacteria, Bioaccumulation, Bioremediation, Biosorption, CRISPR-Cas, Genome editing.

INTRODUCTION

Amidst great apprehension in the 1970s, genetic engineering went on to revolutionize the fields of medical science and agriculture with the production of

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recombinant human insulin and genetically modified crops with enhanced traits like drought-resistant maize and many others [1]. Genetic engineering has also been used to enhance livestock by expression of monoclonal antibodies against specific disease-causing pathogens, thereby inducing disease-resistance in the livestock [2]. However, genetically modified foods (GMF) remain opposed by the public on moral and ethical grounds fuelled by the general suspicion of GMF's ill effects on human health [3]. Moreover, genetic engineering of animals gave rise to limitations that ranged from technical to ethical issues [4]. Even though the genes being transferred are naturally occurring in other species, however, the recipient organism may face risks of altered metabolism and growth rates due to the foreign gene expression. These risks may transfer beyond the genetically modified organism and into the natural environment, creating a risk of exposure to other organisms [5].

Horizontal gene transfer (HGT) is a process of gene transfer that occurs naturally in prokaryotes, eukaryotes and even between prokaryotic symbionts and their eukaryotic hosts. HGT plays a significant role in the variation of gene content and contributes to the adaptation potential of the organisms that take part in HGT [6]. Unfortunately, HGT aids in the transfer of antibiotic-resistant genes in bacteria, which is alarming because multidrug resistance (MDR) threatens human and animal health, environment and food safety [7]. According to the World Health Organisation (WHO), the number of human deaths due to MDR across the world is set to escalate to about 10 million by 2050, which is far more than the estimated number of deaths due to cancer [8].

The restriction-modification (R-M) and CRISPR-Cas systems are prokaryotic defence systems against invading phages and plasmids. These two systems are compatible and act together to defend against attacks on the prokaryotic cell [9]. CRISPR (clustered regularly interspaced palindromic repeats) was first discovered in 1987 as short direct repeats interspaced with short sequences in the genome of *Escherichia coli* [10]. Similarly, it was observed that cas(CRISPR-associated) proteins possess putative nuclease and helicase domains which explain the degradation process of foreign DNA [11]. This mechanism of CRISPR-Cas has revolutionized the field of genome editing. Kang and team in 2015 [12] targeted the chemokine (C-C motif) receptor 5 (CCR5), which is an HIV-1 co-receptor, and CRISPR-Cas9 was used to disrupt expression of *CCR5*, thus protecting the cell from HIV infection. In a similar study, individuals homozygous or heterozygous for the C-C chemokine receptor type 5 gene with 32-bp deletions (CCR5Δ32) seemingly resist or show a slower progression of HIV infection, respectively. This was demonstrated by generating the CCR5Δ32 mutation using CRISPR-Cas9 and transcription activator-like effector nucleases (TALENs) in induced pluripotent stem cells (iPSCs) [13]. In cervical carcinoma cells, CRISPR-

Cas9 was used to inactivate the E6 or E7 oncogene in the human papilloma virus (HPV). E6 and E7 function as disruptors or degraders of tumour suppressor genes like p53 and the retinoblastoma (Rb) protein. Hence, inactivation of these oncogenes leads to cancer cell death [14].

The applications of CRISPR-Cas9 extend beyond therapeutic uses and into the food and agricultural industry. Viral infections in plants reduce their yield and create economic constraints [15]. Geminiviruses are associated with a number of plant infections like the yellow mosaic disease, curly top, leaf curling, stunting, and streaks, which ultimately lead to reduced yields [16]. In a study conducted by Baltes and team, the genome of bean yellow dwarf virus (BeYDV) was targeted at six different regions using the CRISPR-Cas9 mechanism to inhibit the replication process of the virus, thereby conferring resistance to the virus in a plant model [17]. In addition to diseases, plants undergo physical and chemical stresses, which potentially reduce crop yield. Herbicides, although useful in removing unwanted weeds and invasive plants, can sometimes damage crops with low resistance to these chemicals [18]. Acetolactate synthase (ALS) is an enzyme involved in the biosynthesis of branched amino acids like valine, leucine, and isoleucine and is present in many species of higher plants as well as bacteria, fungi, yeasts, and algae [19]. In fact, many commercial herbicide families like sulfonylureas, imidazolinones, triazolopyrimidines, pyrimidinylthio (or oxy)-benzoates and sulfonylamino-carbonyltriazolinones inhibit ALS [20]. In a study conducted on *Brassica napus*, point mutations were created using CRISPR/Cas9-mediated cytosine base-editing technology to produce edited *BnALS* genes which rendered the plant herbicide-resistant [21]. Nutritional improvement of crops is another significant application of CRISPR/Cas9. To promote levels of health-promoting nutrients, proanthocyanidins and anthocyanins in rice, Zhu *et al.* [22] converted three white pericarp varieties into red ones (controlled by the complementary genes, *Rc* and *Rd*). They used a CRISPR/Cas9-mediated method where the recessive *rc* allele was functionally restored to the *Rc* allele through site-specific mutagenesis. Rice rich in amylose and resistant starch (RS) is another desirable nutritional improvement as consumption of RS could lead to a reduced glycemic index that is beneficial in preventing of progression of insulin resistance [23]. High amylose content in rice was achieved by down-regulating starch branching enzymes (SBE), *SBEI* and *SBEIIb* through a CRISPR/Cas9-mediated targeted mutagenesis [24].

ENVIRONMENTAL POLLUTION: CAUSES AND IMPACTS

Environmental pollution is a grave and extensive issue that endangers the lives of every being on this planet and also threatens to destabilise human societies. Anthropological activities have aggravated this problem even further by

CHAPTER 7

Designing the Metabolic Capacities of Environmental Bioprocesses through Genome Editing

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Abstract: The ubiquity of the CRISPR gene system in bacteria and archaea is characterized by the Cas9 protein, which functions in the repression and activation of several genes. This inherent function of the CRISPR system can find application in bioprocess optimization in environmental and health research. Owing to the complex and dynamic nature of microbial communities catalysing the bioremediation of urban and industrial toxic waste effluents in wastewater treatment plant (WWTP)/common effluent treatment plant (CETP), such sites represent a relatively untapped area for applying the CRISPR technique. DNA editing using CRISPR can enable the site-specific enhancement in process efficiency of bacterial remediation, which under normal conditions is hampered by its non-selectivity and saturation of binding sites with multiple non-targeted pollutants. Similarly, under the second generation bio-refinery concept, CRISPR can serve as a powerful tool in strengthening and improving the anaerobic bio-processes by genome editing in microbes for the heterogeneous expression of various genes associated with anaerobic digestion. Not only has the CRISPR system been used to insert desired genes in the host genome but also to regulate the expression of the host-specific genes. The role of methanotrophic and nitrogen metabolizing bacteria in shaping the atmospheric gaseous composition can also be monitored *via* CRISPR aided manipulation so as to regulate the nutritional exchange between the atmosphere and the soil. Additionally, genome editing of targeted organisms and crops has found extensive applications in various areas ranging from the nutrigenomics, food and pharmaceutical industry, diagnostics and therapeutics, health and disease prevention.

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Keywords: Anaerobic bioprocesses, Bioremediation, CRISPR, Gene editing, Gut microbiome.

INTRODUCTION

Clustered regularly interspaced short palindromic repeats (CRISPR) system is widely distributed among bacteria and archaea with 87% of the archaeal and 45% of the bacterial genomes and plasmids showing the presence of CRISPR motifs and Cas proteins [1]. This system has evolved through the battle between bacteria and phages [2, 3]. The presence or absence of CRISPR sequences in a prokaryote is associated with the defence mechanism against the phages, while the presence of more than one array of CRISPR sequences corresponds to the selective maintenance by an organism under pathogenic environmental stress and depends on the ecological niche of the organism [4]. For example, in water and wastewater and their distribution systems, the pathogenic microorganisms mostly reside within the biofilms where warfare-like complex interaction is frequently prevalent amongst the members of different microbial communities like protozoa predation, bacterial and viral lysis *etc*. In such interactions, the phage DNA can be merged into the bacterial genome by horizontal gene transfer resulting in the formation of prophages [5]. Thus, the bacteria that protect themselves from the phages are often encountered with foreign DNA fragments integrated into the location of routinely dissected short palindromic repeats — CRISPR-associated proteins (CRISPR/Cas) as spacers [6]. The functionality of the CRISPR system depends on the presence of the CRIPSR-associated genes (*Cas1* and *Cas2*) in the spacer region of the arrays. The spacer sequences between the CRISPR sequences of bacteria are associated with the foreign genetic material linked to viruses or other mobile genetic components.

Genome editing is a powerful tool in basic biology encasing novel capabilities in microbial genomes. The prokaryotic immune system has given rise to the emerging technology called CRISPR/Cas, which has encouraged researchers to easily alter unique organisms for different applications. The use of CRISPR/Cas systems in microbiome editing is one of the most promising approaches for controlling the gene expression and regulation of metabolites and protein production. CRISPR has become a gene-editing technique of choice that has been proven and widely used to treat or prevent diseases. This approach has been used as an important tool for stress typing in epidemiology for outbreaks and the identification of sources of infection [7, 8]. The established relationship between the exposed surfaces of our body and the production of metabolites, host-immune response, and gut-brain axis make the gut microbiome a potential target for gene therapies [9].

Besides the medical industry, the CRISPR technique has opened new avenues as a powerful genetic engineering tool in protecting, repairing, and saving the environment from harmful anthropogenic activity. The inherent functions of the CRISPR system can be used for many environmental aspects such as biofuel production, bioplastics, biosensing, pesticide reduction, food waste bioremediation, greenhouse gas emissions, water and wastewater [10]. Briefly, wastewater is a cocktail of many pollutants like organic carbon, nutrients (nitrogen and phosphorus), pathogens, and other contaminants. All these components pose a severe threat to human health and environmental integrity, thus making it imperative to clean the water resource. Treatment of such a contaminated resource by microorganisms is the best way owing to the inexpensive and sustainable approach. In view of strengthening and improving the aerobic and anaerobic bioprocesses, exploitation and augmentation of genetically engineered microbes can be a crucial strategy. Second generation bio-refinery caters to the eco-friendly lignocellulose waste disposal *via* anaerobic digestion, simultaneously providing the benefit of biomethane produced. Microbes engineered for heterologous expression of various genes obtained from source organisms can find valuable application in bioaugmentation for hastening the bioprocess operating in bio-refineries.

CRISPR–based genome editing has also been applied across the field of food science. Genome editing has found applications in the targeted engineering of crops, including corn, rice, and tomatoes, for improving their growth and nutrition potential by inserting traits for drought and disease resistance, resistance to insecticides, and survival under low nutrition/fertilizer conditions. Similarly, genome editing has been demonstrated to improve the yield from animal breeds through desirable alteration and herd genetics selection for disease resistant animals [11].

This chapter discusses the significance of gene editing techniques based on CRISPR/Cas in the optimization of various bioprocesses in environmental research. The application of this tool in improving the efficiency of both aerobic and anaerobic bioprocesses, such as wastewater treatment, anaerobic digestion for biogas and volatile fatty acid production, and landfill gas management, has been highlighted.

CRISPR AND BIOREMEDIATION

The global increase in human population and the industrial revolution are the major turning points in human history which have led to a change in society, economy, politics, and particularly in the environment. The large-scale production and use of chemicals over the past few decades, and the unchecked discharge of

CHAPTER 8

Genetic Engineering of Methanotrophs: Methods and Recent Advancements

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Abstract: Methanotrophs use methane gas as their carbon and energy source, but their industrial use has not yet fully been realized due to undiscovered genetic engineering methods that could amend their slow growth rate and economically inefficient product yield. This chapter informs upon genetic engineering approaches taken on methanotrophs so far to enable their widespread use in industry, as well as the reasoning behind these interests. Specific examples of successful engineering performed so far, including conjugation and electroporation methods, CRISPR, genome-scale metabolic modeling, and specific vectors reported as successful, are presented. In addition, the reading provides insights into existing knowledge gaps in the field of methanotrophic engineering and future prospects for optimizing growth and product yield from methanotrophs.

Keywords: Conjugation, Electroporation, Genetic engineering, Methanotroph, Methane monooxygenase (MMO) gene.

INTRODUCTION

Methanotrophs

Methanotrophs use C1 compounds as their sole carbon and energy source. Methanotrophs can be found in many diverse environments and are classified as aerobic or anaerobic, depending on their electron acceptor. Methanotrophs are further classified depending on the pathway they use for formaldehyde assimilation. Type I methanotrophs use the ribulose monophosphate pathway for assimilation of formaldehyde into cellular carbon, Type II methanotrophs use the serine pathway, and Type X methanotrophs have hybrid properties of Type I and

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Type II methanotrophs [1]. Key aspects of methanotrophy include methane assimilation, copper accumulation, and metal-dependent gene expression. Since 1970, genetic engineering approaches have focused on well-studied methanotrophs like *Methylococcus capsulatus* Bath, *Methylosinus trichosporium* OB3b, and *Methylocystis parvus* OBBP [2]. Haloalkaliphilic *Methylo-tuvimicrobium* bacteria like *buryatense* 5GB1C or *alcaliphilum* 20Z have also been of interest for use in the industry because of their fast growth rate [2].

Industrial Use of Methanotrophs

Methanotrophs are of industrial interest because they can produce value-added products from methane, which is a cheap feedstock as well as a greenhouse gas. The most well-studied derived products from methane are methanol, polyhydroxyalkanoates (PHAs), and single-cell protein. Many other chemicals, such as precursors to biofuels, could become industrially relevant through genetic engineering. For example, fatty acids from bacteria are precursors for the production of liquid biofuels, and Type I methanotrophs could be microbial catalysts that substitute methane for sugars as a carbon source.

Why Genetically Engineered Methanotrophs?

For a methanotroph to be as industrially relevant as *E. coli* or yeast, it must be well characterized and tools for its genetic manipulation must be determined. Economically effective methane bioconversion is rare and has only occurred with single-cell protein and PHA products. The slow growth rate is the main problem for industrial use of methanotrophs. Recently spiked interest in methanotrophy has led to the production of new protocols for the genetic engineering of bacteria that are simple and efficient. The high degree of reduction of methane by methanotrophs provides more free electrons for the production of products, and methane bioconversion has a higher carbon conversion efficiency compared to chemical processes (75% compared to 20-50%, respectively) [3]. The ability of Type II methanotrophs to direct high carbon fluxes towards acetoacetyl-CoA under nutrient limited conditions makes them promising for metabolic engineering [4]. As an example, the increased and stable expression of the methane monooxygenase gene (MMO) and methanol dehydrogenase through protein engineering could provide methanotrophic strains with more stable phenotypes, and expression of the MMO in a heterologous host with a higher growth rate could lead to new biotechnologies.

METHODS OF GENETIC ENGINEERING

In General

High-efficiency genome editing requires making a targeted DNA double-strand break in the DNA sequence of interest. Three classes of nucleases can be designed to make this double-strand break at any target. These are zinc-finger nucleases, transcription activator-like effector nucleases, and CRISPR-Cas [5]. A zinc finger nuclease is a hybrid of a DNA cleavage domain from a bacterial protein and a set of zinc fingers that were identified in sequence-specific eukaryotic transcription factors. Transcription activator-like effector nucleases have the same bacterial cleavage domain, but it is instead linked to a DNA recognition module of transcription factors from plant pathogenic bacteria [5]. CRISPR-Cas is a prokaryotic system derived from acquired immunity to invading nucleic acids.

Everything that happens after the targeted double-strand break depends on cellular DNA repair machinery, either by homology-dependent repair or non-homologous end joining [5]. To increase the efficiency of homology-dependent repair machinery, the donor DNA can be designed and linked to the guide RNA, and specific mechanisms that mediate sequence insertions must be considered. Gene-editing systems can enable rapid and high-throughput methanotrophic genetics.

In Methanotrophs

Genome sequences have been published for almost all genera of methanotrophs as either draft or complete sequences [6]. The availability of these sequences provides comparative analyses and allows for regulatory metabolism reconstruction and modelling, as discussed later when genome scale metabolic models (GSMM) are described.

Metabolic engineering in methanotrophs is used to increase metabolic flux and production of end products, as well as to enhance stress tolerance and substrate utilization. The more recent availability of broad-host range plasmids has provided for greater development of genetic techniques for methanotrophs. Protocols for efficient extraction of DNA from methanotrophs as well as plasmid construction and their transfer to *E. colivia* conjugation have been reported [7]. Some vectors, like pCM184, contain antibiotic markers, but unmarked mutants can be created with counterselection systems [6]. Sucrose counterselection using sacB has been successful in *Methylococcus capsulatus* Bath, *Methylomonas* sp. strain 16a, and *Methylomicrobium buryatense*. Insertions and deletions are performed using marker exchange *via* homologous recombination using flanking regions of 500 base pairs of genomic sequence [6].

CHAPTER 9

Genome Editing in *Cyanobacteria*

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Abstract: *Cyanobacteria* are potential organisms being exploited for a wide range of biotechnological applications. They are photosynthetic bacteria and grow in a carbon-free medium and become attractive hosts for biotechnology industries. *Cyanobacteria* can utilize solar energy and atmospheric CO_2 for the growth and synthesis of biomolecules. It is used in many large-scale preparations of various bioproducts such as pharmaceuticals, biofuels, *etc*. *Cyanobacteria* become target organisms for the next generation of biofactories for producing desired products with a low-cost technology. The problem in the metabolic engineering of *Cyanobacteria* is due to ploidy. It has multiple copies of chromosomes ranging from 3-218 copies. There are 12 copies of the genome in *Synechocystis* PCC 6803 and 3 copies in *Synechococcus* PCC 7942. Segregation analysis in the conventional genetic approaches of *Cyanobacteria* becomes laborious due to its polyploidy. Modern genome editing tools such as CRISPR-Cas9 and 12 are available to perform genome editing. CRISPR-Cas9 has been used in a wide range of *Cyanobacteria* such as *Synechococcus elongates* UTEX 2973, *Synechocystis* sp. PCC 6803. To avoid toxic effects caused by Cas-9, a low-level expression system is adopted in *Cyanobacteria*. Cas-9 base genome editing was applied in *Synechococcus* and produced succinate 11-fold higher than the normal. Cas-9 is used to cure plasmids in *Synechocystis* sp. PCC 6803 to develop a shuttle vector for heterologous expression. Another variant of genome editing tool is CRISPR-Cas12a, which is successfully used in *Synechocystis* sp.

Keywords: *Cyanobacteria*, CRISPR-Cas9, Metabolic engineering, *Synechococcus elongates*.

INTRODUCTION

Cyanobacteria are photosynthetic microorganisms living in both marine and freshwater systems [1, 2]. Oxygenic photosynthesis was released approximately

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2.5 billion years ago in primitive *Cyanobacteria* [3]. *Cyanobacteria* are the most promising microorganisms for the sustainable production of a wide range of biotechnological products for the humankind. The free oxygen (O_2) is increased in the atmosphere, that helps in establishing several microorganisms [4, 5]. In present days, *Cyanobacteria* contributes 20%–30% of atmospheric carbon dioxide (CO_2) [6]. *Cyanobacteria* are highly efficient in transforming carbon into a wide range of biomaterials, such as biofuels and commercially important enzymes [7]. *Cyanobacteria* are superior to plants due to many desired features like being capable of synthesizing carbohydrates through photosynthesis [8, 9]. They are capable of growing in unfavourable conditions such as increased temperature, pH and salt concentrations [1, 2]. It can be easily grown on infertile land with the least number of nutrients [10] and is relatively rapid and less expensive in the production of mutants [11]. In addition, chloroplasts are diminished in the internalized *Cyanobacteria* [12] with unique biochemical and physiological features and become an excellent host for producing plant-derived products [13 - 15].

The general features of *Cyanobacteria*, which are desirable for culturing and genetic modification, are listed in Table **1**. In the present years, there is an increasing demand for modifying *Cyanobacteria* for designing markerless selection systems, strong promoters for better expression, reporter proteins and ribosome binding sites. The above modifications made the *Cyanobacteria* a suitable host to transfer genes from desired hosts to make valuable bioproducts. One of the problems with *Cyanobacteria* is the limitation in the growth rate that includes model strains such as *Synechocystis* sp. PCC 6803 (PCC 6803) and *S. elongates* PCC 7942 (PCC 7942). The growth rates of some of them are ca.7 h for PCC6803 [20], compared to 20 min for *Escherichia coli*. In this chapter, we describe many recent developments in the genome engineering of *Cyanobacteria* and their applications in biotechnology. The second problem is the limited number of molecular tools to modify the genome of the Cyanobacterial species.

Table 1. General Features for Growing Genetically Modified *Cyanobacteria*.

S. No.	Desired Features	References
1.	Capable of forming individual colonies on a simple medium.	-
2.	Capable of receiving foreign DNA, either to take native DNA or *via* conjugation or lectroporation.	[28, 29]
3.	Sensitivity to antibiotics for easy selection of transformants.	[30]
4.	Absence of endonucleases that digest foreign DNA. If endonucleases are present, they can be inactivated to improve transformation efficiency.	[31]
5.	Alternatively, specific methylases, restriction inhibitors and liposomes are used during the transfer of DNA.	[32 - 35]

(Table 1) cont.....

S. No.	Desired Features	References
6.	Broad-host-range self-replicating plasmids like RSF1010 can be introduced easily.	-
7.	Existence of homologous recombination (HR) to introduce genetic alterations such as inserting expression cassettes and gene knockouts.	-
8.	Unmarked mutants are important for industrial use and it can be produced by negative selection markers such as sacB and also by CRISPR/Cas.	[11, 36, 37].

There is a rapid progress in the development of modern techniques for *Cyanobacteria*, such as CRISPR/Cas-based tools [16, 17]. It gives an opportunity to design genes with suitable promoters, ribosome binding sites (RBS), coding sequences and terminators [18, 19]. At present, approximately 85 complete genome sequences are available in the form of database such as the CyanoBase database (http://genome.microbedb.jp/cyanobase) [20]. Such databases for *Cyanobacteria* give the opportunity for the production of genome-scale models (GSMs) for a wide range of species, including industrial strains such as *Arthrospira* (*Spirulina platensis* NIES-39) [21, 22]. Metabolic engineering is a process of optimizing the genetic and regulatory functions of the cell to improve the production of various metabolic products [23]. This technique has been successfully applied in *Cyanobacteria* to improve production efficiency [24, 25]. Conventional methods for the improvement of the production of a desired compound are based on random mutations, which take more time to make successful mutations in specific genes [20]. Systems metabolic engineering has given an opportunity to solve the problems associated with random mutagenesis [26]. This systems metabolic engineering involves mathematic models to simulate and predict the output and it is used frequently for the development of a wide range of biomolecules from various microorganisms [27].

Cyanobacteria as a Host for the Heterologous Expression

Cyanobacteria are one of the best hosts for the production of several biomolecules [38]. *Cyanobacteria* are photosynthetic organisms that can fix carbon dioxide to form carbon products which are further converted into valuable compounds [39]. It has become an attractive host for sustainable biotechnological processes, which are in more demand currently [40]. It possesses several advantages over algae and plants, such as easy identification of transformants [41, 42], faster growth and utilizing solar energy for conversion into valuable products [43, 44]. *Cyanobacteria* can be easily cultivated in the absence of arable landmass and potable water [45], and it can also be used to remediate contaminated water, such as removing aromatic hydrocarbons [46, 47]. Cyanobacterial strains have not been much focused to modify to develop for large scale cultivation. *Cyanobacteria* can also synthesize polyhydroxyalkanoates, which are

CHAPTER 10

Genome Editing in *Streptomyces*

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Abstract: *Streptomyces* are Gram-positive, filamentous bacteria belonging to the group actinomycetes. This bacterium is important to the modern industrial world because of the presence of 20-50 biosynthetic gene clusters (BGCs). BGCs contain the genes for the production of industrially important natural products (NP), which includes antibiotics, anti-tumor drugs, anti-depressants, *etc*., naturally originated from this microorganism. Strain improvement is required to enhance the production of these NP in *Streptomyces*. Different methods have been used to enhance NP production and strain improvement. In this chapter, we will be discussing strain improvement of *Streptomyces* species by different genome editing tools. The information, which is put together, includes the basic techniques used for genome editing to the most advanced CRISPR/Cas system associated genome editing in *Streptomyces* (PCR targeting system, Cre-loxP recombination system, I *SceI* meganuclease promoted recombination system and CRISPR/Cas system). The authors have discussed about multiplex automated genome editing (MAGE) tool associated with CRISPR/Cas system.

Keywords: Bacterial gene clusters, CRISPR/Cas, Genetic manipulations, Metabolic engineering, Recombination system, *Streptomyces*.

INTRODUCTION

The genus *Streptomyces* is a Gram-positive bacterium that resembles filamentous fungi and grows in various environmental conditions. Streptomyces are differentiated from other actinomycetes by its filamentous growth and formation of spores in chains. Environmental stress, like nutrient limitation, leading to shifting of *Streptomyces* from the mycelial vegetative phase to the reproductive sporulation phase [1]. *Streptomyces* have linear chromosomes, approximately 8 to 10Mb depending on the species, with high GC content and several linear and circular plasmids. The presence of biosynthetic gene clusters that encodes for enzymes contributes towards the secondary metabolite production having varying

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chemotypes like polyketides, lactams, non-ribosomal peptides, terpenes, *etc.* [2]. These secondary metabolites produced have a wide range of applications like antibiotics, *e.g.,* pristinamycin [3] and daptomycin [4], immune-suppressants (*e.g.,* rapamycin) [5] and FK506 [6], insecticides, *e.g.,* avermectin [7] and milbemycin [8] and anti-tumour drugs daunorubicin [9] and bleomycin [10], which are widely used in agriculture and veterinary/human medicine.

The prolific antibiotic production capability and their significant role in clinical drug production have been exploited. The discovery of natural product (NP) drugs from these now highly exploited bacteria was seriously impaired by conventional screening techniques of synthetic libraries and the low efficiency of conventional top-down screening strategies [2]. The advancement in the next-generation genome sequencing technology and the use of bioinformatics resources to study microbial genomes has lead to a huge leap in unravelling biosynthetic gene clusters for natural products [11 - 13]. Thus has the ability to tap into the possibility of a wide variety of natural products and may lead to drug discovery from the majority of the uncultured microorganisms [14, 15].

Being a significant and most gifted microorganism, *Streptomyces* possesses 20 to 50 biosynthetic gene clusters (BGC) in a single genome [16, 17]. *Streptomyces,* when compared with other common model organisms, like *S. cerevisiae* and *E. coli*, shows poor genetic manipulations and are mostly recalcitrant to genome editing. The natural product (NP) biosynthetic gene clusters are unexplored rich reservoirs for natural compounds, and a majority of these BGCs are either not expressed or poorly expressed hence referred to as silent BGCs [18]. Of late numerous strategies have been developed to activate these BGCs to trigger NP overproduction. These strategies can be grouped into two major groups: (i) induction of BGCs in the native host using gene manipulations (ii) cloning of bacterial gene clusters and subsequent transfer to a surrogate *Streptomyces* host for heterologous expression. In order to achieve the activation of silent BGCs either in native or heterologous *Streptomyces*, highly efficient genome editing techniques are critical as the conventional gene manipulation strategies like DNA deletions, disruption and replacement, use of suicide plasmids with temperature-sensitive replication origin, required selection and screening of single and double cross over recombination events have low efficiency for low genetic engineering at the same time they are time-consuming method [18].

The effect of the low efficiency of conventional gene manipulation was further further compounded by the fact that double cross-over mutations are uncommon in *Streptomyces*, as there is a low level of DNA homologous recombination. Recently, various genome editing technologies (Fig. **1**) adapted from Zhao *et al.* [20], have been developed to overcome these limitations, especially the clustered

regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein (Cas)-based techniques, which have significantly enhanced *Streptomycetes* genetic manipulation and accelerated NP development, strain enhancement, and functional genome works [21, 22].

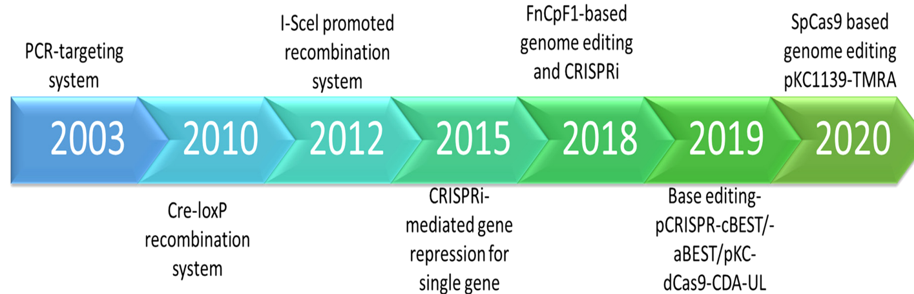

Fig. (1). The evolution of gene-editing technology in *Streptomyces*.

The present chapter aims to look at how genome editing in *streptomyces* has evolved over the years and explore how different works. A brief out line of important steps in different genome editing techniques are discussed along with the pros and cons of using each system for genome editing in *Streptomyces*. The chapter concludes with a brief overview of the possible future prospects of genome editing.

DIFFERENT GENOME EDITING TECHNIQUES

PCR-Targeting System

The PCR-targeting mechanism focuses on high-efficiency recombination between the target region within the *E. coli* genome and a PCR-amplified antibiotic selectable marker flanked on both ends by 40–50 bp homologous extensions [23]. This was first developed for gene knockout in *Escherichia coli* [23]. There are usually three steps in the PCR targeting system 1: The gene within the cosmid is replaced with a disruption cassette bearing a selectable antibiotic marker flanked by FRT or *loxP* site, 2: The mutant cosmid is then transferred into the *S. coelicolor* and screened for mutant strains with double crossover recombination events, 3: The antibiotic-resistant disruption cassette flanked by FRT or *lox P* sites is finally removed by inducing the expression of *tyrosine recombinase FLP* (FLP-FRT) or *Cre* (*Cre-loxP*) to generate unmarked, non-polar mutation (Fig. **2**) [24].

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