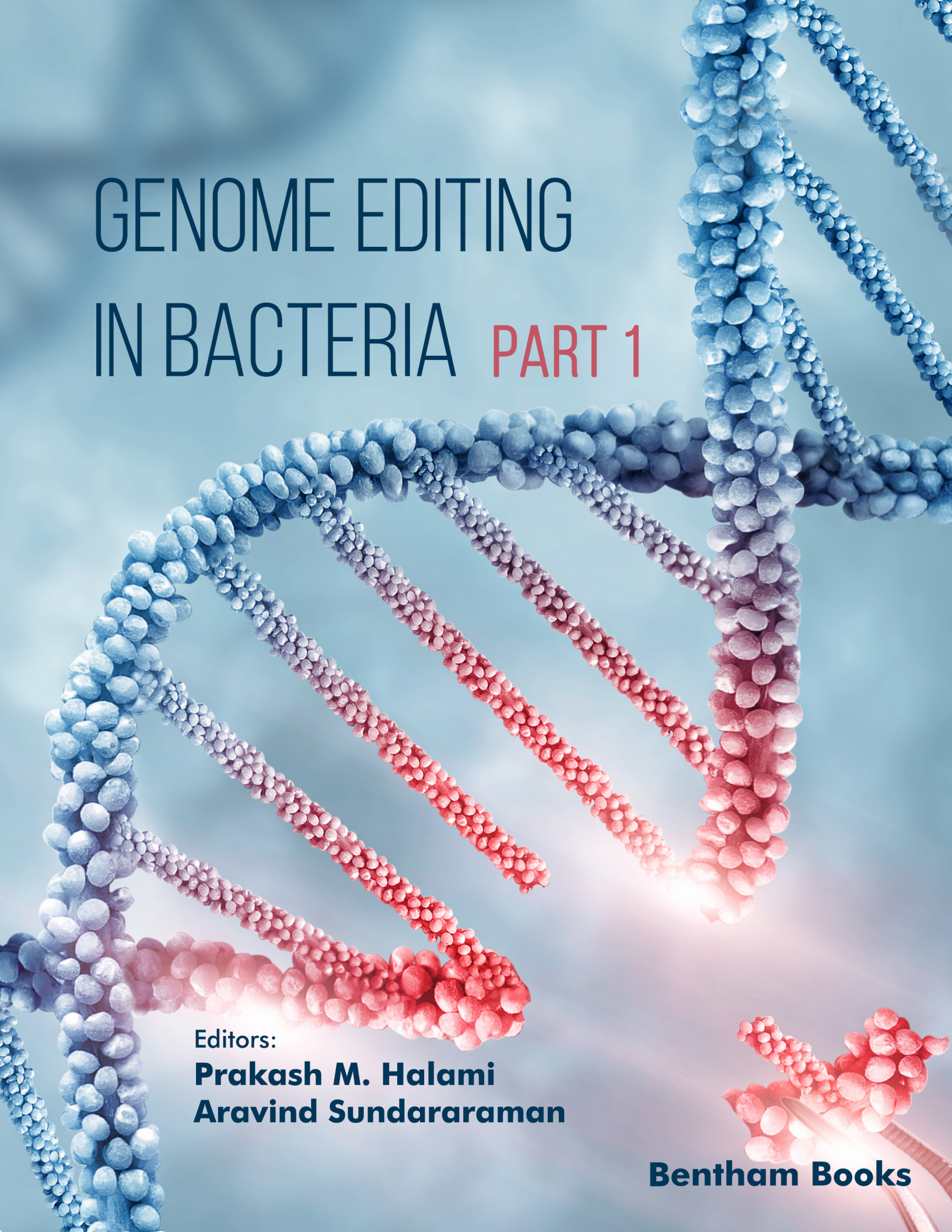
GENOME EDITING IN BACTERIA PART 1



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Genome Editing in Bacteria

(Part 1)

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ISBN (Online): 978-981-5165-67-8

ISBN (Print): 978-981-5165-68-5

ISBN (Paperback): 978-981-5165-69-2

First published in 2024.

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FOREWORD

The importance of Biotechnology and its applications in our life is well known, and with nearly four decades of experience at the University level, I am delighted and glad to write the foreword for the book “Genome Editing in Bacteria” edited by Dr. Prakash Halami and Dr. Aravind Sundararaman. I had known Dr. Halami since 2008, when I was nominated to the Institutional Biosafety Committee of CSIR-CFTRI, Mysuru, by the Dept. of Biotechnology, Govt. of India (New Delhi). I appreciate Dr. Halami abilities in planning, executing, monitoring research projects and dedication in mentoring many researchers in his lab.

Biotechnology is one of the most significant branches of biological sciences that is shaping the century and will continue to flourish and expand to newer frontiers in the present century too. Biotechnology draws parallel developments through research and applications in Microbiology, Genetics, Molecular biology, Bioinformatics, and Nanotechnology. Diverse branches of biotechnology have distinguished niches to well-defined dynamic research areas with both academic and industrial applications. Genome editing is a subject that has turned into a high science topic in our everyday vocabulary over a short period of time. Several positive and negative attributes have been associated with gene delivery techniques to develop transgenic microbes, animals and crops. The field has wide applications, and with every new development reported in leading peer-reviewed journals across the globe, the opportunities only become wider and the hopes brighter.

The editors have done an extraordinary job of bringing out a timely peer-reviewed volume titled "Genome Editing in Bacteria" with contributors spread across different continents. It is quite impressive to note that the editors have identified a wide range and dynamic topics that are organized in the form of a series of captivating articles highlighting different aspects of genetic engineering, both traditional and modern technologies in the field, protocols, advantages, new school of thoughts from around the world associated with some frontier development of biotechnological research. The attractive illustrations for presenting complex theoretical and experimental details and overall production design are sure to win the hearts of enthusiastic readers. The simplicity of the language and presentation style appealed to me a lot.

With years of experience in specific fields, several scientists and researchers have compiled and provided authentic and contemporary information. This compendium will be handy for faculty to update their lectures for the students pursuing higher education. Similarly, this book will be a ready reckoner for researchers working in specific areas to plan their future research.

It is a great pleasure on my part to pen a foreword for this prestigious, multi-authored, peer-reviewed, international publication on a topic that is very contemporary and close to my heart.

I wish the editors great success. I also extend my sincere greetings to all the contributors for their excellent effort in making this book a success.

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PREFACE

Genetic engineering is essential in basic research and Industrial Biotechnology for metabolic and genomic manipulations to regulate microorganisms to produce valuable products. Genome editing is the cornerstone for scientists to interrogate the genetic basis of microorganisms for which the accessibility of the genome along with molecular tools is an essential factor. The classical genetic methods developed for genome editing in bacterial species include culture and transformation. The methods were highly laborious and required the introduction of at least one resistance marker cassette in the genome, which hampers the possibility of producing precise edits like single amino acid mutations. The breakthrough by the discovery of the CRISPR-Cas technology has shed light on the adaptive immune system of prokaryotes to explore tremendous opportunities for targeted genetic engineering approaches in prokaryotes. Here, we discuss the current state-of-the-art approach for gene editing in bacteria and different strategies used in this technology for prokaryotic organisms.

This book has eight chapters, including historical perspectives of genome editing and applications of probiotics and its metabolites. We attempted to update and collate information and research carried out on various applications of bacteria in different industries, such as the food and pharmaceutical industry and their gene regulation for metabolic engineering using genome editing tools. We are grateful to all contributing authors who accepted our invitation to contribute to this book. The contributing authors are well-recognized scientists and researchers with vast experience in the field of bacteriology and molecular biology. We are happy to bring them all together on the same platform to bring out this book. We are grateful to the Bentham Science Group for publishing this comprehensive book, and we hope it will be read by researchers, students, teachers, scientists and food entreprenuers who are interested in the metabolic engineering of bacteria for various health benefits. Although there are hundreds of research articles, review papers, and limited books on genome editing of prokaryotes, this book "Genome Editing in Bacteria" is the first of this kind, a compilation of various applications of bacteria across diverse fields of biotechnology.

We dedicate this book to the creators of the indigenous knowledge of molecular biology and genetic engineering for putting together both an ocean of knowledge and the basis for research to study in-depth genome editing techniques for bacteria.

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CHAPTER 1

Recent Advances in CRISPR-Cas Genome Engineering: An Overview

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Abstract: Bacteria is one of the most primitive organisms on earth. Its high susceptibility to bacteriophages has tailored them to use specific tools to edit their genome and evade the bacteriophages. This defense system has been developed to be the most specific genome editing technology of this current period. Previously, various other tools such as restriction enzymes (RE), zinc finger nucleases (ZNF), and transcription activator-like effector nucleases (TALENS) were utilized. Still, its major limitations led to exploiting the bacterial defense system to edit the genome. CRISPR technology can be applied in various microbiology, pathology, cancer biology, molecular biology, and industrial biotechnology, but its limitations, such as off-target effects due to unspecific alterations, are a major concern. In the future, this effective gene alteration technology will be developed to treat inherited rare genetic disorders. This chapter highlights the discovery, components, applications, limitations, and future prospects of CRISPR-Cas.

Keywords: Bacterial defense system, Cas9, CRISPR-Cas, Genome editing tools, Industrial biotechnology, SgRNA.

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INTRODUCTION

Bacteria are single-celled prokaryotic microorganisms, all belonging to the kingdom of Monera in the system of classification of living organisms [1]. Bacteria are among the oldest living organisms as they were among the first life forms to appear on earth and are present in almost every habitat. We usually associate bacteria with an infectious disease. Nonetheless, every bacterium lives in parasitic relations with plants and animals. A significant number of bacterial species live in symbiotic associations with other living organisms. Bacteria are also prone to infection from specialized viruses called bacteriophages [2]. To evade conditions from bacteriophages, bacteria evolved to use a specialized tool in their genome and clustered regularly interspaced short palindromic repeats [3, 4].

GENOME EDITING

Genome editing is a process where specific changes can be made in the regions of interest with the help of explicit and engineered nucleases by introducing double-stranded breaks (DSB). These breaks can cause site-specific mutations, gene deletions, substitutions, or insertions, and later can be repaired by various mechanisms. Non-homologous end joining (NHEJ) is prone to error, and homology-directed repair (HDR) error-free is the repair mechanism used [5]. Genome editing is a powerful tool for understanding biological roles. It can treat genetic disorders by identifying 'molecular mistakes' and providing appropriate gene therapy. Restriction enzymes (RE) are natural genome editing tool, while transcription activator-like effector nucleases (TALENs) and Zinc Finger Nucleases (ZFNs) are artificial genome editing tools.

GENOME EDITING TOOLS AND THEIR LIMITATIONS

Restriction Enzymes (RE)

The discovery of restriction enzymes in early 1970 heralded a new age in molecular biology. Restriction enzymes or endonucleases are natural genome editing tools that recognize specific nucleotide sequences and cut the DNA at specific sites. The gene of interest could be inserted at a particular location.

The limitation of the restriction enzymes is the difficulty in predicting the location at which the gene of interest could be inserted. The primary reason behind it is that the recognition sequence of most of the restriction enzymes is base pairs long and often arises several times in a genome. The restriction specificity of endonucleases can depend on the environmental conditions.

In contrast, restriction enzymes are used for molecular cloning, DNA mapping, epigenome mapping, and constructing DNA libraries. These enzymes were modified to enhance the specificity of restriction endonucleases like the homing endonuclease systems. They could target specific sequences for genome editing. REs have long recognition sites and tolerate sequence degeneracy within their restriction site, unlike restriction enzymes [6]. One of the examples is meganucleases. It is designed to recognize long DNA sequences.

Zinc Finger Nucleases (ZNFs)

The artificial restriction enzymes consist of a subunit that recognizes desired DNA sequence and the DNA cutting part of restriction enzymes. They can be designed to identify specific DNA sequences and thereby enable targeted cleavage [7]. The hybrid restriction enzymes could be created using a zinc finger DNA binding domain fused to break up the naturally occurring FokI endonuclease domain. FokI, a naturally occurring IIS restriction enzyme, has played a pivotal role in the success of ZFNs. A lot of effort was required to produce a modified ZNF, which was a significant drawback. Thus, research has been done to customize ZNF.

Transcription Activator Like Effector Nucleases

The artificial restriction enzymes (ARE) consist of two components (i) restriction enzyme to cleave DNA and (ii) TAL, effector. TAL effectors comprise 33 repeat sequences, which helps them bind to long lines in the genome. TALENs are preferable over ZNF due to their ease of application. TALENs encode the FokI domain fused to the engineered DNA binding region, and when bound, dimerized FokI endonuclease could form a double-stranded break. The limitation of TALEN is TALE target search process is affected by genomic occlusions.

Discovery and History

The discovery of CRISPR revolutionized gene-editing technology (Fig. **1**). CRISPR was initially discovered in 1987 from the *E. coli* genome, and its role in the adaptive immune system was elucidated in early 2000. In 2020, Prof. Emmanuelle Charpentier and Prof. Jennifer Doudna were honored with the Nobel Prize in chemistry for their discovery of CRISPR-Cas9 technology in *Streptococcus pyogenes,* which is considered an evolution in the fields of medicine, biotechnology, and agriculture [8]. This technique is favorable due to its precision in gene editing [9]. This helps to alter genes efficiently and rapidly. It is also widely used in treating genetic disorders [10].

CHAPTER 2

Overview and Applications of CRISPR/Cas9 Based Genome Editing in Industrial Microorganisms

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Abstract: CRISPR-Cas technology has reshaped the field of microbiology. It has improved the microbial strains for better industrial and therapeutic utilization. In this chapter, we have tried to provide an overview of this technology with special reference to its associated applications in the various fields of interest. We have discussed the origin, classification, and different genome editing methods of CRISPR-Cas to understand its historical significance and the basic mechanism of action. Further, different applications in the area of agriculture, food industry, biotherapeutics, biofuel, and other valuable product synthesis were also explained to highlight the advancement of this system in industrial microbes. We have also tried to review some of the limitations offered by CRISPR and insights into its future perspective.

Keywords: Agriculture, Biofuel, Biotherapeutics, CRISPR/Cas9, Food industry, Genome editing, Industrial applications, Microorganisms.

INTRODUCTION

Microorganisms produce many important enzymes and metabolites. They are the source of a variety of industrially valuable products having applications in areas like food, therapeutics, and agronomy. Over the years, several strategies have been applied to improve the industrially important attributes of these microbes for their effective exploitation. With the emergence of genome editing tools such as Zinc finger nucleases (ZFNs) and Transcription-activator effector nucleases (TALENs), the improvement of bacterial strains appeared possible [1]. But despite their good potential, genetic manipulation is hard to achieve in most cases. The main limitations were the cumbersome process of creating novel nuclease

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pairs for each target site and their inability to target multiple sites at a time. However, a recently emerged genome editing tool, CRISPR-Cas9, can prove to be very important in the exploration and understanding of the genetic basis for physiochemical and metabolic traits in microorganisms. It is a simple, adaptive, and fast technique that has attracted a lot of scientific interest in the field of genome editing. It can easily be used for targeting multiple genes at a single point of time. It has enabled the rapid genome engineering of bacterial strains, transforming them into better cell factories for the production of value-added products, for instance, *Escherichia coli* [2]. Furthermore, it may be utilized to knock out/in genes and alter somatic genes by genome manipulation even in the germline of species. This method has also been utilized to target, activate, and repress specific genes of interest using specific transcription factors. In 2012, researchers from two independent laboratories suggested that the CRISPR-Cas systems possessing biological functions may be created, which cut off individual target DNA sequences allowing scientists to utilize this tool for genomic manipulations [3]. It has also been employed for checking the possible genome editing in mammals. Cong and associates efficaciously knocked-out numerous genes in each human and mouse cell lines by developing CRISPR/Cas systems with the use of *S. pyogenes* Cas9 (SpCas9) [4]. Similarly, Mali and associates extensively utilized Cas9 to purposefully knock-out genes in numerous human mobileular strains [5].

In this chapter, the CRISPR-Cas system is discussed in detail with some of its applications in improving the different industrially important bacterial strains. Various technical aspects and different types of CRISPR-Cas methods have also been described. We have also tried to explain major challenges and future aspects of the technique.

ORIGIN OF CRISPR/Cas9 SYSTEM

CRISPR is a large family of short palindromic repeat sequences found in a wide range of prokaryotes, including bacteria and archaea. The discovery of CRISPR happened accidentally in 1987 when Ishino *et al.*, were working on the *iap* gene in *E. coli*, which encodes alkaline phosphatase [6], and they identified a set of re-occurring DNA sequences in the bacterial genome that differed significantly from other sequences. Later, comparable CRISPR genes from different bacteria and archaea were also cloned [7 - 10]. However, for over a decade, scientists did not understand the function of these unusual repeating sequences and just assumed that they were unique sequences across different bacterial species. But in 1995, Mojica and associates created a plasmid containing the fragments of CRISPR sequences which were used to transform halophilic archaea *Haloferax volcanii*; and found that extra copies of CRISPR sequences caused alteration in the genome

distribution of *H. volcanii* [11]. This, for the first time, revealed incompatibility between external plasmid CRISPR and archaea. However, their actual feature became uncertain till the scientists located CRISPR-related protein (*Cas*) genes and their capabilities in bacterial protection mechanisms. Later, it was discovered that these recurrent DNA sequences might be important components of the "Clustered Regularly Interspaced Short Palindromic Repeats" (CRISPR) family of repetitive DNA sequences [12]. In bacteria, short segments of spacer DNA generated accompany short repeats of DNA sequences from prior exposure to a bacteriophage or plasmid in these CRISPR systems. CRISPR repetitions were discovered to be linked to nucleases or helicases involved in the cleavage or unwinding of particular DNA regions. The primary function of the CRISPR/Cas systems was to protect bacteria from bacteriophage or plasmid invasion. When the system is re-exposed to the same bacteriophage or plasmid, the CRISPR/Cas recognizes it based on its transcribed RNA sequences, and a Cas nuclease is instructed to break the DNA. Cas9 was discovered to be a nuclease with the ability to chop DNA at two active cleaving sites, one for each DNA strand, and was isolated from the bacteria *Streptococcus pyogenes*. A single Cas9 protein may be reused to target and cleave specific locations on bacterial DNA.

CRISPR-Cas Systems Classification

CRISPR-Cas tool is generally categorized into two groups: class 1, including multi-subunit effectors, and class 2, including single protein effectors. Based on their characteristic proteins, these two classes are further categorized into six different types. Three of these have received the most attention: type I, type II, and type III (Fig. **1**), possessing signature proteins Cas3, Cas9, and Cas10, respectively. Most of the CRISPR-Cas systems contain Cas1 and Cas2 proteins, responsible for the integration of the spacer during the adaptation step, in addition to their respective signature proteins [13]. The presence of a protospacer adjacent motif (PAM), corresponding to short conserved sequences, is all that is required for type I and type II systems to target DNA. Furthermore, for the occurrence of DNA interference (DNAi) in all three kinds of CRISPR-Cas systems, a seed sequence with 8–10 base pairs at the 3' end of the guide RNA is required. Type II CRISPR-Cas is the most common and extensively utilized as it relies on a single Cas9 nuclease protein for DNA-induced gene silencing (DNAi). Cas9 is made up of several domains and works in alliance with short RNAs, including mature CRISPR RNA (crRNA) and a trans-acting RNA (tracrRNA) [14]. The HNH system type is also seen in type II systems (also called as Nmeni subtype, for *Neisseria meningitidis* serogroup A str. Z2491). In this system, in addition to the ubiquitous Cas1 and Cas2, the single and extremely big Cas9 protein appears to be ideal for producing CRISPR RNA (crRNA) and slicing the target DNA. Cas9 is made up of two nuclease domains, which are arranged in the order: the RuvC-

CHAPTER 3

Modulating the Gut Microbiome through Genome Editing for Alleviating Gut Dysbiosis

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Abstract: One of the components of the emerging lifestyle shows an exponential rise in the consumption of packaged or high-calorie food. This has caused an increase in the incidences of diseases which are considered to be a consequence of the changing lifestyle. It has been observed that these clinical conditions are linked with gut dysbiosis, and hence it has been proposed that by modulation of the composition of gut microbiota, the risk of such diseases can be lowered. Prebiotics and probiotics, in combination, possess tremendous potential for maintaining the homeostasis in individuals. In this chapter, a comparative assessment of CRISPR-mediated genome editing technique has been discussed with conventional omics tools and modelling approaches. These techniques substantially simplify the modification of target genome in complex microbial communities and could enhance their prebiotic and probiotic potential. The synthetic biology approach to microbiome therapies such as additive, subtractive, and modulatory therapies for curing gut dysbiosis are also discussed. The chapter is aimed at developing a better understanding about the role of CRISPR/Cas as a reliable technology that may be employed as a diagnostic tool for infectious disease diagnosis as well as its treatment. Although, the tool has already demonstrated its use in a wide range of genome editing and genetic engineering applications, additional study into its use in human genome editing and diagnostics is needed considering any potential side effects or ambiguities.

Keywords: Gut microbiome, Gut dysbiosis, Genome editing, Prebiotics and probiotics, CRISPR.

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INTRODUCTION

The concept of genome editing in molecular biology refers to the incorporation of changes in specific DNA sequences by insertion, deletion, or modification of the genome [1, 2]. Genes have been manipulated using this technique in a variety of ways, including modifying their nucleotide sequences and changing their expression. Several enzymes that have been employed for achieving the aforesaid activity include zinc finger nucleases [ZFN], transcription activator-like effector nucleases [TALEN], and homing meganucleases, all of which have demonstrated their efficiency, though they have to be reengineered for each target sequence [3]. However, the drawbacks of homologous recombination (HR) based genome editing, such as the large sample volume requirement and lower editing effectiveness, have restricted their wide-scale application [4]. These drawbacks of the conventional genome editing tools have been overcome by CRISPR (clustered regularly interspaced short palindromic repeats)-mediated genome editing techniques that have substantially simplified the targeted genome modification in complex organisms [1, 5]. Because of its ease of use, cost-effectiveness, and high efficacy of desired targeted changes, the CRISPR/Cas9 system has become the editing tool of choice in recent years in transformed cell cultures and secondary clones [6]. However, considering the inefficiencies and inaccuracies, the efficiencies of genome editing experiments need to be validated by Fragment analysis and/or Sanger sequencing by capillary electrophoresis [CE] [7, 8].

Among the different species of organisms, CRISPR/Cas9 has been utilised in plants, fungi, and mammals for genome editing [9], which has helped us to gain a better understanding of how a gene product contributes to an organism's development and disease [10]. In this chapter, the significance of the CRISPR/Cas system in microbiome editing has been discussed with reference to controlling the gene expression and regulation of metabolites and protein production in the gut. Metabolites such as prebiotics have the capacity to modulate the gut microbial community, which plays a critical role in various illness situations, thereby aiding in the maintenance of homeostasis of an individual. The chapter also highlights the application of CRISPR/Cas in precise diagnostics of diseases arising due to gut dysbiosis, and the detection of microorganisms and their underlying mechanisms suggested to be responsible for the disease conditions. Various platforms based on viruses have been developed for applying the CRISPR-Cas genome editing in gut microbiome studies, such as DNA endonuclease-targeted CRISPR trans reporter [DETECTR] HUDSON, SHERLOCK [Specific high-sensitivity enzymatic reporter unlocking] [11].

CRISPR AND GUT MICROBIOME MANAGEMENT BY PREBIOTICS, PROBIOTICS, AND SYNBIOTICS

The gut microbiome has an impact on the health status of an individual and has been associated with numerous diseases that are manifested in the form of inflammation and immunosenescence, leading to a condition termed gut dysbiosis. These effects are caused due to the immunomodulatory properties of gut microbiota, whose low diversity in aged people is characterized by facultative anaerobes and pathobionts that are responsible for the increase in the inflammatory signals [12 - 15]. During old age, a lower abundance of probiotic beneficial bacteria - *Bacteroides*, *Bifidobacteria*, and *Lactobacilli,* and an increase in abundance of *Enterococci, Coliforms,* and especially *Clostridium perfringens* and *C. difficile* were found to result in a reduction in the relative stability of the gut microbiome [16 - 18]. Similarly, other researchers observed a decrease in *Prevotella, Candida albicans, Streptococcus, Staphylococcus*, and *Faecalibacterium prausnitzii* and an increase in levels of *Ruminococcus* and *Atopobium* that established the relationship between frailty score and microbiota diversity in older people [19, 20]. Considering the above aspects of gut dysbiosis, methods for managing the gut microbiome are of paramount importance for maintaining the homeostasis of an individual. Prebiotics, probiotics, and synbiotics are concepts that have been researched extensively for their potential role in the management of the gut microbiome.

The term “Prebiotics” was coined by Gibson and Roberfroid [21] to describe a nutritional product and/or ingredient that selectively nourishes the gut microbiome, thereby providing health benefits to the host. Sources of natural prebiotics include garlic, onion, chicory root, barley, banana, tomato, and wheat, along with breast milk oligosaccharides that are the third-largest human milk component [22, 23]. Some of the synthetic oligosaccharides include fructooligosaccharides, galactooligosaccharides, xylooligosaccharides, maltooligosaccharides, and inulin [24, 25]. Prebiotics are food for probiotics. The term “Probiotics” which has its origin in Latin, “pro” meaning “for” and “bios” meaning “life”, was coined by noble laureate Elie Winnerat at the beginning of the 20th century. Probiotics consist of live microorganisms that can give health benefits to the host when present in an adequate amount [26, 27]. They are non-pathogenic, non-toxic, and non-allergic [28] and are capable of inducing immunity against various diseases in addition to their ability to provide antimicrobial activity in the gut. Probiotic bacteria are capable of surviving and metabolizing carbohydrates, short-chain fatty acids, and bile acids in the upper gastrointestinal (GI) tract and are characterized by resistance to low pH, bile juice, and gastric acid [29]. Such functional food ingredients and supplements with health-enhancing effects have given rise to the concept of synbiotics that is

CHAPTER 4

Bifidobacterial Genome Editing for Potential Probiotic Development

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Abstract: Genome editing is a promising tool in the era of modern biotechnology that can alter the DNA of many organisms. It is now extensively used in various industries to obtain the well-desired and enhanced characteristics to improve the yield and nutritional quality of products. The positive health attributes of *Bifidobacteria*, such as prevention of diarrhoea, reduction of ulcerative colitis, prevention of necrotizing enterocolitis, *etc*., have shown promising reports in many clinical trials. The potential use of *Bifidobacteria* as starter or adjunct cultures has become popular. Currently, *Bifidobacterium bifidum*, *B. adolescentis, B. breve, B. infantis, B. longum,* and *B. lactis* find a significant role in the development of probiotic fermented dairy products. However, *Bifidobacteria*, one of the first colonizers of the human GI tract and an indicator of the health status of an individual, has opened new avenues for research and, thereby, its application. Besides this, the GRAS/QPS (Generally Regarded as Safe/Qualified Presumption of Safety) status of *Bifidobacteria* makes it safe for use. They belong to the subgroup (which are the fermentative types that are primarily found in the natural cavities of humans and animals) of *Actinomycetes*. *B. lactis* has been used industrially in fermented foods, such as yogurt, cheese, beverages, sausages, infant formulas, and cereals. In the present book chapter, the authors tried to explore the origin, health attributes, and various genetic engineering tools for genome editing of *Bifidobacteria* for the development of starter culture for dairy and non-dairy industrial applications as well as probiotics.

Keywords: *Bifidobacteria*, CRISPR-Cas, Genome editing, IPSD (Inducible Plasmid Self-Destruction), Probiotics.

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INTRODUCTION

Bifidobacterium is most abundant in the human gut, as well as other mammalian guts, though other sources were also reported, for example, recently, a *Bifidobacterium* strain, LMG 28769^T, has been isolated from the household water kefir fermentation process [1]. The exact numbers of *Bifidobacterium* species present in the human gut are not well known. It is hypothesized that this genus comprises most of the human gut microbiota. These bacteria are generally anaerobic in nature, rod-like shaped, gram-positive, non-spore-forming, hetero-fermentative, non-motile, catalase-negative, and belong to the phylum *Actinobacteria* [2]. *Bifidobacteria* are mostly high in GC content, and their genomic size generally varies between 1.73 to 3.12Mb. To date, 2500 genome assemblies of *Bifidobacteria* are available in the NCBI database [3]. This bacterium was first isolated in the year 1899 by Henri Tissier from the isolates of newborn infants' fecal matter though it was then classified as *Bacillus bifidus communis,* which was currently termed *Bifidobacterium bifidum* Ti. 16s rRNA. Pangenome analysis is one of the major techniques providing better insight into their genomic component and inter-species relationship as well as their adaptation in a niche. In Table **1**, a few data regarding GC%, amount of tRNA, rRNA, and CRISPR arrays are given for some *Bifidobacterium* species. A comparative table of genome size, genes, and amount of encoded proteins of some *Bifidobacterium* sp. is presented in Fig. (**1**). Several mobilomes (mobile elements) have been found in Bifidobacterial genomes. These consist of IS (Insertion Sequences), plasmids, prophage, and other prophage-like elements. IS*30* is most abundant in *Bifidobacterium* species, though other Insertion Sequences are also found in the different *Bifidobacterium* species like IS *3*, IS *110*, IS *150*, IS *256*, *etc* [4]. IS are found to be important in bacterial adaptation in an environment where these elements are responsible for gene deletion, rearrangement, or other processes for adaptation. There are more than 2000 ORF (Open Reading Frames) per genome found in the *Bifidobacterium* genus by *in silico* gene prediction [5].

However, plasmids are not common in most *Bifidobacteria*, but some of the species carry a significant number of plasmids. For example, pNAC3, pBLO1, pB44, pMB1, *etc*., plasmids have been found in *B*. *longum* subsp. *longum* [6 - 8]. Like that, pBC1 and pB80 are found in *B*. *catenulatum* and *B*. *bifidum,* respectively [9, 7]. Major replication (Rep) proteins encoded by the Bifidobacterial plasmids are homologous to RCR Rep proteins though some other types, like, homologous to theta replication proteins, are also noticed. There is a lot to further discover in the *Bifidobacterium* genome as it can open new paths for the utilization of these probiotics with improved efficiency and characteristics.

Different species of *Bifidobacteria* have been isolated from different habitats. Mostly they are isolated from human fecal samples, but other sources like animals (like murine, bee, tamarind, marmoset, pig, rabbit, gorilla, chicken, *etc*.), sewage, and food are also reported (shown in Fig. (**2**)), and human origin *Bifidobacterium* species along with their mode of action are listed in Table **2**.

Table 1. Some genome information of different *Bifidobacterium* species.

***Bifidobacterium* Species**	**GC%**	**tRNAs**	**rRNAs**	**CRISPR Arrays**	**Reference**
B. angulatum DSM 20098 = JCM 7096	59.4	53	12	1	[3]
B. breve JCM 7017	58.7	53	6	2	
B. bifidum YIT 10347	62.8	52	9	1	
B. animalis subsp. lactis BLC1	60.5	52	12	1	
B. dentium JCM 1195 = DSM 20436	58.5	56	13	2	
B. adolescentis ATCC 15703	59.2	54	16	1	
B. pseudolongum ASM228291v1	63.4	52	12	1	
B. thermophilum RBL67	60.1	47	12	1	
B. pseudocatenulatum YIT11956	56.5	60	16	1	
B. asteroides PRL2011	60.1	45	6	1	

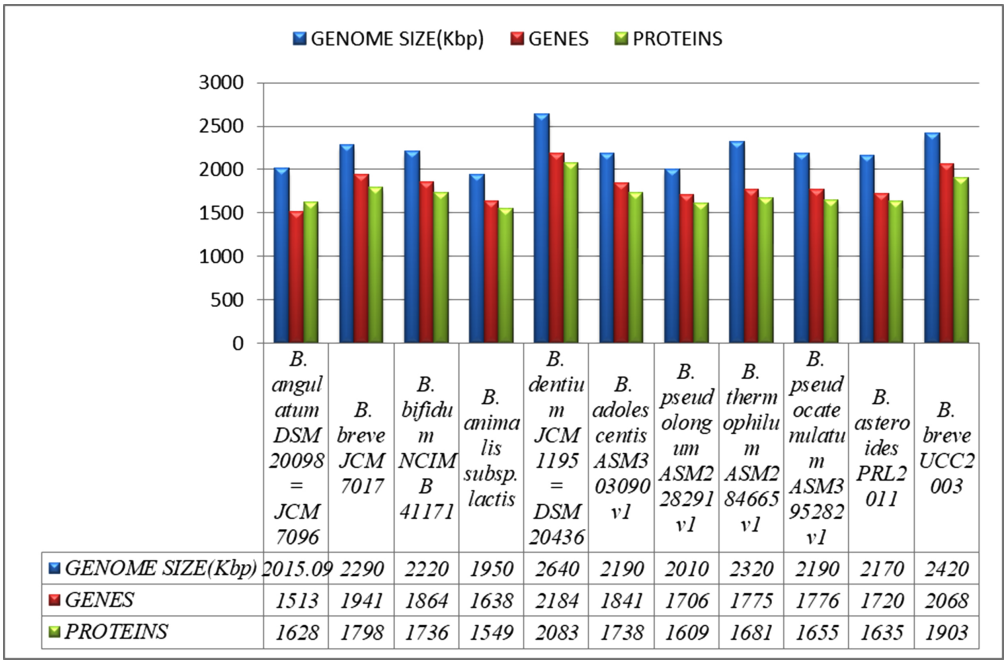

Fig. (1). Comparison of genome size, genes, and protein amount of different *Bifidobacterium* species.

CHAPTER 5

Metabolic Engineering of *Bifidobacterium* sp. Using Genome Editing Techniques

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Abstract: The gut microbiome is significant in maintaining human health by facilitating absorption and digestion in the intestine. Probiotics have diverse and significant applications in the health sector, so probiotic strains require an understanding of the genome-level organizations. Probiotics elucidate various functional parameters that control their metabolic functions. Gut dysbiosis leads to inflammatory bowel disease and other neurological disorders. The application of probiotic bacteria to modulate the gut microbiota prevents diseases and has gained large interest. In a recent decade, the development of modern tools in molecular biology has led to the discovery of genome engineering. Synthetic biology approaches provide information about diverse biosynthetic pathways and also facilitate novel metabolic engineering approaches for probiotic strain improvement. The techniques enable engineering probiotics with the desired functionalities to benefit human health. This chapter describes the recent advances in probiotic strain improvement for diagnostic and therapeutic applications *via* CRISPR-Cas tools. Also, the application of probiotics, current challenges, and future perspectives in disease treatment are discussed.

Keywords: Genome editing, Metabolic engineering, Probiotics, Strain improvement.

INTRODUCTION

The human gastrointestinal tract harbors complex, diverse microbes that regulate the host's physiological functions and well-being. The gut microbiota is linked with many functions, including absorption and fermentation of complex carbohydrates, developing immune functions, and inhibiting pathogen adherence to the intestinal cell wall [1]. The probiotics inhabiting the gastrointestinal tract are influenced by several factors, such as breastfeeding, mode of delivery, age, geography, gender, long-term dietary intake, and drug usage [2].

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Many diseases, such as diabetes, coronary heart disease, inflammatory bowel disease, and obesity, observed dysbiosis in the gut microbiota composition [3]. Thus, gut microbiota is a complex inheritable trait determined by genetic and environmental factors [4].

Bifidobacterium sp. was first described decades ago and is linked with healthy intestinal tracts due to their abundance in breastfed infants. They are considered probiotics as they elicit health benefits to the host by maintaining intestinal microbial balance and have led to wide application as probiotic components, especially in fermented dairy products. *Bifidobacterium* sp. is a Gram-positive, anaerobic, and saccharolytic bacteria found inhabiting the human oral cavity, and the intestinal tract of mammals [5]. The identification and classification of *Bifidobacterium* species are based on DNA-DNA hybridization and whole genome or conserved sequence phylogenetic analysis [6]. *Bifidobacteria* exert their biological activities by producing antimicrobial substances and vitamins. They also have anti-inflammatory, and anti-obesity properties and regulate the immune system [7].

The genus *Bifidobacterium* encodes genes associated with a broad range of non-digestible carbohydrate utilization, including plant fiber and human milk oligosaccharides [8]. *Bifidobacteria* utilize the hexose sugars through the "bifid shunt" pathway with the help of the vital enzyme fructose-6-phosphate phosphoketolase [9]. The ATP-generating pathway produces short-chain fatty acids (SCFAs) that act as antagonists against pathogens and protect from infections [10]. For example, acetate produced by *Bifidobacteria* protects the host against lethal infections and improves intestinal defense mediated by epithelial cells [11].

Several species of *Bifidobacteria*, such as *B. longum, B. breve*, and *B. animalis*, are used to treat inflammatory bowel disease and gastrointestinal disorders [12]. A few strains, such as *B. animalis subsp. lactis* BB-12 and *B. animalis* BF052, are used as major ingredients in probiotic product formulation, indicating that probiotic characteristics are strain-dependent [13]. Therefore, understanding the metabolism of *Bifidobacteria* completely and its adaptation to various nutrient environments is essential.

In the recent decade, efforts have been aimed at understanding the gut microbiota using metabolic modeling, which generates testable hypotheses to elucidate each species' metabolism and interspecies metabolic interactions [14]. Recently, Thiele and co-workers [15] developed a resource AGORA (Assembly of Gut Organisms through Reconstruction and Analysis) to study the human gut microbiome at the genome scale that enables the system-level study. The genome-scale metabolic

models of gut microbes predict the growth phenotypes and link dietary intake and absorption in humans [16]. Further, studies on metabolic reconstruction provide insights into strain-specific metabolic networks and the applications of different strains. In this chapter, we describe the significance of *Bifidobacteria* as a probiotic and their ability to produce metabolites. Moreover, applications of genome engineering in *Bifidobacteria* to improve the production of metabolites to improve human health are discussed.

TYPES OF METABOLITES PRODUCED BY *BIFIDOBACTERIA*

The genera *Bifidobacterium* and *Lactobacillus* are the primary source of probiotics, defined as live microbial food ingredients that exert beneficial effects on health. They are recognized to prevent or treat infections in the large and small intestines. Recent research has demonstrated their antagonistic effect on *H. pylori* diseases [17]. Probiotics have been reported to produce organic acids, immunomodulatory properties, and competitive inhibition for mucous, the binding sites of mucous cells to exert an antagonistic effect on *H. pylori* [18]. The potential uses of probiotic *Bifidobacteria* to combat *H. pylori* infections are less exploited than those reported for *Lactobacilli*.

The antimicrobial metabolites secreted by *Bifidobacteria* are a possible alternative to antibiotic treatment against clinical infections and serve as an adjunct therapy along with simultaneous antibiotic administration (Fig. **1**). The antimicrobial peptides isolated from *Bifidobacteria* could have the prophylactic potential to treat *H. pylori* infections.

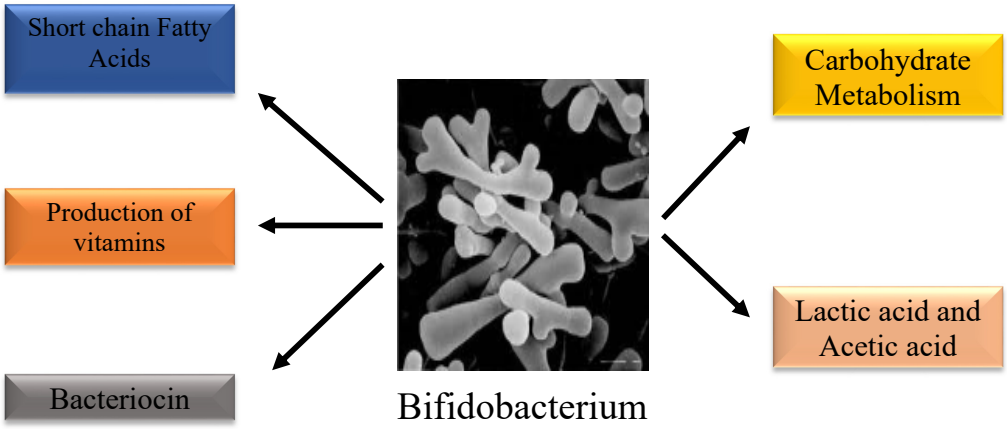

Fig. (1). Potential properties of *Bifidobacterium* used in various applications for genome editing.

CHAPTER 6

Lactic Acid Bacteria as Starter Cultures in Food: Genome Characterization and Comparative Genomics

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Abstract: Fermented food products are consumed by about 30% of the world's population due to their high nutritional value and health properties. The use of LAB in the fermentation process has resulted in a variety of fermented food products derived from both plant and animal sources. LAB have been used as starter cultures for food fermentation both traditionally and industrially, having certain specific characteristics such as rapid growth, product yield, higher biomass and also unique organoleptic properties, and are employed in food fermentation. The advancement of high-throughput genome sequencing methods has resulted in a tremendous improvement in our understanding of LAB physiology and has become more essential in the field of food microbiology. The complete genome sequence of *Lactococcus lactis* in 2001 resulted in a better understanding of metabolic properties and industrial applications of LAB. Genes associated with β-galactosidase, antimicrobial agents, bile salt hydrolase, exopolysaccharide, and GABA producing LAB have received a lot of attention in recent years. Genome editing techniques are required for the development of strains for novel applications and products. They can also play an important part as a research method for acquiring mechanistic insights and identifying new properties. The genome editing of lactic acid bacterial strains has a lot of potential applications for developing functional foods with a favourable influence on the food industries.

Keywords: Functional food, Genes, Genome editing, Lactic acid bacteria, LAB genome, Metagenomics, Sequencing-approaches, Starter culture.

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INTRODUCTION

Fermentation is one of the oldest and cheapest methods of food processing. To date, it is estimated that globally more than 5,000 varieties of different fermented beverages and foods are being consumed. Traditional fermented food products such as cheese, soya sauce, meats, vegetables and wine production have been documented since ancient civilization [1]. The process of fermentation improves the nutritional content and shelf-life, also increasing the functionality and therapeutic effects of the end product. The process further reduces anti-nutritional properties such as tannins and phytic acid, enhancing the bioactive components which exert beneficial health effects along with increasing the flavour, texture and aroma [2, 3]. Bioactive molecules produced during the fermentation of food have gained attention in the research due to their specific health promoting properties, having antioxidant, anti-cholesterol, anti-inflammatory, immunomodulatory, ACE-inhibitory and anti-viral properties [4 - 7].

Lactic acid bacteria (LAB) are Gram-positive, facultative anaerobic, non-sporulating and catalase negative bacteria, involved in the production of fermented foods such as meat, vegetables, cereals and dairy products [8]. Common bacterial genera of LAB include *Lactobacillus, Lactococcus, Enterococcus, Leuconostoc, Bifidobacterium, Pediococcus, Propionibacterium, Streptococcus, Weissella, Tertragenococcus* and *Vagococcus*. According to the new classification of bacterial taxonomy, LAB belongs to the phylum *Firmicutes*, class *Bacilli*, order *Lactobacillales*, and refers to five families, which include *Aerococcaceae*, *Carnobacteriaceae*, *Enterococcaceae*, *Lactobacillaceae* (includes the family *Leuconostocaceae*), and *Streptococcaceae* [9]. LAB are generally considered safe (GRAS) and most of them have probiotic effect [8, 10]. LAB have been used as starters, which is a single and/or mixed culture employed to enhance the rate of the food fermentation process to provide particular characteristics in a controlled manner. LAB are quite frequently used along with fungi and yeast during the process of fermentation, which helps in the release of novel bioactive compounds [11]. Production of lactic acid resulting in acidification of the food is the primary function of starter culture. The use of starter culture has resulted in an increase of food safety by inhibiting the growth of undesirable microorganisms which leads to spoilage of food products and also pathogenic microbes. Since the early 20th century, numerous commercial starter cultures have been employed which possess different metabolic properties, including proteolytic, acidification and antagonistic properties [8].

In the field of food microbiology, microbial genome sequencing has been playing an important role because of its improvement in the speed and quality of sequencing data. Discovery of complete genome sequencing of *Lactococcus lactis*

subsp. *lactis* IL1403 in 2001, have provided new insight and application for LAB [12]. More than 100 complete genomes of LAB have been sequenced and are available in the public domain [13] (Fig. **1**) (Table **1**). Developments of genomics and functional genomics, along with the high throughput technologies in the last decade, have resulted in a greater understanding of metabolic characteristics and industrial use of LAB, thus, making LAB as the most promising microorganisms for food and other industries by large scale production of food ingredients and other products such as polyols, lactic acid and vitamins.

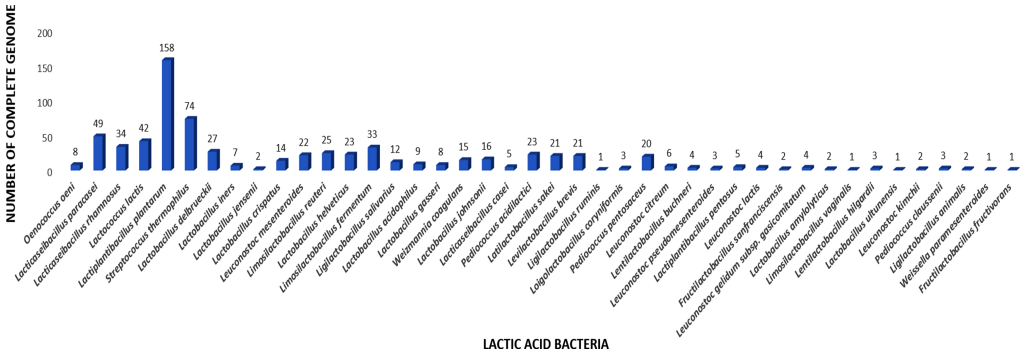

Fig. (1). Number of a complete genome sequence of various LAB available in the public database.

Table 1. Selected LAB species and their genome information retrieved from the available literature.

LAB Species	Genome Size (Mb)	Genes	GC%	References
Lactobacillus acidophilus La-14	1.99	1957	34.7	[85]
Lb. acidophilus NCFM	1.99	1938	34.7	[86]
Levilactobacillus brevis ATCC 367	2.29	2314	46.2	[87]
Lacticaseibacillus casei ATCC 334	2.76	2906	46.7	[87]
Lactobacillus delbrueckii subsp. *bulgaricus* 2038	1.87	1792	49.7	[88]
Lb. delbrueckii subsp. *bulgaricus* ND02	2.13	2183	49.6	[89]
Limosilactobacillus fermentum IFO 3956	2.00	1912	51.0	[90]
Lactobacillus gasseri ATCC 33323	1.95	1898	35	[91]
Lactobacillus helveticus DPC 4571	1.98	1938	37.0	[92]
Lactobacillus johnsonii NCC 533	1.99	1918	34.6	[93]
Lactiplantibacillus plantarum WCFS1	3.31	3135	44.5	[94]
Lacticaseibacillus rhamnosus GG	3.01	2905	46.7	[95]
Latilactobacillus sakei subsp. sakei 23k	1.90	1963	41.2	[96]
Ligilactobacillus salivarius UCC118	1.83	1864	32.9	[97]

CHAPTER 7

L. Plantarum of Vegetable Origin - Genome Editing and Applications

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Abstract: *Lactobacillus plantarum* is a widespread, versatile bacterium that plays a vital role in the preservation of innumerable fermented foods. These strains are commonly employed as silage additives and starter cultures of fermented goods. Genome editing could provide an added benefit by improving the fermentation profile and quality, as well as the accompanying therapeutic benefits.

Genome editing of various strains of *L. plantarum* can be used commercially to produce L-ribulose or succinic acid, direct lactic acid production, and increased ethanol production. *L. plantarum* strains or recombinant strains can help restore intestinal flora homeostasis, reduce the number of pathogenic organisms, and could even be employed as vaccine carriers. Food products such as raw and fermented vegetables, olives, and cereals inoculated with probiotic microbes have shown encouraging benefits as people now seek non-dairy based probiotics. *L. plantarum* of vegetable or plant origin, as well as applications of genome edited strains, are discussed in this book chapter.

Keywords: Fermentation, Genome rditing, *Lactiplantibacillus plantarum*, *Lactobacilli*, Probiotics.

INTRODUCTION

Lactic acid bacteria are one of the most significant food-grade microorganisms, with nutritional and fermentative properties [1]. *L. plantarum* is a member of the *Lactobacillus casei – Pediococcus* phylogenetic group [2], specifically the *L. plantarum* taxonomic subgroup. Many metabolic processes distinguish this species, allowing it to colonise a variety of environments, including dairy products, pickled vegetables, fish products, silage, wine, and mammalian intestinal tracts [3].

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L. plantarum strains are utilised as a starting culture in sourdough bread, meat products, and wine because they are associated with favourable qualities in many fermented foods [4] and are added to a number of them to improve their quality or associated health benefits, as a result, paving way for the availability of a variety of bio-therapeutic products [5]. They play an essential role in the preservation of food and fermented food products such as vegetables, sausages, silages, brine olives, sauerkraut, cassava, and kimchi [6]. Due to its higher acid tolerance than other lactic acid bacteria, it can significantly improve the latter stages of fruit and vegetable fermentations [7]. As a result, these bacteria are frequently used in industrial fermentation and are "generally recognised as safe" (GRAS), which qualifies the safety presupposition [8].

Various functional features of microorganisms depend on its genes. Dissecting biochemical processes that drive food fermentation, as well as identifying and characterisation of health-promoting traits that have a favourable impact on the composition and roles of microbiomes in human health, are all dependent on the genome of the microbe. Our ability to manipulate genomes, given the recent advancements in molecular biology, has made easier to tinker with the genes and gain the desired modifications.

Genome editing precisely defined as a genetic engineering principle which deals with the alterations of genome of an organism at single base pair levels in order to manipulate or add on or enhance particular features of the organism [9]. Genome editing has emerged as a widely practised engineering tool in various microorganisms like *Escherichia coli, Staphylococcus aureus, Lactobacillus reuteri, Clostridium beijerinckii, Streptococcus pneumonia,* and *Saccharomyces cerevisiae etc*. as it is a highly efficient and simple tool for site specific manipulation of genes when compared to Zinc Finger Nucleases (ZFNs) and Transcription Activator-Like Effector Nucleases (TALENs) techniques [10].

CRISPR-Cas9 genome-editing mechanism has evolved naturally in prokaryotes like bacteria as a protective mechanism against bacteriophages. CRISPR arrays are DNA segments created by bacteria that catch fragments of DNA from invading viruses [11]. CRISPR-Cas9 assisted double-stranded DNA and single-stranded DNA recombination engineering in *L. plantarum* WCFS1 was used to edit the genome, including gene knockouts, insertions, and point mutations [12]. Most of the *L. plantarum* have the type II CRISPR-Cas system, comprising four genes- Cas1, Cas2, Cas9, and csn2 [13].

With the development of clustered, regularly interspaced short palindromic repeat (CRISPR) based technologies, we are on the verge of a broad-scale genome editing revolution. Various genome editing tools and technologies, such as

CRISPR-associated enzyme genome editing, single-stranded DNA recombinant engineering, and bacteriophage modification, have paved the way for the development of next-generation bio therapeutic agents with improved genotypes and health-promoting functional features.

Genetic manipulation of *Lactobacillus sp.* could be beneficial to get an improved fermentation profile and or enhance medically important characters, *etc* [14]. The main aim of the book chapter is to put emphasis on the various applications of genome editing in *L. plantarum.*

LACTIPLANTIBACILLUS PLANTARUM

Morphology

L. plantarum is a nomadic, non-pathogenic gram-positive lactic acid bacterium previously named *Streptobacterium plantarum* [15]. *L. plantarum* is named after the Latin word "*plantarum"* which means "plant species." They are non-motile, non-sporing forming, catalase negative, rod-shaped and appear individually or in pairs, or in short chains. When haem is present, certain strains exhibit pseudo-catalase activity or true catalase activity.

The scientific classification of *L. plantarum* is given as follows (Basonym: *Lactobacillus plantarum*) in Table **1**. It has two main subspecies associated with it - *L. plantarum* (subsp. *plantarum* and subsp. *argentoratensis*) [16].

Table 1. Classification of *L. plantarum* [18].

Domain	Bacteria
Phylum	*Firmicutes*
Class	*Bacilli*
Order	*Lactobacillales*
Family	*Lactobacillaceae*
Genus	*Lactiplantibacillus*
Species	*L. plantarum*
Subspecies	*L. plantarum* subsp. *Argentoratensis* *L. plantarum* subsp. *Plantarum*

L. plantarum is an aero-tolerant *bacteriium* that grows well at 15°C [17] in a 4% NaCl concentration but not much at 45°C, according to research. It can convert hexoses into both D and L isomers of lactic acid *via* fermentation, as well as pentoses and/or gluconate, into acetic and lactic acids. It also has the ability to ferment malic acid to lactic acid and carbon dioxide, as well as citric acid to

CHAPTER 8

Genome Editing in *Bacillus Licheniformis*: Current Approaches and Applications

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Abstract: *Bacillus licheniformis* has been regarded as an exceptional microbial cell factory for the production of biochemicals and enzymes. The complete genome sequencing and annotation of the genomes of industrially-relevant *Bacillus* species has uplifted our understanding of their properties and helped in the progress of genetic manipulations in other *Bacillus* species. The genome sequence analysis has given information on the different genes and their functional importance. Post-genomic studies require simple and highly efficient tools to enable genetic manipulation. With the developments of complete genome sequences and simple genetic manipulation tools, the metabolic pathways of *B. licheniformis* could be rewired for the efficient production of interest chemicals. However, gene editing (such as gene knockout) is laborious and time consuming using conventional methods. Recently, useful tools for the genetic engineering of *Bacillus* species have emerged from the fields of systems and synthetic biology. The recent progress in genetic engineering strategies as well as the available genetic tools that have been developed in *Bacillus licheniformis* species, has conveniently enabled multiple modifications in the genomes of *Bacillus* species and thereby improved its use in the industrial sector.

Keywords: *Bacillus licheniformis*, Complete genome sequence, CRISPR-Cas9, Gene editing, Genome engineering tools.

INTRODUCTION

The finding of genes as the information carrier made it the focus of experimental research and investigation for its applications in mankind and their betterment. The discovery of DNA along with the hereditary role of genes, leads to the research on the methods to manipulate site-specific editing in the genome [1]. The engineering of nucleases could create site-specific double-strand breaks in DNA,

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which stimulated the homologous recombination [2]. From then, the genome editing field showed a tremendous change from a niche research technique to a universal genetic research tool. In genome editing, natural cellular pathways were exploited to repair DNA breaks introduced in the genome by various types of nucleases like zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and engineered meganucleases [3]. These are the old techniques used to produce specific DNA breaks in the desired DNA sequence. The CRISPR-Cas system is one of the latest nuclease platforms, which is now being used extensively for gene editing [4]. This technique is based on the adaptive immune mechanism shown by bacteria and archaea, where it uses RNA guided nucleases to degrade the specific sequence of the viral DNA [5]. The Cas9 endonuclease (Cas9-gRNA ribonucleoprotein (RNP) complex) of this system depends on a 100 nucleotide sgRNA (single guide RNA) for cleaving the chromosomal target, which is activated by the hybridization of the sgRNA on the target DNA [6]. Because of its specificity, speed and cost effectiveness, it is the most efficient and simple technique in the field of gene editing [7].

The latest applications of genome editing, along with its latest tools mentioned above, made it a keen area of interest for researchers. Such advanced gene editing tools have made it possible that any gene can be deleted or incorporated into the genome of any organism. It also allows system level identification and exposing the functionality of the genome along with direct editing and modulation of the DNA functions of any organism of choice [8]. The advancement in this area created a revolution in the food and biopharmaceutical industries and also can cure innumerable genetic diseases. Apart from its clinical use, it can also be used for the production of recombinant therapeutic protein products by engineering yeast and CHO cells [9].

Besides the enormous therapeutic applications of gene editing in eukaryotes, it can also achieve specific deletions, insertions and point mutations in the bacterial genome, making it relevant in the industrial sector. Such metabolic engineering in bacterial cells can manipulate their cellular functions to achieve an increased yield and productivity of the targeted value-added biochemicals [10]. The ease and efficacy in the genetic manipulation of *E. coli* species are attributable to its extensive application in research and industrial fields [11]. This made it one of the most extensively used cellular factories for the making of industrially important enzymes and biochemicals [12].

Recently, the species *Bacillus* also received improved importance because of their application in genome editing and for the synthesis of heterologous proteins, vitamins, antibiotics, valuable enzymes and chemicals [13]. The *Bacillus* species, which are non-pathogenic and have a history of secure exploitation in foods, are

being used in fermentation and industrial level production. From the global and gene specific level regulation and modification of metabolic pathways, numerous cellular phenotypes can be obtained in *Bacillus* species. For such genomic alteration of *Bacillus*, various valuable tools have been recently developed, which helped realize the potential of this bacterium [14].

The whole genome sequence data of *Bacillus* species helped in understanding the metabolic pathways along with supplying an overview of protein machinery. The enhanced understanding of the strains allowed advances in genetic manipulations in the same and related species [13]. And thus, efficient and simple genetic tools emerged from the fields of systems and synthetic biology to enable multiple modifications in the bacterial genome conveniently. In view of this, the current chapter is discussing the progress in the development of genetic tools and the strategies for the genetic modification in *Bacillus* species with an emphasis on *Bacillus licheniformis*.

CHARACTERISTICS OF *BACILLUS* SPP.

The genus *Bacillus* comprises rod-shaped, chemo heterotrophic, Gram-positive bacteria which have peritrichous flagella for their motility and have no capsules. They are catalase positive aerobic or facultative anaerobic organisms [15]. They are distinguished by their ability to produce dormant endospores aerobically when they are grown in unfavourable conditions. These spores can be round, cylindrical, oval or kidney shaped. They are dormant, rigid, non-reproductive structures with high resistance to heat, cold, disinfectants, desiccation, radiation and drying and can remain for a longer period [16].

They are majorly free –living non-parasitic saprophytes, which are seen ubiquitously in soil but are also isolated from other environments like water, air, food and vegetables and human and animal gut [17]. Though some of the *Bacillus* species are pathogenic in nature to humans and animals, they have a crucial role in balancing the ecological systems. They are the most heterogenous group in terms of phenotypic and genotypic characteristics. They typically exhibit large, flat colonies on non-selective media and are often beta-hemolytic [18].

The genus *Bacillus* is classified taxonomically in the family Bacillaceae, order Bacillales, class Bacilli, phylum Firmicutes, and domain Bacteria. Representing 1 of the 27 genera within the family Bacillaceae, the genus *Bacillus* comprises more than 140 recognized species, including nonpathogenic saprophytes, vertebrate pathogens, and invertebrate parasites [19].

The significance of *Bacillus* species relies on their ability to synthesize antibiotics/metabolites, which shows antagonistic effects on pathogenic microbes

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