

# METHODS AND APPLICATIONS OF GENETIC ENGINEERING

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□ FOR THOUSANDS OF YEARS, humans have realized that the selective propagation of chosen individual animals and plants can increase food production and quality. In recent decades, this technique has been extended to bacteria and fungi in an intensified fashion and has contributed to the development of strong fermentation technologies in the pharmaceutical, food, and brewing industries. Thus, attempts to improve productivity or product quality by the alteration and selective propagation of the producing organism are not new. In the past 10 years, new techniques—e.g., recombinant DNA, cell fusion, plant tissue culture, etc.—have been developed which extend the capabilities of these efforts. Collectively referred to as genetic engineering or genetic technology, these techniques add unprecedented scope and precision to the efforts to modify animals, plants, and microorganisms to increase their usefulness. The application of these methods will cause truly revolutionary advances in such areas as health care, food production and processing, and industrial chemistry. This article will examine the basic experimental methods of genetic engineering research and some possible applications to the food industry.

Genetic engineering is the alteration of the hereditary material, the deoxyribonucleic acid (DNA), of a living cell so that the cell can produce more or different chemicals or perform completely new functions (OTA, 1981). A major technique used in genetic engineering is that of recombinant DNA, which is the joining of pieces of DNA from different organisms to produce a hybrid molecule. An understanding of the methods and capabilities of these techniques requires familiarity with the structure and mode of action of the hereditary material.

## GENE AND PROTEIN STRUCTURE AND FUNCTION

Living cells may be considered as collections of chemical reactions responsible for structure, growth, reproduction, response to environmental stimuli, and the capture and utilization of energy. All of these reactions occur through the action of cellular proteins, which either are directly involved in these events or synthesize nonprotein molecules essential to these functions. A major group of proteins, the enzymes, have catalytic activities and thereby cause the multitude of essential chemical reactions to occur at reasonable rates at standard temperature, pressure, and reactant concentrations. Other proteins have structural, regulatory, or information-transfer functions. All biological traits result from the action of one or more proteins. The key to the regulation of the properties of a cell, therefore, is the alteration of its proteins, such as by changing the amount or function of a protein or by introducing proteins not originally present in that organism (not all organisms or all cells within a multicellular organism possess all known proteins).

Proteins are composed of one or more linear chains (polypeptides) of amino acids (Fig. 1). Adjacent amino acids

are joined by amide bonds between their amino and carboxyl groups. The amino acid sequence of a polypeptide determines how it folds in space to form a three-dimensional structure containing specific recognition sites, substrate binding sites, catalytic centers, and other structures upon which activity depends. Thus, the function, efficiency, and specificity of a protein is determined by the sequence of amino acids in its peptide chain(s). This sequence is determined by the structure of an analogous region in the DNA of the cell.

DNA is a double-stranded polymer of four deoxyribonucleotide phosphates (Fig. 1): deoxyadenosine phosphate (A), deoxyguanosine phosphate (G), deoxycytidine phosphate (C), and deoxythymidine phosphate (T). Adjacent nucleotides in a DNA strand are covalently joined by phosphodiester linkages between their deoxyribose components. The two strands of a DNA molecule are held together by weak hydrogen bonds which join Gs to Cs and As to Ts. DNA is the template for protein synthesis. Thus, the sequence of nucleotides in DNA determines the sequence of amino acids in the proteins of a cell. A stretch of DNA sufficient to encode an intact polypeptide is termed a gene. The average size of a gene is approximately 1,000 nucleotide pairs.

Proteins are not directly synthesized on the DNA template. Rather, by the process of *transcription*, the weak forces joining DNA strands to one another are temporarily broken, and one of the strands serves as a template for the polymerization of a complementary polynucleotide chain, ribonucleic acid (RNA). RNA is a single-stranded polymer of four nucleotide phosphates. These differ from the nucleotides in DNA only in that they contain ribose instead of deoxyribose, and deoxythymidine instead of uridine. The order of nucleotides in an RNA molecule is determined by the order of deoxyribonucleotides in the corresponding template DNA and is based, as in DNA strand pairing, on weak, temporary G-C and A-U hydrogen bonds formed between the DNA strand and the monomer nucleotides to be incorporated into RNA. Transcription does not proceed along the full length of the DNA molecule to produce an RNA as long as the entire genome. Rather, termination signals occur after each gene or after a group of genes, resulting in production of RNAs of corresponding lengths and genetic content. A major proportion of RNA is messenger RNA (mRNA), which is the template for the process of protein synthesis, *translation*.

During translation (Fig. 1), amino acids are joined to form proteins. The order of nucleotides in an mRNA determines the amino acid sequence of the protein whose synthesis it determines. Nucleotides are "read" in groups of three, known as codons. For example, the codon A-A-A causes the addition of lysine to the end of the growing amino acid chain. Thus, by the intermediary action of mRNA, the sequence of nucleotides in DNA determines the amino acid sequences of proteins.

In addition to regions encoding protein structure, genes contain DNA sequences which facilitate and regulate gene expression (Fig. 1). Specific nucleotide sequences are vital to protein synthesis: (1) codons signaling the sites of initiation and termination of synthesis of a polypeptide chain border the coding region of each gene, sometimes occurring repeat-

edly in tandem; (2) upstream of the initiation codon lie specific base sequences which facilitate binding of the protein synthesis apparatus (the ribosome) to mRNA prior to the initiation of protein synthesis; and (3) other flanking sequences modulate the stability of mRNA within the cell. Variations in the nucleotide sequence of any of these regions can alter the rate of production of a protein.

Specific DNA sequences also signal the appropriate positions for the initiation and termination of RNA synthesis by the enzyme RNA polymerase. Since mRNA and protein synthesis are energy-requiring processes, it is advantageous to modulate them so that only those proteins which are of use to the cell are synthesized at any given time. Thus, controls are exerted on the rates of transcription and translation. Since translation is dependent upon the presence of the mRNA product of transcription, transcriptional controls also regulate protein synthesis. Transcriptional control appears to be the most common form of control of gene expression. Each gene or coordinately expressed group of genes has its own transcription regulation sequences. The extent of regulation varies, with some genes being transcribed continuously at constant rates while the level of activity of others varies greatly. Elegant and precise patterns of induction and repression of gene expression have been described (Miller and Reznikoff, 1980; Rosenberg and Court, 1979).

The template-product relationship between DNA and protein dictates that alterations in the DNA nucleotide sequence are the basis of alterations in the biological properties of an organism. Single or multiple changes in the DNA base sequence (mutations) within the structural portion of a gene can alter or destroy the physical and biochemical properties of a protein. Mutations in the regulatory regions of a gene can alter the rate and timing of synthesis of the corresponding protein or the identities of the compounds which will serve as inducers or repressors of mRNA and protein synthesis. The cumulative effects of several such changes within a gene can be the production of a protein with a completely different set of catalytic properties or a new pattern of gene expression.

Further information on the structure and action of genes is given by Watson (1976) and Lewin (1974; 1980).

Historically, alterations in a trait of an organism have been obtained in three ways: (1) the detection, within a population, of those individuals which had undergone desirable changes; since spontaneous mutation rates are usually low (one per million or more cells), this is an inefficient, slow process; (2) the enhancement of the mutation rate by treatment with chemicals that alter the nucleotide sequence of DNA and the screening of the treated organisms for variants displaying the desired trait; and (3) the discovery of new organisms which naturally possess the desired quality. Significant contributions have been achieved with these methods. However, they are time-consuming and inefficient, since the mutation event is generally random across the entire genome, there is little way to design a mutagenesis so as to generate a high proportion of the desired type of mutant, and the examination of thousands of organisms is often required. It is apparent that progress in this area would be accelerated by the development of techniques allowing the determination and precise alteration of DNA nucleotide sequence and the transfer of genes to organisms which do not naturally possess them. The observation was the stimulus for the development of recombinant DNA and related technologies.

## BASIC GENETIC ENGINEERING TECHNOLOGIES

Recombinant DNA (rDNA) technology involves the use of microorganisms and specific microbiological and biochemical techniques.

● **Organisms.** The several thousand genes of a representative bacterial cell are distributed along a DNA strand at least a millimeter in length which is tightly folded within the organism, which is itself only about one one-thousandth of a millimeter in diameter. Animal and plant genomes are orders of magnitude larger than this. Because of their relative genetic and biochemical simplicity, ease of manip-

ulation, and rapid growth rates, bacteria have been thoroughly studied by the classical techniques of microbiology. The resulting knowledge and methods have been the foundation for rDNA and other genetic technologies. The gut bacterium *Escherichia coli* is the most thoroughly characterized microorganism and has been the most frequently used in these studies. Significant work has also been done with other genera of bacteria, including *Bacillus*, *Pseudomonas*, *Streptococcus*, *Streptomyces*, and others. Progress is being made in work with fungi, higher animals, and plants, but in these cases, particularly the latter two, progress is limited by the greater complexity of the organisms and lack of fundamental knowledge. The methods of DNA isolation, analysis, rearrangement, and modification are essentially the same for all these organisms. However, the techniques of genetic analysis, gene transfer, cell propagation, and aspects of gene expression and regulation increase in difficulty as the genetic complexity increases.

Although *E. coli* is the organism most frequently used in rDNA research, it produces toxins and is therefore not always the preferred organism for rDNA experiments with genes of interest to the food industry. Efforts are underway to apply rDNA methods to organisms accepted as safe by this industry (Kondo and McKay, 1982; Davies and Gasson, 1981).

Most rDNA projects begin with the cloning of the gene(s) of interest, i.e., separation from other genes of the host cell and transfer to a suitable organism. This allows the determination of the genetic activity of a DNA fragment in a characterized genetic background, the production of sufficient quantities of DNA for nucleotide sequence analysis or alteration, and the isolation of significant amounts of the proteins encoded by the DNA. The suitability of selected organisms for the insertion of DNA from other sources has been enhanced by the isolation of variants with advantageous mutations. For example, strains of *E. coli* have been isolated which lack a normal complement of DNA-degrading enzymes. These bacteria accept foreign DNA more efficiently than their wild-type progenitors and are routinely used as DNA recipients.

Fig. 2 depicts the fundamental steps of a gene-cloning operation.

● **DNA Isolation.** DNA is readily isolated, after gentle cell lysis, by extraction of contaminating substances with phenol, followed by precipitation with ethanol. A variety of lysis protocols have been developed to compensate for the variety of organisms from which DNA may be isolated (animals, plants, fungi, Gram-positive and Gram-negative bacteria, and viruses). Each cell type poses unique challenges, requiring the development of special methods for efficient cell lysis and the release of DNA (Meyers et al., 1976; Humphreys et al., 1975; Kolodner and Tewari, 1975). Ultracentrifugation in dye-buoyant density gradients is often used to separate DNA into fractions of different physical structure (covalently closed circular vs linear), one of which may be enriched for the genes of interest (Humphreys et al., 1975). Precautions must be taken to avoid the shearing forces to which the long, thin DNA molecule is sensitive and to minimize the action of endogenous cellular nucleases, which degrade DNA.

● **DNA Fragmentation.** Physical shearing (e.g., repeated pipetting, drawing through a syringe needle, exposure to ultrasonic vibration) reduces DNA to short lengths, some of which may contain the gene of interest. However, these forces cause random, irreproducible cleavage of DNA and facilitate neither the subsequent analysis of gene structure nor gene reconstruction. Digestion with restriction endonucleases is the preferred method of reducing the complexity of DNA. These enzymes, more than 250 of which have been described, cleave DNA at or near specific recognition sequences (Roberts, 1981). These recognition sequences differ, although some enzymes with the same specificity have been described. Table 1 lists some common restriction endonucleases and their recognition and cleavage sites. Many of these enzymes are commercially available as preparations free from contaminating nucleases. Such purity is essential to efficient cloning. Since the distribution of

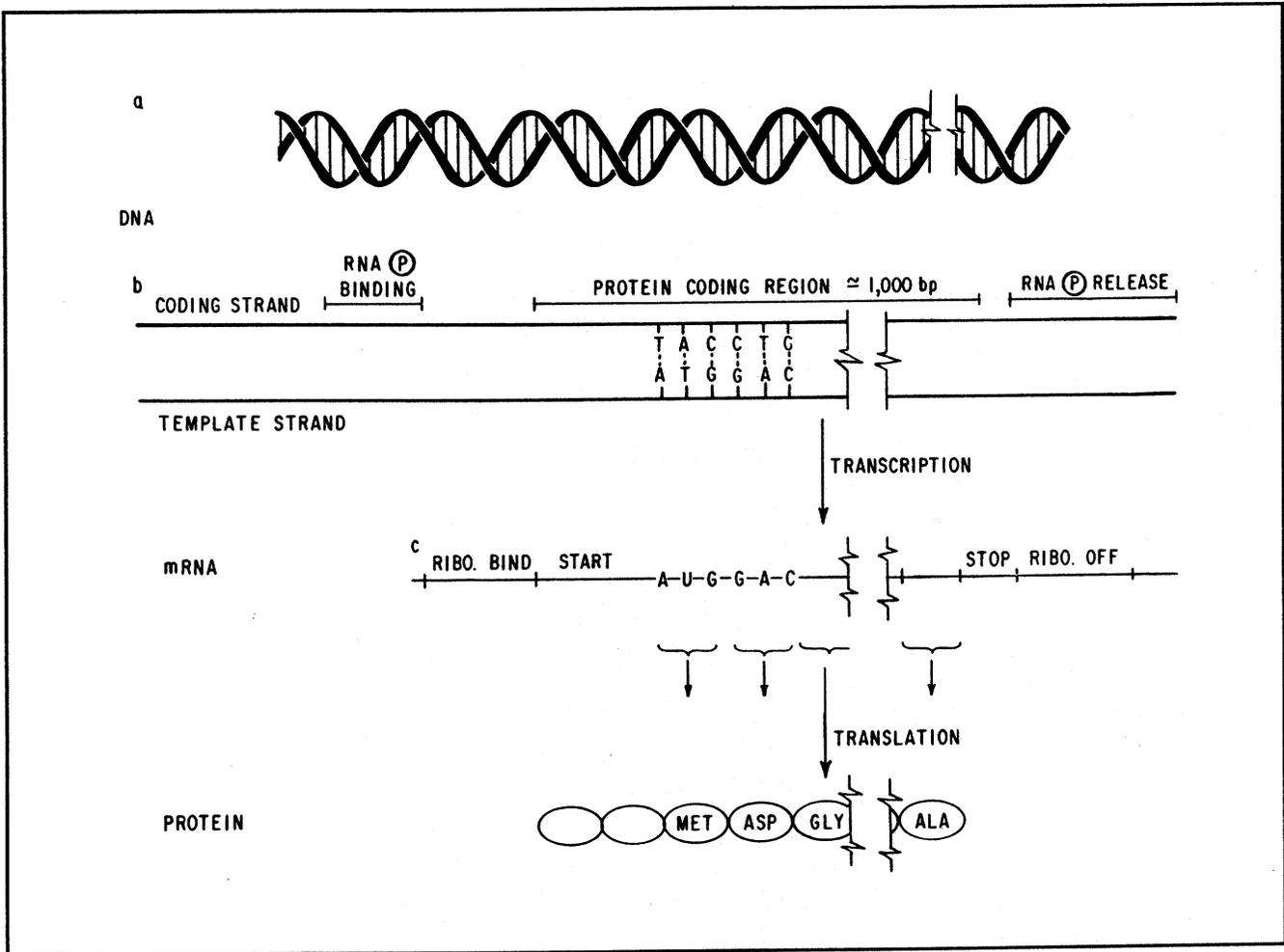


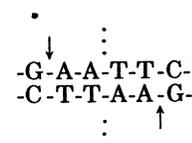
Fig. 1—THE STORAGE AND TRANSMISSION of genetic information in the cell. Genetic information is stored in the structure of the double helix of DNA (a). Each strand of the helix is a chain of nucleotides, each nucleotide containing a deoxyribose group and a phosphate group, which form the strand's backbone, as well as one of four bases: adenine (A), guanine (G), thymine (T), and cytosine (C). The information is encoded in the sequence of the bases along a strand. The complementarity of the bases (A always pairs with T, and G with C) is the basis of the replication of DNA from generation to generation and of its expression as protein. Expression begins with the transcription of the DNA base sequence (b) into a strand of messenger RNA (c), which is complementary to the coding strand of the DNA except for the fact that uracil (U) replaces thymine. Transcription into RNA and translation into protein are regulated by special sequences in the DNA and RNA, respectively. One such sequence causes the binding of RNA polymerase, the enzyme that synthesizes RNA, at the appropriate site "upstream" of the protein coding region of the DNA. Beyond the end of the structural gene a termination region causes RNA polymerase to cease transcription and dissociate from the DNA. In some genes an operator sequence, which lies between the polymerase binding site and the coding region of the DNA and can bind a repressor molecule, provides an extra control. Messenger RNA (mRNA) is translated on cellular organelles called ribosomes. Each triplet of bases (codon) specifies the incorporation of a particular amino acid into the growing protein chain. A ribosomal binding site on the RNA allows translation to begin at a "start" codon. Translation proceeds until a "stop" codon is reached, which signals the end of translation and detachment of the completed protein chain from the ribosome

restriction sites within a DNA molecule is usually random, digestion of a population of identical DNAs produces a family of product molecules of different sizes. These can be separated by electrophoresis (Southern, 1979) and isolated, resulting in a DNA sample enriched for the gene of interest.

If a gene contains the appropriate recognition sequences, it will be cleaved upon treatment with a restriction endonuclease, destroying its genetic continuity. Initial cloning experiments, therefore, often involve digestion of different aliquots of DNA with separate restriction enzymes in an attempt to produce the smallest DNA fragment necessary and sufficient to encode the desired protein. Once cloned, ample DNA can be produced to allow determination of the "restriction map" of a DNA fragment, i.e., the relative location of cleavage sites for each of a battery of restriction enzymes. After further restriction enzyme treatments, electrophoretic separation, isolation, and recloning of the DNA fragments, it is possible to identify the exact location of a gene on the restriction map of the cloned DNA fragment on

which it lies (Fig. 3). This allows preparative-scale isolation of the gene on the smallest DNA fragment containing it, for further study or modification. Restriction endonuclease analysis can also be of diagnostic value in determining the locations of DNA deletions, rearrangements, additions, or nucleotide sequence alterations in variant molecules isolated following mutagenesis.

The recognition and cleavage sites of the most useful restriction endonucleases display axes of rotational symmetry and staggered DNA strand cleavage, as exemplified by the EcoRI site.



where the dotted line indicates the axes of symmetry and the arrows designate the sites of DNA-strand cleavage. The

result of these properties is that DNA fragments generated by the same restriction enzyme will associate with one another end to end on the basis of the G-C and A-T nucleotide pair attractions described above. This facilitates the joining of DNA restriction fragments.

● **Cloning Vectors.** Specific proteins and DNA sequences are required for intracellular DNA replication. These sequences and the genes for the required proteins are not common in the genome. Thus, to stably establish a DNA fragment in a cell, that fragment must usually be attached to another DNA, a vector, which contains the regions encoding DNA replication and inheritance. Several such vectors have been developed and elegantly engineered, some for use in *E. coli* (Bolivar and Backman, 1979), others for gene cloning in yeasts, higher animals, and plants (Struhl et al., 1979; Purchio and Fareed, 1979; Ream and Gordon, 1982). Vectors are designed to confer upon cells bearing them a unique property, often resistance to an antibiotic (Fig. 3), which facilitates the selective enrichment of a bacterial population for organisms containing the vector. Cloning vectors are small, 1-3% of the size of the bacterial chromosome. They are also usually closed circular molecules (plasmids) which do not normally integrate into the chromosome. These features facilitate their separation from the chromosome and isolation (Radloff et al., 1967) and protect them from cellular exonucleases, which degrade DNA inward from free ends. Useful vectors have also been developed from viruses (Old and Primrose, 1980). An attractive feature of some vectors is that they possess a high copy number, i.e., each cell possesses multiple copies of the vector, sometimes 50 or more. This increases the efficiency of isolation of the vector and the production of large amounts of the proteins whose genes it contains. Table 2 lists some of the common cloning vectors and their properties.

● **DNA Ligation.** DNA fragments to be cloned (passengers) must be covalently linked to their cloning vector in order to survive in the cell. The enzyme DNA ligase catalyzes the covalent joining of the free ends of DNA molecules. Cloning vectors are often engineered to contain a single recognition site for one or more restriction endonucleases (Fig. 3). Digestion by such an enzyme linearizes the vector while preserving its genetic continuity at positions other than the single cleavage site. The staggered ends generated on the vector by restriction endonuclease treatment are homologous with the ends of passenger molecules generated by treatment with the same restriction enzyme. This facilitates vector-passenger pair formation, as discussed above, and reformation of the covalent DNA structures by DNA ligase, with the regeneration of a circular molecule.

● **Introducing rDNA into a Host.** It is necessary to introduce DNA into a suitable organism to obtain gene expression or sufficient DNA for further analysis. This is most frequently done by the process of *transformation*, whereby cells are induced to incorporate free DNA to which they are exposed. Few organisms are naturally "competent," i.e., capable of incorporating exogenous DNA without degrading it. Competence can be induced in some organisms, most significantly *E. coli*, by treatments with magnesium sulfate and calcium chloride (Davis et al., 1980). DNA which is cloned using a bacterial virus as a vector can be introduced into cells by viral *transfection* (Maniatis et al., 1978). In other instances, the uptake of DNA is facilitated by removal of the bacterial or plant cell wall (Hinnen et al., 1978; Hopwood, 1981; Matthews and Cress, 1981). Advances have also been made in the transformation of mammalian cells (Pellicer et al., 1980). Efficiencies as high as  $10^6$ - $10^7$  transformed cells per microgram of input DNA have been reported.

● **Selection and Screening.** Following transformation or transfection, it is necessary to identify cells which have received the target gene. The success of a cloning project is often dependent upon the efficiency of the screening or selection method employed. The significance of this step becomes evident when one considers that in the attempted cloning of a particular gene, millions of different DNA fragments may be present at the transformation stage of the

experiment, producing millions of transformants, only a small portion of which contain the target gene.

The presence of antibiotic-resistance determinants on the cloning vector allows enrichment for the transformants in a bacterial population. Some of the more common methods for the detection of those transformants carrying the gene of interest are:

1. Assaying selected cells for the presence of the biological activity (enzymatic, hormonal etc.) encoded by the target gene.

2. Using selective media which support the growth only of organisms which have taken up the target gene. For example, transformants harboring the gene for  $\beta$ -galactosidase, which is required for lactose metabolism, may be selected by growing a mixed population of transformants on media whose sole carbon source is lactose.

3. Immunologic detection of the product encoded by the gene of interest.

4. Screening transformants for the presence of the target gene. This requires a probe, which can be either the gene of interest or its complementary RNA. Transformant DNA is denatured to single strands and allowed to reanneal with single-stranded probe DNA or RNA by the formation of complementary nucleotide pairs. The presence of double-stranded hybrids is then determined.

● **Methods of DNA Sequence Analysis, Modification, and Synthesis.** Since gene expression and protein function are dependent upon DNA base sequence, a full understanding of the relationship between gene structure and protein function awaited the development of methods to determine the nucleotide sequence of DNA (Maxam and Gilbert, 1980; Sanger et al., 1977). The knowledge generated by the application of these techniques has been substantial and has given insights into gene structure, function, and regulation. Further discoveries are anticipated.

It is also possible to synthesize a DNA molecule of defined nucleotide sequence (Narang et al., 1979; Itakura and Riggs, 1980) and to insert it into desired positions within a gene, modifying its expression or the function of its protein product. The technique of mutagenesis has also been refined, allowing the targeting of the mutagenic event to a particular region within a gene and the generation of desired types of nucleotide sequence change (Oka et al., 1982). It is now feasible to consider the use of such methods in the synthesis of genes and gene fragments encoding proteins with novel stabilities, catalytic activities, substrate ranges, etc. (Ulmer, 1983).

● **Protoplast Fusion Technology.** Mechanisms of transformation do not exist for all organisms. Furthermore, the genes encoding the enzymes of multistep biochemical reactions may be dispersed throughout the chromosomes of an organism, complicating their successful cloning as a group. In such situations, it is practical to combine the entire genomes of two organisms, allow genetic reassortment and stabilization, and select from the resulting mixture of progeny those organisms expressing the desired traits. This technique relies on the observation that once the rigid cell walls are removed from bacterial, fungal, or plant cells, fusion of the resulting protoplasts can be induced. This method has been applied to the union of undefined and multistep biochemical pathways present in different organisms (Stewart et al., 1984; Shepard et al., 1983; Hopwood, 1981).

● **Higher Animals and Plants.** Because of their greater genetic complexity, multicellular compositions, differentiation, slow growth rates, and the dearth of knowledge about them, genetic manipulation of the plants and higher animals is not as well developed as is genetic manipulation of the bacteria. The transfer of individual genes from these organisms to bacteria has allowed the high-level production of rare and beneficial molecules such as growth hormones, insulin, seed storage proteins, and interferons (natural defense proteins of higher animals). However, the improvement of plants and animals by the manipulation of their genomes is still in its infancy. Refinement of the techniques required to perform genetic manipulation in these organisms and further growth in the knowledge base should

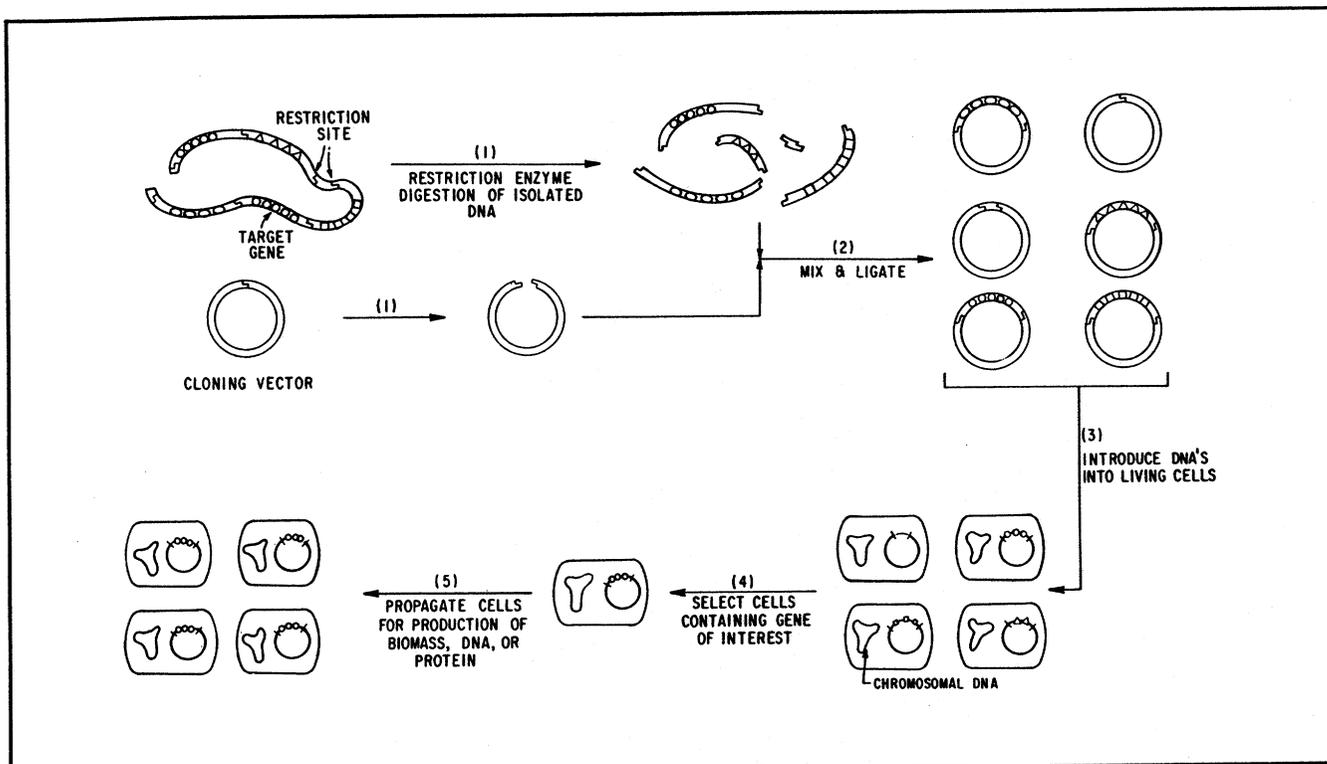


Fig. 2—THE FUNDAMENTAL STEPS in the simplest type of gene cloning experiment consist of: (1) isolation of genomic DNA from an organism which bears the gene of interest and digestion of that DNA with a restriction endonuclease to generate a family of DNA fragments, some of which contain the target gene; a sample of cloning vector is also treated with the restriction enzyme to prepare it to receive the genomic DNA fragments; (2) restriction endonuclease-digested vector and genomic DNAs are joined by treatment with DNA ligase; (3) ligated DNAs are introduced into a suitable recipient organism, often by the transformation process; (4) transformant cells are screened to select those having received the target gene; and (5) such cells are then propagated for further analysis, modification, DNA production, or the production of the target gene product

facilitate advances in these areas (Sharp, 1984; Barton and Brill, 1983).

#### APPLICATIONS IN THE FOOD INDUSTRY

Any biological reaction or trait should be amenable to modification by the application of genetic engineering techniques. However, the amount of effort and time required to achieve certain goals may be extensive, and some goals are not attainable with current knowledge and methods. Nonetheless, the range of topics which can be successfully addressed with these methods is very broad.

Several organizations specialize in the development and application of genetic technology. Industrial firms desiring to use this technology often enter joint-venture arrangements with these companies. Alternatively, several manufacturers, particularly in the pharmaceuticals trade, support in-house genetic technology groups.

The applications of genetic engineering fall into two broad categories: (1) modification of the structure and/or amplification of the production of scarce biological products by cloning the relevant genes in bacteria (this amounts to the use of bacteria as factories for the production of target compounds), and (2) alteration of selected organisms to improve their utility in a desired environment, such as the development of a bacterium which imparts novel texture or flavor when used in cheese production.

Table 3 lists some compounds of relevance to the food industry whose production could be increased by the application of genetic technology. This list is representative, not all-inclusive. Selected topics are discussed below. An underlying principle in considering the range of topics to which genetic engineering may be applied is that one's imagination may limit achievement as much as any other factor.

● **Enzymes.** Approximately \$160 million worth of enzymes are consumed annually by the food and detergent

Table 1—RESTRICTION ENDONUCLEASES commonly used for gene cloning and characterization

Enzyme	Source	Recognition sequence and DNA cleavage sites
EcoRI	<i>Escherichia coli</i> Ry 13	↓ GAATTC CTTAAG ↑
HindIII	<i>Haemophilus influenzae</i> Rd	↓ AAGCTT TTCGAA ↑
BamHI	<i>Bacillus amyloliquefaciens</i> H	↓ GGATCC CCTAGG ↑
HpaII	<i>Haemophilus parainfluenzae</i>	↓ CCGG GGCC ↑
PstI	<i>Providencia stuartii</i> 164	↓ CTGCAG GACGTC ↑
TaqI	<i>Thermus aquaticus</i> YT1	↓ TCGA AGCT ↑



ing organisms may increase the production rate and final concentration of the products or expand the range of substances used as carbon sources during the fermentations. Increased production efficiencies will result in lowered prices for these amino acids. All 105,000 tons of methionine produced annually are chemically synthesized. Microbial synthesis of methionine is an attractive goal but has not been achieved because of the inability of conventional mutagenesis and selection techniques to generate mutants producing significant levels of methionine. The successful use of rDNA techniques to elevate methionine biosynthesis would lead to economical microbial production of this amino acid.

● **Organic Chemicals.** The use of organic chemicals as preservatives, acidulants, flavorants, precursors, and texturizers is widespread in the food industry. Many of these compounds can be produced by fermentation, e.g., acetic, citric, and propionic acids, glycerol, citronellal, etc. (OTA, 1981). In some instances the efficiencies of production are quite high. Genetic engineering research may achieve increases in the production rates or allow changes in the feedstocks used to support fermentation. In other, less efficient fermentations, the overall efficiency may be increased. Microbiological routes for the production of some organic chemicals have been known for more than 50 years but were supplanted by chemical synthesis from petroleum products when these became readily available following World War II. With recent increases in petrochemical prices and advances in the techniques of gene manipulation, it may once again become economically feasible to produce some organic chemicals by fermentation.

● **Vitamins.** The current annual market value of vitamins is at least \$670 million (OTA, 1981). Some vitamins, notably B-2, B-12, C, and D, are made in part or in total by microbial processes. Progress toward the more efficient synthesis of these compounds should be achieved by rDNA research. However, this progress may be slow, since many vitamins are structurally complex molecules with multiple enzyme biosynthetic routes. Elucidation of the rate-controlling steps in these paths and elimination of bottlenecks may not be straightforward. Vitamin E is presently obtained by the extraction of agricultural products. Algae have been isolated which also produce vitamin E. By characterization and alteration of the microbial biosynthetic pathway for vitamin E, it may be possible to produce this compound fermentatively.

● **Flavorants and Pigments.** In addition to glutamate, an amino acid consumed in large amounts, other compounds used as flavorants are, or can be, obtained from biological sources. Although aspartame was not originally isolated from a biological source, the direct precursor of this sweetener can be produced in bacteria. Doel et al. (1980) synthesized a gene which codes for a protein composed of multiple repeating units of the dipeptide found in aspartame; proteolytic cleavage of this molecule yields the free dipeptide. The extremely sweet peptides monellin and the thaumatins are produced by West African plants (van der Wel and Loeve, 1972; 1973; Morris et al., 1973); their cloning and production in bacteria could be useful in producing enough material for commercial use or for studies of the mechanisms of taste perception. The flavorants inosinic acid and guanylic acid are presently produced by extraction of yeast. Because of the complexity of yeast

Table 2—REPRESENTATIVE VECTORS for gene cloning

Vector	Molecular type	Host range <sup>a</sup>	Comments
pBR322	Plasmid	<i>Escherichia coli</i>	The most frequently used general-purpose vector. Can be amplified to very high numbers of copies per cell for efficient DNA isolation
RP4	Plasmid	Most Gram-negative bacteria	Possesses one of the widest known host ranges. Low copy number per cell
pC194.2	Plasmid	<i>E. coli</i> ; <i>Bacillus subtilis</i>	A "shuttle" vector: a hybrid vector containing DNA sequences which allow DNA replication in two usually incompatible bacterial strains
YRp7, YEplPT	Plasmid	<i>E. coli</i> ; <i>Saccharomyces cerevisiae</i> (yeast)	A bacterial/yeast shuttle vector
Ti	Plasmid	<i>Agrobacterium tumefaciens</i> ; some plants	A portion of the Ti plasmid becomes stably integrated into the genes of plants infected by <i>A. tumefaciens</i> , a plant pathogen. Currently the only effective vehicle for cloning in plants
pRK646	Plasmid	<i>E. coli</i>	A "containment" vector unable to replicate except in specially constructed bacteria that survive only under laboratory conditions. Minimizes the dangers of environmental biocontamination
λ Charon 16A	Bacterial virus	Susceptible <i>E. coli</i>	Allows cloning in a virus. Facilitates very high-level expression of the cloned genes
M13	Bacterial virus	Susceptible <i>E. coli</i>	High copy number facilitates DNA isolation. DNA is produced in a single-stranded form, the required substrate for a common nucleotide sequence determination methodology

<sup>a</sup>Unless otherwise indicated, these are bacteria

genetics and difficulties in the genetic manipulation of yeast, it may prove most feasible to use rDNA technology to amplify the production of these compounds in bacteria. Alternatively, it may be possible to obtain other nucleotides with flavor properties in quantities sufficient for industrial use. The fermentative production of natural pigments, such as the carotenoids, may also become more feasible if rDNA methods are applied to enhance the activity of their biosynthetic paths (Morris, 1981). The biosynthesis of pigments is currently too expensive to be competitive. The economical production of flavorings, spices, and other desirable compounds by bacteria or by plant cell cultures is also a possibility.

● **Polysaccharides.** Polysaccharides are used as stabilizers and texturizers. Some of these are plant products whose supply would become more reliable if produced by bacteria. The genes coding for their synthesis may be transferred to microorganisms, although this may be a complicated undertaking because of the structural complexity of the polysaccharides. Xanthan gum is a commonly used polysaccharide produced by the bacterium *Xanthomonas campestris*. Its production may be enhanced through genetic modification. The recent introduction of the genes for lactose metabolism into *X. campestris*, allowing the growth of this organism on the lactose found in inexpensive whey, may improve the economy of xanthan synthesis (Walsh et al., 1984) and represents another way in which genetic technology can be applied to organisms and reactions of interest to the food industry.

● **Starter Cultures.** The baking, brewing, winemaking, and food fermentation industries utilize microbial metabolism in their processes. The genetic manipulation of starter cultures could enhance the efficiencies of these reactions and the quality of the products. Such improvements have been sought and slowly attained for centuries. Genetic engineering represents a powerful tool with which these goals may be attained more rapidly. The introduction of novel lipases or proteases into dairy starter cultures may result in more palatable or novel products. If the genes for vitamin production were transferred to these organisms, a naturally fortified product might result. Resistance to bacteriophage infection is a genetic trait that could also be engineered into starter cultures. Fundamental knowledge of the molecular biology of the organisms used in the dairy industry is being accumulated (Kondo and McKay, 1982; Lee et al., 1982; Davies and Gasson, 1981), and application of this knowledge to enhance the fermentation process should be forthcoming.

In the brewing industry, protoplast fusion has been used to isolate derivatives of standard brewing yeasts able to ferment most dextrans, thus decreasing the caloric content of the product (Stewart et al., 1984). Similar methods might augment the gains achieved by conventional mutagenesis and screening in increasing the ethanol tolerance, fermentation efficiency, and sedimentation rates of winemaking yeasts.

The baking industry could profit from the production of strains with improved stability, faster fermentation rates, or novel carbohydrate and protein modification capabilities.

● **Animal and Plant Productivity.** The genetic alteration of living organisms to improve food production is another area in which major contributions are anticipated. Improvements in livestock could include increased feed efficiency, fertility, milk and egg output, disease resistance, growth rate, and meat quality. The application of rDNA to achieve improvements of these types awaits characterization of the genetics of the traits. Therefore, near-term progress toward these goals will be slow.

Plant productivity will also be enhanced by genetic technologies (Sharp, 1984; Barton and Brill, 1983). Increased yield, fertilizer and photosynthetic efficiencies, disease and herbicide resistance, tolerance to saline soils, nutritional quality, and a reduction in undesirable plant constituents (Teutonico and Knorr, 1984) are anticipated. The most intense areas of plant molecular biology research today are the development of techniques to introduce DNA

Table 3—COMPOUNDS AND MICROORGANISMS which are currently or potentially useful in the food industry whose production may be enhanced by genetic engineering<sup>a</sup>

Product category	Example	End use
Amino acids	Cysteine	Food enrichment
	Glutamate	Flavorant, food enrichment
	Lysine	Food enrichment
	Methionine	Food enrichment
Biopolymers	Polyamylose	Biodegradable wrapping material
	Xanthan gum	Food texturizer
Enzymes/Proteins	Amylases	Flour supplement, mashing agent, baby foods
	Bromelin	Meat tenderizer
	Glucose isomerase	Production of high-fructose corn syrup
	Lipases	Flavor production
	Monellin	Sweetener
	Proteases	Meat tenderizer, chill-proofing beer, cheese flavor production
	Thaumatococcus	Sweetener
Microorganisms	Yeast	Brewing, baking, winemaking
	Lactobacilli, streptococci	Dairy and meat fermentations
	<i>Methylophilus methylotrophus</i>	Single-cell protein for consumption
Vitamins	B-2, B-12, D, E, nicotinic acid	Food enrichment
	C	Food enrichment, antioxidant
Miscellaneous	Aspartame	Sweetener
	Carotenoids	Colorant
	Geraniol	Flavorant
	Glycerol	Texturizer
	Isobutylene	Flavorant
	Linalool	Flavorant
	Nerol	Flavorant
	Nucleotides	Flavorants
	Organic acids: acetic, benzoic, citric, gluconic, lactic, propionic, etc.	Preservatives, flavorants
	Propylene glycol	Antioxidant
	Sorbitol	Anticaking agent

<sup>a</sup>From OTA (1981)

into plants (Ream and Gordon, 1982; Howell et al., 1981; Krens et al., 1982) and the study of the nitrogen-fixation reactions whereby atmospheric nitrogen is converted to the forms required for metabolism (Roberts and Brill, 1981; Barton and Brill, 1983). Efficient nitrogen fixation by plants would greatly reduce the need for added fertilizers. It is beyond the scope of this review to further discuss the extensive research underway in regard to the improvement of animal and plant productivity.

● **Single-Cell Protein.** Efforts to develop nontraditional foods have also been stimulated by rDNA technology. Microbial cells have long been considered as a possible source of dietary protein, particularly for animals. By transferring the genes for more efficient nitrogen assimilation from *E. coli* into a *Methylophilus methylotrophus* strain used in this capacity, researchers have already attained a 4-7% increase in the assimilation of carbon (Windass et al., 1980; Gelfand, 1980). One problem in the

use of microorganisms as single-cell protein (SCP) is that the relatively high levels of nucleic acids in these organisms are poorly metabolized and can cause gout in the animals consuming them. By modifying the SCP organisms, this difficulty may be resolved. This could be achieved by introducing the genes for the appropriate nucleic acid-degrading enzymes and timing their expression so that DNA and RNA breakdown could be induced just before harvest of the cells.

## POTENTIAL IS GREAT

This article has only touched on the highlights of the vast capabilities of the new genetic technologies. It has also only indirectly alluded to the immense data base which will have to be acquired before some of the more imaginative gene manipulations can be achieved, particularly with regard to plants and animals. Nonetheless, the potential of this area is difficult to overestimate, particularly since it offers efficient means to achieve goals formerly attained primarily by chance, if attained at all. The tremendous advances which have been made in the short time that the rDNA techniques have been in existence indicate that the future holds profound possibilities.

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