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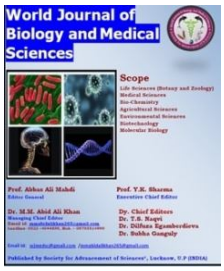

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REVIEW ARTICLE

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The Use of Gene Vectors in Molecular Biology

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ABSTRACT

Bacterial transformation may be referred to as a stable genetic change brought about by the uptake of naked DNA (DNA without associated cells or proteins) to increase DNA quantity and competence refers to the state of being able to take up exogenous DNA from the environment. There are two forms of transformation and competence: natural and artificial in molecular biology, transformation is the genetic alteration of a cell resulting from the direct uptake and incorporation of exogenous genetic material (exogenous DNA) from its surroundings and taken up through the cell membrane(s). Transformation occurs naturally in some species of bacteria, but it can also be affected by artificial means in other cells. For transformation to happen, bacteria must be in a state of competence, which might occur as a time-limited response to environmental conditions such as starvation and cell density

Keywords: Gene, Vectors and Biology

INTRODUCTION

Transformation is one of three processes by which exogenous genetic material may be introduced into a bacterial cell, the other two being conjugation (transfer of genetic material between two bacterial cells in direct contact) and transduction (injection of foreign DNA by a bacteriophage virus into the host bacterium). "Transformation" may also be used to describe the insertion of new genetic material into nonbacterial cells, including animal and plant cells; however, because "transformation" has a special meaning in relation to animal cells,

indicating progression to a cancerous state, the term should be avoided for animal cells when describing introduction of exogenous genetic material. Introduction of foreign DNA into eukaryotic cells is often called "transfection. Transformation was first demonstrated in 1928 by British bacteriologist Frederick Griffith. Griffith discovered that a harmless strain of Streptococcus pneumoniae could be made virulent after being exposed to heat-killed virulent strains. Griffith hypothesized that some "transforming principle" from the heat-killed strain was responsible for making the harmless strain virulent. In 1944 this "transforming principle" was identified as being genetic by Oswald Avery, Colin MacLeod, and Maclyn McCarty. They isolated DNA from a virulent strain of S. pneumoniae and using just this DNA was able to make a harmless strain virulent. They called this uptake and incorporation of DNA by bacteria "transformation" (See Avery-MacLeod-McCarty experiment). The results of Avery et al.'s experiments were at first skeptically received by the scientific community and it was not until the development of genetic markers and the discovery of other methods of genetic transfer (conjugation in 1947 and transduction in 1953) by Joshua Lederberg that Avery's experiments were accepted.

It was originally thought that Escherichia coli, a commonly used laboratory organism, were refractory to transformation. However, in 1970, Morton Mandel and Akiko Higa showed that E. coli may be induced to take up DNA from bacteriophage λ without the use of helper phage after treatment with calcium chloride solution. Two years later in 1972, Stanley Cohen, Annie Chang and Leslie Hsu showed that CaCl2 treatment is also effective for transformation of plasmid DNA. The method of transformation by Mandel and Higa was later improved upon by Douglas Hanahan. The discovery of artificially induced competence in E. coli created an efficient and convenient procedure for transforming bacteria which allows for simpler molecular cloning methods in biotechnology and research, and it is now a routinely used laboratory procedure. Transformation using electroporation was developed in the late 1980s, increasing the efficiency of in-vitro transformation and increasing the number of bacterial strains that could be transformed. Transformation of animal and plant cells was also investigated with the first transgenic mouse being created by injecting a gene for a rat growth hormone into a mouse embryo in 1982. In 1907 a bacterium that caused plant tumors, Agrobacterium tumefaciens, was discovered and in the early 1970s the tumor inducing agent was found to be a DNA plasmid called the Ti plasmid. By removing the genes in the plasmid that caused the tumor and adding in novel genes researchers were able to infect plants with A. tumefaciens and let the bacteria insert their chosen DNA into the genomes of the plants. Not all plant cells are susceptible to infection by A. tumefaciens so other methods were developed including electroporation and micro-injection. Particle bombardment was made possible with the invention of the Biolistic Particle Delivery System (gene gun) by John Sanford in the 1980s.

Both prokaryotic and eukaryotic host-vector systems can be used in GM work. This section initially concentrates on prokaryotic host-vector systems with some details of eukaryotic ones being given later.

A prokaryotic vector should

1. be capable of autonomous replication independent of the main bacterial

chromosome, i.e. possess an origin of replication (ori).
2. be easy to isolate, i.e. small.
3. be non-toxic to host cells.
4. Have space for foreign inserts.
5. Have unique restriction sites for common restriction enzymes.
6. Have convenient markers for selection of transformants, e.g. antibiotic resistance genes.
7. Be relaxed, i.e. multiple copies in a host cell.

Most prokaryotic vectors are based on:
1. Plasmids
2. Bacteriophages
3. Cosmids (artificial constructions)

1. PLASMID VECTORS

Plasmids are:

Circular, autonomous molecules of DNA

Found naturally in most bacterial (and some other) species.
Size: 1.5 - 300 kilobases.
Function: carry non-essential (dispensable) genes, e.g. antibiotic resistance, toxin production.
But "cryptic" plasmids have no known function!
Plasmids can be conjugative or non-conjugative (conjugation is generally not required in GM).
Plasmids can be mobilizable or non-mobilizable (non-mobilizable plasmids are preferred as they are less likely to "escape" from host cells).
Plasmids can be relaxed (multiple copies per host cell) or stringent (1-3 copies per host cell).
For GM work we want: small, relaxed, non-conjugative, non-mobilizable plasmids with good markers and unique restriction sites.

Table 1. Three Examples of Natural Plasmids.

PLASMID	SIZE (kb)	RELAXED (AMPLIFIED)	SINGLE SITES FOR RESTRICTION ENZYMES	MARKER GENES FOR SELECTING TRANSFORMANTS	ADDITIONAL MARKER GENES SHOWING INSERTIONAL INACTIVATION
pSC101	6.5	NO	XhoI, EcoRI, PvuII, HincII	Tetracycline resistance	-
			HindIII, BamHI, SalI	-	Tetracycline resistance
ColE1	8.0	YES	EcoRI	Immunity to colicin E1	Colicin E1 production
RSF2124	11.0	YES	EcoRI, BamHI	Ampicillin resistance	Colicin E1 production

1st natural plasmid is stringent (not relaxed).
2nd natural plasmid has poor marker genes.
3rd natural plasmid is too large.

The perfect plasmid doesn't exist in nature!!!!
However, some early GM experiments were done with natural plasmids.
e.g. Morrow et al. (1974)
Used frog oocyte 5S ribosome DNA (multiple copies of this gene in the oocyte cell).
DNA cut with EcoRI restriction endonuclease.
Then inserted into pSC101 plasmid.
E. coli cells transformed and transfected.
Tetracycline-resistant clones/colonies selected on tetracycline agar (these cells would contain the plasmid but not necessarily the insert).
13 out of 55 clones were transformants and contained the insert - a good result.
Therefore, eukaryotic DNA could be propagated in prokaryotic cells.
A great breakthrough!!!!!
However, natural plasmids like pSC101 were found unsuitable for much other GM work for the reasons already given.
Solution??? Construct artificial plasmids with the best features derived from different natural plasmids.

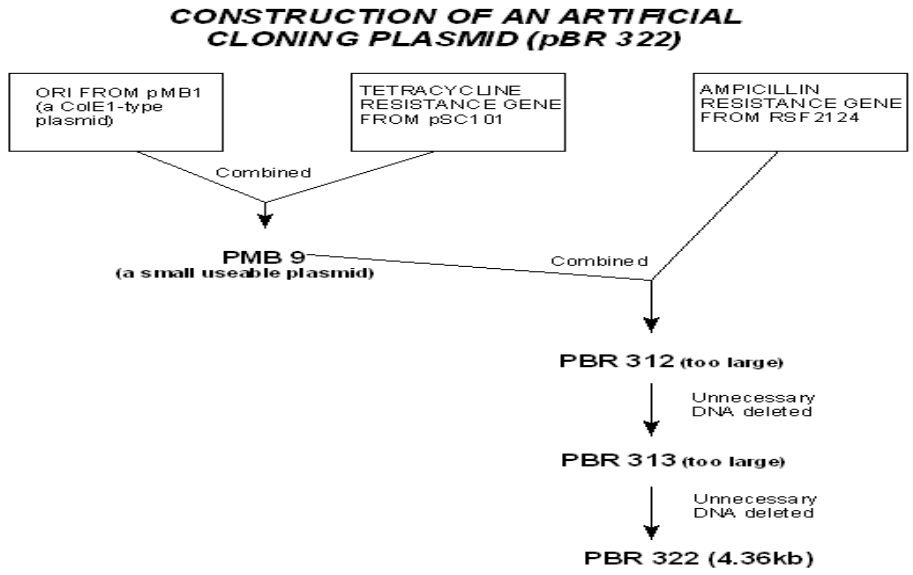


Fig 1. Showing cloning plasmid.

pBR322
An example of an artificial plasmid cloning vector.
Cleavage sites shown are for those restriction enzymes that cut the plasmid only once insertional inactivation (gene disruption). The exception is EcoRI!!! pBR322 has been completely sequenced - every base is known. pBR322 has over 30 known unique restriction sites (for clarity only 4 are shown in the diagram but for a more detailed map click here). Some of these sites are within the tetracycline (Tcr) or ampicillin resistance (Apr) genes insertional inactivation.

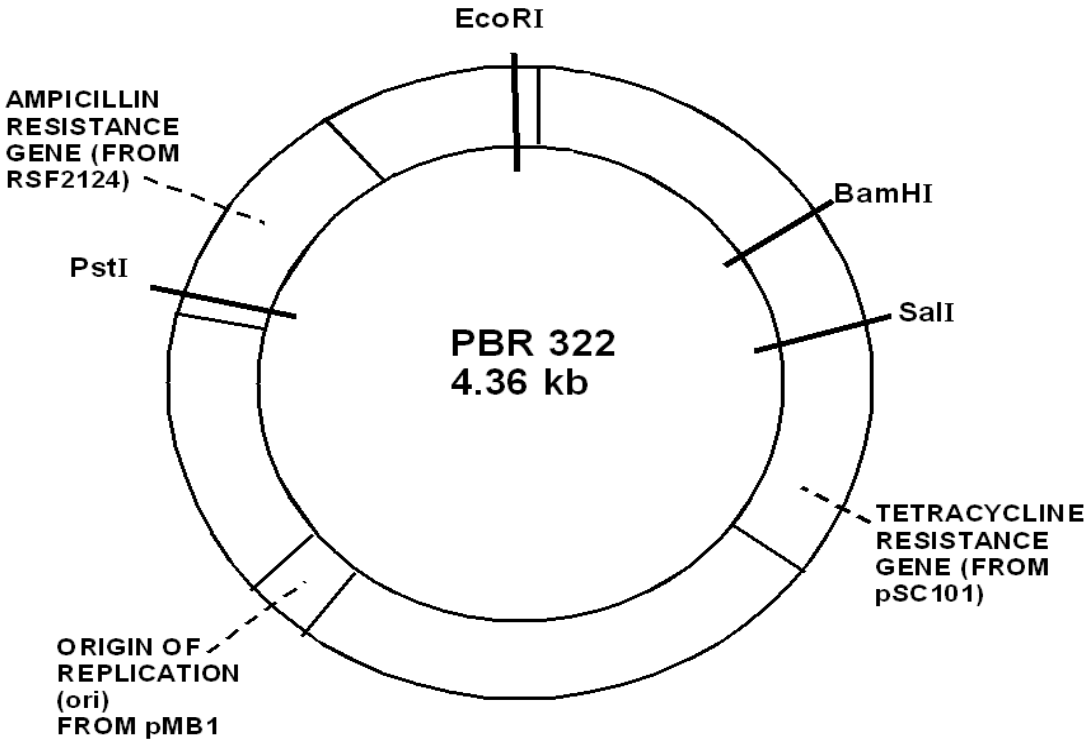


Fig 2. An example of an artificial plasmid cloning vector.

Unfortunately, using restriction sites, e.g. EcoRI, outside the 2 genes for insertion does not lead to insertional inactivation of ampicillin or tetracycline gene and, therefore, selection of desired transformants is more difficult, although not impossible. But if cloning with BamHI enzyme, inserts lead to: Apr Tcs transformants (because tetracycline resistance gene is inactivated by the presence of the insert).

3 types of bacterial host cell are possible after transformation and transfection:

1. Cells with no plasmid (not required)
2. Cells with plasmid but no insert (not required)
3. Cells with plasmid and insert (required)

Therefore, select transformants on ampicillin agar plate (selects out cells not containing the plasmid, i.e. type 1 above).

Also replica plate onto tetracycline agar. Colonies which grow on the ampicillin plate could be either type 2 or type 3 but those that also do not grow on the tetracycline plate are the transformants that are required. They can then be picked off from the ampicillin plate - being in the same position. These colonies come from cells containing an insert at the BamHI site, i.e. these cells are transformants and contain plasmid and insert (i.e. type 3 above). Click here for more detailed examples of cloning and selection using pBR322 plasmid.

There are improved derivatives of pBR322, e.g. pBR325 - has an additional chloramphenicol resistance gene containing a unique EcoRI site. But it's a bit big. e.g. pBR328 - as pBR325, i.e. 3 antibiotic resistance genes but smaller (4.9 kb).

pBR plasmids have been used extensively but they require replica plating and negative selection (i.e. absence of growth). Other plasmid vectors have since been constructed that use positive selection.

e.g. pUC plasmids

These contain several restriction sites within a galactosidase [lac z] gene from the lac operon (actually only part of it to save space). The natural substrate for the enzyme galactosidase is the disaccharide sugar, lactose. These plasmids also contain an ampicillin resistance gene (Apr) for selection of host cells containing plasmid from those not containing plasmid.

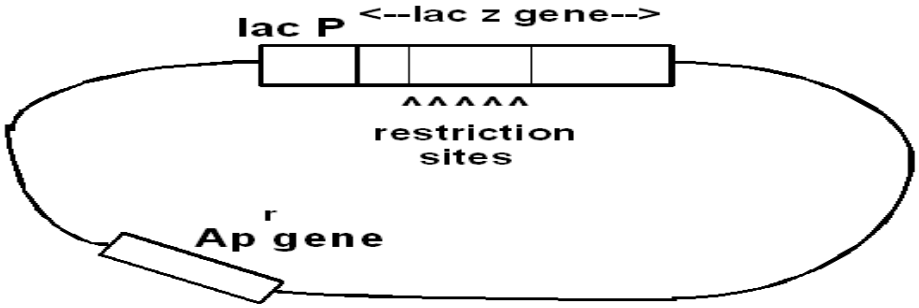


Fig 3. Showing restriction.

pUC PLASMID

Insertion of foreign DNA into this gene leads to loss of ك-galactosidase activity by insertional inactivation. How can this be detected? For a more detailed map of this type of plasmid (pUC18/19) click here.

e.g. pTR262 plasmid

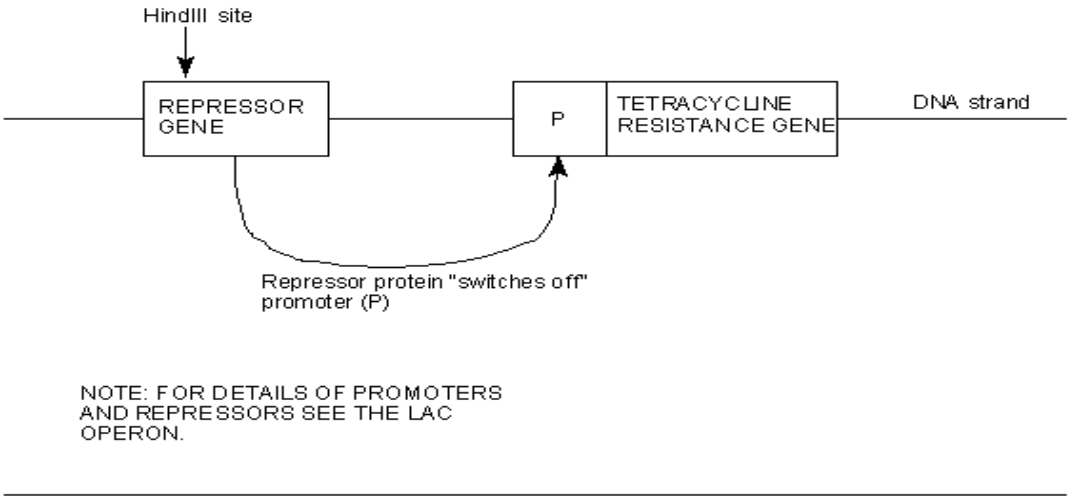


Fig 4. Showing repressor gene.

Here the repressor protein coded for by the repressor gene 'switches off' the promoter. RNA polymerase cannot now bind to the promoter and expression of the tetracycline gene does not occur. The host cell is, therefore, tetracycline sensitive and will not grow on agar plates containing this antibiotic. However, if an insert is added at the Hind III site, this will lead to insertional inactivation of the repressor gene and no repressor protein will be produced. This in turn will lead to expression of the tetracycline gene and the host cell will now be tetracycline resistant. Any colonies growing on tetracycline agar will be transformants containing both plasmid and insert. This is another example of a positive selection vector and has the advantage over vectors like pBR322 that replica plating is not required. However, this particular plasmid has only one useable restriction site, which could be a disadvantage.

e.g. pET plasmids

pET plasmids are part of a powerful host-vector system for the cloning and expression of recombinant proteins in Escherichia coli. They use the strong promoter from T7 bacteriophage. There are over 30 different types of pET vector with different characteristics and different marker genes. Over 10 different E. coli host strains can be used. A target gene is inserted into a pET plasmid under the control of strong bacteriophage T7 transcription and translation signals. Expression is induced by providing a source of T7 RNA polymerase in the host cell. T7 RNA polymerase is so selective and active that almost the cell's entire metabolism is switched to expression of the target gene. After only a few hours, the desired product can comprise more than 50% of the total cell protein.

USES OF PLASMID VECTORS

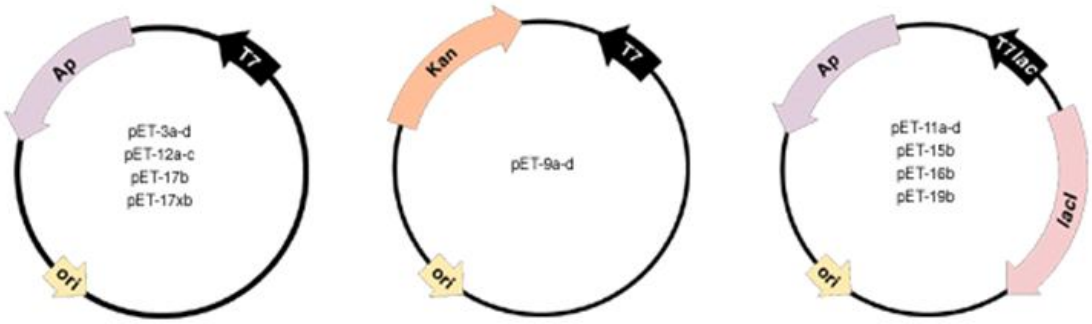


Fig 5. Use of plasmid vectors.

1. Gene expression, i.e. production of gene products.

e.g. The first mammalian product produced in bacteria was somatostatin (1977). This was done using pBR322 and E. coli. Other useful pharmaceutical products such as human insulin can also be synthesized using plasmid vectors. Click here for more details.

e.g. Gene expression using the pET system.

2. Gene cloning. 3. Cloning of complementary DNA (cDNA), i.e. DNA synthesized using mRNA as a template and reverse transcriptase. Cloned DNA can be used for base sequencing and for probes.

LIMITATIONS OF PLASMIDS

1. As size of insert increases fall in transformation frequency. 2. Plasmids with large inserts are often unstable deletion of insert during growth. Inserts >10 kb are generally impractical.

2. BACTERIOPHAGE VECTORS

e.g. lambda (l) phage, a bacterial virus that infects E. coli

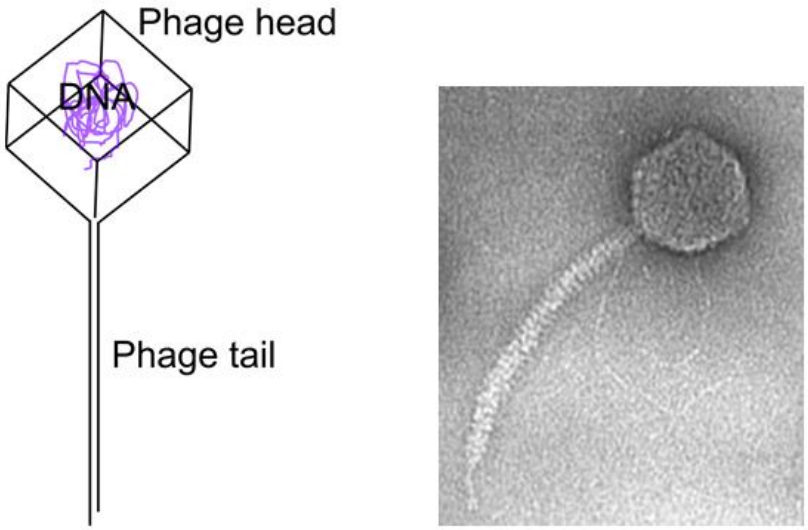


Fig 6. Showing phage head.

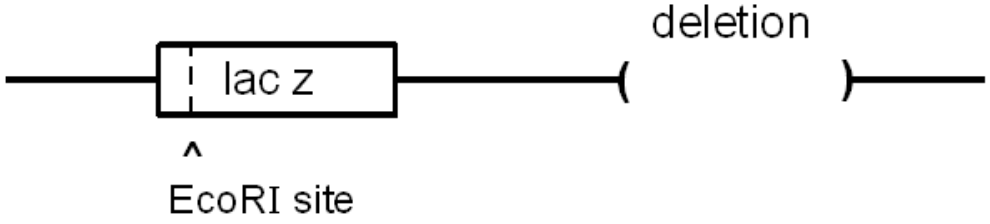
Well understood life cycle (lytic & lysogenic phases). Phage particle adsorbs to bacterial cell wall and DNA in head is injected via tail. In the lytic cycle DNA then circularizes and replicates. More phage particles are made and host cell then lyses to release them. Virus infection spreads to other bacterial cells plaque (clearing in a bacterial lawn).

LAMBDA (l) VECTORS

Wild type l has multiple restriction sites and is, therefore, no use for GM (except as a standard for gel electrophoresis). Several types of artificial l are now available which have:

1. 1 or 2 restriction sites only.
2. Deletions to allow room for inserts.
3. Selection system(s)., With replacement vectors the DNA is cut twice and piece removed allowing space for insert. Insertion vectors have a deletion which leaves space for an insert. Up to 20 kb size inserts are possible.

e.g. Charon 16A



Selection is on Xgal plates. Blue plaques - phage DNA but no insert White plaques - phage DNA with insert (lac z gene has been disrupted). This type of vector is named after a boatman in Greek mythology who ferried the souls of the dead across the river Styx into the underworld (Hades or Hell). To find out more about Charon click here. Also, click on the following thumbnail image to see a painting of Charon in his boat with some dead souls and with Cerberus, the three-headed dog, waiting on the other side of the river.

IN VITRO PACKAGING

Very useful feature of the l system.

With in vivo packaging: Transduction using l 105 plaques per µg DNA (transduction frequency). But after manipulation this can fall 103 - 104 plaques per µg DNA. With in vitro packaging: Mix together: phage DNA (with appropriate cos ends and insert)

+ head protein

+ tail protein

+ packaging enzymes

Complete phage particles these artificially produced phages are then able to infect host cells. In vitro packaging usually gives a higher transduction frequency (i.e. number of plaques produced per µg DNA) than in vivo packaging.

ADVANTAGES OF THE l SYSTEM

1. Large inserts possible (up to 20 kb).
2. Stable propagation (C.F. plasmids where large inserts may get deleted).
3. Efficient entry into cell using in vitro packaging.
4. Multiple copies of insert and strong promoters.

DISADVANTAGES OF THE l SYSTEM

1. Plaques are produced not colonies.

Useful for cloning DNA but no use for gene expression, e.g. production of gene products in biotechnology.

2. 20 kb is still not large enough for many eukaryotic genes. For larger inserts cosmids or eukaryotic vectors can be used.

USES OF THE l SYSTEM

1. for cloning DNA for further analysis.
2. for use to store DNA in gene and cDNA libraries (random collection of fragments of all the genome/mRNA of a species in a host-vector system).

CONTAINMENT

Most l vectors are disabled and will only grow in special host cell strains. e.g. amber (nonsense) mutations present in essential genes. Only host bacteria with a nonsense suppressor can support growth of the phage vector. Non-laboratory strains do not have the suppressor gene and, therefore, will not support phage growth.

M13 PHAGE

M13 is a small filamentous E. coli bacteriophage cloning vector which behaves like a plasmid for part of its replication cycleIt is rather unusual in that it produces both double-stranded (dsDNA) and single-stranded DNA (ssDNA) in different phases of its replication cycle. When in replicative form (RF) inside the host cell, M13 acts rather like a plasmid, the DNA being double stranded (ds), but, when packaged up in capsids to form phage particles to be released from the host cell, the DNA is single stranded. M13 (unlike lambda phage in its lytic cycle) does not cause lysis and death of the E. coli host cells. Instead, infected host cells excrete M13 phage particles containing ssDNA in large numbers. But can only take relatively small inserts up to 0.5kb in size.

P1 PHAGE

Vectors based on this bacteriophage can take larger inserts than lambda vectors shoehorning up to 110kb of DNA into the capsid.

COSMID VECTORS

Consist of the cos ends of phage with plasmid DNA. In vitro packaging is used to introduce the DNA into a host cell. Any DNA between 2 cos sites will be packaged in the phage head. Phage injects the DNA which then behaves like a plasmid once inside the host cell. But such "phages" are not virulent and do not cause lysis or produce plaques. Colonies are produced. Therefore gene expression and production is possible. Inserts up to 40 kb can be inserted. Selection can be by adding ampicillin resistance gene to insert - positive selection on ampicillin plates. However, cosmids can be unstable.

OTHER HOST-VECTOR SYSTEMS

PHASMIDS

Artificial constructions of plasmid and l attachment site. The plasmid can insert into the l phage genome. Reversal of the process releases the plasmid vector. Phasmids can exist in bacterial cells as a plasmid or as a phage.

BACTERIAL ARTIFICIAL CHROMOSOMES (BACs)

This type of vector is based on the F (fertility) plasmid involved in conjugation in some bacterial species. BACs can take inserts up to 300kb in size.

P1-DERIVED ARTIFICIAL CHROMOSOMES (PACs)

This type of vector combines features of P1 phage vectors with BACs and, like the latter, can take inserts up to 300kb in size.

YEAST VECTORS

e.g. Yeast episomal plasmids (2 µm) YIP's (yeast integrative plasmids - bacterial plasmids which integrate into yeast chromosome) YRP's (yeast replicative plasmids - include an ori and can replicate independently of the main yeast chromosome) YAC's (yeast artificial chromosomes - can accept very large inserts (e.g. 2000kb) and become more stable the larger the insert - unlike virtually every other type of vector!) "Shuttle vectors" can replicate in two types of cell, e.g. both yeast and bacterial cells. They can ferry DNA between the two. Yeasts are eukaryotic and, therefore, often better than prokaryotic vectors for the expression of eukaryotic genes. Factors that should be considered in the choice of a host-vector system for gene expression (i.e. if a product is required) and especially if a eukaryotic gene is inserted into a prokaryotic host-vector system include:

- Size of insert (inserted foreign gene may be too large for the vector - use another type of vector such as a YAC)
- Vector limitations (in addition to insert-size limitations, some vectors are quite unsuitable for gene expression, e.g. lambda phage causes lysis of its host cells - dead cells cannot synthesize a product!)
- Small peptide degradation (occurs in some prokaryotic host cells - solution: increase size of gene insert to produce a larger 'fused' product as in somatostatin example)
- Reading frame (this must be taken into account after each manipulation of the DNA)
- Glycosylation (eukaryotic cells often modify proteins after translation, e.g. by adding sugar groups, but prokaryotic host cells are generally not capable of these post-translational modifications, in which case the final product may be different from the original eukaryotic one)
- Polyadenylation (eukaryotic mRNA has a tail of adenine bases, not found in prokaryotes, that acts as a signal for export of the mRNA into the cytoplasm and also increases its stability)

- Introns (common in the genes of eukaryotes but rarer in prokaryotes - can 'confuse' a prokaryotic host cell)
- promoter (must be of the right type for the host cell and also be in the correct position - often expression of a eukaryotic gene is more likely to be successful if the vector possesses a eukaryotic promoter)
- Codon differences (there are differences in codon bias and usage between prokaryotes and eukaryotes which may cause problems if expression of a eukaryotic gene is attempted in a prokaryotic host cell)
- Toxicity (the foreign gene product may be harmful to the host cell)
- Product export (post-translational modifications of some eukaryotic gene products are required for export out of the cell and a prokaryotic host is generally not capable of carrying these out)

Some potential problems in achieving successful gene expression are highlighted by the early example of somatostatin synthesis. You should look up further details about gene expression and production in one of the recommended textbooks.

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