

Screening for productive strains and strain improvement in biotechnological organisms



1. Sources of Microorganisms Used in Biotechnology

1.1 Literature Search and Culture Collection Support

Sources of information on potentially useful microbial cultures

- The website
- The literature
 - Patent literature,
 - Contact with one or more of the established culture collections
 - The regulatory offices dealing with patents

Table 1 Some microbial metabolites with non-antibiotic pharmacological activity

<i>Compound</i>	<i>Activity</i>	<i>Producing Microorganism</i>
Aspergillilic acid	Antihypertensive	<i>Aspergillus</i> sp
Astromentin	Sommoth muscle relaxant	<i>Monascus</i> sp
Siolipn	Acceleration of fibrin clot	<i>Streptomyces sioyaensis</i>
Azaserine	Antidiuretic, antitumor	<i>Streptomyces fragilis</i>
Ovalicin	Immunosuppressive, antitumor	<i>Pseudeurotum ovalis</i>
Candicidin (and other polyene Macrolides)	Cholesterol lowering	<i>Streptomyces noursei</i>
Streptozotocin	Hyperglycemic, antitumor	<i>Streptomyces achromogenes</i>
Zygosporin A	Anti-inflammatory	<i>Cephalosporium acremonium</i>
Fusaric acid	Hypotensive	<i>Fusarium oxysporum</i>
Leupeptin family	Plasmin inhibitor	<i>Bacillus</i> sp
Pepstatin	Pepsin inhibitor	<i>Aspergillus niger</i>
Oosponol	Dopamine β -hydroxlyase inhibitor	<i>Oospora adringens</i>
Fumagallin	Angiogenesis inhibitor	<i>Aspergillus fumigatus</i>

1.2 Isolation *de novo* of Organisms Producing Metabolites of Economic Importance

Although the well-known ubiquity of microorganism implies that almost any natural ecological entity—water, air, leaves, tree trunks – may provide microorganisms, the soil is the preferred source for isolating organisms, because it is a vast reservoir of diverse organisms. Indeed microorganisms capable of utilizing virtually any carbon source will be found in soil if adequate screening methods are used. In recent times, other ‘new’ habitats, especially the marine environment, have been included in habitats to be studied in searches for bioactive microbial metabolites or ‘bio-mining’. Some general screening methods are described below. Detailed methods for the discovery of new antibiotics and other bioactive metabolites will be discussed in Chapter 21 and Chapter 28.

1.2.1 Enrichment with the substrate utilized by the organism being sought

If the organism being sought is one which utilizes a particular substrate, then soil is incubated with that substrate for a period of time. The conditions of the incubation can also be used to select a specific organism. Thus, if a thermophilic organism attacking the substrate is required, then the soil is incubated at an elevated temperature. After a period of incubation, a dilution of the incubated soil is plated on a medium containing the substrate and incubated at the previous temperature (i.e., elevated for thermophile search). Organisms can then be picked out especially if some means has been devised to select them. Selection could, for instance, be based on the ability to cause clear zones in an agar plate as a result of the dissolution of particles of the substrate in the agar. In the search for α -amylase producers, the soil may be enriched with starch and subsequently suitable soil dilutions are plated on agar containing starch as the sole carbon source. Clear halos form around starch-splitting colonies against a blue background when iodine is introduced in the plate.

Continuous culture (Chapter 9) methods are a particularly convenient means of enriching for organisms from a natural source. The constant flow of nutrients over material from a natural habitat such as soil will encourage, and after a time, select for organisms able to utilize the substrate in the nutrient solution. Conditions such as pH, temperature, etc., may also be adjusted to select the organisms which will utilize the desired substrate under the given conditions. Agar platings of the outflow from the continuous culture setup are made at regular intervals to determine when an optimum population of the desired organism has developed.

1.2.2 Enrichment with toxic analogues of the substrate utilized by the organism being sought

Toxic analogues of the material where utilization is being sought may be used for enrichment, and incubated with soil. The toxic analogue will kill many organisms which utilize it. The surviving organisms are then grown on the medium with the non-toxic substrate. Under the new conditions of growth many organisms surviving from exposure to toxic analogues over-produce the desired end-products. The physiological basis of this phenomenon was discussed earlier in Chapter 6.

1.2.3 Testing microbial metabolites for bioactive activity

(i) Testing for anti-microbial activity

For the isolation of antibiotic producing organisms the metabolites of the test organism are tested for anti-microbial activity against test organisms. One of the commonest starting point is to place a soil suspension or soil particles on agar seeded with the test organism(s). Colonies around which cleared zones occur are isolated, purified, and further studied.

(ii) Testing for enzyme inhibition

Microorganisms whose broth cultures are able to inhibit enzymes associated with certain disease may be isolated and tested for the ability to produce drugs for combating the disease. Enzyme inhibition may be determined using one of the two methods.

In the first method the product of the reaction between an enzyme and its substrate is measured using spectroscopic methods. The quantity of the inhibitor in the test sample is obtained by measuring

- (a) the product in the reaction mixture without the inhibitor and
- (b) the product in the mixture with the inhibitor (i.e., a broth or suitable fraction of the broth whose inhibitory potency is being tested).

The percentage inhibition (if any) is calculated by the formula

$$\frac{(a - b)}{a} \times 100$$

The second method determines the quantity of the unreacted substrate. For this determination the following measurements of the substrate are made: (a) with the enzyme and without the inhibitor (i.e., broth being tested); (b) with the enzyme and with the inhibitor and; (c) without the enzyme and without the inhibitor. Percentage inhibition (if any) is determined by $(c-a) - (c-b) \times 100$. The results obtained above enable the assessment of the existence of enzyme inhibitors and facilitate the comparison of the inhibitory ability of broths from several sources.

(iii) Testing for morphological changes in fungal test organisms

The effect on spore germination or change in hyphal morphology may be used to detect the presence of pharmacologically active products in the broth of a test organism. This method does not rely on the death or inhibition of microbial growth, which has been so widely used for detecting antibiotic presence in broths.

(iv) Conducting animal tests on the microbial metabolites

The effect of broth on various animal body activities such as blood pressure, immunosuppressive action, anti-coagulant activity are carried out in animals to determine the content of potentially useful drugs in the broth. This method is discussed extensively in Chapter 21, which discusses details of the search for the production of bioactive metabolites from microorganisms.

2. Strain Improvement

Several options are open to an industrial microbiology organization seeking to maximize its profits in the face of its competitors' race for the same market. The organization may undertake more aggressive marketing tactics, including more attractive packaging while leaving its technical procedures unchanged. It may use its human resources more efficiently and hence reduce costs, or it may adopt a more efficient extraction system for obtaining the material from the fermentation broth. The operations in the fermentor may also be improved by its use of a more productive medium, better environmental conditions, better engineering control of the fermentor processes, or it may genetically improve the productivity of the microbial strain it is using. Of all the above options, strain improvement appears to be the one single factor with the greatest potential for contributing to greater profitability.

While realizing the importance of strain improvement, it must be borne in mind that an improved strain could bring with it previously non-existent problems. For example, a more highly yielding strain may require greater aeration or need more intensive foam control; the products may pose new extraction challenges, or may even require an entirely new fermentation medium. The use of a more productive strain must therefore be weighed against possible increased costs resulting from higher investments in extraction, richer media, more expensive fermentor operations and other hitherto nonexistent problems. This possibility notwithstanding, strain improvement is usually part of the program of an industrial microbiology organization.

To appreciate the basis of strain improvement it is important to remember that the ability of any organism to make any particular product is predicated on its capability for the secretion of a particular set of enzymes. The production of the enzymes, themselves depends ultimately on the genetic make-up of the organisms.

Improvement of strains can therefore be put down in simple term as follows:

- (i) regulating the activity of the enzymes secreted by the organisms;
- (ii) in the case of metabolites secreted extracellularly, increasing the permeability of the organism so that the microbial products can find these way more easily outside the cell;
- (iii) selecting suitable producing strains from a natural population;
- (iv) manipulation of the existing genetic apparatus in a producing organism;
- (v) introducing new genetic properties into the organism by recombinant DNA technology or genetic engineering.

Items (i) and (ii) above have been discussed in Chapter 6. The other possible procedures, namely selection from natural variants, modification of the genetic apparatus without the introduction of foreign DNA and the use of foreign DNA will be discussed below (

2.1 Selection from Naturally Occurring Variants

In selection of this type, naturally occurring variants which over-produce the desired product are sought. Strains which were encountered but not selected should not be automatically discarded; the better ones are usually kept as stock cultures in the organization's culture collection for possible use in future genetic manipulations. Selection from natural variants is a regular feature of industrial microbiology and biotechnology. For example, in the early days of antibiotic production the initial increase in yield was obtained in both penicillin and griseofulvin by natural variants producing higher yields in submerged rather than in surface culture. Another example is lager beer manufacture where the constant selection of yeasts that flocculate eventually gave rise to strains which are now used for the production of the beverage. Similarly in wine fermentation yeasts were repeatedly taken from the best vats until yeasts of suitable properties were obtained.

Selection of this type is not only slow but its course is largely outside the control of the biotechnologist, an intolerable condition in the highly competitive world of modern industry. Strain improvement is therefore mostly achieved by other means described below.

2.2 Manipulation of the Genome of Industrial Organisms in Strain Improvement

The manipulation of the genome for increased productivity may be done in one of two general procedures as shown

Table 2 Methods of manipulating the genetic apparatus of industrial organisms

A. Methods not involving foreign DNA

1. Conventional mutation

B. Methods involving DNA foreign to the organism (i.e. recombination)

2. Transduction
 3. Conjugation
 4. Transformation
 5. Heterokaryosis
 6. Protoplast fusion
 7. Genetic engineering
 8. Metabolic engineering
 9. Site-directed mutation
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(a) manipulations not involving foreign DNA;

(b) manipulations involving foreign DNA .

2.2.1 Genome manipulations not involving Foreign DNA or Bases: Conventional Mutation

Nature of conventional mutation

The properties of any microorganism depend on the sequence of the four nucleic acid bases on its genome: adenine (A), thymine (T), cytosine (C), and guanine (G). The arrangement of these DNA bases dictates the distribution of genes and hence the nature of proteins synthesized. A mutation can therefore be described as a change in the sequence of the bases in DNA (or RNA, in RNA viruses). It is clear that since it is the sequence of these bases which is responsible for the type of proteins (and hence enzymes) synthesized, any change in the sequence will lead ultimately to a change in the properties of the organism.

Mutations occur spontaneously at a low rate in a population of microorganisms. It is this low rate of mutations which is partly responsible for the variation found in natural populations. An increased rate can however be induced by mutagens, (or mutagenic agents) which can either be physical or chemical.

2.2.1.1 Physical agents

- (i) ionizing radiations
- (ii) ultraviolet light

(i) Ionizing radiations:

X-rays, gamma rays, alpha-particles and fast neutrons are ionizing radiations and have all been successfully used to induce mutation. X-rays are produced by commercially available machines as well as van de Graaf generators.

Gamma rays are emitted by the decay of radioactive materials such as Cobalt₆₀. Fast neutrons are produced by a cyclotron or an atomic pile. Ionizing radiations are so called because they knock off the outer electrons in the atoms of biological materials (including DNA) thereby causing ionization in the molecules of DNA. As a result, highly reactive radicals are produced and these cause changes in the DNA. Some authors do not advise the use of ionizing radiations unless all other methods fail. This is partly because the equipment is expensive and hence not always readily available, but also because ionizing radiations are apt to cause breakage in chromosomes.

(ii) *Ultraviolet light.*

The mutagenic range of ultraviolet light lies between wave length 200 and 300 nm. 'Low pressure' UV lamps used for mutagenesis emit most of their rays in the 254 nm region. The suspension of cells or spores to be mutagenized is placed in a Petri dish 2-3 cm below a 15 watt lamp and stirred either by a rocking mechanism or by a magnetic stirrer. The organisms are exposed for varying periods lasting from about 300 seconds to about 20 minutes depending on the sensitivity of the organisms. Since UV damage can be repaired by exposure to light in a process known as photo-reactivation all manipulations should be conducted under a special light source such as 25 watt yellow or red bulbs. A proportion of the organisms ranging from about 60–99.9% should be killed by the radiation. The preference of workers as to the amount of kill varies, but the higher the kill the more the likelihood of producing desirable mutants. Furthermore, the higher the kill, the less likely it is that the killing is due to overheating consequent on having the organism too close to the lamp. The initial concentration of the organisms should also be in the order of 10^7 per ml.

The main effect of ultraviolet light on DNA is the formation of covalent bonds between adjacent pyrimidine (thymine and cytosine) bases. Thymine is mainly affected, and hence the major effect of UV light is thymine dimerization, although it can also cause thymine-cytosine and cytosine-cytosine dimers. Dimerization causes a distortion of the DNA double strand and the ultimate effect is to inhibit transcription and finally the organism dies (Fig. 1).

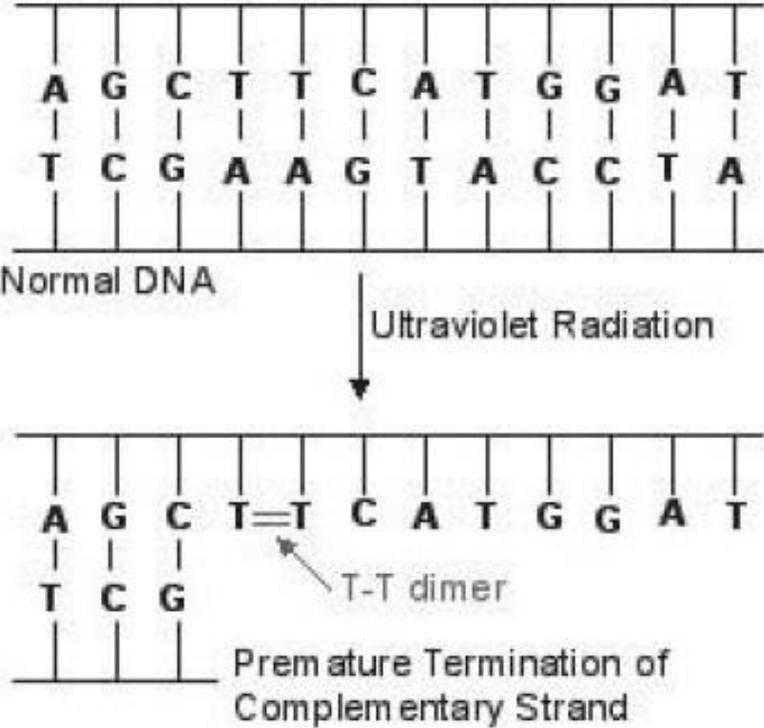


Fig. 1 Schematic Representation of Thymine Dimerization by UV Light on DNA

2.2.1.2 Chemical mutagens

These may be divided into three groups:

- (i) Those that act on DNA of resting or non-dividing organisms;
- (ii) DNA analogues which may be incorporated into DNA during replication;
- (iii) Those that cause frame-shift mutations.

(i) *Chemicals acting on resting DNA*

Some chemical mutagens, such as nitrous acid and nitrosoguanidine work by causing chemical modifications of purine and pyrimidine bases that alter their hydrogenbonding properties. For example, nitrous acid converts cytosine to uracil which then forms hydrogen bonds with adenine rather than guanine. These chemicals act on the non-dividing cell and include nitrous acid, alkylating agents and nitrosoguanidine (NTG) (also known as MNNG).

- (a) Nitrous acid
- (b) Alkylating agents:
- (c) NTG – nitrosoguanidine
- (d) Nitrogen mustards:

(ii) *Base analogues*

These are compounds which because they are similar to base nucleotides in composition may be incorporated into a dividing DNA in place of the natural base. However, this incorporation takes place only in special conditions. The best examples include 2-amino purine, a compound that resembles adenine, and 5-bromouracil (5BU), a compound that resembles thymine. The base analogs, however, do not have the hydrogen-bonding properties of the natural base. Base analogues are not useful as routine mutagens because suitable conditions for their use may be difficult to achieve. For example, with BU, incorporation occurs only when the organisms is starved of thymine.

(iii) *Frameshift mutagens (also known as intercalating agents)*

Frameshift or intercalating agents are planar three-ringed molecules that are about the same size as a nucleotide base pair. During DNA replication, these compounds can insert or intercalate between adjacent base pairs thus pushing the nucleotides far enough apart that an extra nucleotide is often added to the growing chain during DNA replication. A mutation of this sort changes all the amino acids downstream and is very likely to create a nonfunctional product since it may differ greatly from the normal protein.

Furthermore, reading frames (i.e., the DNA base sequences) other than the correct one often contain stop codons which will truncate the mutant protein prematurely. Acridines are among the best known of these mutagens, which cause a displacement or shift in the sequence of the bases. Although strongly mutagenic for some bacteriophages, acridines have not been found useful for bacteria. However, certain compounds, ICR (Institute for Cancer Research), (eg, ICR191) compounds in which an acridine nucleus is linked to an alkylating side chain, induce mutations in bacteria.

Acridine, $C_{13}H_9N$, is an organic compound consisting of three fused benzene rings (Fig. 7.3). Acridine is colorless and was first isolated from crude coal tar. It is a raw material for the production of dyes. Acridines and their derivatives are DNA and RNA binding compounds due to their intercalation abilities. Acridine Orange (3,6-dimethylaminoacridine) is a nucleic acid selective metachromatic stain useful for cell cycle determination. Another example is ethidium bromide, which is also used as a DNA dye.

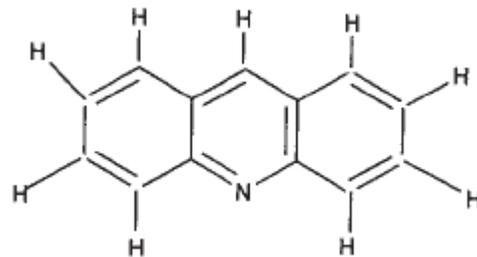
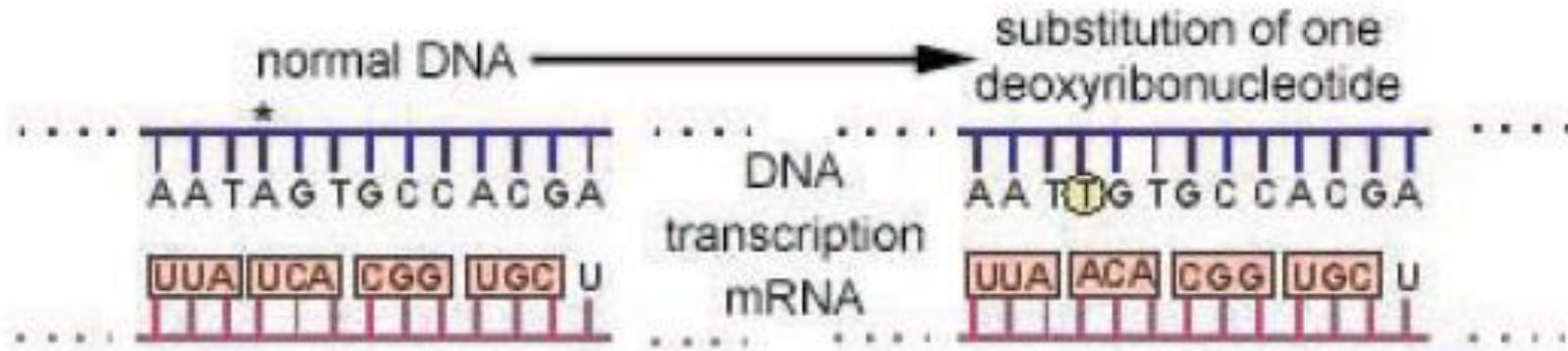


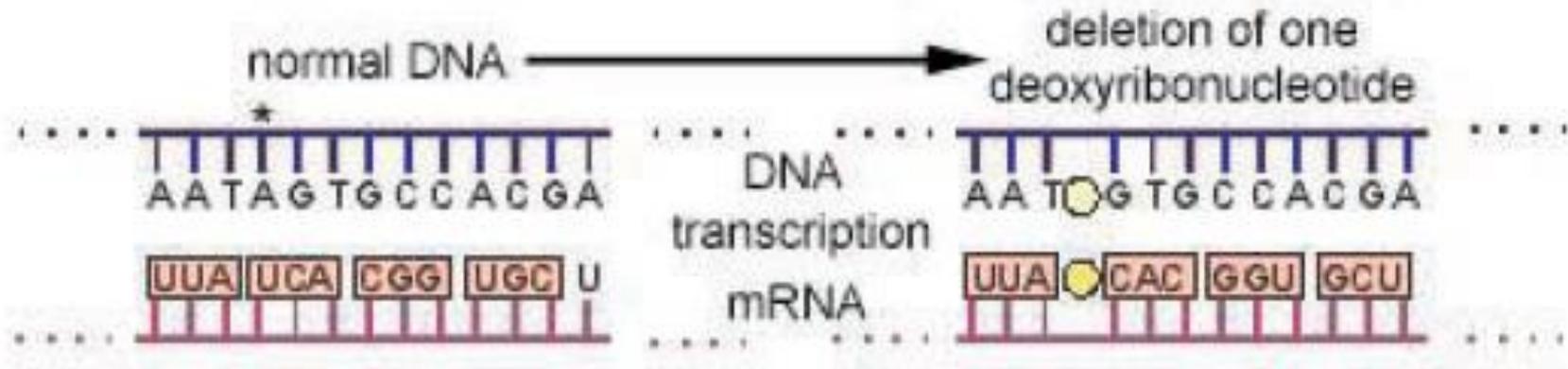
Fig. 7.3 Acridine

Different Types of Mutation

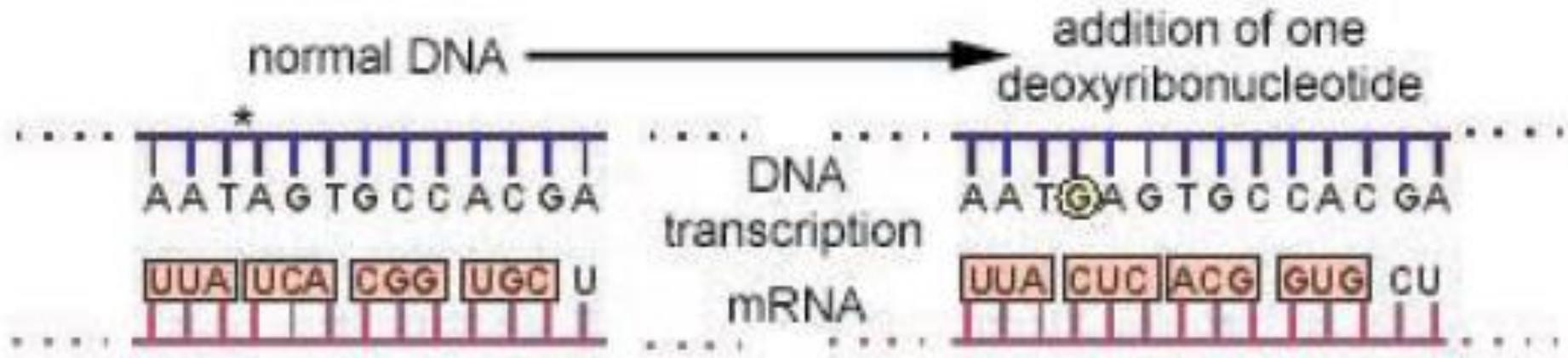
i) Point Mutation or Substitution of a Nucleotide



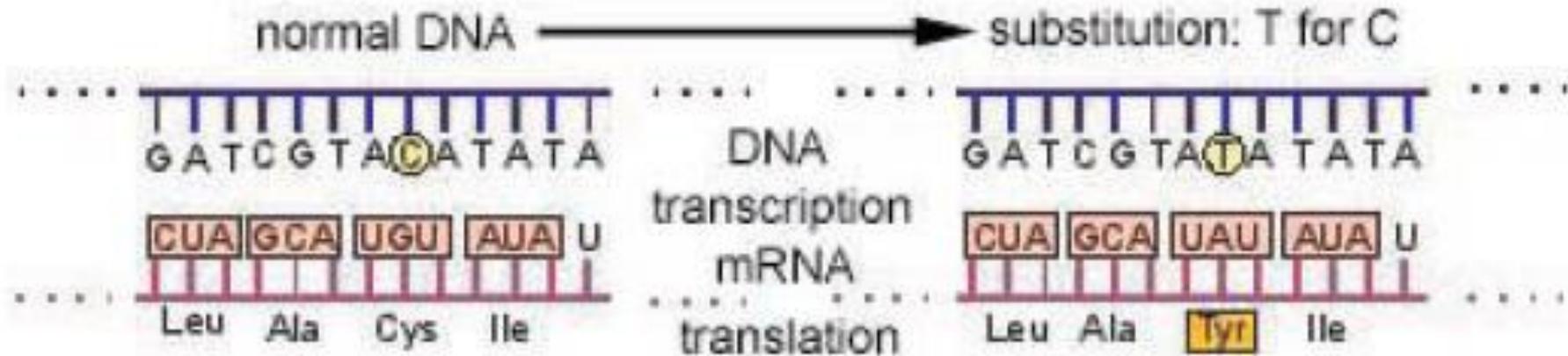
ii) Deletion of a nucleotide



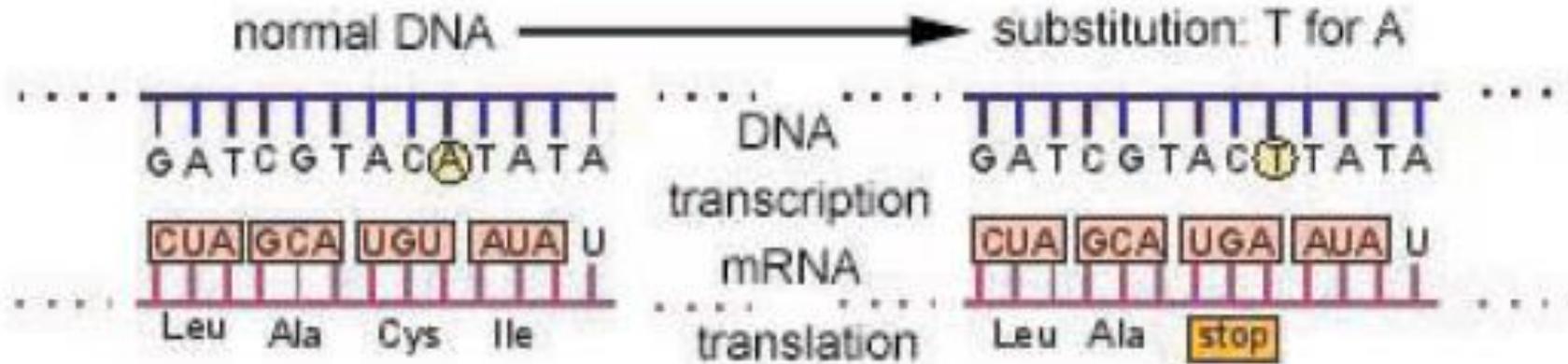
iii) Addition of a Nucleotide



iv) Substitution of a nucleotide: Results in one wrong codon and one wrong amino acid



v) **Substitution of a nucleotide: Results in a 'stop' codon and premature termination of the protein**



vi) **Frameshift mutation: Results in a reading frame shift. All codons.**



2.2.1.3 Choice of mutagen

Mutagenic agents are numerous but not necessarily equally effective in all organisms. Should one agent fail to produce mutations then another should be tried. Other factors besides effectiveness to be borne in mind are (a) the safety of the mutagen: many mutagens are carcinogens, (b) simplicity of technique, and (c) ready availability of the necessary equipment and chemicals.

Among physical agents, UV is to be preferred since it does not require much equipment, and is relatively effective and has been widely used in industry. Chemical methods other than NTG are probably best used in combination with UV. The disadvantage of UV is that it is absorbed by glass; it is also not effective in opaque or colored organisms.

2.2.1.4 The practical isolation of mutants

There are three stages before a mutant can come into use: the organisms must be exposed to a suitable mutagen under suitable conditions; the treated cells must be exposed to conditions which ideally select for the mutant; and finally, the mutant must then be tested for productivity.

(i) Exposing organisms to the mutagen:

The organism undergoing mutation should be in the haploid stage during the exposure. Bacterial cells are haploid; in fungi and actinomycetes the haploid stage is found in the spores. However, in non-sporing strains of these organisms hyphae, preferably the tips, may be used. The use of haploid is essential because many mutant genes are recessive in comparison to the parent or wild-type gene.

(ii) Selection for mutants:

Following exposure to the mutagen the cells should be suitably diluted and plated out to yield 50 – 100 colonies per plate. The selection of mutants is greatly facilitated by relying on the morphology of the mutants or on some selectivity in-built into the medium on which the treated cells or spores are plated.

When morphological mutants are selected, it is in the hope that the desired mutation is pleotropic (i.e., a mutation in which change in one property is linked with a mutation in another character). The classic example of a pleotropic mutation is to be seen in the development of penicillin-yielding strains of *Penicillium chrysogenum*. It was found in the early days of the development work on penicillin production that after irradiation, strains of *Penicillium chrysogenum* with smaller colonies and which also sporulated poorly were better producers of penicillin. Similar increases of metabolite production associated with a morphological change have been observed in organisms producing other antibiotics: cycloheximide, nystatin, and tetracyclines. In citric acid production it was observed that mutants with color in the conidia produced more of the acid; in some bacteria strains overproducing nucleic acid had a different morphological characteristic from those which did not.

In-built selectivity of the medium for mutants over the parent cells may be achieved by manipulating the medium. If, for example, it is desired to select for mutants able to stand a higher concentration of alcohol, an antibiotic, or some other chemical substance, then the desired level of the material is added to the medium on which the organisms are plated. Only mutants able to survive the higher concentration will develop. Toxic analogues may also be incorporated. Mutants resisting the analogues develop and may, for reasons discussed in Chapter 6, be higher yielding than the parent.

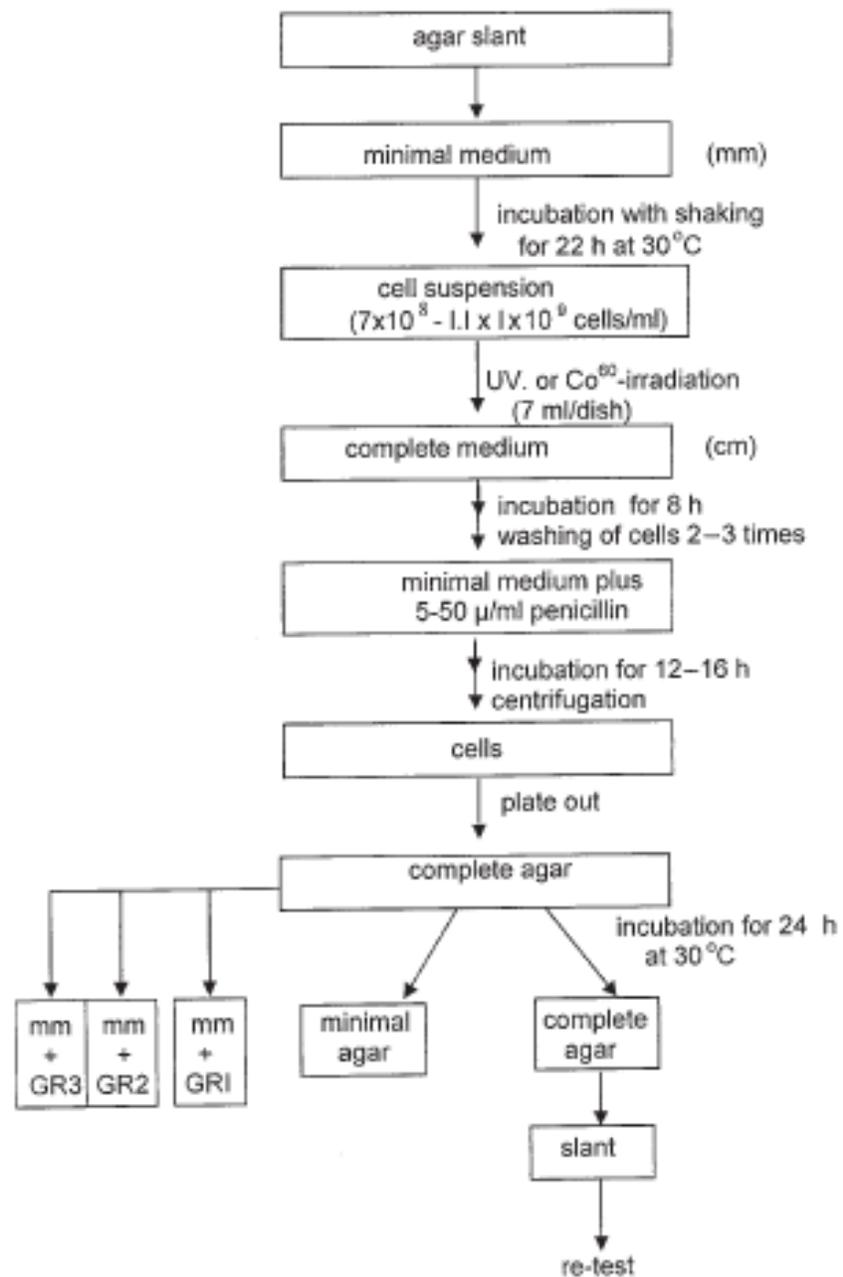
(iii) *Screening.*

Screening must be carefully carried out with statistically organized experimentation to enable one to accept with confidence any apparent improvement in a producing organism. Shake cultures are preferred and about 6 of these of 500 ml capacity should be used. Accurate methods of identifying the desired product among a possible multitude of others should be worked out. It may also be better in industrial practice where time is important to carry out as soon as possible a series of mutations using ultraviolet, and a combination of ultraviolet and chemicals and then to test all the mutants.

Isolation of auxotrophic mutants

Auxotrophic mutants are those which lack the enzymes to manufacture certain required nutrients; consequently, such nutrients must therefore be added to the growth medium. In contrast the wild-type or prototrophic organisms possess all the enzymes needed to synthesize all growth requirements. As auxotrophic mutants are often used in industrial microbiology, e.g., for the production of amino acids, nucleotides, etc., their production will be described briefly below.

A procedure for producing auxotrophic mutants is illustrated in Fig. 7.4. The organism (prototroph) is transferred from a slant to a broth of the minimal medium (mm) which is the basic medium that will support the growth of the prototroph but not that of the auxotroph. The auxotroph will only grow on the complete medium, i.e., the minimal medium plus the growth factor, amino-acid or vitamin which the auxotroph cannot synthesize. The prototroph is shaken in the minimal broth for 22–24 hours, at the end of which period it is subjected to mutagenic treatment. The mutagenized cells are now grown on the complete medium for about 8 hours after which they are washed several times. The washed cells are then shaken again in minimal medium to which penicillin is added. The reason for the addition of penicillin is that the antibiotic kills only dividing cells; as only prototrophs will grow in the minimal medium these are killed off leaving the auxotrophs. The cells are washed and plated out on the complete agar medium.



In order to determine the growth factor or compound which the auxotroph cannot manufacture, an agar culture is replica-plated on to each of several plates which contain the minimal medium and various growth factors either single or mixed. The composition of the medium on which the auxotroph will grow indicates the metabolite it cannot synthesize; for example when the auxotroph requires lysine it is designated a 'lysineless' mutant (Table 7.3).

Table 7.3 Growth of various mutants, produced after treatment of a wild-type organism

<i>S/N</i>	<i>Complete medium (cm)</i>	<i>Minimal medium (mm)</i>	<i>mm + lysine</i>	<i>Growth on mm + biotin</i>	<i>mm + valine</i>	<i>Remarks</i>
1	+	-	-	+	-	Biotin-less mutant
2	+	-	+	-	-	Lysine-less mutant
3	+	-	-	-	+	Valine-less mutant
4	+	-	-	+	+	Biotin-and valine-less
5	+	+	+	+	+	Parent Prototype

Key: + = growth
 - = no growth

2.2.2 Strain Improvement Methods Involving Foreign DNA or Bases

2.2.2.1 Transduction

2.2.2.2 Transformation

2.2.2.3 Conjugation

2.2.2.4 Parasexual recombination

2.2.2.5 Protoplast fusion

2.2.2.6 Site-directed mutation

2.2.2.7 Metabolic engineering

2.2.2.8 Genetic engineering

1. Dissection of a portion of the DNA of the donor organism
2. The attachment of the spliced piece of DNA to a vector
3. Plasmids
4. Phages
5. Cosmids
6. Transfer of the vector along with the attached DNA into the host cell
7. Recognizing the transformed cell
8. Gene transfer into organisms other than *E. coli*, including plants and animals
9. Application of genetic engineering in industrial microbiology and biotechnology in general
10. Genetically engineered plants
11. Transgenic animals and plants as biological fermentors (or Bioreactors)

2.2.2.1 Transduction

Transduction is the transfer of bacterial DNA from one bacterial cell to another by means of a bacteriophage. In this process a phage attaches to, and lyses, the cell wall of its host. It then injects its DNA (or RNA) into the host.

Once inside the cell the viral genome may become attached to the host DNA or remain unattached forming a plasmid. Such a phage, which does not lyse the cell, is a temperate phage and the situation is known as lysogeny. Sometimes the viral genome may direct the host DNA to produce hundreds of copies of the phage. At the end of this manufacture the host is lysed releasing the viral particles into the medium; the new phages carry portions of the host DNA. If one of these viral particles now invades another bacterium, but is lysogenic in the new host, the new host will acquire some nucleic acid, and hence, some properties from the previous bacterial host. This process of the acquisition of new DNA from another bacterium through a phage is transduction.

Transduction is two broad types: *general transduction* and *specialized transduction*. In general transduction, host DNA from any part of the host's genetic apparatus is

integrated into the virus DNA; in specialized transduction, which occurs only in some temperate phages, DNA from a specific region of the host DNA is integrated into the viral DNA and replaces some of the virus' genes.

It is now possible by methods which will be discussed later under the section on genetic engineering to excise genes responsible for producing certain enzymes and attach them on the special mutant viral particles, which do not cause the lysis of their hosts. Several hundreds of virus particles carrying the attached gene may therefore be present in one single bacterial cell following viral replication in it. The result is that the enzyme specified by the attached gene may be produced up to 1,000-fold. Gene amplification by phage is much higher than that obtained by plasmids (see below). The method is a well-established research tool in bacteria including actinomycetes but prospects for its use in fungi appear limited.

2.2.2.2 Transformation

Transformation is a change in genetic property of a bacterium which is brought about when foreign DNA is absorbed by, and integrates with the genome of, the donor cell. Cells in which transformation can occur are 'competent' cells. In some cases competence is artificially induced by treatment with a calcium salt. The transforming DNA must have a certain minimum length before it can be transformed. It is cut by enzymes, endonucleases, produced by the host before it is absorbed.

Reports of transformation in *Streptomyces* spp have been made. Transformation has been used to introduce streptomycin production into *Streptomyces olivaceus* with DNA from *Streptomyces griseus*. Oxytetracycline producing ability was transformed into irradiated wild-type *S. rimosus*, using DNA from a wild-type strain. The technique has also been used to transform the production of the antifungal antibiotic thiolutin from *S. pimirin* to a chlortetracycline producing *S. aureofaciens* which subsequently produced both antibiotics. An inactive strain of *Bacillus* was transformed to one producing the antibiotic bacitracin with the same method. The method has also been used to increase the level of protease and amylase production in *Bacillus* spp. The method therefore has good industrial potential.

2.2.2.3 Conjugation

Conjugation involves cell to cell contact or through sex pili (*singular*, pilus) and the transfer of plasmids. Conjugation involves a donor cell which contains a particular type of conjugative plasmid, and a recipient cell which does not. The donor strain's plasmid must possess a sex factor as a prerequisite for conjugation; only donor cells produce pili. The sex factor may on occasion transfer part of the hosts' DNA. Mycelial 'conjugation' takes place among actinomycetes with DNA transfer as in the case of eubacteria. Among sex plasmids of actinomycetes, perhaps the two best known are plasmids SCP1 and SCP2. Plasmids play an important role in the formation of some industrial products, including many antibiotics. Plasmids will be discussed in more detail later in this chapter.

2.2.2.4 Parasexual recombination

Parasexuality is a rare form of sexual reproduction which occurs in some fungi. In parasexual recombination of nuclei in hyphae from different strains fuse, resulting in the formation of new genes. Parasexuality is important in those fungi such as *Penicillium chrysogenum* and *Aspergillus niger* in which no sexual cycles have been observed. It has been used to select organisms with higher yields of various industrial product such as phenoxy methyl penicillin, citric acid, and gluconic acid. Parasexuality has not become widely successful in industry because the diploid strains are unstable and tend to revert to their lower-yielding wild-type parents. More importantly is that the diploids are not always as high yielding as the parents.

2.2.2.5 Protoplast fusion

Protoplasts are formed from bacteria, fungi, yeasts and actinomycetes when dividing cells are caused to lose their cell walls. Protoplasts may be produced in bacteria with the enzyme lysozyme, an enzyme found in tears and saliva, and capable of breaking the -1-4 bonds linking the building blocks of the bacterial cell wall. Protoplast fusion enables recombination in strains without efficient means of conjugation such as actinomycetes. It has also been used previously to produce plant recombinants. The technique involves the formation of stable protoplasts, fusion of protoplasts and subsequent regeneration of viable cells from the protoplasts. Fusion from mixed populations of protoplasts is greatly enhanced by the use of polyethylene glycol (PEG). Protoplast fusion has been successfully done with *Bacillus subtilis* and *B. megaterium* and among several species of *Streptomyces* (*S. coeli-color*, *S. acrimycini*, *S. olividans*, *S. pravulies*) has been done between the fungi *Geotrichum* and *Aspergillus*. The method has great industrial potential and experimentally has been used to achieve higher yields of antibiotics through fusion with protoplasts from different fungi.

2.2.2.6 Site-directed mutation

The outcome of conventional mutation which we have discussed so far, is random, the result being totally unpredictable. Recombinant DNA technology and the use of synthetic DNA now make it possible to have mutations at specific sites on the genome of the organism in a technique known as **Site-Directed Mutagenesis**. The mutation is caused by *in vitro* change directed at a specific site in a DNA molecule. The most common method involves use of a chemically synthesized oligonucleotide mutant which can hybridize with the DNA target molecule; the resulting mismatch-carrying DNA duplex may then be transfected into a bacterial cell line and the mutant strands recovered. The DNA of the specific gene to be mutated is isolated, and the sequence of bases in the gene determined (Chapter 3). Certain pre-determined bases are replaced and the 'new' gene is reinserted into the organism. Site-directed mutagenesis creates specific, well-defined mutations (i.e., specific changes in the protein product). It has helped to raise the industrial production of enzymes, as well as to produce specific enzymes.

2.2.2.7 Metabolic engineering

Metabolic engineering is the science which enables the rational designing or redesigning of metabolic pathways of an organism through the manipulation of the genes so as to maximize the production of biotechnological goods. In metabolic engineering, existing pathways are modified, or entirely new ones introduced through the manipulation of the genes so as to improve the yields of the microbial product, eliminate or reduce undesirable side products or shift to the production of an entirely new product. It is a modern evolution of an existing procedure which as described earlier in Chapter 6, is used to induce over production of products by blocking some pathways so as to shunt productivity through another. In the older procedure the pathways are shut off by producing mutants in which the pathways are lacking using the various mutation methods described earlier. In metabolic engineering the desired genes are isolated, modified and reintroduced into the organism. Metabolic engineering is the logical end of site-directed mutagenesis. It has been used to overproduce the amino acid isoleucine in *Corynebacterium glutamicum*, and ethanol by *E. coli* and has been employed to introduce the gene for utilizing lactose into *Corynebacterium glutamicum* thus making it possible for the organism to utilize whey which is plentiful and cheap. Through metabolic engineering the gene for the utilization of xylose was introduced into *Klebsiella* sp making it possible for the bacterium to utilize the wood sugar.

It is equally applicable to primary and secondary metabolites alike. Among primary metabolites the alcohol producing *adhB* gene from the high alcohol yielding bacterium, *Zymomonas mobilis* was introduced into *E. coli* and *Klebsiella oxytoca*, enabling these organisms to produce alcohol from a wide range of sugars, hexose and pentose. Other primary metabolites which have been produced in other organisms by introducing genes from extraneous sources are carotenoids, the intermediates in the manufacture of vitamin A in the animal body, and 1,3 propanediol (1,3 PD) an intermediate in the synthesis of polyesters. 1,3 PD is currently derived from petroleum and is expensive to produce.

1,3 PD has been produced by *E. coli* carrying genes from *Klebsiella pneumoniae* able to anaerobically produce the diol.

Among secondary metabolites, increase in the production of existing antibiotics, and the production of new antibiotics and anti-tumor agents have been enabled by metabolic engineering. The transfer of genes from *Streptomyces erythreus* to *Strep lividans* facilitated the production of erythromycin in the latter organism. In the field of anti-tumor drugs, epirubicin has less cardiotoxicity than others such as the more frequently prescribed doxorubicin. The chemical production of epirubicin is complicated and requires seven steps. However using a metabolic engineering method in which the erythromycin biosynthetic gene was introduced into *Strep peucetius* it has been possible to produce it directly by fermentation.

2.2.2.8 Genetic engineering

Genetic engineering, also known as recombinant DNA technology, molecular cloning or gene cloning. has been defined as the formation of new combinations of heritable material by the insertion of nucleic acid molecules produced by whatever means outside the cell, into any virus, bacterial plasmid or other vector system so as to allow their incorporation into host organisms in which they do not naturally occur but in which they are capable of continued propagation

The DNA to be inserted into the host bacterium may come from a eucaryotic cell, a prokaryotic cell or may even be synthesized chemically. The vector-foreign DNA complex which is introduced into the host DNA is sometimes known as a DNA chimera after the Chimera of classical Greek mythology which had the head of lion, the body of a goat and the tail of a snake.

A species has been described as a group of organisms which can mate and produce fertile offspring. A dog cannot mate with a cat; even if they did the offspring would not be fertile. A horse and the donkey are not the same species. Although they can mate, the offspring the mule, is not fertile. Genetic engineering has enabled the crossing of the species barrier, in that DNA from one organism can now be introduced into another where such exchange would not be possible under natural conditions. With this technology engineered cells are now capable of producing metabolic products vastly different from those of the unaltered natural recipient.

Procedure for the Transfer of the Gene in Recombinant DNA Technology (Genetic Engineering)

In broad terms the following are the steps involved in *in vitro* recombination or genetic engineering. The bulk of the work done so far has been with *E. coli* as the recipient organism

1. Dissecting a specific portion from the DNA of the donor organism.
2. Attachment of the spliced DNA piece to a replicating piece of DNA (or vector), which can be from either a bacteriophage or a plasmid.
3. Transfer of the vector along with the attached DNA (i.e., the DNA chimera) into the host cell.
4. Isolation (or recognition) of cells successfully receiving and maintaining the vector and its attached DNA.

1. Dissection of a portion of the DNA of the donor organism
2. The attachment of the spliced piece of DNA to a vector
3. Plasmids
4. Phages
5. Cosmids
6. Transfer of the vector along with the attached DNA into the host cell
7. Recognizing the transformed cell
8. Gene transfer into organisms other than *E. coli*, including plants and animals
9. Application of genetic engineering in industrial microbiology and biotechnology in general
10. Genetically engineered plants
11. Transgenic animals and plants as biological fermentors (or Bioreactors)

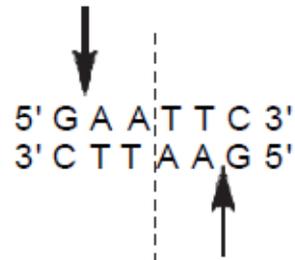
1. Dissection of a portion of the DNA of the donor organism

The donor DNA may come from a plant, an animal, a microorganisms or may even be synthesized in the laboratory.

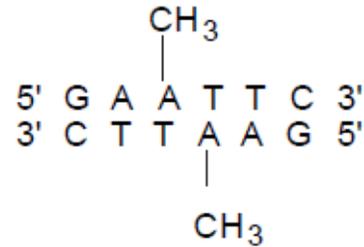
The dissection of DNA at specific sites is done by enzymes obtained from various bacteria and known as *restriction endonucleases*. They will be discussed briefly below.

(i) Nature and Types of restriction endonucleases

Restriction endonucleases are nucleic acid-splitting enzymes and are termed 'restriction' because they help a host cell destroy or restrict foreign DNA which enter the cell. The host protects its DNA from its own restriction endonucleases by the introduction of methyl groups at recognition sites where the cleavage of the DNA occurs. The host DNA so protected is said to be 'modified.' For every restriction enzyme there is a modification one hence the enzymes exist as restriction-modification complexes. Their discovery was an important landmark in molecular biology. Daniel Nathans and Hamilton Smith received the 1978 Nobel Prize in Physiology and Medicine for their isolation of restriction endonucleases, which are able to cut DNA at specific sites.



Restriction of DNA

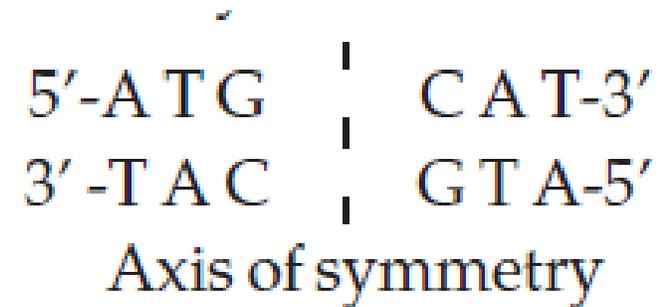


Modification of DNA

There are four different types of restriction endonucleases: Types I, II, III and IV (Type IV is designated Type II S by some authors), but only Type II is used extensively in gene manipulations. In Types I and III, one enzyme is involved for recognition of specific DNA sequences for cleavage and methylation, but the cutting positions are at variable distances from these sites (sometimes up to 1000 base pairs (bps)) away from these sites. Type IV cuts only methylated DNA. As most molecular biology work is done with Type II endonucleases and only they will be discussed.

Type II endonucleases have the following advantages over the others. Firstly in Type II systems, restriction and modification are brought about by different enzymes and hence it is possible to cut DNA in the absence of modification (note that in Types I and III a single enzyme is involved); secondly, Type II enzymes are easier to use because they do not require enzyme cofactors. Finally as will be seen below they recognize a defined symmetrical sequence and cut within this sequence.

Type II restriction endonucleases recognize and cut DNA within particular sequences of 4 to 8 nucleotides in an axis of symmetry in such a way that the sequences of the top strand when read backwards are exactly like the bottom on the other side of the axis thus:



Such sequences are referred to as palindromes. Type II restriction endonucleases were discovered in *Haemophilus influenzae* in 1970. About 3,000 of these enzymes have now been discovered and they cut in about 200 patterns; many of them are available commercially

(ii) Nomenclature of restriction endonucleases

The nomenclature of restriction endonucleases is based on the proposals of Smith and Nathans and the currently adopted procedure is as follows:

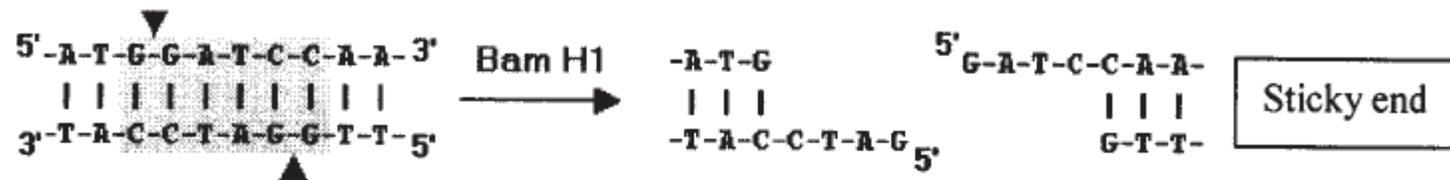
- (a) The species name of the host organisms is identified by the first letter of the genus name and the first two letters of the species name to form a three-letter abbreviation written in italics. For example, *E. coli* is *Eco* and *Haemophilus influenzae*, *Hin*.
- (b) Strain or type identification is supposed to be written as a subscript. Thus, *E. coli* strain K, *Eco*_K. In practice it is all written in one line *Eco*_K.
- (c) Where a particular host has several different restriction and modification systems, these are identified by Roman numerals. Thus, those from *H. influenzae* strain Rd. would be *Hind* I, *Hind* II, *Hind* III, in the order of their discovery.
- (d) Restriction enzymes have the general name endonuclease R and in addition carry the system name, thus endonuclease *R. Hind* I. Modification enzymes are named methylase M; thus the modification enzyme from *H. influenzae Rd.* is named methylase M. *Hind* I. Where the context makes it clear that restriction enzymes are being discussed, 'endonuclease R' is left out leaving *Hind* I as in the example quoted above.

(iii) Cutting DNA by Type II endonucleases

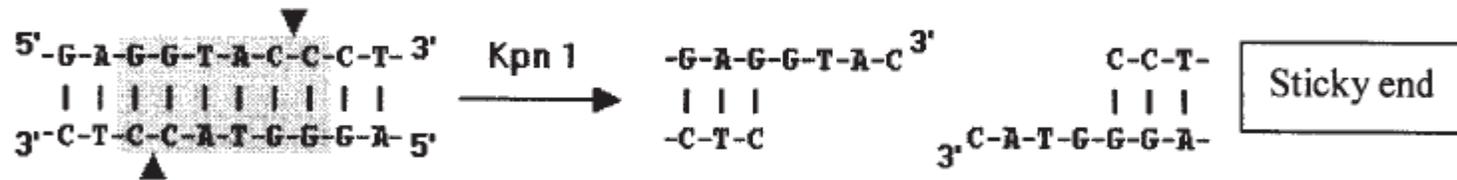
Type II endonucleases recognize and break DNA within particular sequences of four, five, six, or seven nucleotides (Table 7.4A, B) which have a symmetry along a central axis.

A: Patterns of Endonucleases Cutting);

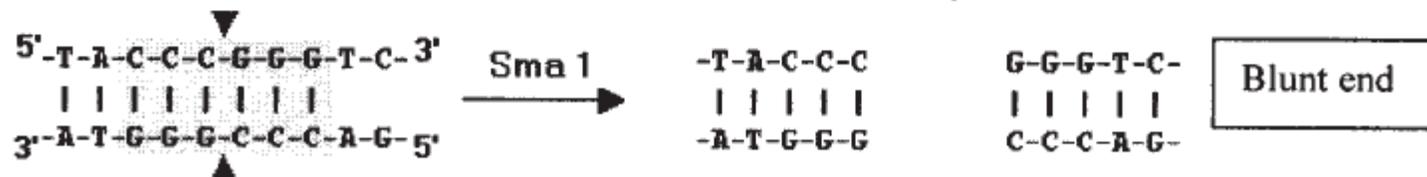
1 **5' overhangs**: The enzyme cuts asymmetrically within the recognition site such that a short single-stranded segment extends from the 5' ends as with BamHI.



2 **3' overhangs**: Again, we see asymmetrical cutting within the recognition site, but the result is a single-stranded overhang from the two 3' ends as KpnI does.



3 **Blunts**: Enzymes that cut at precisely opposite sites in the two strands of DNA generate blunt ends without overhangs. SmaI cuts in this way.



B: Some Restriction Endonucleases and their Recognition Sequences

ORGANISM	ENZYME	RECOGNITION SEQUENCES
<i>Bacillus subtilis</i>	<i>BsuRI</i>	CC / CC [*]
<i>Brevibacterium albidum</i>	<i>BalI</i>	TGG / CCA [*]
<i>Escherichia coli</i>	<i>EcoRI</i>	G / AATTC [*]
<i>Haemophilus influenzae</i>	<i>HindII</i>	GTPy / PuAC [*]
<i>Nocardia otitidis-caviarum</i>	<i>NotI</i>	GC / GGCCGC [*]

The same restriction endonuclease is used to cut the foreign DNA to be inserted into a vector, as well as the vector itself, in order to open it up.

Restriction enzymes cut DNA between deoxyribose and phosphate groups, leaving a phosphate at 5' end and an OH group at the 3'. The restriction enzymes used in genetic engineering cut within their recognition sites and generate one of three types of ends (see Table 74.4A):

- Single-stranded, "sticky" or cohesive ends as cut by *Bam* H1 (1, 5' overhangs).
- Single-stranded, "sticky" or cohesive ends as cut by *Kpn* 1 (2, 3' overhangs).
- Double-stranded, "blunt" ends as cut by *Sma* 1. (3, Blunts)

The single-stranded sticky or cohesive ends of DNA ends (Table 7.4A and Fig. 7.5) will join (anneal) with any DNA with sticky ends, having complimentary bases no matter the origin of the DNA, provided that both DNA samples have been cut with the same restriction enzyme.

Some restriction endonucleases and their recognition sequences are given in Table 7.4B.

2 The attachment of the spliced piece of DNA to a vector

(i) Joining DNA molecules:

Three methods are used for the *in vivo* 'tying' of DNA molecules. The first method uses an enzyme DNA ligase to tie sticky ends produced by restriction endonucleases; the second is the use of another DNA ligase produced by *E. coli* infected by T4 bacteriophages to link blunt ended DNA fragments. The third method uses an enzyme terminal deoxynucleotidyl –transferase isolated from calf thymus to introduce single-stranded complimentary tails to two different DNA populations after which they anneal when mixed. Only the first, method, i.e., the use of DNA ligase, will be discussed, because this has been used extensively.

(ii) *The use of DNA ligase to join foreign DNA to the vector.* High concentrations of the DNA of the previously circular vector (usually a plasmid) and of the foreign DNA to be cloned onto the vector, are mixed. Both DNA types have sticky ends having been treated with the same restriction endonuclease: in the case of the foreign DNA to cut it from its source and in the case of the vector, to open it up. Complimentary sticky ends from the foreign DNA and the vector anneal leaving however gaps created by the absence of a few base pairs in opposite strands (Fig. 7.5). The enzyme DNA ligase can repair these gaps to create an intact duplex. DNA ligase is produced by *E. coli* and phage T4. The ligase from T4 can, however, join blunt-ended DNA whereas that from *E. coli* cannot. The vector-foreign DNA chimaera is then introduced into the bacterial cell by transformation. To prevent recircularization of the linearized vector, it may be treated with alkaline phosphatase. When it is so treated circularization can only occur when a foreign DNA is introduced. A gap is left at each joint. These gaps are closed after transformation by the hosts' repair system.

(iii) *Vectors used in recombinant DNA work:*

Two broad groups of cloning vehicles have been used, namely plasmids and lamda phages. Both have replication systems that are independent of that of the host cell.

3 Plasmids

Plasmids are circular DNA molecules with molecular weights ranging from a few million to a few hundred million Daltons. Plasmids appear to be associated with virtually all known bacterial genera. They replicate within the cell. Some of the larger plasmids, known as *conjugative plasmids*, carry a set of genes which promote their own transfer in a sexual process known as conjugation which has already been discussed. Smaller plasmids are usually non-conjugative but their transfer can usually be promoted by the presence of a conjugative plasmid in the same cell.

Besides genes for sexual transfer, plasmids usually carry genes for antibiotic or heavy metal resistance. They often also carry genes for the production of toxins, bacteriocins, Two important features of plasmids to be used in genetic experiments may be compared by examining two plasmids. Plasmid psC101 has only two to five copies per cell and replicates with its host DNA. It is said to be under 'stringent' control. However, another plasmid pCol E 1 is found in about 25–30 copies per cell. It has a 'relaxed control' independent of the host and replicates without reference to the host DNA.

When the host cell is starved of amino-acids or its protein synthesis is inhibited in some other manner, such as with the use of chloramphenicol, the Col E 1 plasmid continues to replicate for several hours until there are 1,000 to 3,000 copies per cell. Due to this high level of gene dosage (also referred to as gene amplification), products synthesized because of the presence of these plasmids are produced in extremely high amounts, a property of immense importance in biotechnology and industrial microbiology. Generally conjugative plasmids are large, exhibit stringent control of DNA replication, and are present in low copy numbers; on the other hand, non-conjugative plasmids are small, show relaxed DNA replication, and are present in high numbers. Many other plasmid vectors exist, some constructed in the laboratory (Table 7.5).

Table 7.5 Some commonly used plasmid cloning vehicles

<i>Plasmid</i>	<i>Molecular weight (x 10⁻⁶)</i>	<i>Marker*</i>	<i>Single restriction sites</i>
pSC101	5.8	Tc	BamHI, EcoRI, HindIII, HpaI, Sall, SmaI
Col E1	4.2	Colimm	EcoRI
pMB9	3.6	Tc ^r , Colimm	BamHI, EcoRI, HindIII, HpaI, Sall, SmaI
pBR313	5.8	Tc ^r , Ap ^r Colimm	BamHI, EcoRI, HindIII, HpaI, Sall, SmaI
pBR322	2.6		BamHI, EcoRI, HindIII, pstI, Sall

*Tc^r: tetracycline resistance. Ap^r: ampicillin resistance
colimm: colicin immunity

(i) *Ideal properties in a plasmid used as a vector*

A plasmid to be used in genetic engineering should ideally have the following properties:

- (a) the plasmid should be as small as possible so the unwanted genes are not transmitted, as well as to facilitate handling;
- (b) it should have an *origin of replication*, the site where DNA replication initiates;
- (c) it should have a relaxed mode of replication;
- (d) it should have sites for several restriction enzymes;
- (e) it should carry, preferably, two marker genes. Marker genes are those which express characteristics by which the plasmid can be identified. Such characteristics include resistance to one or more antibiotics. A marker of great importance is the ability to satisfy auxotrophy, i.e., the ability to produce an amino acid or other nutritional component which the host's chromosome is incapable of producing.
- (f) the nucleotide sequence of the plasmid should be known;
- (g) for safety reasons the plasmid should not be able to replicate at mammalian body temperatures so that should it enter the human body and be able to produce deleterious substances, it should fail to replicate;
- (h) for safety reasons also, it should not be highly transmissible by conjugation if it controls the production of any material harmful to the mammalian body;
- (i) the plasmid as a cloning vehicle should have a site for inducing transcription across the inserted fragment. The plasmid-initiated transcription should be controlled by the host (by induction or repression). Uncontrolled transcription could be harmful to the host.

Table 7.5 shows some commonly used plasmid cloning vehicles. They carry various markers based on tetracycline or ampicillin resistance or immunity against colicin attack. The marker may be carried either on the plasmid or on the inserted DNA. If neither of them carries a marker then DNA carrying a marker can be grafted on to either the vector or the insert.

(ii) *Plasmids currently in use for cloning*

In the early years of genetic engineering, naturally occurring plasmids such as Col E1 and pSC 101 were used as cloning vectors. They were small and had single sites for the common endonucleases. However they lacked markers which would help select transformed organisms. New plasmids were therefore developed. The best and most commonly used is pBR322 developed by Francisco Bolivar. (In naming plasmids p is used to show it is a plasmid; p is followed by the initials of the worker who isolated or developed the plasmid; numbers are used to denote the particular strain). Plasmid pBR322 has all the properties expected in a plasmid vector: low molecular weight, two markers, (resistance to ampicillin, Ap_R and tetracycline, Tc_R) an origin of replication, and several single-cut replication sites. (see map of pBR322 in Fig 7.6). Modifications of the original pBR322 have been made to suit special purposes, and consequently many variants exist in the pBR322 family. A widely used variant of pBR322 is pAT153, which some consider a better vector than its parent because it is present in more copies per cell than pBR322. Another series of popular vectors is the pUC family of vectors (Fig. 7.7). It has several unique restriction sites in a short stretch of DNA, which is an advantage in some kinds of work.

4 Phages

Two types of phages have been developed for cloning, lamda, and M13. Most of the phages used for cloning are derivatives of the lamda phage of *E. coli* because so much is already known about this phage. Derivatives are used because the wild-type phage is not suitable as a vector as it has several targets of sites for most of the most commonly used endonucleases. The chromosome of phage must be folded and encapsulated into the head of the virus in order to provide a mature virion. The amount of DNA that can enter the head is limited, and hence the available DNA in a phage is also limited. Therefore unwanted phage DNA must be removed as well as all but one of restriction targets for the chosen enzyme.

The DNA of phage lambda when it is isolated from the phage particle is linear and double-stranded. At each end of the chain are single-stranded portions which are complimentary to each other, much like the 'sticky ends' produced from DNA cutting by restriction endonucleases (Fig. 7.9). These lambda DNA pieces are able to circularize and replicate independently within the host.

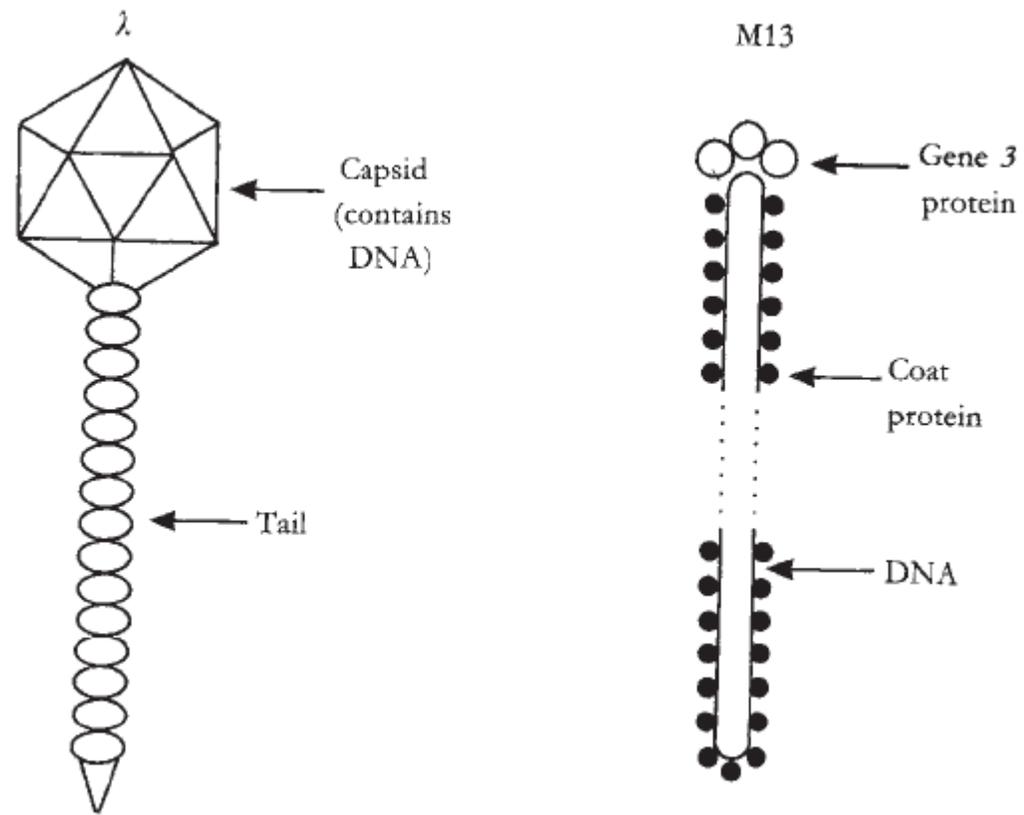


Fig. 7.8 Structure of λ and M13 Bacteriophages

The middle portion of the linear double-stranded phage DNA is non-essential for phage growth and it is here that the foreign DNA is introduced. The more distal positions carry genes which code for essential components such as the head, tail of the bacteriophage and the host lysis (Fig. 7.9)

(i) ***Transfection***: The linear chimera can be introduced by transformation. (When virus DNA is transformed the process is known as transfection.) However, much of introduced chimeras are restricted in comparison to when pure phage DNA is transfected.

(ii) ***Packaging the chimeras into virus heads***: The recombinant DNA or chimera may be packaged into a virus head and a tail attached by *in vitro* means. The procedure for this packaging is outside the scope of this book but may be found elsewhere. Once packaged, the synthetic virus can then inject its DNA into the host in the usual way.

5 Cosmids

Cosmids are plasmids constructed from phage DNA by circularization at the ‘sticky,’ single stranded ends or *cos sites*. Foreign DNA is attached to the cosmid which is then packaged into a phage. When the cosmid is injected it circularizes like other virus DNA but it does not behave as a phage, rather it replicates like a plasmid. Drug resistance markers carried on it help to identify it.

A number of commercially available vectors are based on phages and some are shown in Table 7.6

Table 7.6 Phage-based vectors

<i>Vector</i>	<i>Features</i>	<i>Applications</i>
pBR322	Ap ^r Tc ^r Single cloning sites	General cloning and sub-cloning in <i>E. coli</i>
pAT 153	Ap ^r Tc ^r Single cloning sites	General cloning and sub-cloning in <i>E. coli</i>
pGEM TM -32	Ap ^r MCS SP6/T7 promoters lacZ α -peptide	General cloning and <i>in vitro</i> transcription in <i>E. coli</i> and mammalian cells
pCI TM	Ap ^r MCS T7 promoter	Expression of genes in mammalian cells
pCMV-Script TM	CMV enhance/promoter Neo ^r Large MCS CMV enhancer/promoter	Expression of genes in mammalian cells Cloning of PCR products

6 Transfer of the vector along with the attached DNA into the host cell

The vector once spliced with the endonuclease cannot reform into a circular structure unless a suitable fragment of the foreign DNA with a complementary 'sticky end' fits in. Foreign DNA digests produced by physical inactivation may also be used. If a large enough amount of foreign DNA digest is used the probability is that a piece with the appropriate complementary end will fit in. The new hybrid DNA is introduced into the host cell by transformation. Transformation is facilitated by treating the host cells in calcium salts, after washing them in magnesium salts.

7 Recognizing the transformed cell

The introduction of the new property into the host may be detected by growing the cells in a medium containing antibiotics whose resistance is specified in the introduced foreign DNA. Growth should occur if the resistance gene was transferred. If genes for the synthesis of some products were introduced via the chimera, the transformed bacteria should grow on the selective medium. The products are then examined for the synthesis of the new compound. For the introduced gene to lead to protein synthesis a suitable promoter must be present; it may be introduced from other organisms if an appropriate one is not indigenous.

8 Gene transfer into organisms other than *E. coli*, including plants and animals

The methods discussed above are those developed primarily for *E. coli*. The discussion below will look at the introduction of DNA into bacteria other than *E. coli* as well into other organisms, including plants and animals. Some of the methods to be discussed are also used on *E. coli*.

(i) *Delivery into bacteria other than E. coli*

(a) **Electroporation**

In the process of electroporation, cells into which DNA is to be introduced (i.e., cells to be **transfected**) are exposed to high-voltage electric pulses. This creates temporary holes in the cell membrane through which DNA can pass. Electroporation can be used for transfecting cells of *Bacilli* spp and actinomycetes, especially when protoplasts are produced from the cells. Electroporation is the short form for electric field-mediated membrane permeabilization. It is still used for *E. coli*, especially when chimera longer than about 100 kilobases (100 kb) are to be used. In general electroporation can be used for transfecting bacteria and archae, after the appropriate electric voltage and other parameters have been worked out. As will be seen below, it is also used for transfecting plant cells.

(b) Conjugation

In some bacteria where other means of introducing DNA appear difficult or have failed, the natural means of transferring DNA by plasmid mediated conjugation has been exploited. A conjugative plasmid which is carrying the insert and which has the genes for its own transfer is used. However where this is not possible, a conjugative plasmid with its own transfer gene may first be introduced, followed by the non-conjugative (i.e., does not promote the transfer of DNA through pili) plasmid carrying the DNA insert. This has been used in some strains of *Pseudomonas*.

(c) Use of Liposomes

When the DNA to be introduced is first entrapped in phospholipid droplets known as liposomes, it enhances the entry of the DNA into protoplasts of Gram-positive *Bacillus* and actinomycetes. Liposomes have also been used for delivering DNA into animal cells. Liposomes are microscopic, fluid-filled vesicles whose walls are made of layers of *phospholipids* identical to the phospholipids that make up cell membranes. The outer layer of the vesicle is hydrophobic, while the inner layer is hydrophilic; this enables the liposome to carry water soluble materials within it. They can be designed so that they have cationic, anionic or neutral charges at the hydrophobic end depending on the purpose for which they are meant. They are used for introducing DNA into animal, plant or bacterial cells. When used for introducing DNA into plant cells, such cells must have their cell walls removed, yielding protoplasts; with bacteria, the cell walls must also be removed to yield sphaeroplasts. Liposomes have been used experimentally to carry normal genes into a cell to replace defective, disease-causing genes in gene therapy. Liposomes are used to deliver certain vaccines, enzymes, or drugs (e.g., insulin and some cancer drugs) to the animal body. Liposomes are sometimes used in cosmetics because of their ability to penetrate the skin.

(ii) *Delivery of DNA into Plant cells*

Plants are peculiar in that most single plant cells can be caused to develop into the entire plants. Successfully transfecting (i.e., introducing foreign DNA into) a plant cell will result in having the foreign DNA as part of the genetic apparatus of the transfected plant. The introduction of foreign DNA into plants is done for the improvement of the agricultural, ornamental, nutritional, or horticultural value of the plant. It is also done to convert the plants into 'living fermentors' which with the appropriate genes can manufacture cheaply, some industrially important materials, which it may not even be possible to produce by chemical means. Several methods are available for the delivery of DNA into plants

(a) Plant Transfection with Ti plasmid of *Agrobacterium tumefaciens*

(b) Use of Viruses

(c) Electroporation

(d) Biollistic or Microprojectile methods

(e) Microinjection

(iii) *Delivery of DNA into Animal cells*

Genetic engineering in plants differs in at least two respects from that in animals. Firstly while plant cells are mostly totipotent (i.e., most plant cells are able to give rise to a new plant), animal cells cannot give rise to whole animals once differentiated into specialized cells. In animals the cells that become reproductive cells separate early from those that are ordinary body (somatic) cells. Somatic cells do not give rise to new animals. To create transgenic animals the foreign DNA must be introduced into cells while they are still totipotent and differentiation has not occurred. Generally this involves introducing the DNA into stem cells (yet undifferentiated cells), an egg, the fertilized egg, (oocyte or zygote) or early embryo.

Some of the methods discussed above for introducing foreign DNA into bacteria and plants are also applicable to animal cells: electroporation, biolistic methods and microinjection have all been successfully used in animals. In addition the liposome (phospholipid) delivery seen in bacteria is also used in animal cells.

Genes are introduced into animal cells as well as *in vivo* by transduction via viruses in gene therapy. Four groups of viral vectors are used for gene therapy in humans: adenoviruses, baculoviruses, herpesvirus vectors, and retroviruses.

Changing the genetic make-up of animals, in large domesticated mammals such as cows, pigs and sheep, allows a number of commercial applications. These applications include the production of animals which express large quantities of exogenous proteins in an easily harvested form (e.g., expression into the milk), the production of animals which are resistant to infection by specific microorganisms and the production of animals having enhanced growth rates or reproductive performance.

Most of the work on transgenic animals has been done with mice on account of their small size and low cost of housing in comparison to that for larger vertebrates, their short generation time, and their fairly well defined genetics. Foreign DNA is introduced in mice in one of the following ways: DNA microinjection, embryonic stem cell-mediated gene transfer and retrovirus-mediated gene transfer, sperm-mediated transfer, transfer into unfertilized ova.

- (a) DNA microinjection**
- (b) Embryonic stem cell-mediated gene transfer**
- (c) Retrovirus-mediated gene transfer**
- (d) Sperm-mediated Gene Transfer**

9 Application of genetic engineering in industrial microbiology and biotechnology in general

The unparalleled ability of DNA to replicate and reproduce itself is truly remarkable. What this means is that, put crudely, DNA of a given sequence coding for the production of a polypeptide or protein in organism A will lead to the production of the same polypeptide or protein if the same sequence is put into organism B. This is the basic assumption underlying the numerous advances in our manipulation of the biotic world for the benefit of humans. This section looks only at some of the numerous positive changes recombinant DNA technology has contributed to spreading a better quality of life to millions of people around the world through improvements in agriculture, health care delivery and industrial productivity.

(i) Production of Industrial Enzymes

Genetically engineered bulk enzymes are used mostly in the food industry (baking, starch manufacture, fruit juices), the animal feed industry, in textile manufacture, and in detergents. A leading manufacturer of these enzymes among world manufacturer is Novo Enzymes of Denmark.

The advantages of using engineered enzymes are as follows:

- (a) such enzymes have a higher specificity and purity;
- (b) it is possible to obtain enzymes which would otherwise not be available due to economical, occupational health or environmental reasons;
- (c) on account of the higher production efficiency there is an additional environmental benefit through reducing energy consumption and waste from the production plants;
- (d) for enzymes used in the food industry particular benefits are for example a better use of raw materials (juice industry), better shelf life of the final food and thereby less wastage of food (baking industry) and a reduced use of chemicals in the production process (starch industry);
- (e) for enzymes used in the animal feed industry particular benefits include a significant reduction in the amount of phosphorus released to the environment from farming.

(ii) Enhancing the activities of Industrial Enzymes

Through protein engineering it has been possible to enhance the properties of proteins to make them more stable to denaturation, more active in their biocatalytic ability and even to design new properties in existing enzymes. The properties of proteins are due to their conformation which is a result of their amino acid sequence. Certain amino acids in a protein play important parts in determining the stability of the protein to high temperatures, specificity and stability to acidity. In protein engineering changes are caused to occur in the protein by changes in the nucleotide sequence; a change of even a single nucleotide could lead to a drastic change in a protein. Many industrial processes are carried out at elevated temperatures, which can unfold the proteins and cause them to denature. The addition of disulphide bonds helps to stabilize them. Disulphide bonds are usually added by engineering cysteine in positions where it is desired to have the disulphide bonds. The addition of disulphide bonds not only increases stability towards elevated temperatures, but in some instances also increases stability towards organic solvents and extremes of pH. An example of the increase of stability to elevated temperatures due to the addition of disulphide bonds is seen in xylanase.

Xylanase is produced from *Bacillus circulans*. During paper manufacture, wood pulp is treated with chemicals to remove hemicelluloses. This treatment however leads to the release of undesirable toxic effluents. It is possible to use xylanase to breakdown the hemicellulose. However, at the time when bleaching is done, the pulp is highly acidic as a result of the acid used to digest the wood chips to produce wood pulp. The acid is neutralized with alkali, but the temperature is still high and would denature native xylanase. *In silico* (i.e. computer) modeling showed the sites where disulphide bonds can be added without affecting the enzyme's activity. The introduction of the disulphide bonds did increase the thermostability of the enzyme, making it possible to keep 85% of its activity after 2 hours at 60°C whereas the native enzyme lost its activity after about 30 minutes at the same temperature.

Another way in which enzyme activity can be enhanced by protein engineering is to actually increase the activity of the enzyme. This can be done only with an enzyme whose conformation, including the active sites, is thoroughly understood. Using *in silico* modeling, it is possible to predict the effect of changing amino acids at the active site of an enzyme. This has been done with the enzyme tRNA synthase from *Bacillus stearothermophilus*.

Various other properties have been engineered into proteins including a modification of the metal co-factor and even a change in the specificity of enzymes.

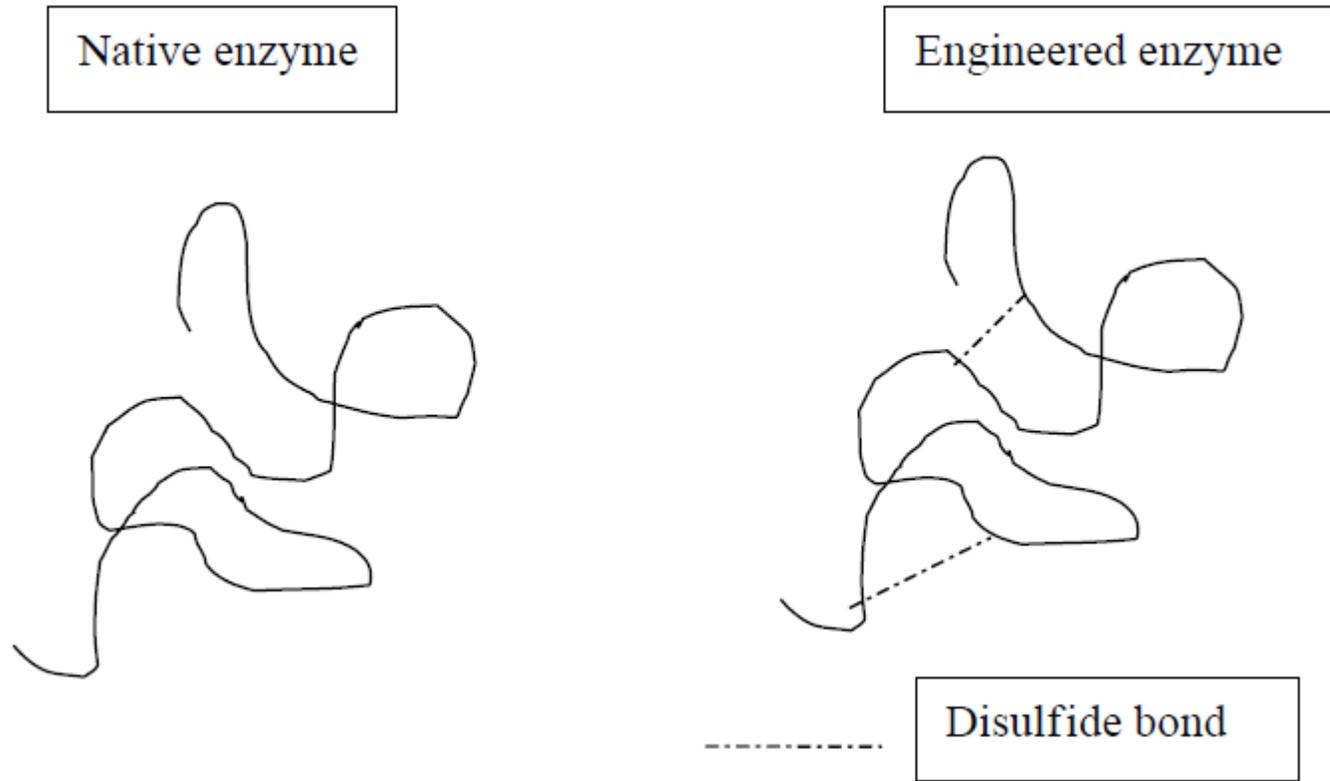


Fig. 7.12 Stabilizing Enzymes through the Introduction of Disulfide Bonds

(iii) Engineered Products or Activities Used for the Enhancement of Human Health

Engineered health care products and activities can be divided into: a) those used to replace or supplement proteins produced by the human body in insufficient quantities or not produced at all; b) those involving the replacement of a defective gene; c). those that are used to treat disease, d) those that are used for prophylaxis or prevention of disease, i.e., vaccines, or e) those that are used for the diagnosis of disease (Table 7.8).

Table 7.8 Some genetically engineered health related products

<i>Product</i>	<i>Application</i>
Hormones	
Insulin	Treatment of diabetes
Human growth hormone (somatotropin)	Treatment of dwarfism
Follicle stimulating hormone	Treatment of some disorders of the reproductive system
Immune System Participants	
Tumor necrosis factor	Anti-tumor agent
Interleukin 2	Treatment of some cancers
Lysozyme	Anti-inflammatory agent
A-Interferon	Antiviral
Blood components	
Erythropoietin	Treatment of anemia and cancers
Tissue plasminogen activate	Dissolves blood clots
Factor VIII	Treating hemophilia
Enzymes	
Human DNase I	Treatment of cystic fibrosis

10 Genetically engineered plants

Plants have been engineered for the introduction of many new desirable properties. Collectively these attainments represent a major triumph of biotechnology, enabling us to achieve in a few years what would take traditional plant breeding decades to attain, if at all. Some genetic engineering achievements would be impossible with traditional plant breeding methods since in the latter, the introduction of new genetic properties occurs only through the exchange of sexual materials (in the pollen grains) of the same species. In genetic engineering the natural species barrier is not recognized since the DNA sequence introduced into a plant can come from another plant of a different species or even from a non-plant source such as a bacterium, and indeed may even be synthesized. The introduction of some genetically engineered foods has met with public resistance, although many have been shown to be safe. What is required is continued public education about their safety before their introduction, and constant sensitivity to public opinion thereafter.

The ensuing discussion will be under two headings,

- a) improving field, production or agronomic traits
- b) b) modification of consumer products.

Improving Field or Production Characteristics

(i) Engineering Plants for Herbicide Resistance

An estimated US \$10 billion is spent annually on weed killers. In spite of this about 10% of world crop production is lost to weeds. Herbicides (weed killers) target processes that are essential and unique to plants. These processes are however important to plants and weeds alike, and getting methods that are selective for either is difficult. One method that is used is to engineer crops so they become resistant to the weed killer. Plants can become resistant to herbicides in one of the four following ways:

- (a) overproduction of the herbicide sensitive target, so that some is still left for the proper cell function despite presence of the herbicide in the cell;
- (b) reduction of the ability of the herbicide-sensitive target protein to bind to the herbicide;
- (c) engineering into plants the ability to metabolically inhibit the herbicide;
- (d) inhibition of the uptake of the herbicide.

(ii) Engineering Plants for Pathogenic Microbe Resistance

The majority of microbes attacking plants are fungi, but some bacterial diseases of plants do exist. Plants are conventionally sprayed with chemicals to eliminate fungal pathogens. Such chemicals sometimes are not always easily biodegradable, and they may also find their way into food. A genetic approach which bypasses this problem is to engineer into plants anti-fungal proteins such as the gene coding for chitinase, an enzyme which hydrolyzes chitin, a polymer of the amino sugar N-acetyl glucosamine. Chitinase gene from bean has been cloned into tobacco where chitinase stopped the attack by the fungus, *Rhizoctonia solani*. Chitinase is one of the 'pathogen-related proteins' (PRs) synthesized by plants; they also synthesize ant-fungal peptides known as defensins. Genes coding for these are sought from source of high productivity and cloned into plant to protect them.

Plant resistance to bacterial disease has also been genetically engineered. For example, the -thionin gene from barley has been shown to confer resistance to a bacterial pathogen, *Pseudomonas syringae* in transgenic tobacco.

With regard to engineering plants against viruses, when the viral coat of a plant virus is engineered into a plant, that plant usually becomes resistant to the virus from which the coat comes. Often the plant is also resistant against other unrelated viruses.

(iii) Engineering Plants for Insect Resistance

Insect pests are devastating to crops, about US \$5 billion are currently being used to control them annually with chemicals. The advantages of using biological means of controlling insect pest have been highlighted in Chapter 17. The methods described relate to the use of biological insecticides which are sprayed on plants. Such sprayed insecticides have the disadvantage that they are inactivated by ultraviolet rays from the sun or may be washed away by the rain. Genetic engineering of crops for resistance against insect pests has the advantage that the active constituents are protected from the environment and remain within the plant.

The major strategy of producing plants resistant to insect pests is to engineer the gene for producing the toxic crystals of *Bacillus thuringiensis* (Bt) into plants. These crystals are produced in Bt but in no other *Bacillus* sp. They are small proteins and are highly specific against given insects. In such susceptible insects they bind to receptors in the gut lining of the insects, dissolve in the alkali milieu therein and create holes in the gut lining through which gut contents leak out, leading to death. The gene for Bt toxin has been engineered into cotton, tomatoes and numerous other plants (Fig. 7.15).

Alternative strategies which have been inspired by the fact that Bt toxins do not affect some insects, is to engineer into plants two groups of enzymes which inhibit digestive enzymes in the insect gut: amylase inhibitors and protease inhibitors. In effect the insect starves to death.

Another strategy for developing insect resistance in plants is to engineer into the plant the gene for cholesterol oxidase, which is present in many bacteria. Cholesterol oxidase catalyzes 3-hydroxysteroids to ketosteroids and hydrogen peroxide (Chapter 26). Small amounts of this enzyme are very lethal to the larvae of boll weevil which attacks cotton. It is possible that the cholesterol oxidase acts by disrupting the insect larva's alimentary canal epithelium leading to its death.

(iv) **Genetically Engineering Plants to Survive Water and Salt Stress**

Many parts of the world have desert or near desert conditions where water is in short supply. Added to this is the fact that salt used for treating ice in the winter finds its way into agricultural land. These factors create conditions which bring plants into conditions of water (drought) and salt stress. To survive under these conditions, many plants synthesize compounds known as osmoprotectants. They help the plant increase its water uptake as well as retain the water absorbed. Osmoprotectants include sugars, alcohols and quaternary ammonium compounds. The quaternary ammonium compound, betaine, is a powerful osmoprotectant and the gene encoding it obtained from *E. coli* has enabled plants into which it was cloned survive drought better than un-engineered plants.

Modification of Plant Consumer Products

(i) Maintenance of Hardness and Delayed Ripeness in Fruits

During post-harvest transportation of fruits to supermarkets these fruits sometimes ripen and become soft due to the natural processes which go on within the fruit. These natural processes include the production of polygalacturonase (PG) (which hydrolyzes pectin) and cellulases by the fruit.

Two biotechnological strategies have been pursued to control ethylene action on fruit ripening. One approach taken in tomato was designed to inhibit biosynthesis of ethylene within the plant by the use of antisense expression of ACC synthase. In a second approach, a mutated ethylene receptor from *Arabidopsis* was introduced into tomato and petunia. This resulted in delayed fruit ripening.

(ii) Engineering Sweetness into Foods

The taste of fresh tomatoes and lettuce is well known in sandwiches. Some enjoy these items with greater relish with the addition of sweet tasting tomato ketchup. Sweet taste has been engineered into tomatoes and lettuce by cloning into them the synthesized gene coding for monellin. Monellin is a protein which is 3,000 times sweeter than sucrose by weight; it is naturally obtained from the red berries of the West African plant, *Dioscoreophyllum comminsii* Diels, and has been expressed in yeast. A major attraction of sweetening tomatoes and lettuce with this protein is that it is 'weight-friendly'. Several sweet proteins which might be similarly engineered into foods are shown in Table 7.11.

(iii) Modification of Starch for Industrial Purposes

Starch consists of amylose in which the glucose molecules are configured in a straight chain in the -1-4 linkage, and the branched chain amylopectin which has -1,4 and -1,6 linkages (Chapter 4). Starches from different plants have different percentages of amylose and amylopectin, but generally in the order of 30% amylose to 70 to 80% amylopectin. Starch is used for making several industrial products such as glue, gelling agent or thickener. For some purposes it may be desirable to have starch that has a preponderance of amylose. When that is the case, antisense technology has been used to block the formation of the amylopectin component of starch, giving rise to a product with only about 20%.

(iv) Modifying Flower Pigmentation and Delaying Wilting and Abscission in Flowers

The flower business is of the order of many billions of dollars annually. Most of the market centers around four flowers: roses, carnations, tulips, and chrysanthemums. Hundreds of different flowers differing in shape, size, color, fragrance, and structure have become available through traditional plant breeding. But the usual shortcomings have also affected this industry: the slow pace of the plant breeding, the uncertainty of the results of the efforts and the limitation imposed by the paucity of the genes available in traditional plant breeding.

Genetic engineering has now been introduced and has helped to extend the range of the variety of flowers. A group of flavonoids, anthocyanins (Chapter 22) are commonest pigments in flowers. Anthocyanins are glucosides of phenolic compounds produced in plants, some being colorless, while many are responsible for the colors in plants. The aglycone (non-sugar) portions of anthocyanins are derived from the amino acid phenylalanine. The color which they bear is determined by the chemical nature of the side chain substituent. By blocking some of the genes in the pathway of anthocyanin synthesis using anti-sense technology or introducing totally new genes it is possible to create flowers with new colors

(v) Modification of Nutritional Capabilities of Crops

Genetic engineering has enabled the introduction of new nutritional capabilities in crops, in a much shorter time and in a range of qualities impossible with traditional breeding. Unlike genetic engineering which can cross the species barrier, plant breeding deals with the collection of genes within the species. The amino acid content of foods, the lipid composition, the amylose/amylopectin ratio of starch, the vitamin contents and even the mineral contents of foods have all been modified by genetic engineering.

(a) Engineering Vitamin A into Rice

(b) Engineering Amino Acids into Legumes and Cereals

(c) Modifying Fats and Oils for Various Purposes

11 Transgenic animals and plants as biological fermentors (or Bioreactors)

Transgenic animals and plants have been used to produce high-quality pharmaceutical substances or diagnostics. The procedure is known as 'pharming' from a parody of the word pharmaceutical; it is also known as 'molecular farming' or 'gene pharming' and the transgenic plants or animals used are sometimes referred to as animal or plant 'bioreactors' or 'fermentors'. Therapeutically active proteins already on the market are usually produced in bacteria, fungi, or animal cell cultures. However microorganisms usually produce comparatively simple proteins; furthermore microorganisms are not always able to correctly assemble and fold complex proteins. If the protein structure is very complicated, such microorganisms may produce defective clumps.

In pharming, transgenic animals are mostly used to make human proteins that have medicinal value. The protein encoded by the transgene is secreted into the animal's milk, eggs or blood or even urine, and then collected and purified. Livestock such as cattle, sheep, goats, chickens, rabbits and pigs have already been modified in this way to produce several useful proteins and drugs. Some human proteins that are used as drugs require biological modifications that only the cells of mammals, such as cows, goats and sheep, can provide. For these drugs, production in transgenic animals is a good option. Using farm animals for drug production has many advantages: they are reproducible, have flexible production, and are easily maintained. Since the mammary gland and milk are not part of the main life support systems of the animal, there is not much risk of harm to the animal making the transgenic protein. To ensure that the protein coded in the transgene is secreted in the milk, the transgene is attached to a promoter which is only active in the mammary gland. Although the transgene is present in every cell of the animal, it is only active where the milk is made. Some examples of the drugs currently being tested for production in animals are antithrombin III and tissue plasminogen activator used to treat blood clots, erythropoietin for anemia, blood clotting factors VIII and IX for hemophilia, and alpha-1-antitrypsin for emphysema and cystic fibrosis.

The advantage of using biological fermentors have been put as follows: lower drug prices for consumers, production of drugs unavailable any other way, new value-added products for farmers, and inexpensive vaccines for the developing world.

- (a) Lower drug costs**
- (b) Faster, more flexible manufacturing**
- (c) Drugs unavailable any other way**
- (d) New value-added agricultural products**

Special Advantages of Plants as Biological Fermentors

Apart from the advantages accruing in the use of plants and animals as bioreactors, a sector of the biotechnology industry views plants (in comparison with animals) as preferable for protein production for the following reasons.

1. Plants are less controversial than animal production systems

Plants do not normally transmit animal pathogens (that can cause diseases like mad cow). Products obtained by 'pharming' plants are promoted as safer than animalsourced proteins. It must, however, be borne in mind that plants as bioreactors may also present their own risks of product contamination from mycotoxins, pesticides, herbicides or endogenous plant secondary metabolites such as nicotine and glycoalkaloids. Using plants as bioreactors also avoids any animal welfare and certain ethical concerns associated with cloning animals and using them as bioreactors

2. Inexpensive, easily delivered vaccines

Food plants engineered to contain pieces of disease agents can function as orally administered vaccines, avoiding the need for injection and syringes. Currently, tomatoes and other vegetables are under development for that purpose. Although the vaccines would still have to be standardized for dose and delivered to patients one at a time, it is hoped that the lower production costs and the convenience of avoiding refrigeration would make the products attractive to the developing world.



