

Introduction of DNA into living cells

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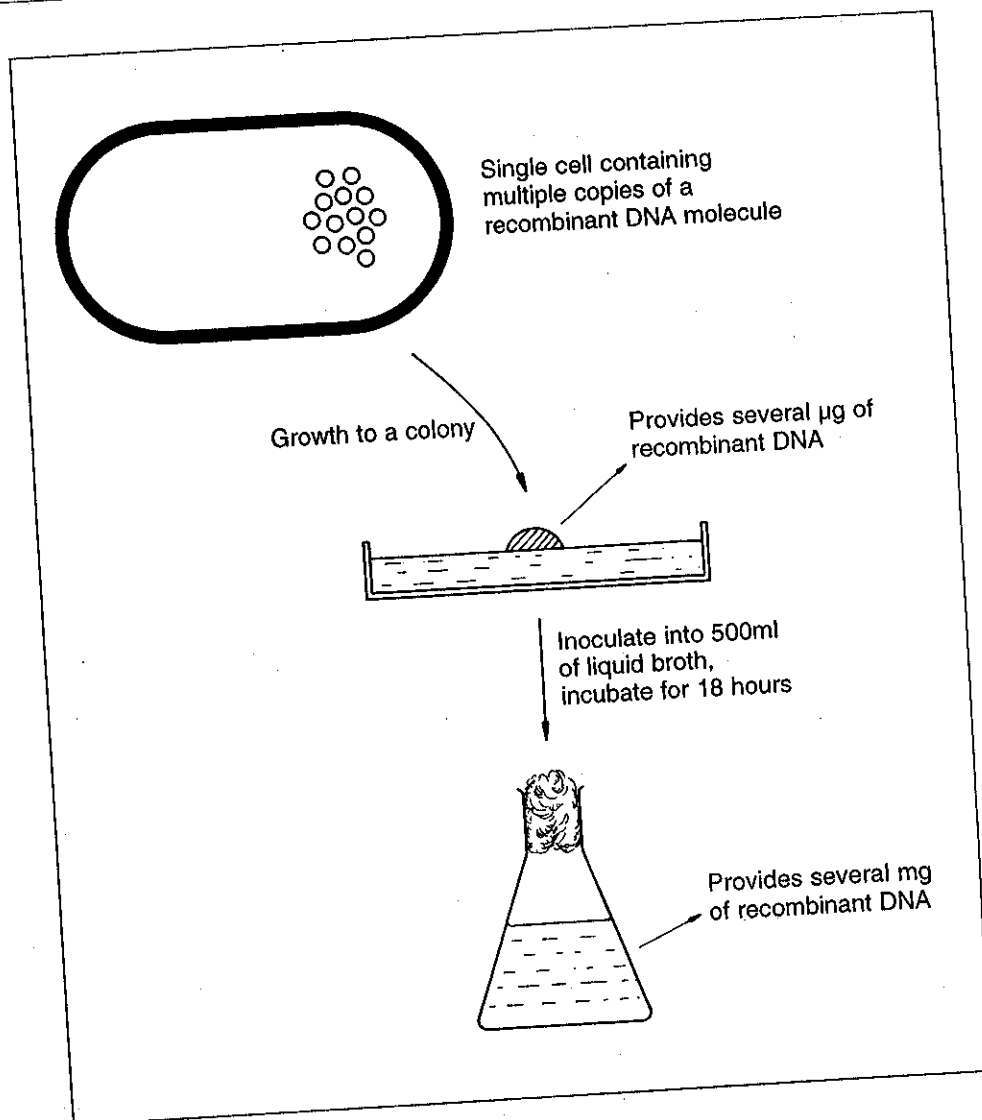
The manipulations described in Chapter 4 allow the molecular biologist to create novel recombinant DNA molecules. The next step in a gene cloning experiment is to introduce these molecules into living cells, usually bacteria, which will then grow and divide to produce clones (Figure 1.1). Strictly speaking, the word 'cloning' refers only to the later stages of the procedure, and not to the construction of the recombinant DNA molecule itself.

Cloning serves two main purposes. Firstly, it allows a large number of recombinant DNA molecules to be produced from a limited amount of starting material. At the outset only a few nanograms of recombinant DNA may be available, but each bacterium that takes up a plasmid will divide numerous times to produce a colony, each cell of which contains multiple copies of the molecule. Several micrograms of recombinant DNA can usually be prepared from a single bacterial colony, representing a thousandfold increase over the starting amount (Figure 5.1). If the colony is used not as a source of DNA, but as an inoculum for a liquid culture, then the resulting cells may provide milligrams of DNA, a millionfold increase in yield. In this way cloning can supply the large amounts of DNA needed for molecular biological studies of gene structure and expression (Chapters 9 and 10).

The second important function of cloning can be described as purification. The manipulations that result in a recombinant DNA molecule can only rarely be controlled to the extent that no other DNA molecules are present at the end of the procedure. The ligation mixture may contain, in addition to the desired recombinant molecule, any number of the following (Figure 5.2(a)):

1. unligated vector molecules;
2. unligated DNA fragments;
3. vector molecules that have recircularized without new DNA being inserted ('self-ligated' vector);
4. recombinant DNA molecules that carry the wrong inserted DNA fragment.

Figure 5.1 Cloning can supply large amounts of recombinant DNA.



Unligated molecules rarely cause a problem because, even though they may be taken up by bacterial cells, only under exceptional circumstances will they be replicated. It is much more likely that the host enzymes will degrade these pieces of DNA. On the other hand, self-ligated vector molecules and incorrect recombinant plasmids will be replicated just as efficiently as the desired molecule (Figure 5.2(b)). However, purification of the desired molecule can still be achieved through cloning because it is extremely unusual for any one cell to take up more than one DNA molecule. Each cell gives rise to a single colony, so each of the resulting clones consists of cells that all contain the same molecule. Of course different colonies contain different molecules: some contain the desired recombinant DNA molecule, some have different recombinant molecules, and some self-ligated vector. The problem therefore becomes a question of identifying the colonies that contain the correct recombinant plasmids.

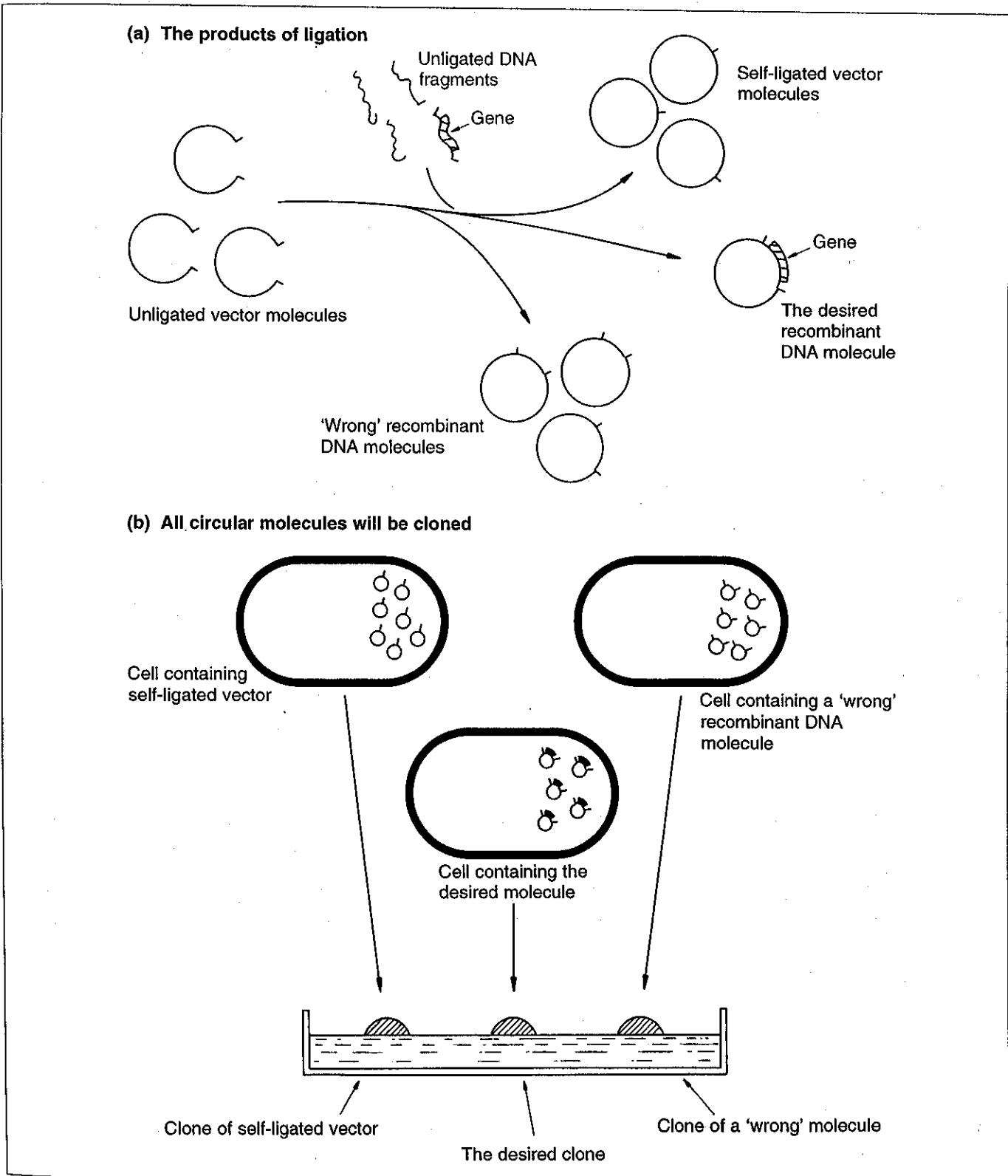



Figure 5.2 Cloning is analogous to purification. From a mixture of different molecules, clones containing copies of just one molecule can be obtained.

This chapter is concerned with the way in which plasmid and phage vectors, and recombinant molecules derived from them, are introduced into bacterial cells. During the course of the chapter it will become apparent that selection for colonies containing recombinant molecules, as opposed to colonies containing self-ligated vector, is relatively easy. The more difficult proposition of how to distinguish clones containing the correct recombinant DNA molecule from all the other recombinant clones will be tackled in Chapter 8.



Most species of bacteria are able to take up DNA molecules from the medium in which they grow. Often a DNA molecule taken up in this way will be degraded, but occasionally it will be able to survive and replicate in the host cell. In particular this will happen if the DNA molecule is a plasmid with an origin of replication recognized by the host.

Uptake and stable retention of a plasmid is usually detected by looking for expression of the genes carried by the plasmid (p. 13). For example, *E. coli* cells are normally sensitive to the growth inhibitory effects of the antibiotics ampicillin and tetracycline. However, cells that contain the plasmid pBR322 (p. 108), one of the first cloning vectors to be developed back in the 1970s, are resistant to these antibiotics. This is because pBR322 carries two sets of genes, one gene that codes for a β -lactamase enzyme that modifies ampicillin into a form that is non-toxic to the bacterium, and a second set of genes that code for enzymes that detoxify tetracycline. Uptake of pBR322 can be detected because the *E. coli* cells are transformed from ampicillin- and tetracycline-sensitive ($\text{amp}^{\text{S}}\text{tet}^{\text{S}}$) to ampicillin- and tetracycline-resistant ($\text{amp}^{\text{R}}\text{tet}^{\text{R}}$).

In recent years the term **transformation** has been extended to include uptake of any DNA molecule by any type of cell, regardless of whether the uptake results in a detectable change in the cell, or whether the cell involved is bacterial, fungal, animal or plant.

5.1.1 Not all species of bacteria are equally efficient at DNA uptake

In nature, transformation is probably not a major process by which bacteria obtain genetic information. This is reflected by the fact that in the laboratory only a few species (notably members of the genera *Bacillus* and *Streptococcus*) can be transformed with ease. Close

study of these organisms has revealed that they possess sophisticated mechanisms for DNA binding and uptake.

Most species of bacteria, including *E. coli*, take up only limited amounts of DNA under normal circumstances. In order to transform these species efficiently, the bacteria have to undergo some form of physical and/or chemical treatment that enhances their ability to take up DNA. Cells that have undergone this treatment are said to be **competent**.

5.1.2 Preparation of competent *E. coli* cells

As with many breakthroughs in recombinant DNA technology, the key development as far as transformation was concerned occurred in the early 1970s, when it was observed that *E. coli* cells that had been soaked in an ice-cold salt solution were more efficient at DNA uptake than unsoaked cells. A solution of 50 mM calcium chloride is traditionally used, although other salts, notably rubidium chloride, are also effective.

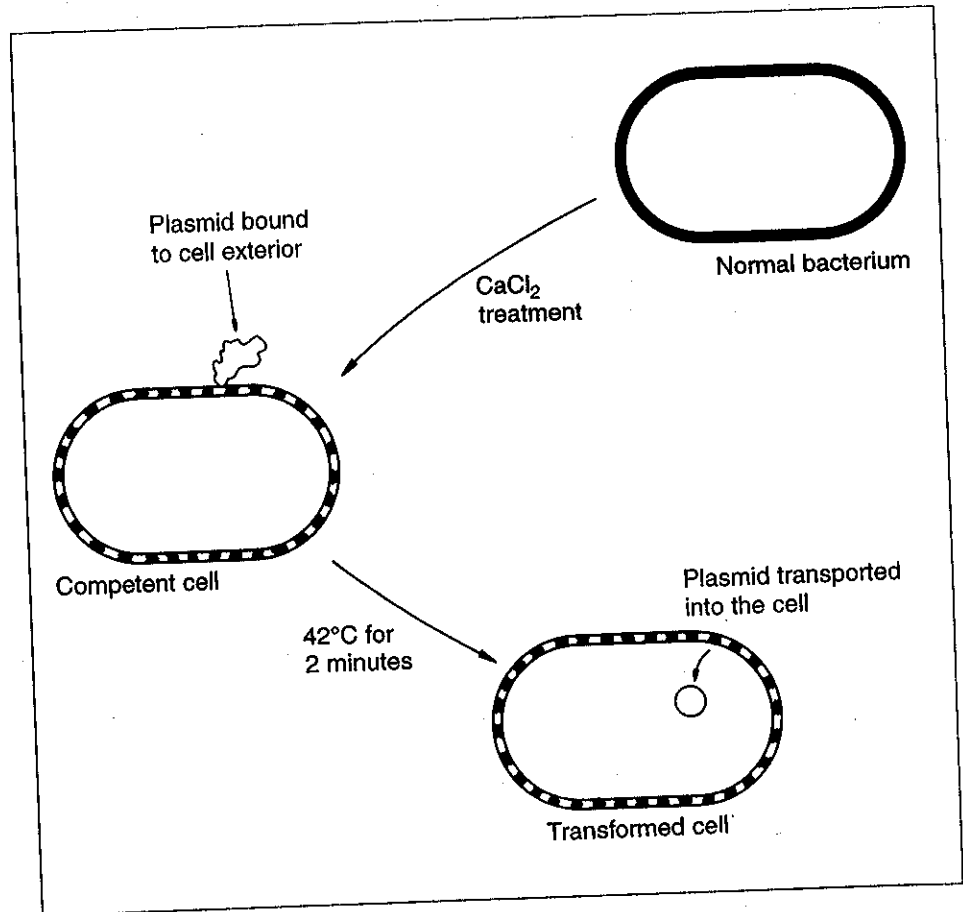
Exactly why this treatment works is not understood. Possibly CaCl_2 causes the DNA to precipitate on to the outside of the cells, or perhaps the salt is responsible for some kind of change in the cell wall that improves DNA binding. In any case, soaking in CaCl_2 affects only DNA binding, and not the actual uptake into the cell. When DNA is added to treated cells, it remains attached to the cell exterior, and is not at this stage transported into the cytoplasm (Figure 5.3). The actual movement of DNA into competent cells is stimulated by briefly raising the temperature to 42°C. Once again, the exact reason why this heat-shock is effective is not understood.

5.1.3 Selection for transformed cells

Transformation of competent cells is an inefficient procedure, however carefully the cells have been prepared. Although 1 ng of pUC8 can yield 1000–10 000 transformants, this represents the uptake of only 0.01% of all the available molecules. Furthermore, 10 000 transformants is only a very small proportion of the total number of cells that are present in a competent culture. This last fact means that some way must be found to distinguish a cell that has taken up a plasmid from the many thousands that have not been transformed.

The answer is to make use of a **selectable marker** carried by the plasmid. A selectable marker is simply a gene that provides a transformed cell with a new characteristic, one that is not possessed by a non-transformant. A good example of a selectable marker is the ampicillin resistance gene of pBR322. After a transformation experiment with pBR322, only those *E. coli* cells that have taken up a plasmid will be $\text{amp}^{\text{R}}\text{tet}^{\text{R}}$ and able to form

Figure 5.3 The binding and uptake of DNA by a competent bacterial cell.



colonies on an agar medium that contains ampicillin or tetracycline (Figure 5.4); non-transformants, which are still $amp^s tet^s$, will not produce colonies on the selective medium. Transformants and non-transformants are therefore easily distinguished.

Most plasmid cloning vectors carry at least one gene that confers antibiotic resistance on the host cells, with selection of transformants being achieved by plating on to an agar medium that contains the relevant antibiotic. Bear in mind, however, that resistance to the antibiotic is not due merely to the presence of the plasmid in the transformed cells. The resistance gene on the plasmid must also be expressed, so that the enzyme that detoxifies the antibiotic is synthesized. Expression of the resistance gene begins immediately after transformation, but it will be a few minutes before the cell contains enough of the enzyme to be able to withstand the toxic effects of the antibiotic. For this reason the transformed bacteria should not be plated on to the selective medium immediately after the heat-shock treatment, but first placed in a small volume of liquid medium, in the absence of antibiotic, and incubated for a short time. Plasmid replication and expression can then get started, so that when the cells are plated out and encounter the antibiotic, they will already have synthesized sufficient resistance enzymes to be able to survive (Figure 5.5).

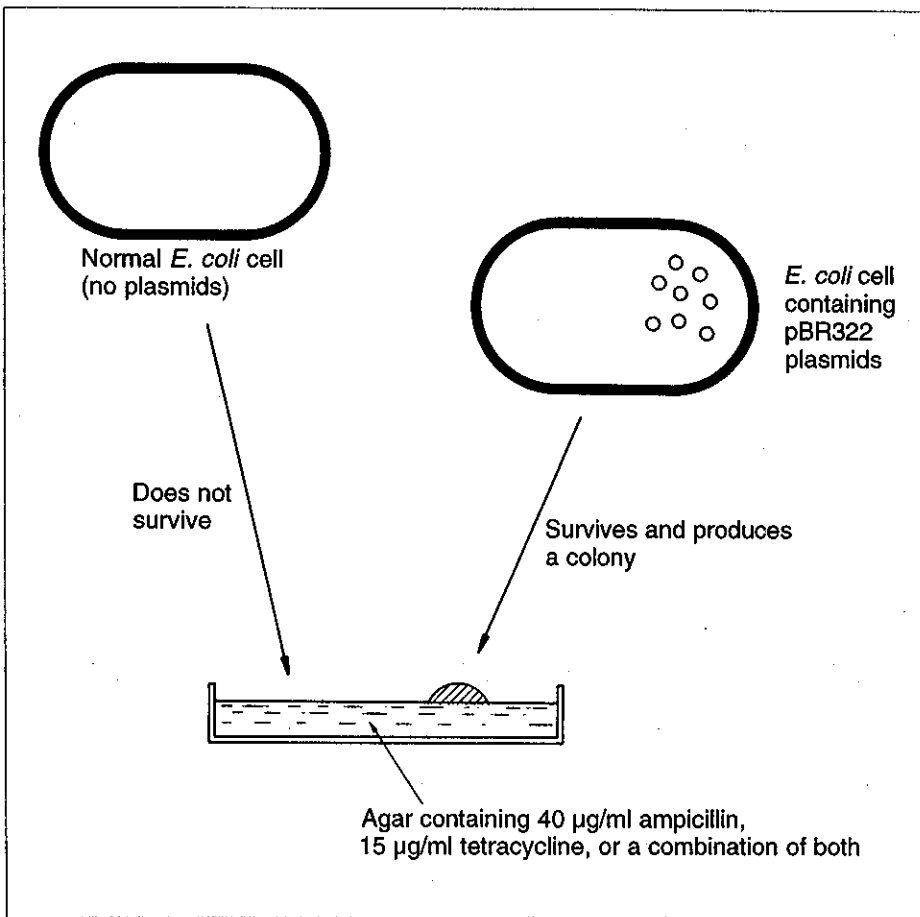


Figure 5.4 Selecting cells that contain pBR322 plasmids by plating on to agar medium containing ampicillin and/or tetracycline.

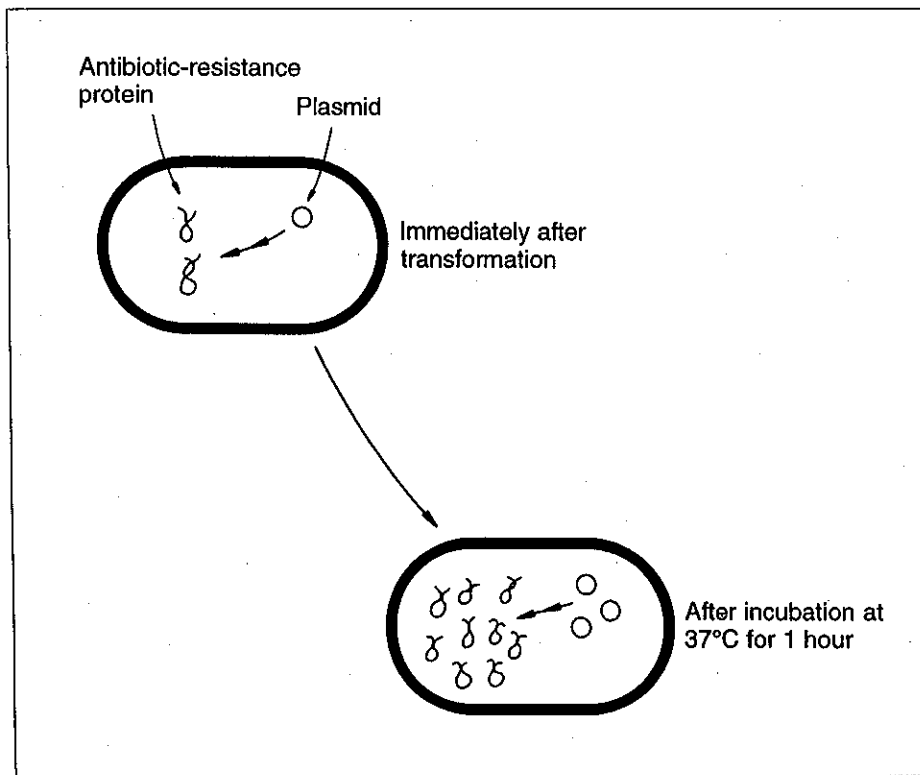
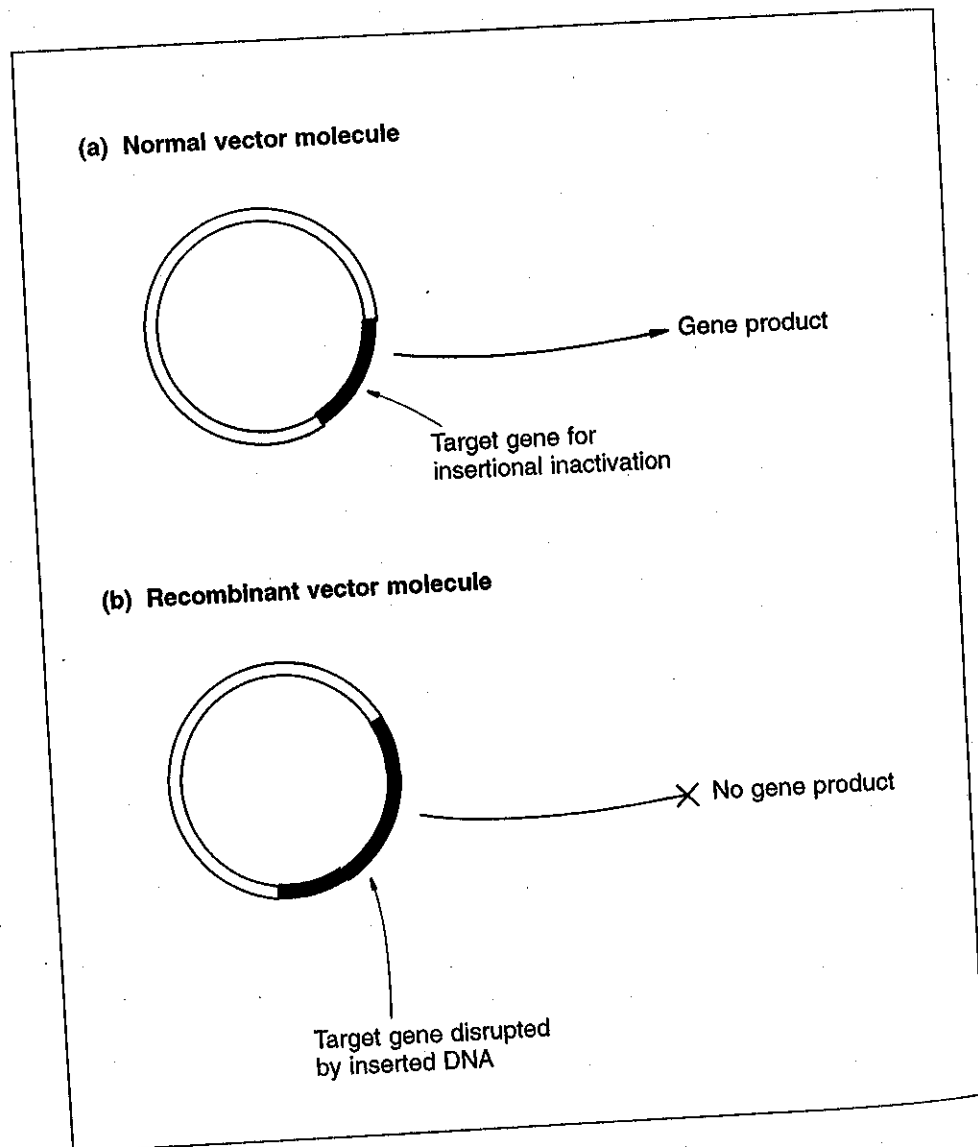


Figure 5.5 Phenotypic expression. Incubation at 37°C for 1 h before plating out improves the survival of the transformants on selective medium, because the bacteria have had time to begin synthesis of the antibiotic resistance enzymes.

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Plating on to a selective medium enables transformants to be distinguished from non-transformants. The next problem is to determine which of the transformed colonies comprise cells that contain recombinant DNA molecules, and which contain self-ligated vector molecules (see Figure 5.2). With most cloning vectors insertion of a DNA fragment into the plasmid destroys the integrity of one of the genes present on the molecule. **Recombinants** can therefore be identified because the characteristic coded by the inactivated gene is no longer displayed by the host cells (Figure 5.6). The general principles of **insertional inactivation** are illustrated by a typical cloning experiment using pBR322 as the vector.

Figure 5.6 Insertional inactivation. (a) The normal, non-recombinant vector molecule carries a gene whose product confers a selectable or identifiable characteristic on the host cell. (b) This gene is disrupted when new DNA is inserted into the vector; as a result the recombinant host does not display the relevant characteristic.



5.2.1 Recombinant selection with pBR322 – insertional inactivation of an antibiotic resistance gene

pBR322 has several unique restriction sites that can be used to open up the vector before insertion of a new DNA fragment (Figure 5.7(a)). *Bam*HI, for example, cuts pBR322 at just one position, within the cluster of genes that code for resistance to tetracycline. A recombinant pBR322 molecule, one that carries an extra piece of DNA in the *Bam*HI site (Figure 5.7(b)), is no longer able to confer tetracycline resistance on its host, as one of the necessary genes is now disrupted by the inserted DNA. Cells containing this recombinant pBR322 molecule will still be resistant to ampicillin, but sensitive to tetracycline ($\text{amp}^{\text{R}}\text{tet}^{\text{S}}$).

Screening for pBR322 recombinants is performed in the following way. After transformation the cells are plated on to ampicillin medium and incubated until colonies appear (Figure 5.8(a)). All of these colonies are transformants (remember, untransformed cells are amp^{S} so do not produce colonies on the selective medium), but

Figure 5.7 The cloning vector pBR322. (a) The normal vector molecule. (b) A recombinant molecule containing an extra piece of DNA inserted into the *Bam*HI site. For a more detailed map of pBR322 see Figure 6.1.

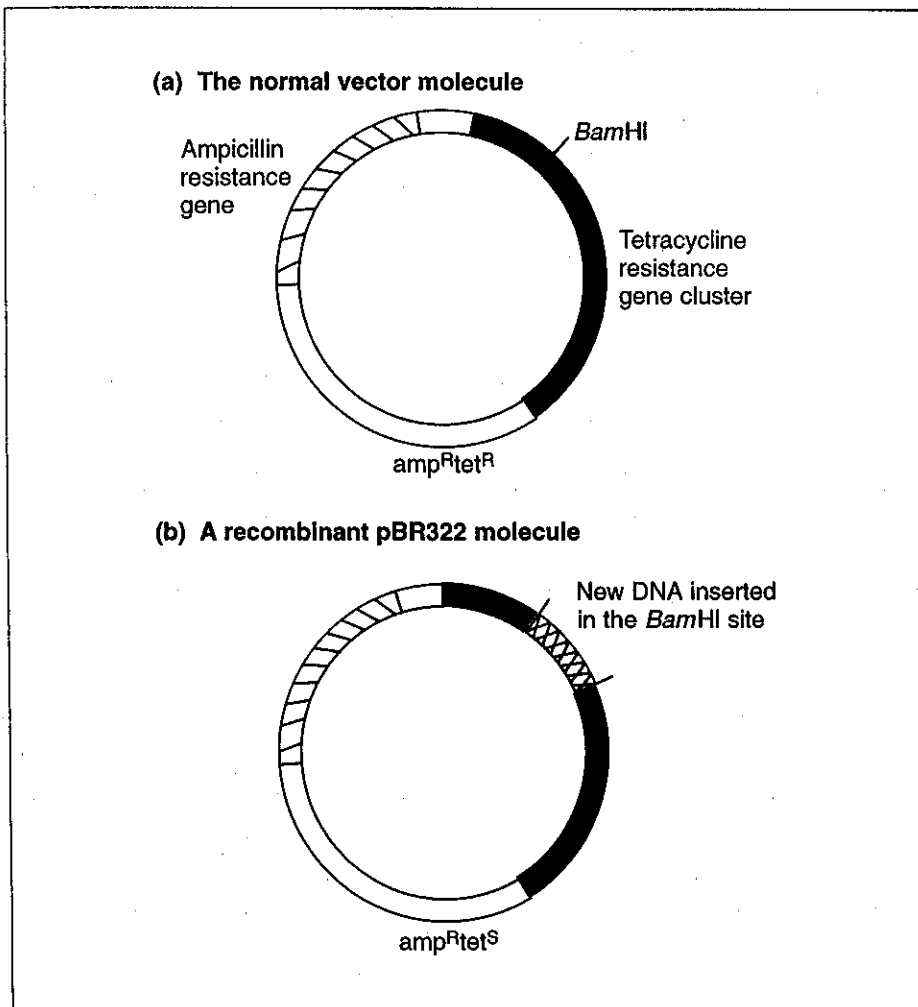
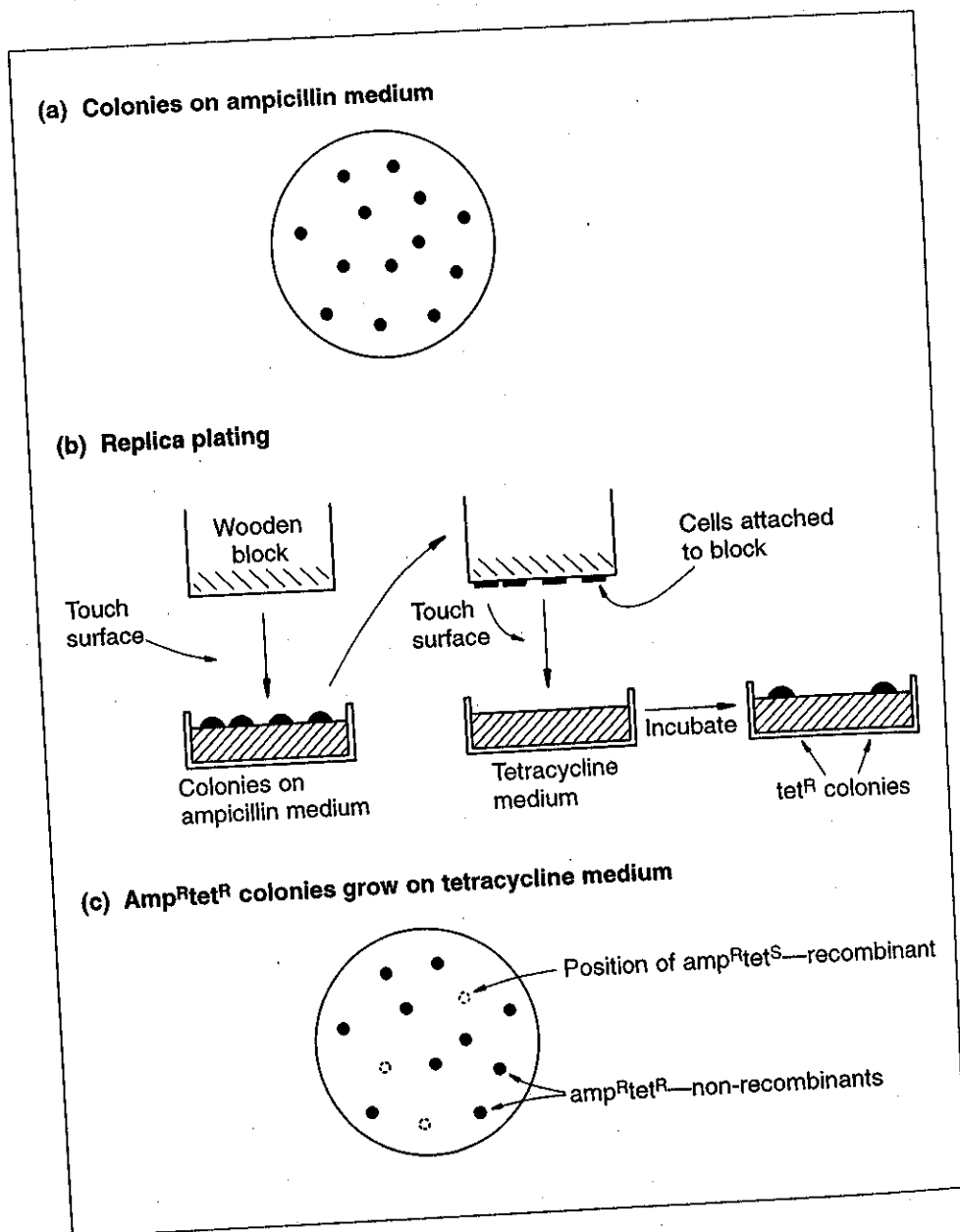


Figure 5.8 Screening for pBR322 recombinants by insertional inactivation of the tetracycline resistance gene. (a) Cells are plated on to ampicillin agar: all the transformants produce colonies. (b) The colonies are replica-plated on to tetracycline medium. (c) The colonies that grow on tetracycline medium are $\text{amp}^{\text{R}}\text{tet}^{\text{R}}$ and therefore non-recombinants. Recombinants ($\text{amp}^{\text{R}}\text{tet}^{\text{S}}$) do not grow, but their position on the ampicillin plate is now known.



only a few contain recombinant pBR322 molecules, most contain the normal, self-ligated plasmid. To identify the recombinants the colonies are **replica-plated** on to agar medium that contains tetracycline (Figure 5.8(b)). After incubation, some of the original colonies will regrow, others will not (Figure 5.8(c)). Those that do grow consist of cells that carry the normal pBR322 with no inserted DNA and therefore a functional tetracycline resistance gene cluster ($\text{amp}^{\text{R}}\text{tet}^{\text{R}}$). The colonies that do not grow on tetracycline agar are recombinants ($\text{amp}^{\text{R}}\text{tet}^{\text{S}}$); once their positions are known samples for further study can be recovered from the original ampicillin agar plate.

5.2.2 Insertional inactivation does not always involve antibiotic resistance

Although insertional inactivation of an antibiotic resistance gene provides a convenient means of recombinant identification, several important plasmid cloning vectors make use of a different system. An example is pUC8 (Figure 5.9(a)), which carries the ampicillin resistance gene and a gene called *lacZ'*, which codes for part of the enzyme β -galactosidase. Cloning with pUC8 involves insertional inactivation of the *lacZ'* gene, with recombinants identified because of their inability to synthesize β -galactosidase (Figure 5.9(b)).

β -galactosidase is one of a series of enzymes involved in the breakdown of lactose to glucose plus galactose. It is normally coded by the gene *lacZ*, which resides on the *E. coli* chromosome. Some strains of *E. coli* have a modified *lacZ* gene, one that lacks the segment referred to as *lacZ'* and coding for the α -peptide portion of β -galactosidase (Figure 5.10(a)). These mutants can synthesize

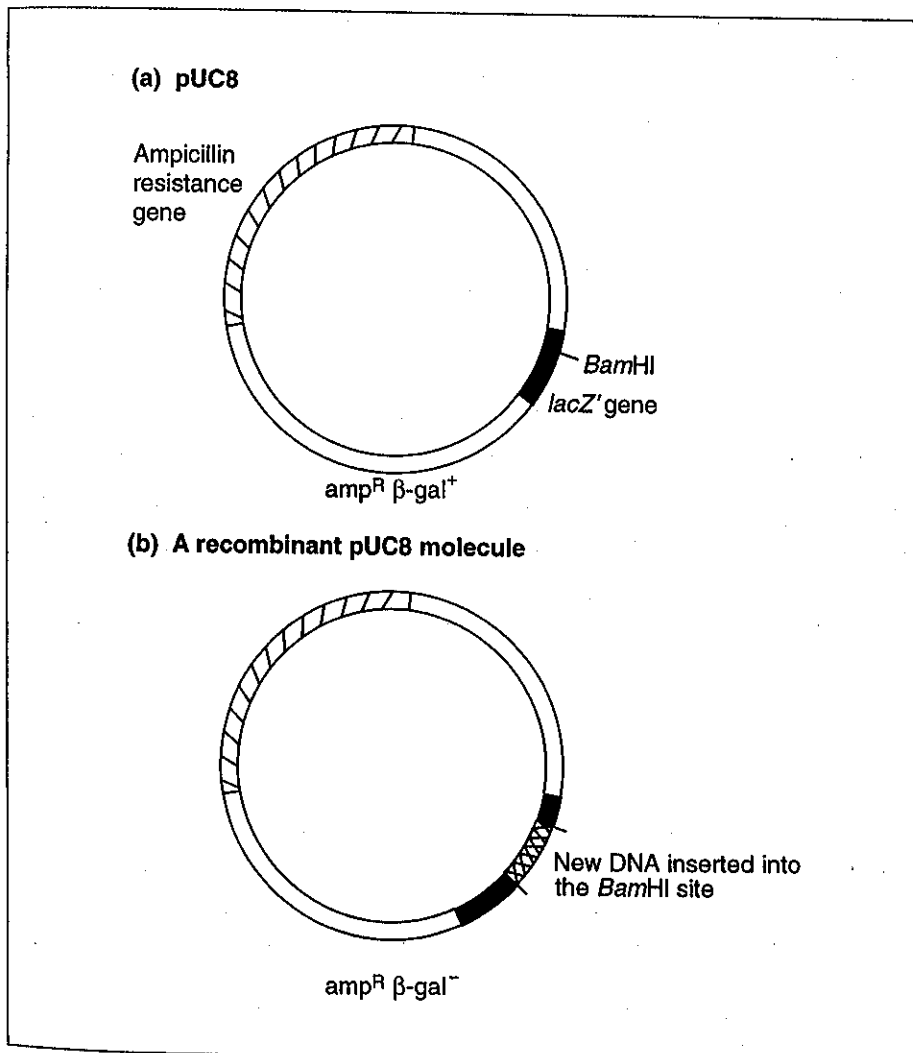
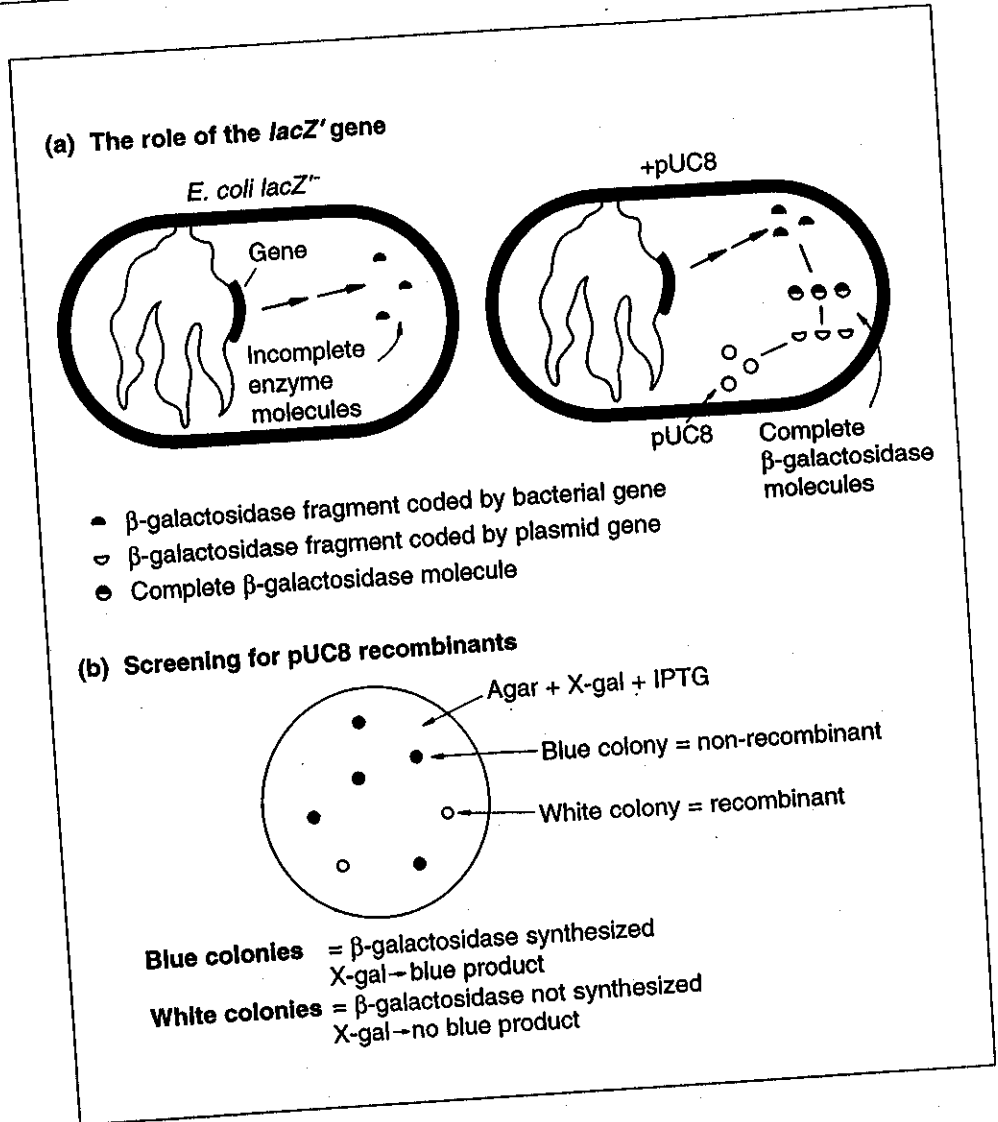


Figure 5.9 The cloning vector pUC8. (a) The normal vector molecule. (b) A recombinant molecule containing an extra piece of DNA inserted into the *Bam*HI site. For more detailed maps of pUC8 see Figures 6.3 and 6.4.

Figure 5.10 The rationale behind insertional inactivation of the *lacZ'* gene carried by pUC8. (a) The bacterial and plasmid genes complement each other to produce a functional β -galactosidase molecule. (b) Recombinants are screened by plating on to agar containing X-gal + IPTG.



the enzyme only when they harbour a plasmid, such as pUC8, that carries the missing *lacZ'* segment of the gene.

A cloning experiment with pUC8 involves selection of transformants on ampicillin agar followed by screening for β -galactosidase activity to identify recombinants. Cells that harbour a normal pUC8 plasmid are amp^R and able to synthesize β -galactosidase (Figure 5.9(a)); recombinants are also amp^R but unable to make β -galactosidase (Figure 5.9(b)).

Screening for β -galactosidase presence or absence is in fact quite easy. Rather than assay for lactose being split to glucose and galactose, a slightly different reaction catalysed by the enzyme is tested for. This involves a lactose analogue called X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) which is broken down by β -galactosidase to a product that is coloured deep blue. If X-gal (plus an inducer of the enzyme such as isopropyl-thiogalactoside, IPTG) is added to the agar, along with ampicillin, then non-recombinant colonies, the cells of which synthesize β -galactosidase, will

be coloured blue, whereas recombinants with a disrupted *lacZ'* gene and unable to make β -galactosidase, will be white. This system, which is called **Lac selection**, is summarized in Figure 5.10(b)).



There are two different methods by which a recombinant DNA molecule constructed with a phage vector can be introduced into a bacterial cell: transfection and *in vitro* packaging.

(a) Transfection This process is equivalent to transformation, the only difference being that phage DNA rather than a plasmid is involved. Just as with a plasmid, the purified phage DNA, or recombinant phage molecule, is mixed with competent *E. coli* cells and DNA uptake induced by heat-shock.

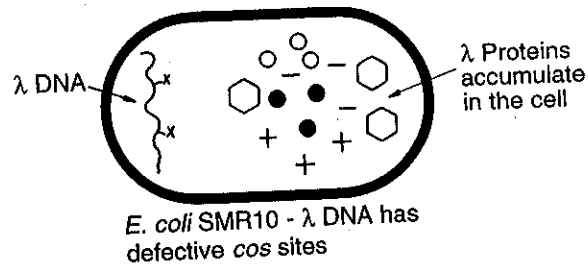
(b) *In vitro* packaging Transfection with λ DNA molecules is not a very efficient process when compared with the infection of a culture of cells with mature λ phage particles. It would therefore be useful if recombinant λ molecules could be packaged into the λ head-and-tail structures in the test-tube.

This may sound difficult but in fact is relatively easy to achieve. Packaging requires a number of different proteins coded by the λ genome, but these can be prepared at a high concentration from cells infected with defective λ phage strains. Two different systems are in use. With the single-strain system the defective λ phage carries a mutation in the *cos* sites, so that these are not recognized by the endonuclease that normally cleaves the λ concatamers during phage replication (p. 23). The defective phage cannot therefore replicate, though it does direct synthesis of all the proteins needed for packaging. The proteins accumulate in the bacterium and can be purified from cultures of *E. coli* infected with the mutated λ , and used for *in vitro* packaging of recombinant λ molecules (Figure 5.11(a)).

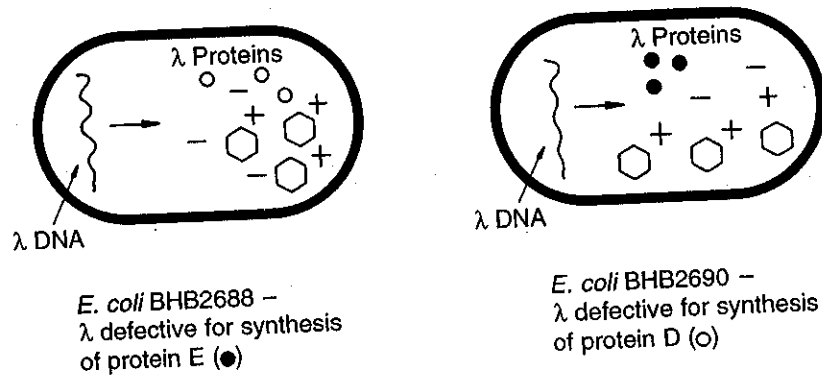
With the second system two defective λ strains are needed. Both of these strains carry a mutation in a gene for one of the components of the phage protein coat: with one strain the mutation is in gene *D*, and with the second strain it is in gene *E* (see Figure 2.9). Neither strain is able to complete an infection cycle in *E. coli* as in the absence of the product of the mutated gene the complete capsid structure cannot be made. Instead the products of all the other coat protein genes accumulate (Figure 5.11(b)). An *in vitro* packaging mix can therefore be prepared by combining lysates of two

Figure 5.11 *In vitro* packaging.
 (a) Synthesis of λ capsid proteins by *E. coli* strain SMR10, which carries a λ phage that has defective *cos* sites.
 (b) Synthesis of incomplete sets of λ capsid proteins by *E. coli* strains BHB2688 and BHB2690. (c) A mixture of cell lysates provides the complete set of capsid proteins and can package λ DNA molecules in the test-tube.

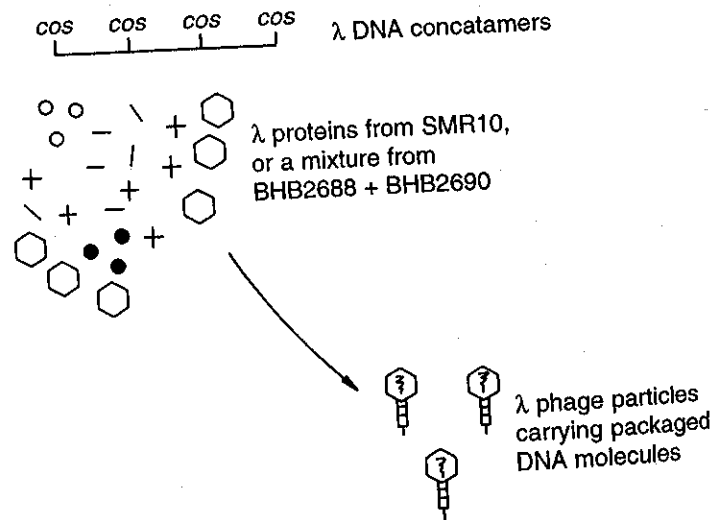
(a) A single-strain packaging system



(b) A two-strain packaging system



(c) *In vitro* packaging



cultures of cells, one infected with the λ *D*⁻ strain, the other infected with the *E*⁻ strain. The mixture now contains all the necessary components and will package recombinant λ molecules into mature phage particles (Figure 5.11(c)). With both systems, pack-

aged molecules can be introduced into *E. coli* cells simply by adding the assembled phage to the bacterial culture and allowing the normal λ infective process to take place.

5.3.1 Phage infection is visualized as plaques on an agar medium

The final-stage of the phage infection cycle is cell lysis (p. 19). If infected cells are spread on to a solid agar medium immediately after addition of the phage, or immediately after transfection with phage DNA, then cell lysis can be visualized as **plaques** on a lawn of bacteria (Figure 5.12(a)). Each plaque is a zone of clearing produced as the phage lyse the cells and move on to infect and eventually lyse the neighbouring bacteria (Figure 5.12(b)).

Both λ and M13 form plaques. With λ these are true plaques, produced by cell lysis. However, M13 plaques are slightly

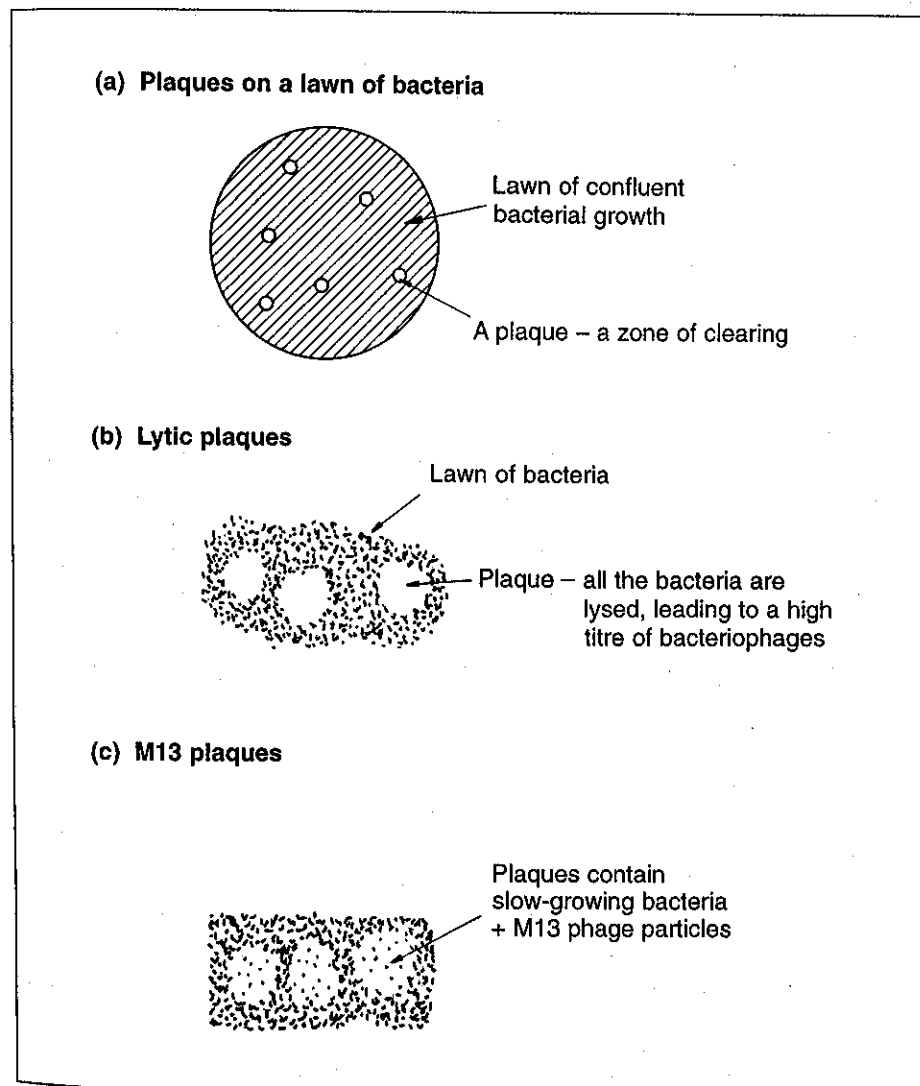



Figure 5.12 Bacteriophage plaques. (a) The appearance of plaques on a lawn of bacteria. (b) Plaques produced by a phage that lyses the host cell (e.g. λ in the lytic infection cycle); the plaques contain lysed cells plus many phage particles. (c) Plaques produced by M13; these plaques contain slow-growing bacteria plus many M13 phage particles.

different as M13 does not lyse the host cells (p. 20). Instead M13 causes a decrease in the growth rate of infected cells sufficient to produce a zone of relative clearing on a bacterial lawn. Although not a true plaque, these zones of clearing are visually identical to normal phage plaques (Figure 5.12(c)).

The end result of a gene cloning experiment using a λ or M13 vector is therefore an agar plate covered in phage plaques. Each plaque is derived from a single transfected or infected cell and therefore contains identical phage particles. These may contain self-ligated vector molecules, or they may be recombinants.



A variety of ways of distinguishing recombinant plaques have been devised, the following being the most important.

5.4.1 Insertional inactivation of a *lacZ'* gene carried by the phage vector

All M13 cloning vectors (p. 115), as well as several λ vectors, carry a copy of the *lacZ'* gene. Insertion of new DNA into this gene inactivates β -galactosidase synthesis, just as with the plasmid vector pUC8. Recombinants are distinguished by plating cells on to X-gal agar: plaques comprising normal phage are blue, recombinant plaques are clear (Figure 5.13(a)).

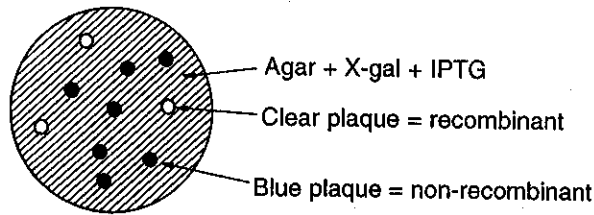
5.4.2 Insertional inactivation of the λ *cI* gene

Several types of λ cloning vector have unique restriction sites in the *cI* gene (map position 38 on Figure 2.9). Insertional inactivation of this gene causes a change in plaque morphology. Normal plaques appear 'turbid', whereas recombinants with a disrupted *cI* gene are 'clear' (Figure 5.13(b)). The difference is readily apparent to the experienced eye.

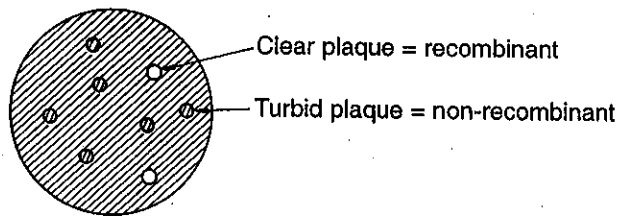
5.4.3 Selection using the *Spi* phenotype

λ phage cannot normally infect *E. coli* cells that already possess an integrated form of a related phage called P2. λ is therefore said to be *Spi*⁺ (sensitive to P2 prophage inhibition). Some λ cloning vectors are designed so that insertion of new DNA causes a change from *Spi*⁺ to *Spi*⁻, thus the recombinants can infect cells that carry P2 prophages. Such cells are used as the host for cloning experiments with these vectors; only recombinants are *Spi*⁻ so only recombinants form plaques (Figure 5.13(c)).

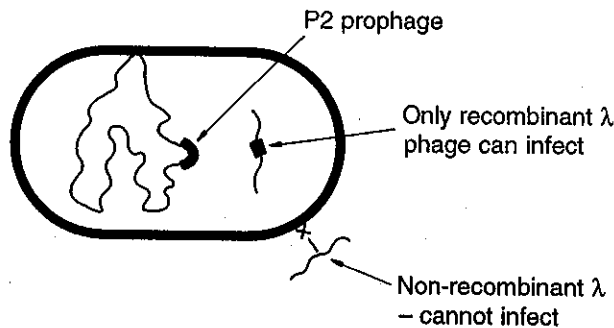
(a) Insertional inactivation of the *lacZ'* gene



(b) Insertional inactivation of the λ *ci* gene



(c) Selection using the Spi phenotype



(d) Selection on the basis of λ genome size

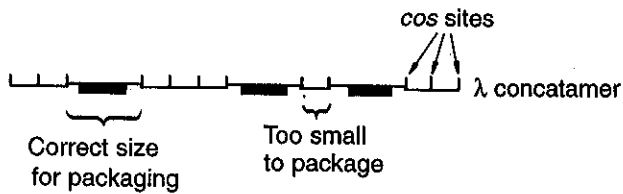


Figure 5.13 Strategies for the selection of recombinant phage.

5.4.4 Selection on the basis of λ genome size

The λ packaging system, which assembles the mature phage particles, can only insert DNA molecules of between 37 and 52 kb into the head structure. Anything less than 37 kb will not be packaged.

Many λ vectors have been constructed by deleting large segments of the λ DNA molecule (p. 123) and so are less than 37 kb in length. These will only package into mature phage particles after extra DNA has been inserted, bringing the total genome size up to 37 kb or more (Figure 5.13(d)). Therefore with these vectors only recombinant phage are able to replicate.

TRANSFORMATION OF NON-BACTERIAL CELLS

Ways of introducing DNA into yeast, fungi, animals and plants are also needed if these organisms are to be used as the hosts for gene cloning. In general terms, soaking cells in salt is effective only with a few species of bacteria, although treatment with lithium chloride or lithium acetate does enhance DNA uptake by yeast cells, and is frequently used in the transformation of *Saccharomyces cerevisiae*. However, for most higher organisms more sophisticated methods are needed.

5.5.1 Transformation of individual cells

With most organisms the main barrier to DNA uptake is the cell wall. Cultured animal cells, which usually lack cell walls, are easily transformed, especially if the DNA is precipitated on to the cell surface with calcium phosphate (Figure 5.14(a)). For other types of cell the answer is often to remove the cell wall. Enzymes that degrade yeast, fungal and plant cell walls are available, and under the right conditions intact **protoplasts** can be obtained (Figure 5.14(b)). Protoplasts generally take up DNA quite readily; alternatively transformation can be stimulated by special techniques such as **electroporation**, during which the cells are subjected to a short electrical pulse, thought to induce the transient formation of pores in the cell membrane through which the DNA molecules can enter the cell. After transformation the protoplasts are washed to remove the degradative enzymes and the cell wall spontaneously reforms.

In contrast to the transformation systems described so far there are two physical methods for introducing DNA into cells. The first of these is **microinjection**, which makes use of a very fine pipette to inject DNA molecules directly into the nucleus of the cells to be transformed (Figure 5.15(a)). This technique was initially applied to animal cells but has subsequently been successful with plant cells. The second method involves bombardment of the cells with high-velocity microprojectiles, usually particles of gold or tungsten that have been coated with DNA. These microprojectiles are fired at the cells from a particle gun (Figure 5.15(b)). This unusual

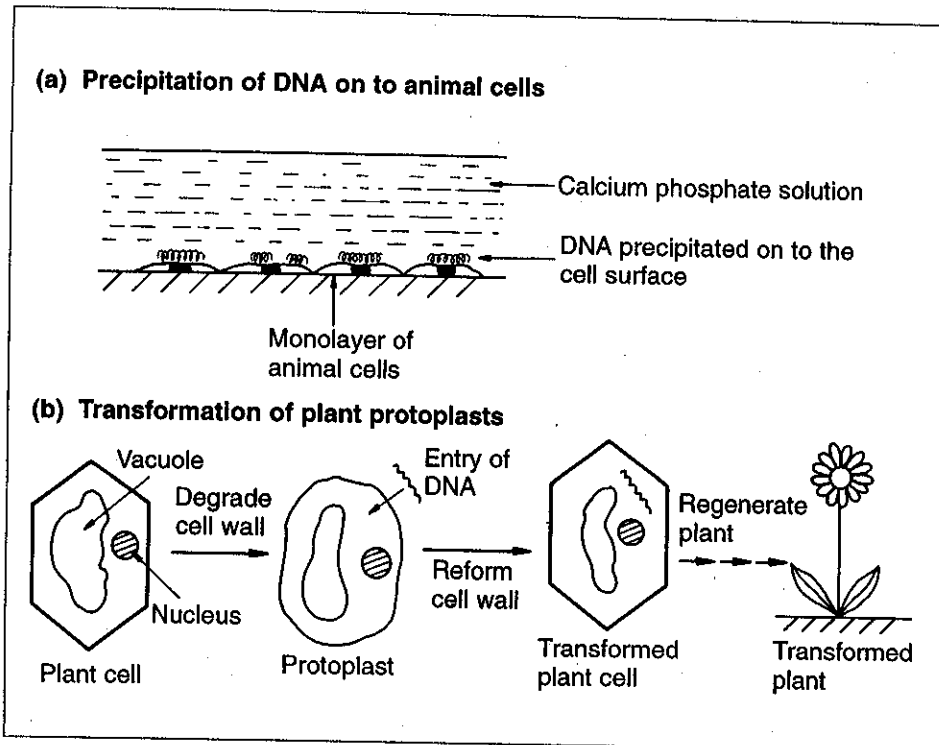


Figure 5.14 Strategies for introducing new DNA into animal and plant cells.

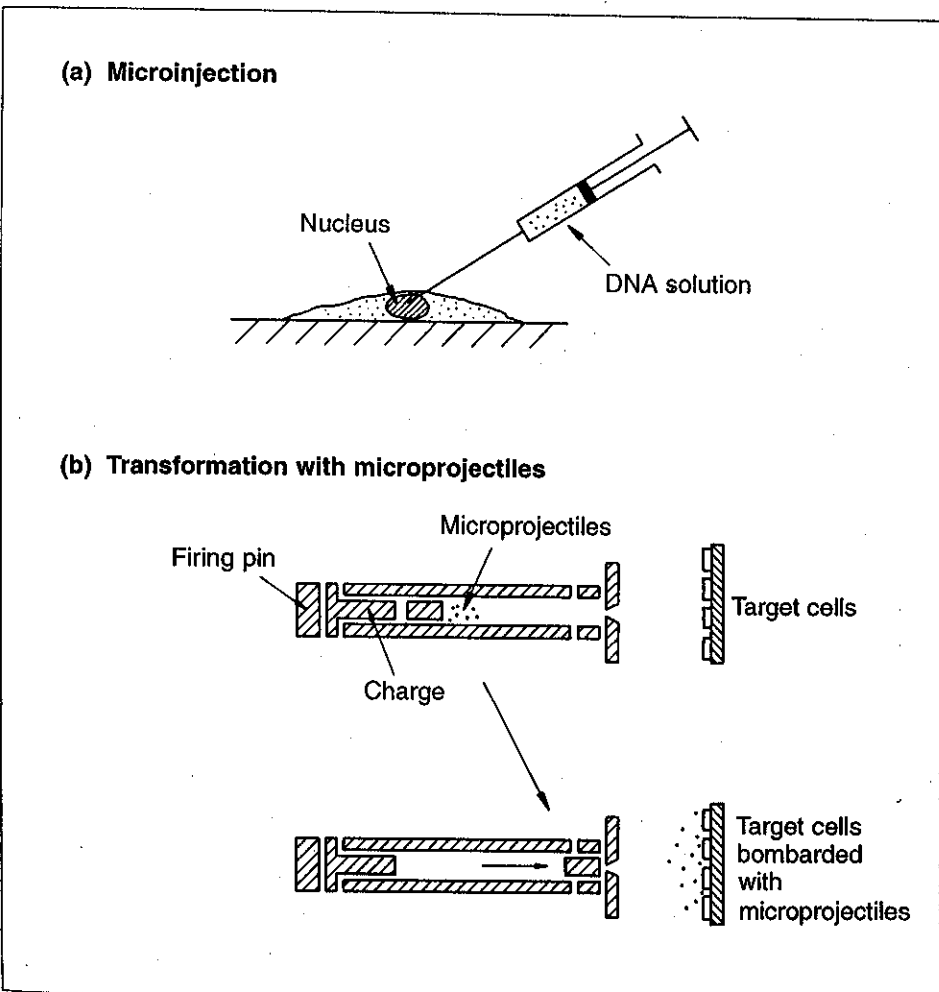


Figure 5.15 Two physical methods for introducing DNA into cells.

technique is termed **biolistics** and has been used with a number of different types of cell.

5.5.2 Transformation of whole organisms

With animals and plants the desired end-product may not be transformed cells, but a transformed organism. Plants are relatively easy to regenerate from cultured cells, though problems have been experienced in developing regeneration procedures for monocotyledonous species such as cereals and grasses. A single transformed plant cell can therefore give rise to a transformed plant, which will carry the cloned DNA in every cell, and will pass the cloned DNA on to its progeny following flowering and seed formation (Figure 7.13(b)). Animals of course cannot be regenerated from cultured cells, so obtaining transformed animals requires a rather more subtle approach. The standard technique with mammals such as mice is to remove fertilized eggs from the oviduct, to microinject DNA, and then to re-implant the transformed cells into the mother's reproductive tract.

FURTHER READING

- Cohen, S. N. *et al.* (1972) Nonchromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R-factor DNA. *Proceedings of the National Academy of Sciences USA*, **69**, 2110-14 - transformation of a bacterium with a plasmid.
- Mandel, M. and Higa, A. (1970) Calcium-dependent bacteriophage DNA infection. *Journal of Molecular Biology*, **53**, 154-62 - transfection.
- Hohn, B. and Murray, K. (1977) Packaging recombinant DNA molecules into bacteriophage particles *in vitro*. *Proceedings of the National Academy of Sciences USA*, **74**, 3259-63 - *in vitro* packaging.
- Calvin, N. M. and Hanawalt, P. C. (1988) High-efficiency transformation of bacterial cells by electroporation. *Journal of Bacteriology*, **170**, 2796-801.
- Capecchi, M. R. (1980) High efficiency transformation by direct microinjection of DNA into cultured mammalian cells. *Cell*, **22**, 479-88.
- Klein, T. M. *et al.* (1987) High velocity microprojectiles for delivering nucleic acids into living cells. *Nature*, **327**, 70-3 - biolistics.
- Hammer, R. E., Pursel, V. G., Rexroad, C. E., Wall, R. J., Bolt, D. J., Ebert, K. M., Palmiter, R. D. and Brinster, R. L. (1985) Production of transgenic rabbits, sheep and pigs by microinjection. *Nature*, **315**, 680-3.