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Production of Remedial Proteins through Genetically Modified Bacteria

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Abstract

Recombinant DNA technology has created biological organisms with advanced genetic sequences and has been extensively used to express multiple genes for therapeutic purposes when expressed in a suitable host. Microbial systems such as prokaryotic bacteria has been successfully utilized as a heterologous systems showing high therapeutic potency for various human diseases. Bioengineered bacteria have been successfully utilized for producing therapeutic proteins, treating infectious diseases, and disease arise due to increasing resistance to antibiotics. Prominently *E. coli* found to be the most widely used expression system for recombinant therapeutic protein production i.e. hormones, enzymes and antibodies. Besides *E. coli,* non-pathogenic lactic acid bacteria has also been considered as an excellent candidate for live mucosal vaccine. Likewise, *S. typhimurium* has been deployed as attenuated type of vaccination as well as in treatment strategy of various cancers due to its ability of wide progression in tumors. The present article is a summarized view of the main achievements and current developments in the field of recombinant therapeutics using bacterial strains focusing on their usability in therapeutics and future potential.

Introduction

The twentieth century has brought a lot of developments in the science and technology. It has been found to be dominated by Virchow, Lister, Koch, and Pasteur who brought advancements in modern medicine and therapeutics. Furthermore, with the advent of molecular techniques and Biotechnology, learning about the science of life using biological organisms and systems has improved a lot. Biotechnology includes a lot of disciplines i.e. seed biotechnology, recombinant gene development, functional genomics, applied immunology, as well as development of Biopharmaceuticals and diagnostic approaches [1].

An Increased understanding of the cell physiology and heterologous gene expression system empowered the use of living cells i.e. microorganisms, plants and animal cells as Biological factories for the development of recombinant molecule [2]. The idea of recombinant or Chimeric DNA was first proposed by Peter Lobban, a graduate student in the Biochemistry Department at Stanford University Medical School. The DNA sequences for constructing chimeric DNA (recombinant DNA) can be taken from any specie. For that purpose human DNA can be joined to fungal DNA and plant DNA can be joined to bacterial DNA for specific gene expressions. Such expressions required specialized expression vector which act as a vehicle to carry foreign genes needed to be expressed.

Among various specialized vectors, bacterial plasmid is considered as the most promising vector in recombinant DNA technology [3]. Bacterial Plasmid has been found to be more significant than any other vector in use like viral vectors. Besides bacteria, these plasmid vectors are also found in few fungal strains. During the last four decades, these plasmids have played a crucial role in the recombinant technology as a molecular tool widely used for the gene expressions in microorganisms and making these recombinant microbes beneficent for mankind [4].

With the advent of time, recombinant DNA technology has rapidly become a choice of scientists for treating various diseases. Using microbial genomes (plasmids and viral genome) as a vector, various successful attempts have been done which are playing significant roles in bringing improvements in human health. Bacterial attempts for therapeutic purposes has been initiated from last four decades [5]. A large number of studies have been done that prove recombinant Bacteria an excellent therapeutic agent. Various practical applications can be found in pharmaceutical industry including production of various human therapeutic proteins and various classes of antibiotics through molecular cloning. In addition to that, recombinant bacteria has also been focused to find solution to various drug resistance diseases, to study the therapeutic efficacy of various strains in anti-cancer therapies as well as to find their efficacy as live mucosal vaccines. Many more researches are currently under study, expecting to bring advancement in science and technology, however due to the increasing need of innovative therapeutics, a lot of work is still need to be done in future.

Methods

For writing the article recombinant bacteria in therapeutics, different search engine like, PubMed, NCBI and Google Scholar were used. Different keywords i.e., enzyme production in bacteria, hormone production, vaccine production and antibiotic development in recombinant bacteria were used. A total of 137 articles were collected from these search engines and 62 were selected and cited in writing this article.

Discussion

THERAPEUTIC PROTEIN PRODUCTION

Human cells produce a large number of different proteins or polypeptides that get integrated into complex physiological network to perform specialized functions such as catalysts, signaling agents, structural components as well as regulators of inter and intracellular interactions [2]. The genetic mutations or abnormal amino acid sequences eventually cause the absence or dysfunctioning of respective cellular protein which ultimately resulted in severe diseases. Few of them are diabetes, dwarfism, cystic fibrosis, thalassemia, and impaired blood clotting [2,6]. Such diseases can be treated by providing missing proteins from external sources to reach their required concentration in the human body. To overcome the difficulty in obtaining such proteins from their natural sources, recombinant DNA (rDNA) technology has used bacterial strains as a biological framework. Using this approach several recombinant proteins with therapeutic applications have been developed prominently including insulin and human growth hormone [7]. For the recombinant

protein production, wide and growing spectrum of expression systems has been available [8]. *E. coli* is found to be the most widely used expression system for heterologous protein production due to its easiest, least expensive and quickest reproduction. Recent advancement in the understanding of Cell's central dogma i.e. transcription, translation and protein folding along with improved genetic tools has made this bacterium more valuable in Microbial therapeutics. It has the ability to accumulate recombinant proteins up to 80% of its dry weight and can survive under variety of environmental conditions [9].

Moreover different strains of Gram positive Lactic acid bacteria (LAB) have also been modified for therapeutic purposes [10,11]. Various strains have been successfully used for the production of recombinant proteins like *Lactococcus lactis* strains which do not produce endotoxins and have been successfully used for the production of more than 100 recombinant proteins including expression of various potential vaccine components and many others [12]. Studies have also shown the importance of *Salmonella typhimurium* in cancer treatment as a genetically modified bacteria for producing anti-cancer drugs [13].

Hormones production

Diabetic patients were treated with insulin purified from the bovine or porcine pancreas, since the early 1920s. In 1978, the gene encoding for human insulin was first cloned and expressed in *E. coli* introducing recombinant human insulin as a first licensed drug. Now a days, *E. coli* and a fungi *Saccharomyces cerevisiae* have been used primarily for producing human insulin. *E. coli* expression system involves the formation of insulin precursors which are produced as inclusion bodies and become fully functional polypeptides after their solubilization and refolding. This functional polypeptide, insulin has been approved by FDA for therapeutic applications in humans [7]. Similarly *E. coli* expression system has also been extensively used for the production of recombinant hormones. Human growth hormone (hGH) containing 191 amino acid residues is a single chain polypeptide that is synthesized in the pituitary gland. Due to its pivotal role in a variety of biological functions i.e. cell proliferation and metabolisms, it is considered one of the most important hormones in the body. Studies reported on GH deficient patients showed that it plays a crucial role in maintaining mental and emotional stability by maintaining high energy levels [14]. For therapeutic purposes, exogenous recombinant hGH has been significantly expressed by prokaryotic expression system *E. coli* under the control of its promoter [15]. This exogenous hGH can be used to treat patients with cognitive impairment that results due to GH deficiency [16].

Enzyme production

Enzymes are biocatalysts of cell's metabolism that accelerate all biological reactions in the body. Any malfunction in the single critical enzyme can lead to a diseased condition. For instance absence of enzyme phenylalanine hydroxylase eventually resulted in a severe disorder called Phenylketonuria and causes mental retardation if not treated. Therefore, several enzymes have been produced using microbial expression system which cannot be synthesized in sufficient amount naturally. Recombinant enzymes such as factor VIII and Urokinase have been successfully produced through microbial expression system and provided to the patients as a drug, if their sufficient amount is not produced in the blood. They act as a thrombolytic agents in the treatment of thrombosis and myocardial infarction [17].

Streptokinase, an enzyme secreted by many *Streptococcus* species, is found to be a significant therapeutic agent [18]. Being widely used as a thrombolytic agent in treatment of acute myocardial infarction it has being expressed in various biological heterologous expression system like *E. coli, Bacillus subtilis, Proteus mirabilis, Streptococcus lactis* and *Streptococcus sanguis*. However, the recombinant streptokinase produced by these bacterial strains undergoes degradation easily [19]. *L. lactis* has been used to produce recombinant streptokinase by using its p170 expression system with a reduced degradation of that enzyme. Streptokinase gene was cloned in *L. lactis* and protein expression was controlled by changes in the medium composition and by pH. The expression and stability of recombinant streptokinase in *L. lactis* was found to be significantly increased by suppressing the development of ATR (acid tolerance response) in it,

which was basically controlled by adjusting extracellular pH and phosphate concentrations [18].

Therapeutic antibiotic production

Antibiotic, one of the most successful forms of chemotherapy has contributed a lot towards the field of medicine. From 1950s to 1970s the period was considered as a golden era for the discovery of new classes of antibiotics. However, after 1970, with the drop of new drug discovery the increase in resistance of infectious bacteria towards the existing antibiotics was also observed. Many scientists observed the enzymatic degradation of antibiotics by microbes leading to the increase in multiple diseases caused by the multidrug resistant bacteria [20]. Moreover, the clearance of many pathogenic microbes by the use of broad spectrum antibiotics also causes the elimination of commensal microorganisms. These reasons lead towards various infections caused by opportunistic pathogens such as a gut infection caused by *Clostridium difficile* [21] and various other infections caused due to increased drug resistance in *Staphylococcus aureus, Klebsiella pneumoniae* and *Mycobacterium tuberculosis etc.* [22]. These growing antibiotic resistant problems and the reports of CDC on the risks of antibiotic resistance enforced the thought of discovering new antimicrobial therapies. [23]. In the past, synthetic biological approaches were used for producing new antibiotics through recombinant microbes. Clustered chromosomal genes coding for the enzymes of whole antibiotic biosynthetic pathways were cloned specially in bacteria and expressed at higher level. Modified and hybrid antibiotics were obtained by introducing desired genes into the non-producing strains and into the producers of other antibiotics [24]. Such antibiotics successfully gained importance in medicine market however hurdles in the treatment of microbial diseases due to growing bacterial resistance enforced the researchers to work on the development of new microbial therapeutic. Probiotics, have been engineered to produce targeted antimicrobials. These are specially designed for controlled release of these antimicrobials on the onset of infection. Such modifications include production of antimicrobial peptides, production of toxin receptor mimics and production of such antimicrobials that prevent virulent gene expression by disrupting Quorum sensing signals.

RECOMBINANT BACTERIA TOWARDS ANTIBIOTIC RESISTANCE

Disruption of quorum sensing signals

Many pathogenic bacteria have the ability to synchronize with other bacteria by producing and sensing specific extracellular chemical signals. This chemical signaling called Quorum sensing stimulates the virulence regulatory genes, a coordinated behavior of bacteria that is dependent on their high microbial density. Researchers find the disruption of these QS signals an attractive target for prevention of diseases [25].

One of the recent works done by Duan and his co-worker through genetic engineering approach was the inhibition of virulence expression of bacterial strain *Vibrio cholera*, a causative agent of intestinal disease Cholera. The non-pathogenic strain of *E. coli* was engineered to express cholera auto inducer CAI-1 whose high concentration in *V. cholera* infected murine model caused the repression of its virulence factors. This significantly reduced its colonization and thus was found to have potential to prevent disease [26].

Toxin receptor mimics

Many non-pathogenic microbes have been genetically modified to display host receptor mimics for binding bacterial toxins. Recombinant probiotics were developed as a therapeutic strategy for prevention and treatment of enteric diseases through molecular mimicry of toxin receptors [27]. In one trial done by Paton and his co-workers, chimeric lipopolysaccharide expressed by the harmless recombinant *E. coli* strain was found capable to bind with heat-labile enterotoxins produced by a diarrhea causing *E. coli* strain. Results showed the considerable potential of toxin binding probiotics as a prophylaxis and treatment of enteric disease [28]. Likewise, in another trial, molecular mimicry of host receptors for Cholera Toxins were developed. The toxin receptor mimics were expressed by transforming the glycosyltransferase genes (from *Campylobacter jejuni* and *Neisseria gonorrhoeae*) in non-pathogenic *E. coli* for the production of chimeric surface lipopolysaccharide expressed a mimic of the ganglioside GM [1]. This recombinant bacteria was found capable of binding cholera toxins with high avidity in mouse even after establishment of infection [29].

Antimicrobial peptides

Many commensal bacteria have also been genetically modified to detect disease causing microbes and inhibit their growth by producing antimicrobial peptides [30]. In a recent work done by Borrero and his co-workers, recombinant lactic acid bacteria was designed to detect *Enterococcus faecalis*, a multi-resistant enterococci that is one of the dominant pathogens in Human GI-tract and respond by producing enterococcus peptides. *L. lactis* was engineered to detect enterococcal sex pheromone cCF10 and express genes for three bacteriocins, enterocin A, hiracin JM79 and enterocin P that showed antimicrobial activity against *E. faecalis* [31].

In another attempt, lactic acid bacteria was modified to produce antimicrobial peptides for gram negative pathogenic *E.coli* and *Salmonella* strains. Antimicrobial peptides A3APO and Alyteserin were selected on the basis of their high activity against *E.coli* and *Salmonella* strains. When these peptides were expressed in *L. lactis,* considerable reduction in pathogenic growth of these bacteria was observed [32]. In another work researchers successfully co-produced antimicrobial peptides nisin and colicin V through recombinant *L. lactis* and found them to be effective against both gram-positive (nisin) and gram negative bacteria (colicin V) [33]. Different trials have also been conducted on engineering those bacterial species that can stably colonize the cervicovaginal tract for treating diseases like HIV. Women are at higher risks for HIV infections. The production of an effective vaccine against HIV is somehow difficult to achieve due to the ability of rapid mutation and its evasion in the immune system. Researches were conducted on engineering a human vaginal isolate of *Lactobacillus jensenii* to secrete the HIV-1 inhibitors. In one trial Vangelista and her co-workers engineered *L. jensenii* strain to secrete anti HIV-1 chemokine RANTES and C1C5 RANTES. Both RANTES variants, having correctly folded conformation were shown to inhibit HIV-1 infection in T cells and macrophages. This recombinant bacterial strain was not only found to inhibit the HIV-1infection but also found to display strong activity against different HIV-1 genetic subtypes [34]. Another research work was conducted using the same vaginal strain *L. jensenii* to develop a live microbicide that produce antiviral peptide, cyanovirin-N (HIV inhibitor). It was tested on macaques model. In this study, macaques were repeatedly colonized with recombinant *L. jensenii* strain before each viral challenge. That strain was shown to colonize and secrete CV-N for at least 6 weeks after administration. It was found to reduce the rate of HIV acquisition in the macaque model with a capacity to provide more durable protection with relatively infrequent re-colonization. As healthy women have much more higher levels of *Lactobacillus* colonization in the cervical mucosa than female rhesus macaques, this approach can lead to greater protection in humans [35]. In another trial, a highly colonizing strain of *E. coli* was modified for the purpose of producing an antiviral hybrid peptide, HIV-gp41-hemolysin A hybrid peptides that has the ability to block the entry of HIV into the target cells by blocking its fusion [36].

RECOMBINANT BACTERIA AS VACCINES

Genetically modified bacteria have been used not only for the treatment (therapeutic) but also at the same time for the prevention (prophylactics) of diseases. Many recombinant bacterial strains have been proved as effective live vaccines through heterologous antigens production and therefore have been found to evoke immune responses in various trials [23].

Mucosal vaccination through recombinant bacterial strains has been found to be an effective approach towards immunization as it offers many advantages over systemic delivery system. Mucosal vaccination has been found to show reduced secondary effects as compared to systemic vaccination [10].

Salmonella, *Listeria* and *Bordetella* have been reported to be successfully used as live attenuated vaccines for delivering heterologous vaccines**.** In recent trials, a *S. typhimurium* vaccine strain generated specific immune responses in mice and protect it against virulent *S. pneumonia* by expressing several pneumococcal surface proteins including PsaA, PspA, PspC and Ply [37]. It has been also found to elicit immune responses against HPV 16 by expressing its antigens and further found to cause regression of HPV associated cervical tumors by expressing certain HPV associated fusion proteins [38]. *Listeria spp*. has been also found to show therapeutic and prophylactic affects against HPV associated cancers [39]. Kong and her co-workers done study on utilizing

pathogenic strain for antigen delivery and have shown attenuated recombinant pathogenic strains to be an attractive candidate vaccines [40]. However few safety concerns are associated with them while testing with human as these attenuated strains have the potential to become virulent at any stage. Non-pathogenic, genetically modifiable, Lactic acid bacteria (LAB) have been found to be a better approach towards mucosal therapeutic vaccine delivery as compared to the live attenuated pathogens. LAB has been intensively studied as a candidate vector of live mucosal vaccine and large number of successful researches have been conducted on *Lactobacillus* and *Lactococcus spp.* [41].

The first successful attempt to find killed recombinant *Lactococci* as a mucosal vaccine was done in 1990 against *S. mutan* infection. Later on, the use of live recombinant *Lactococci* against tetanus toxins was reported. Similarly from late 90,s large number of peer-reviewed publications confirmed recombinant *Lactobacilli* as an effective therapeutic vaccine [42]. Very promising therapeutic and prophylactic results have been shown by GM LAB in mouse models against HPV type 16 which is a major cause of cervical cancer, a second most common type of cancer in Women [38]. Successful results have been shown against HPV16 E7antigen. GM *L. lactis* strain expressing HPV 16 E7 antigen has shown promising results in many trials and when co-administrated with IL-12 showed strong therapeutic immune responses [43]. Studies conducted on *L. plantarum* against HPV 16 E7 have shown less immune responses but more rapid regression of tumor than *L. lactis* strains [44]. Another strain *L. casei* expressing E7 antigen has been successfully evaluated in human clinical trials as therapeutic vaccines [45]. *L. casei* has also shown immune responses and promising antitumor effects in *L. casei* PgSA-E6 treated mice [46]. Besides this, *Lactobacillus sp.* expressing VLPs (virus like particles) of HPV has opened the door for the development of a new type of vaccine [47]. Like L2 of HPV 16 when expressed only give immune response against HPV 16 but also found to show cross-neutralizing activity against HPV-18,-45 and -58 [48].

In the past decade, recombinant *L. casei* has been reported to provide immunity against Salmonella enteric serovar Enteridis and against SARS (Severe Acute Respiratory Syndrome) associated coronavirus in mice by expressing their respective antigens [49]. Oral administration of *L. casei* expressing enterogenic *E.coli* (ETEC) K99, K88 fibril proteins providing protection against ETEC in mice and secretion of bioactive murine Interleukin-1β to give adjuvant effects are few other examples [50,51]. Recombinant *L. lactis* has been reported to provide immunity against *Yersinia pseudotuberculosis* infections by secreting LcrV, a *Yersinia pseudotuberculosis* low-calcium response V (LcrV) antigen for mucosal vaccination against Yersinia infections. It has been also reported to provide immunity against Rotavirus infection by expressing its Vp8 antigen and also found to have immune response against various parasites [43]. Besides this recombinant *L. lactis* has also been found to induce protective immunity against different pneumococcal serotypes in mice by expressing its various antigens and against another disease, caused by *Helicobacter pylori* by expressing its antigen UreB with human IL-2 as adjuvant [37,52]. To generate passive immunity by producing antibodies various other LAB strains have been engineered successfully. A single-chain antibody fragment (ScFv) produced by recombinant *L. zeae* strain has shown to have potential to target *S. mutans* and *Porphyromonas gingivali* (causing periodontitis) antigens [43].

THERAPEUTICS IN CANCER TREATMENT

Cancer, a major cause of morbidity and mortality worldwide**,** is an uncontrolled and invasive cell growth that can spread to the other parts of the body, a condition called metastasis. Conventional anti-cancer therapies are effective up to certain level, mostly at early stages but cannot cure metastatic condition [2]**.** Bacteria have been utilized from many years as anticancer agent and studied a lot due to their intrinsic antitumor effect as well as a vector system to provide therapeutic compounds into tumors [53,54]. Genetically modified non-pathogenic and attenuated bacterial strains have been found to treat cancer either by providing direct tumoricidal effects or by delivering tumoricidal molecules [55]. Attenuated *Salmonella typhimurium* is the most extensively studied antitumor vector due to its natural ability to accumulate and replicate in a wide variety of solid tumors [56]. Non pathogenicity resulting from deletion of two of its gene *msbB* and *purl* and purine dependence nature make this bacterium a good vector for antitumor therapies. Due to its purine

dependence growth, it don't show growth in tissues other than tumor cells. It can also target metastatic lesions. Genetically engineered *S. typhimurium* strains expressing certain murine cytokines have been found to retard the growth of certain melanomas. Il-2 encoding *Salmonella* organisms can suppress the growth of tumor more effectively than non-modified strains [57]. It has been studied largely as a vector for various genes coding IL-12, GM-CSF, IL-4, IL-18 for targeting lung cancer, breast cancer and various melanomas when provided through oral route [5]. Attenuated *S. typhimurium* has also been genetically modified to act as an imaginable therapeutic probe for cancer. They are bioengineered to generate bioluminescence signals which are used to monitor their migration to tumor cells to predict their therapeutic efficacy. Besides this non-pathogenic *E.coli* strains are also playing important role in anti-cancer therapies by providing a large number of antitumor proteins. Among 24% of all the biopharmaceutical proteins available in markets are the antitumor proteins used in cancer treatment and among the all true antitumor drugs 69% of the drugs are produced in *E.coli* expression system consisting of engineered and fusion proteins [58].

Many other attenuated and engineered bacterial strains belonging to genus *Clostridium, Bifidobacterium*, *Salmonella*, *Mycobacterium*, *Bacillus* and *Listeria*, have also been found to colonize hypoxic and necrotic regions, present in solid tumors and can thus specifically act as antitumor agents [59,60].

Different efforts have also been done to block tumor angiogenesis through modified bacteria. For this purpose, different approaches have been utilized for the usage of such bacteria as anti-cancer therapeutics. This include bactofection, a Bacteria-mediated gene transfer and DNA vaccination which depend upon using bacterial vector [61]. Another technique is alternative gene therapy, which is basically a bacterial protein delivery mechanism for therapeutic purpose [62]. A bacterial *S. choleraesuis* strain bearing endostatin (angiogenesis inhibitor) and encoding eukaryotic expression plasmid was studied and successfully found to produce dual tumoricidal and antiangiogenic effects [5].

Conclusion

Bacteria hold great importance in the field of medical sciences not only as a disease causing agents but have been found to serve mankind through several natural and synthetic ways. With the advent of recombinant DNA technology, genetic engineering has changed the face of pharmacology and medicine industry. The physiological diversity in the microbial world has provided a large number of biological hosts. Among the growing spectrum of emerging hosts, few microorganisms display most appealing properties as biological hosts in therapeutic applications. Bacterial cells have been successfully modified for the production of therapeutically important drugs, for treating various genetic, cancerous and multi drug resistant diseases. Thus genetic engineering has served as a great source of therapeutic agents.

Future Prospects: The need of improved drugs and innovative therapies have prompt the researchers to focus more on curing chronic and complex acute diseases by working more in this field. They are constantly challenged to improve and optimize the existing microbial expression systems to develop more advanced therapeutically approaches to bring further advancement in medical sciences in forthcoming future i.e., slow releasing injectable recombinant proteins, chimeric protein as future drug to treat different problem with same drug. As protein degrades quickly because of their low molecular weight, the recombinant techniques can attach two different kind of molecules together to increase molecular weight, thus half-life can be increased.

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