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Genetically Modified Microorganisms

Development and Applications

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1. Introduction

With the development of recombinant deoxyribonucleic acid (DNA) technology, the metabolic potentials of microorganisms are being explored and harnessed in a variety of new ways. Today, genetically modified microorganisms (GMMs) have found applications in human health, agriculture, and bioremediation and in industries such as food, paper, and textiles. Genetic engineering offers the advantages over traditional methods of increasing molecular diversity and improving chemical selectivity. In addition, genetic engineering offers sufficient supplies of desired products, cheaper product production, and safe handling of otherwise dangerous agents. This chapter delineates several molecular tools and strategies to engineer microorganisms; the advantages and limitations of the methods are addressed. The final part of this chapter reviews and evaluates several applications of GMMs currently employed in commercial ventures.

2. Molecular Tools for Genetic Engineering of Microorganisms

A number of molecular tools are needed to manipulate microorganisms for the expression of desired traits. These include (1) gene transfer methods to deliver the selected genes into desired hosts; (2) cloning vectors; (3) promoters to control the expression of the desired genes; and (4) selectable marker genes to identify recombinant microorganisms.

2.1. Gene Transfer Methods

Table 1 *(1–23)* lists the gene transfer methods commonly used to introduce DNA into commercially important microorganisms. The most frequently used method is *transformation*. In this process, uptake of plasmid DNA by recipient microorganisms is accomplished when they are in a physiological stage of competence, which usually occurs at a specific growth stage *(24)*. However, DNA uptake based on naturally occurring competence is usually inefficient. Competence can be induced by treating bacterial cells with chemicals to facilitate DNA uptake. For *Escherichia coli*, an organism used commonly as a cloning host and a "bioreactor" for the commercial production

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Table 1
Gene Transfer Methods Used With Several Commercially Important Microorganisms

Type of Organism	Industrial Applications	Gene Transfer Methods	Reference
Aspergillus	Food fermentations	Protoplast transformation	*1*
		Electroporation	*1*
		Biolistic transformation	*1*
Yeasts	Food and beverage fermentations	Protoplast transformation	*2*
		Electroporation	*2*
Bacillus	Industrial enzymes	Transformation of competent cells	*3*
	Fine chemicals	Protoplast transformation	*4*
	Antibiotics	Electroporation	*5,6*
	Insecticides		
Corynebacterium	Amino acids	Protoplast transformation	*7*
		Conjugation	*8*
		Electroporation	*9*
Escherichia coli	Therapeutic protein production	Transformation of competent cells	*10*
	Biodegradable plastics		
Lactic acid bacteria	Food fermentations	Electroporation	*11*
	Organic acids	Protoplast transformation	*11*
Pseudomonas	Plant biological control agents	Electroporation	*12,13*
	Bioremediation	Conjugation	*14*
Streptomyces	Antibiotics, antitumor and antiparasitic agents	Protoplast transformation	*15*
		Electroporation	*16–18*
	Herbicides	Conjugation	*19–21*
			22,23

of numerous therapeutic proteins, the uptake of plasmid DNA is achieved when cells are first treated with calcium chloride or rubidium chloride *(10)*.

For many microorganisms, such as the antibiotic producing *Streptomyces*, transformation of plasmid DNA is a more complicated process. For these organisms, transformation involves preparation of protoplasts using lysozyme to remove most of the cell wall. Protoplasts are mixed with plasmid DNA in the presence of polyethylene glycol to promote the uptake of DNA. Growth medium, growth phase, ionic composition of transformation buffers, and polyethylene glycol molecular weight, concentration, and treatment time are variables that must be studied to identify the optimum conditions for protoplast formation and regeneration.

Electroporation is an alternative method to transform DNA into microorganisms. This method, originally used to transform eukaryotic cells, relies on brief high-voltage pulses to make recipient cells electrocompetent *(25)*. Transient pores are formed in the cell membrane as a result of an electroshock, thereby allowing DNA uptake. Growth phase, cell density, growth medium, and electroporation parameters must be optimized to achieve desirable efficiency. The main advantage of this method is that it bypasses the need to develop conditions for protoplast formation and regeneration of cell wall. Electroporation is often used when the efficiency of protoplast transformation is insufficient or ineffective. Several reports have documented the application of this method to industrially important *Streptomyces (16–18)*, *Corynebacterium (9)*, and *Bacillus (5,6)*. Electroporation is also the primary method of choice for transferring DNA into lactic acid bacteria *(11,26,27)*. In addition to using purified DNA for electroporation procedures, methods have been developed to transfer DNA directly from DNA-harboring cells into a recipient without DNA isolation *(28)*.

Conjugation is another method used to introduce plasmid DNA into microorganisms. This method involves a donor strain that contains both the gene of interest and the origin of transfer (*oriT*) on a plasmid and the genes encoding transfer functions on the chromosome *(29)*. Upon brief contact between donor and recipient, DNA transfer occurs. After conjugation takes place, donor cells are eliminated with an antibiotic to which the recipient cells are resistant. Recipient cells containing the transferred plasmid are identified based on the selectable marker gene carried by the plasmid. One advantage of this method is that it does not rely on the development of procedures for protoplast formation and regeneration of cell wall. In addition, this method offers the possibility of bypassing restriction barriers by transferring single-stranded plasmid DNA *(21)*. Introducing DNA by conjugation from donor *E. coli* has proven useful with *Streptomyces* and *Corynebacterium (8,19–23)*.

2.2. Vectors

Selection of a cloning vector to carry out genetic modifications depends on the choice of the gene transfer method, the desired outcome of the modification, and the application of the modified microorganism. Several classes of vectors exist, and the choice of which to use must be made carefully. Replicating vectors of high or low copy numbers are commonly used to express the desired genes in heterologous hosts for manufacturing expressed proteins. Replicating vectors are also used to increase the dosage of the rate-limiting gene of a biosynthetic pathway, such as that used for an amino acid, to enhance the production of the metabolite. Cosmid and bacterial artificial chromosome

vectors, which accept DNA fragments as large as 100 kb, are necessary when cloning a large piece of DNA into a heterologous host for manipulation and high-level metabolite production *(30,31)*. Conjugal vectors facilitate gene transfer from an easily manipulated organism such as *E. coli* into a desired organism that is usually more difficult to transform. Gene replacement vectors allow stable integration of the gene of interest. Food-grade vectors differ from the conventional cloning vectors in that they do not carry antibiotic resistance marker genes.

Special consideration must be given when constructing GMMs for industrial applications. If a GMM is to be released into the environment as a biological control agent, conjugal vectors should be avoided to prevent the horizontal transfer of the vectors and the genes into indigenous microorganisms. If a GMM is used as a starter culture for food fermentation, conjugal vectors should also be avoided *(32)*, and food-grade vectors should be developed and used for genetic manipulation *(27,33)*.

2.3. Promoters

A *promoter* is a segment of DNA that regulates the expression of the gene under its control. Constitutive promoters are continuously active; inducible promoters become activated only when certain conditions, such as the presence of an inducer, are met. It is important to select an appropriate promoter to optimize the expression of the target genes for desired timing and level of expression. A strong constitutive promoter is used when continuous expression of a target gene is desirable. For example, constitutive promoters were used to drive the expression of selectable marker genes to achieve complete elimination of nontransformed cells *(34)*. However, inducible promoters are often chosen when it is necessary to control the timing of target gene expression. This is especially true when expressing foreign genes, including toxin genes in *E. coli*.

The most commonly used inducible promoter for target gene expression in *E. coli*, the *lac* promoter, is turned on when the nonhydrolyzable lactose analog isopropyl-β-D-1-thiogalactopyranoside (IPTG) is added to the growing culture *(35,36)*. This promoter is relatively weak and therefore is often suitable for expressing genes encoding toxic proteins *(35)*. Promoters dependent on IPTG induction are usually undesirable for large-scale production of therapeutic proteins because of the high cost of the inducer and potential toxicity *(37)*.

Alternative promoters have also been developed for this purpose. The arabinose promoter is induced upon addition of L-arabinose to the medium *(35,38)*. L-Arabinose is a good alternative to IPTG because it is a less-expensive compound and thus is cost-effective in large-scale fermentations. The cold-shock promoter based on the *cspA* gene of *E. coli* is induced upon temperature downshift *(39)*. However, this promoter becomes repressed within 2 hr after temperature downshift and is therefore unable to achieve high-level accumulation of desired proteins. This problem is overcome by using an *E. coli* strain carrying a null mutation in the *rbfA* gene and thereby allowing continuous expression of target genes *(40)*. The *phoA* and *trp* promoters are turned upon with phosphate or tryptophan depletion, respectively, in the medium *(35,36)*. These inducible expression systems are inexpensive to implement and therefore are worth consideration for industrial applications. Promoters controlled by pH, dissolved oxygen concentration, or osmolarity in the medium are possible attractive alternatives *(36,41)*.

2.4. Selectable Marker Genes

Selectable marker genes, which often encode proteins conferring resistance to antibiotics, are an important part of cloning vectors and are required for identification of transformed cells. Application of selection pressure is necessary because the number of transformed cells is often significantly less than the number of nontransformed cells. Transformed cells are identified using a toxic concentration of the selection agent to inhibit the growth of the nontransformed cells. Usually, high-level expression of a selectable marker gene is necessary to ensure complete elimination of nontransformed cells.

Antibiotic resistance marker genes, although routinely used, are not generally acceptable for the construction of recombinant organisms such as lactic acid bacteria and yeasts used for food fermentation *(34,42)*. For lactic acid bacteria, alternative selection systems based on plasmid-linked properties of the organism itself, including lactose metabolism, proteolytic activity, DNA synthesis, and bacteriocin resistance, have been developed and incorporated into cloning vectors *(11,42)*. One problem associated with these selection systems is that they tend to give more nontransformed background cells than the antibiotic resistance marker gene-based selection systems *(11)*.

For constructing recombinant yeast acceptable for food fermentations, a number of selection systems based on yeast genes instead of heterologous antibiotic resistance marker genes were developed *(43)*. One such system is based on the *YAP1* gene, which is responsible for stress adaptation in yeast *(34)*. Overexpression of this gene under the control of the constitutive yeast gene (*PGK*) promoter confers resistance in cells to the fatty acid synthesis inhibitor cerulenin and the protein synthesis inhibitor cycloheximide. An added advantage of this dual selection system is that it almost completely eliminates nontransformed background cells.

3. Strategies for Genetic Engineering of Microorganisms

Several strategies have been developed to create GMMs for desired traits. They include (1) disruption or complete removal of the target gene or pathway; (2) overexpression of the target gene in its native host or in a heterologous host; and (3) alteration of gene sequence, and thereby the amino acid sequence of the corresponding protein.

3.1. Disruption of Undesirable Gene Functions

Disruption of a gene function can be achieved by cloning a DNA fragment internal to the target gene into a suitable vector. Upon introducing the recombinant plasmid into the host organism, the internal fragment of the gene, along with the vector, is integrated into the host chromosome via single-crossover recombination. The integration results in the formation of two incomplete copies of the same gene separated by the inserted vector sequence, thereby disrupting the function of the target gene. However, such integration is unstable because of the presence of identical DNA sequences on either side of the vector. The recombinant strain often undergoes a second recombination that will "loop" out the recombinant plasmid from the chromosome, thus restoring normal function of the target gene.

To create a stable recombinant strain blocked in the unwanted gene function, a gene replacement plasmid carrying two selectable marker genes is required. The first selectable marker gene, originating from the cloning vector, is used to select the transformed

cells, whereas the second selectable marker gene is inserted into the target gene. The recombinant plasmid is introduced into the host organism, followed by the selection of transformed cells based on the first selectable marker gene. Upon double-crossover recombination, the second selectable marker gene, now inserted into the target gene on the host chromosome, disrupts the sequence of the target gene and destroys gene function. The recombinant strain is selected based on its resistance to the second selectable marker gene product and its sensitivity to the first selectable marker gene product.

Another approach to disrupting gene functions relies on antisense technology. The technology is based on antisense ribonucleic acid (RNA) or DNA sequences that are complementary to the messenger RNAs (mRNAs) of the target genes *(44)*. The binding of an antisense molecule to its complementary mRNA results in the formation of a duplex RNA structure. The activity of the target gene is inhibited by the duplex RNA structure because of (1) an inaccessible ribosomal-binding site that prevents translation; (2) rapid degradation of mRNA; or (3) premature termination that prevents transcription *(45)*. Antisense technology has been used to downregulate target gene activities in bacteria *(45)*. The main advantages of this approach are rapid implementation and simultaneous downregulation of multiple target genes. In addition, this method is ideal for downregulation of primary metabolic gene activities without creation of lethal events.

3.2. Overexpression of Desired Genes

High-level expression of a target gene may be achieved by employing a high copy number vector. Eggeling et al. *(46)* constructed several *Corynebacterium glutamicum* recombinant strains containing increased copy numbers of *dapA*, a gene encoding dihydrodipicolinate synthase at the branch point of the lysine and methionine/threonine pathway. Lysine titer was higher in the recombinant strain containing one extra copy of *dapA* than the wild-type strain and was highest in the recombinant strain containing the highest copy number of the same gene.

However, gene expression systems based on high copy number vectors have a number of drawbacks. One is the segregational instability of recombinant plasmids, which results in the loss of recombinant plasmids and therefore loss of the desired traits. For example, expression of the *Bacillus thuringiensis* (*Bt*) toxin gene from a high copy number vector in *Pseudomonas fluorescens* was undetectable because of plasmid instability *(47)*. Segregational instability of plasmids is usually resolved by maintaining recombinant strains under selective pressures, usually by means of antibiotics. However, concerns about the use, release, and horizontal transfer of antibiotic resistance marker genes suggest that other means of maintaining plasmid stability need to be developed.

Baneyx *(35)* outlined a few options to achieve this goal. One method relies on creating a mutation in a critical chromosomal gene that is complemented with a functional copy of the same gene on the plasmid. As long as the plasmid housing the critical gene is present, the recombinant strain will survive. Major disadvantages of this method are the need to create a mutation in an essential gene of the host organism, the need to develop a specific growth medium, and the need to introduce an additional plasmid-encoded gene to complement the deficiency *(35)*.

Another concern about the use of high copy number vectors for high-level protein production in bacterial cells, especially in *E. coli*, is the formation of insoluble protein aggregates known as *inclusion bodies*. Inclusion bodies are biologically inactive because of protein misfolding, which is a consequence of rapid intracellular protein accumulation *(35,48)*. Although methods exist to isolate and renature inclusion bodies *(49–51)*, these systems are often inefficient and add steps in the purification of active proteins. Also, in the process of renaturation of proteins, a significant percentage of the proteins remains denatured and inactive *(52)*.

In most cases, the goal of protein production is to achieve acceptable levels of accumulation of desired proteins that retain biological activity. To achieve this goal, the rate of recombinant protein synthesis needs to be optimized *(36)*. An effective method to accomplish this is by lowering the growth temperature or by altering medium composition *(53)*. A molecular approach to maximizing active protein production is to coexpress the genes that facilitate protein folding and improve transportation of the recombinant protein out of the cell to decrease the intracellular concentration of the protein *(36,54,55)*. Another molecular approach is to carefully select a vector or promoter system that does not overwhelm the cell's capacity to produce active proteins *(56)*. However, both methods result in GMMs that harbor plasmids.

A different approach, which ensures expression of target genes at desired levels and avoids plasmid segregational instability and production of inclusion bodies, is to integrate target genes into the host's chromosome *(57)*. Although integration of a single copy of target genes may not be enough to achieve the desired level of protein production, integration of multiple copies of target genes has yielded very encouraging results *(58–60)*.

3.3. Improving Protein Properties

Site-directed mutagenesis and DNA shuffling are two powerful technologies that alter gene sequence in vitro to produce proteins that have improved characteristics. Site-directed mutagenesis is a technique used to change one or more specific nucleotides within a cloned gene to create an altered form of a protein via change in a specific amino acid *(61)*. This technique has been used successfully to identify catalytically important residues in new proteins. Two examples include the identification of catalytically essential residues in the *Aspergillus oryzae* Taka-amylase A *(62)* and the identification of the active site residue in the *Clostridium thermosulfurogenes* xylose isomerase *(63)*.

DNA shuffling, a technology introduced in 1994, is based on error-prone polymerase chain reaction and random recombination of DNA fragments *(64)*. DNA shuffling may involve a single gene or multiple genes of the same family. Family gene shuffling is more powerful than shuffling of single genes because it takes advantage of the natural diversity that already exists within homologous genes *(65,66)*.

Site-directed mutagenesis and DNA shuffling have been applied successfully for the improvement of numerous commercially important enzymes, notably the enzymes used in laundry detergents *(67,68)*. The goals sought commonly include altered substrate specificity, improved enzyme activity under broad washing conditions such as pH and temperature, enhanced resistance to detergent additives such as bleach, and longer shelf life. To improve enzyme characteristics using site-directed mutagenesis, prior knowledge regarding the enzyme, such as its active site and substrate-binding site, is required.

The advantage of site-directed mutagenesis is that only a limited number of recombinants will be screened. DNA shuffling, on the other hand, does not require specific knowledge about the enzymes of interest and can create new variants containing multiple beneficial mutations in the gene sequence for maximum benefit. High-throughput screening assays for identifying desired recombinants are necessary for using this method.

3.4. Approaches to Enhancing Product Yield

Usually, production levels of metabolites of commercial value, such as amino acids, vitamins, and antibiotics, by unaltered natural-producing microorganisms are quite low. Enhancing metabolite yield is therefore essential for meeting the product demands and for maintaining an economically viable process. Several approaches have proven successful in increasing the production of the desired products through manipulating the producing microorganisms. They include (1) overcoming rate-limiting steps; (2) eliminating feedback regulation; (3) manipulating regulatory genes; (4) perturbing central metabolism; (5) removing competing pathways; and (6) enhancing product transport.

3.4.1. Overcoming Rate-Limiting Steps

Rate-limiting steps refer to the steps in a biosynthetic pathway that restrain the flow of intermediates and thereby limit the overall production of the final product. The classical approach to identify rate-limiting steps is to feed pathway intermediates to the producing strain. If the intermediate is not converted to the final product, assuming it is transported into the cell, one or more steps between the intermediate and the final product are limiting. Once rate-limiting steps are identified, modification of the genes that encode the limiting pathway enzymes by either increasing gene dosage (amplification) or placing the gene under the control of a strong promoter often leads to relief of the bottleneck.

Ikeda et al. *(69)* successfully increased phenylalanine production in *C. glutamicum* by increasing the copy numbers of the genes encoding the rate-limiting enzymes in the phenylalanine pathway. Kennedy and Turner *(70)* increased penicillin production in *Aspergillus nidulans* by replacing the native promoter of the rate-limiting δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthase gene with a strong inducible ethanol dehydrogenase promoter.

3.4.2. Eliminating Feedback Regulation

One mechanism by which microorganisms control the production of essential metabolites, such as amino acids, is feedback regulation *(71)*. Genetic engineering offers a promising solution to overcome feedback regulation and satisfy increasing demand. An excellent example is isoleucine, an amino acid produced by *C. glutamicum*. In *C. glutamicum*, threonine dehydratase, the first committed enzyme in the isoleucine biosynthetic pathway, is sensitive to inhibition by the end product isoleucine. Isoleucine production was increased by relieving the feedback inhibition either by amplifying the native gene (*ilvA*) encoding the enzyme or by expressing the *E. coli* gene *tcdB*, which is insensitive to isoleucine feedback inhibition, in *C. glutamicum (72)*.

3.4.3. Manipulating Transcription Regulatory Genes

Manipulation of transcription regulatory genes is another way of achieving increased production. There are two types of regulatory genes, categorized by the effect they have

on the genes they control. Positive regulators turn on the expression of the genes they control; negative regulators repress the expression of the genes under their control. Regulatory genes control many biological processes, including antibiotic biosynthesis. Genes involved in antibiotic biosynthesis are often linked to form a cluster that usually contains pathway-specific regulatory genes *(73)*. Such regulatory genes encode proteins that directly bind to the promoter region of the biosynthetic gene for that antibiotic. This binding results in either an increase or decrease in the expression levels of the biosynthetic gene, which in turn either boost or hamper the production of the antibiotic. Manipulation of regulatory genes has proven to be rewarding in achieving increased production of the desired products *(73)*. Pathway-specific regulators are often controlled by higher-level regulators, called *global regulators*, that coordinate many metabolic activities. Manipulation of global regulators can be very fruitful, although their identification and the understanding of their functions is a time-consuming and labor-intensive task. Tatarko and Romeo *(74)* engineered a high phenylalanine-producing *E. coli* strain by disrupting the global regulatory gene *csrA*. Disruption of this gene enhanced gluconeogenesis and decreased glycolysis, which in turn resulted in increased accumulation of phosphoenoylpyruvate, one of the two starting molecules for phenylalanine biosynthesis.

3.4.4. Perturbing Central Metabolism

Central metabolism provides primary metabolites and energy to support the survival of microorganisms. In addition, central metabolism, under special growth conditions, contributes a small fraction of primary metabolites to pathways that produce secondary metabolites such as antibiotics. Perturbing central metabolism is complicated. Complications may arise from situations in which (1) disturbing the balance of metabolic activities is detrimental to the host or (2) the host is resistant to unnatural alternations imposed on it.

Despite these difficulties and complications, manipulation of central metabolism has proven to be rewarding. Butler et al. *(75)* engineered a superior actinorhodin-producing strain by deleting the genes responsible for either of the first two steps in the pentose phosphate pathway. Peters-Wendisch et al. *(76)* constructed a lysine-overproducing strain by expressing the *pyc* gene encoding pyruvate carboxylase at high levels. Apparently, overexpression of the gene increased the availability of oxaloacetate, the precursor for the starting material, aspartate, of the lysine pathway. Increased availability of oxaloacetate in turn enhanced lysine production.

3.4.5. Removing Competing Pathways

Competing pathways utilize the precursor or intermediate of the desired pathway. Two approaches may be used to remove competing pathways: (1) deletion of the entire competing pathway or (2) knocking out the function of the first gene of the competing pathway. Many antibiotic producers accumulate several compounds structurally related to the antibiotic of interest. Because these compounds do not usually possess useful biological activity, their synthesis is considered a waste of cellular energy, precursors, and intermediates. In addition, the presence of these compounds may complicate downstream purification. In this case, knocking out the pathways leading to the synthesis of the unwanted derivatives would simplify downstream processing and, more importantly, would redirect the precursor or intermediates toward the synthesis of the desired

product. Backman et al. *(77)* engineered an elegant system in which tyrosine synthesis, competing for the common intermediate used for phenylalanine production, is interrupted during the phenylalanine production phase.

3.4.6. Enhancing Product Transport

Enhancing product transport across the cytoplasmic membrane is another commonly used approach. Most microorganisms producing metabolites of commercial interest have means of transporting the products across the membrane. Reinscheid et al. *(78)* showed that the intracellular concentration of threonine in *C. glutamicum* is proportional to the copy number of the deregulated threonine pathway genes and extracellular concentrations of the same amino acid remained unchanged, indicating the importance of optimizing export machinery. Existence of an active transport system in *C. glutamicum* involved in lysine secretion was identified through cloning and characterization of the *lysE* gene *(79)*. The *lysE* knockout mutant lost the ability to secrete lysine, and the *lysE*-overexpressed mutant was able to export the amino acid at an accelerated rate, thereby increasing extracellular lysine concentration.

3.5. Genomic Approaches

New technologies and novel approaches are revolutionizing the way microorganisms are engineered. Rapid advancements in sequencing technologies make it possible to unlock the genetic code of a target organism in a relatively short period of time. This wealth of information offers clues to the genes directly involved in the synthesis of the product of commercial interest. The information also provides insight into the metabolic potential of the organism, such as pathways supplying precursors or cofactors necessary for the production of the metabolite of interest, pathways competing for valuable precursors or intermediates, and global regulatory networks that may control metabolite production.

Genomic information is beginning to modernize the engineering of recombinant microorganisms. Ohnishi et al. *(80)* reported on the genetic engineering of a hyperlysine-producing strain of *C. glutamicum*. The group identified the mutations carried by the lysine-producing strain derived from the conventional mutagenesis and random screening approach through comparative genomic analysis of the *C. glutamicum* wild-type strain and the lysine-producing strain. Introducing the mutations into the wild-type strain produced a mutant that was better than the classically derived lysine-producing strain in both lysine production and growth.

4. Applications of GMM-Derived Products

4.1. Human Health

4.1.1. Recombinant Therapeutic Proteins

Several proteins, such as insulin, interferons (IFNs), and interleukins, are now produced by GMMs for therapeutic use. The traditional method of supplying these proteins to patients requires purification of the proteins from cells, tissues, or organs of humans, cows, or pigs. Because it was impractical to treat diabetes with human insulin from cadaver sources, cow and pig insulin, which are somewhat different from human insulin, were substituted *(81)*. The problems with obtaining the proteins directly from animal sources included the limited supply and potential immunological responses *(81)*.

Limited supply translates into higher cost for the medication. Further concerns arose that therapeutic proteins of animal origin may be contaminated with viruses or other toxic substances *(81)*.

These problems can sometimes be avoided by producing the proteins in microorganisms. Human insulin, the first recombinant therapeutic protein approved by the Food and Drug Administration (FDA) in 1982, was produced by genetically engineered *E. coli* containing the human insulin genes *(81)*. Human growth hormone, approved by the FDA in 1985, was produced by a modified *E. coli* strain containing the native human growth hormone gene *(82)*. Recombinant IFN γ, under the trade name Actimmune® in North America or Imukin® in Europe, was jointly developed by Genentech and Boehringer Ingelheim *(83)*.

The gene encoding IFN γ was introduced into *E. coli* under the control of the tryptophan promoter and operator cassette. Therefore, IFN γ production is repressed in the presence of tryptophan in the medium during the first phase of fermentation and becomes derepressed when a tryptophan analog is added to the medium during the second phase of fermentation *(83)*. With this system, recombinant IFN γ production is regulated to ensure adequate accumulation of cell mass before production begins. Other examples of recombinant therapeutic proteins, such as IFN α-2a *(84)*, IFN β-1b *(85)*, and granulocyte-macrophage colony-stimulating factor *(86)*, are also produced using recombinant *E. coli* strains as production factories. Production of these therapeutic proteins in a fast-growing and easily manipulated organism ensures sufficient supply, free of contamination, reduced cost, and safe and consistent production.

4.1.2. Recombinant Vaccine

Hepatitis B is a serious disease caused by hepatitis B virus that attacks the liver. The first vaccine against hepatitis B was prepared with the purified hepatitis B surface antigen (HBsAg) extracted from blood samples of infected individuals *(87)*. This process is unsafe because of the risk in handling the infectious agent and expensive because of the required animal testing. In addition, the vaccine may be contaminated with other infectious agents. The second generation of hepatitis B vaccine was produced by expressing the gene coding for hepatitis B surface antigen in *Saccharomyces cerevisiae (87)*, common baker's yeast. The recombinant vaccine, under the trade name Engerix®-B, is identical to the first-generation hepatitis B vaccine and is produced safely, consistently, and economically.

4.2. Animal Health

4.2.1. Recombinant Proteins

Proteins benefiting animal health are also produced by recombinant microorganisms. Bovine somatotropin (bST), a natural protein hormone produced in the pituitary glands of cattle, regulates both animal growth and milk production in lactating dairy cows. Injection of pituitary extracts into lactating cows boosts milk production; however, pituitary glands from as many as 25 cows are needed to provide sufficient bST to supplement 1 cow for 1 day (http://www.monsantodairy.com/about/history).

To increase bST production for commercial use, the gene encoding bST was expressed in *E. coli*. The recombinant bST was approved by the FDA in 1994 and is marketed under the trade name Posilac™. The recombinant protein has the same chemi-

cal structure and biological activity as the native bST *(88)*. Studies showed that lactating dairy cows supplemented with recombinant bST produced 10–15% more milk. The same method has since been applied to produce a variety of animal growth hormones for other animals, including sheep, pig, buffalo, and goat for both meat and milk production *(89)*.

Another protein produced by a GMM and used to benefit animal health is phytase. Phytase catalyzes the release of phosphate from phytate, the primary storage form of phosphorus in plants *(90)*. The enzyme is present in ruminants, but is absent or nearly absent in nonruminants such as poultry and swine. Therefore, nonruminants are unable to obtain phosphate, an essential nutrient, from phytate present in the feed of plant sources. The lack of phosphate can be corrected by supplementing rock phosphate in animal feed. However, this method generates excessive amounts of released phosphate, both from the phosphate supplement and from the unused phytate, which is excreted in the animal's manure *(91)*. Excessive amounts of phosphate released to the environment contribute significantly to water pollution *(90,92)*.

One solution to the problem is to supplement phytase in animal feed if the enzyme can be produced economically. To achieve this goal, the gene encoding phytase was isolated from its natural host, *Aspergillus niger*, and placed under the control of the constitutively expressed glucoamylase gene promoter for high-level expression *(93)*. The recombinant gene cassette was introduced into an industrial strain of *A. niger* for phytase production and was found to integrate randomly at multiple locations in the host genome. With this system, phytase is produced in large quantities and is suitable for commercial application. Studies have shown that use of the recombinant phytase reduces the phosphate level in the feed by 20%. In addition, the level of phosphate in manure is reduced by 25–30% *(93)*.

4.2.2. Recombinant Vaccine to Eradicate Rabies

Rabies, a viral disease encountered by humans and other mammals, leads to more than 35,000 human deaths and several million animal deaths worldwide every year *(94,95)*. The rabies virus reservoir is primarily in wild animals, including fox, skunks, raccoons, wolves, mongooses, and raccoon-dogs. Humans normally become infected with the virus through bites from infected animals. Upon exposure to the virus, the current method to prevent development of rabies in humans and domestic animals is to inoculate with rabies vaccine prepared from an attenuated strain of rabies virus. This is normally in addition to treatment using antirabies γ-globulin. However, this method is impractical to eradicate rabies in wild animals.

A safe and cost-effective method to achieve vaccine production was through the development of a recombinant vaccinia virus expressing the glycoprotein G of rabies virus *(95)*. The recombinant virus is amplified in animal cells for preparation of vaccine suspensions, which are placed in animal baits for release into the wild. The recombinant vaccine is currently used in eradication programs in Europe and North America *(94)*.

4.3. Textile Industry

Microbial enzymes have been used in the textile industry since the early 1900s. To commercialize the enzymes, they must be produced at high levels. Conventional methods to enhance production include optimizing medium composition, growth conditions, and

the fermentation process *(67)*. Random mutagenesis and screening commonly is used to achieve high yields. Genetic engineering offers a possibility in which high-level enzyme production is achieved in a heterologous host to overcome the limitations of the natural producing organism. Two examples are α-amylase of *Bacillus stearothermophilus (96)* and cellulase of the alkaliphilic *Bacillus* BCE103 *(97)*. Amylases have been used for many years to remove starch sizes from fabrics, known as *desizing*. Originally, amylases from plant or animal sources were used. Later, they were replaced by amylases of bacterial origin.

The first bacterial enzyme for desizing was α-amylase from *Bacillus subtilis*, which was commercialized in the early 1950s *(96)*. A novel α-amylase naturally produced by *B. stearothermophilus* is heat stable and is active over a broad pH range *(96)*. These characteristics make this new α-amylase an attractive alternative. However, the new α-amylase is produced at low levels by its natural producing organism. To increase the production of this new enzyme for commercial use, the gene encoding the enzyme was cloned into a heterologous host, *Bacillus licheniformis (96)*.

Cellulases prevent and remove fuzz and pills and provide color brightening of cellulose-based fabrics such as cotton. A novel cellulase, active under alkaline detergent conditions, is an attractive alternative *(97)*. It is also produced in low levels by its native producing organism, an extremophile, because of poor growth. The gene encoding the cellulase was cloned into a heterologous host, *B. subtilis*, for high-level expression and enzyme production. In both cases, the desired enzymes were produced efficiently from heterologous hosts during fermentation. In addition, the enzymes are secreted directly into the culture media, which simplifies the recovery of the respective enzymes.

4.4. Food Industry

Enzymes manufactured by GMMs have been used in the food industry for more than 15 years *(98)*. Well-known examples include the use of chymosin for cheese making and pectinases for fruit and beverage processing. Traditionally, cheese making requires chymosin-containing rennet from calf stomachs to provide the essential proteolytic activity for coagulation of milk proteins *(93)*. However, chymosin preparations could have animal sources of contaminants.

In the early 1980s, Gist-brocades began investigating the possibility of producing chymosin from a microorganism using the genetic engineering approach *(93)*. The gene encoding calf stomach chymosin was cloned and expressed in an industrial strain of *Kluyveromyces lactis*, a yeast that had been used for many years in the safe production of food ingredients. To facilitate prochymosin secretion into the culture medium for recovery, the yeast α-factor leader sequence was used. This system efficiently secreted prochymosin into the culture medium along with very few endogenous proteins. On fermentation, prochymosin is converted into active chymosin via a simple autolysis step, followed by recovery of the final product. Chymosin produced through this genetically engineered yeast strain has the same chemical and biological properties as that from calf rennet. The chymosin preparation, registered under the brand-name Maxiren®, has been commercially produced since 1988.

Production of pectinases via the genetic engineering approach focuses on economic enzyme production, enhanced enzyme purity, and environmentally friendly production

processes *(98)*. Total pectin methyl esterase I (PME I) from *Aspergillus aculeatus* represents less than 1% of the total cellular protein *(99)*. In addition, this fungus accumulates a wide range of pectinolytic enzymes in the culture, making it difficult to acquire pure PME I *(99)*. These features make the natural producing organism less than ideal for commercial production of PME I.

The limitations were overcome by expressing the full-length cDNA (complementary DNA) encoding the enzyme in a heterologous host, *A. oryzae (99)*. The recombinant PME I represented 20–30% of the total cellular protein and was secreted directly into the culture medium for simplified purification. Complete pectin degradation was achieved by recombinant PME I in the presence of polygalacturonases *(99)*.

Complete degradation of pectin, a natural substance found in all fruits, is important for the beverage industry. The reason is that complete degradation of pectin increases juice extraction from fruits, enhances juice clarification, and helps the filtration step of the process *(98)*. Several enzyme preparations made by GMMs are currently used in the beverage industry. NovoShape™, containing a pure pectinesterase, helps retain the original shape and structure of individual fruit pieces during processing and thereby offers a finished product that is more appealing (http://www.novozymes.com). Pectinex® SMASH, containing a variety of different pectinases, is used for treating apple and pear mash for higher yield and capacity (http://www.novozymes.com).

4.5. Diagnostic Tools

Acquired immunodeficiency syndrome (AIDS) immunological tests are used for diagnosing the disease and for testing donated blood samples. The first generation of AIDS tests, commercialized in 1985, was based on inactivated human immunodeficiency virus (HIV) grown in tissue culture *(100)*. This production method is both expensive and, more importantly, hazardous because of the risk from handling the infectious agent. Further, this first-generation AIDS test was subject to false-positive reactions because of the cellular debris from virus-producing human cells. These problems were overcome by cloning the gene encoding the relevant antigenic coat protein of the virus into *E. coli* for large-scale production of the protein *(100)*.

Other diagnostic tests that have been developed using GMMs include one for diagnosing Alzheimer's disease. Noninvasive diagnosis of Alzheimer's disease was not possible until the development of an enzyme-linked immunosorbent assay kit in the mid-1990s *(101)*. The test kit, marketed under the trade name INNOTEST hTAU Antigen and used for the detection of tau proteins in human cerebrospinal fluid, is based on an Alzheimer's antigen produced by a modified *E. coli*.

4.6. Biodegradable Plastics

Conventionally, plastics polymers are made via petroleum-based processes. Because of the growing concerns over the environmental impact of petroleum-derived polymers, alternative methods to synthesize the polymers are under investigation. Many microorganisms naturally produce polyhydroxyalkanoates (PHAs) in the form of granules that the organisms use as an energy storage material *(102)*. PHAs are genuine polyester thermoplastics with properties similar to the petroleum-derived polymers. In addition, PHAs are degradable by depolymerase, an enzyme family widely distributed among bacteria and fungi *(103)*. These characteristics make PHAs an attractive

replacement for the petroleum-based polymers. However, the microorganisms that naturally produce PHAs are not necessarily suitable for commercial PHA production, mostly because of slow growth and low yields.

PHA accumulation is achieved in *E. coli*, a microbe lacking PHA biosynthetic machinery, after receiving the PHA pathway genes through transformation. The advantages of producing PHAs in *E. coli* are (1) the organism is robust in growth; (2) the organism's metabolism is well characterized; and (3) the organism lacks PHA depolymerase, the enzyme that degrades PHAs. In 2002, Metabolix (Cambridge, MA; http://www.metabolix.com) demonstrated high-yield, commercial-scale manufacture of PHAs using *E. coli* as a host.

5. Applications of GMMs

5.1. Agriculture

5.1.1. Biological Control of Frost Injury in Plants

Frost damage is a major agricultural problem affecting many annual crops, deciduous fruit trees, and subtropical plants. In the United States alone, annual losses because of plant frost injury can reach over $1 billion *(104)*. In addition to the losses caused by frost injury, hundreds of millions of dollars are spent every year to reduce plant frost injury mechanically. These methods are both costly and ineffective *(104)*.

Frost damage is initiated by bacteria belonging to the genera *Pseudomonas*, *Xanthomonas*, and *Erwinia*, collectively called *ice-nucleating bacteria (105)*. The bacteria, living on the surface of the plants, possess a membrane protein that acts as an ice nucleus for initiation of ice crystal formation *(105)*. Ice crystals disrupt plant cell membranes, thus causing cell damage. The biological route of controlling the nucleating bacteria is through seed or foliar applications of non-ice-nucleating bacteria to outcompete ice-nucleating bacteria *(106)*. The non-ice-nucleating bacteria were isolated by treating the ice-nucleating bacteria with chemical mutagens *(107)*.

One disadvantage of the chemically induced mutants is that they often harbor multiple mutations that may adversely impact their genetic stability and ecological fitness. To avoid multiple mutations, ice-nucleation-deficient mutants of *Pseudomonas syringae* were constructed by deleting the genes conferring ice nucleation *(108)*. These genetically engineered mutants were able to compete successfully with ice-nucleating *P. syringae* for the colonization of plant leaf surfaces. Field tests showed that plants treated with ice-nucleation-deficient *P. syringae* suffered significantly less frost damage than the untreated control plants *(109)*.

5.1.2. Biological Control of Insect Pests

Bt, a naturally occurring soil-borne bacterium, produces unique crystal-like proteins that have larvicidal activities against different insect species and pose no harm to mammals, birds, or fish *(110)*. The crystal-like proteins bind to specific receptors on the intestinal lining of susceptible insects, causing the cells to rupture. Because of these unique features, *Bt* has been used as a safe alternative to chemical pesticides for several decades *(111)*. However, natural *Bt*-based products do possess some shortcomings, including instability in the natural environment, narrow host range, need for multiple applications, and difficulty in reaching the crop's internal regions where larvae feed *(111)*.

One way to overcome these problems is to use plant-associated bacteria as hosts for delivering the toxins. *Bt* toxin genes have been introduced successfully into several plant-associated bacteria, including *Clavibacter xyli* subsp *cynodontis (112)* and *Ancylobacter aquaticus (113)*. Genetically modified *C. xyli* subsp *cynodontis* containing the *Bt* toxin gene integrated into the chromosome showed moderate control of European corn borer *(112)*. The modified *A. aquaticus* strain expressing *Bt* toxin genes, introduced by electroporation, exhibited significant toxicity toward mosquito larvae, thus demonstrating its potential in mosquito control *(113)*.

5.1.3. Biological Control of Plant Disease

Plant pathogens, including fungi and bacteria, damage crops and thereby reduce crop yield. Plant diseases are conventionally fought with chemicals, a strategy that is expensive, inconvenient, potentially environmentally unfriendly, and sometimes ineffective. An alternative method is to develop biological control agents in which microorganisms are modified to deliver the desired chemicals.

Agrobacterium tumefaciens causes crown gall disease in a wide range of broad-leaved plants by transferring part of its DNA (T-DNA), located on a large tumor-inducing (Ti) plasmid, into the plant cell *(114)*. Upon integration of T-DNA into the plant host's chromosome, the genes on T-DNA are expressed, resulting in overproduction of plant growth hormones and opines *(115)*. Overproduction of plant growth hormones causes cancerous growth, whereas opines are believed to serve as nutrients for the bacterium. *Agrobacterium radiobacter* K84 produces a bacteriocin, agrocin 84, to which pathogenic *A. tumefaciens* strains are susceptible *(116)*. In addition, K84 competes for the nutrients on which the pathogen thrives. Therefore, *A. radiobacter* K84 became the first commercial biological control agent against crown gall disease.

One potential problem that threatens the continued success of this biological control agent is that the genes responsible for agrocin 84 production and resistance are located on a plasmid harbored by K84 *(115)*. Horizontal transfer of the plasmid from K84 to pathogenic *A. tumefaciens* allows the pathogen to acquire resistance against the toxic effect of agrocin 84 and therefore survive in the presence of K84. To prevent plasmid transfer, part of the transfer region was deleted from the plasmid. The genetically modified strain was as effective as the nonmodified K84 strain in preventing crown gall disease and was commercialized for use in Australia in 1989 *(115,117)*.

Other plant disease control methods using GMMs include (1) overproduction of oomycin A *(118)*; (2) synthesis of phenazine-1-carboxylic acid in a heterologous host *(119)*; and (3) heterologous expression of a lytic enzyme gene *(120)*.

5.1.4. Soil Improvements

Genetic modifications to improve soil fertility have also been developed. *Medicago sativa* (alfalfa), grown in soils with a high nitrogen concentration, has been shown to undergo better root nodulation when exposed to a genetically modified *Sinorhizobium* (*Rhizobium*) *meliloti* expressing the *Klebsiella pneumonia nif* A gene than plants in the same environment exposed to wild-type *S. meliloti (121)*. Another study showed that the recombinant *S. meliloti* significantly increased plant biomass when compared to the wild-type strain *(122)*.

5.2. Bioremediation

Bioremediation refers to the utilization of biological systems to detoxify environments contaminated with heavy metals such as mercury and lead, organic compounds such as petroleum hydrocarbons, radionuclides such as plutonium and uranium, and other compounds, including explosives, pesticides, and plastics *(123)*. The first field release of a GMM for bioremediation was *Pseudomonas fluorescens* HK44 for naphthalene degradation *(124)*. Strain HK44 was derived from *P. fluorescens* isolated from a site heavily contaminated with polyaromatic hydrocarbons. HK44 contains a plasmid capable of naphthalene catabolism. In addition, this genetically modified strain harbors a bioluminescence-producing reporter gene (*lux*) fused with the promoter that controls the naphthalene catabolic genes. Therefore, in the presence of naphthalene, the naphthalene genes are expressed, resulting in naphthalene degradation and emission of luminescence from the recombinant strain. The presence of the reporter system facilitates real-time monitoring of the bioremediation processes.

Despite the success, bioremediation based on GMMs is still limited to academic research. Commercial remediation currently relies on naturally occurring microbes identified at the contaminated sites. There are many issues surrounding the application of GMMs for use in bioremediation, including (1) their effectiveness compared with their counterparts present in nature; (2) their influence on indigenous microorganisms; (3) their fitness in nature; and (4) their containment. Until these issues are clarified, the future use of GMMs in bioremediation will remain uncertain.

6. Conclusion

GMMs have been developed to benefit human health, agriculture and the environment. Advances in functional genomics and bioinformatics tools, combined with existing recombinant DNA technologies, will help us better understand the physiology and metabolic potential of the organisms we study, and in turn, will lead to the development of GMMs best suited to our needs. In addition, functional genomics and bioinformatics can be applied for risk analysis of GMMs. A comprehensive safety assessment of GMMs is important both in addressing public concerns and in ensuring faster industrial applications of GMMs.

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