

GENETIC MANIPULATION OF BACTERIA

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Summary

The discovery of type II restriction endonucleases, and the fact that fragments produced by cutting with such enzymes could be rejoined in the presence of DNA ligase in a random order dictated only by the need for the fragments to have identical ends, initiated the process of *in vitro* cloning and manipulation of genes in bacteria and other systems.

Plasmids and bacteriophages have been engineered to make gene cloning simpler, by incorporation of multiple cloning sites and markers that allow the bacteria carrying such vectors to be screened for incorporation of DNA fragments. While whole genome random sequencing depends on high copy number vectors carrying small fragments,

gene libraries can be now be created with very large inserts in stable low copy number vectors allowing one to screen for phenotypic determinants spread over tens of kilobases.

However, the availability of whole genome sequences also allows one to select regions to clone on the basis of prior knowledge of the sequence and then to use polymerase chain reaction (PCR) to amplify and clone segments defined by short DNA sequences which act as primers for thermostable DNA polymerase.

Regions coding for specific proteins can then be selected and manipulated for over-expression of the gene product, for production of an altered gene product or to produce a mutant gene which can be reintroduced back into the chromosome from which it came, so as to produce an altered bacterial strain.

Such changes can be used to produce bacteria with novel physiological properties, novel pathways for production of antibiotics or degradation of an organic pollutant or with properties that can be exploited in devices such as biosensors.

Release of bacteria into the environment can be useful for processes such as bioremediation, and further manipulation can be used to minimise the chance of such bacteria from displacing the indigenous bacterial population, as well as providing the ability to monitor the survival of bacteria in the environment.

1. Introduction

This section summarises methods and applications for genetic manipulation of the DNA content of bacteria. It is written from a context in which there is already a complete genome sequence (see also - *Molecular Biology*) for at least one strain of many of the species with which we work.

It is not intended as a detailed methods guide (see also - *Methods in genetical engineering*), but rather its aim is to provide definitions of the key elements of the subject and impart understanding of those elements.

The purpose of genetic manipulation is to create a new strain with precisely defined genetic properties (an altered genotype). In the first place the phenotypic consequences of the genotypic changes may not be known - this is the research stage when one is trying to define the biological system under investigation.

Later, in a biotechnological context the aim may be to create genotypic changes that result in a desired phenotypic change. Both goals however involve similar experimental approaches.

The first stage is to obtain the DNA segment to be manipulated, the second stage is to manipulate it and the third stage is to introduce it into the desired context and determine the phenotypic consequences.

2. Genetic Manipulation in Bacteria

2.1 Vectors

Because DNA fragments will generally not replicate on their own when introduced into a bacterium they need to be joined to a DNA molecule that can replicate - a vector. Vectors need to be small enough to be manipulated conveniently and also to allow the joined DNA to be easily analysed. Vectors are derived either from plasmids or bacterial viruses - bacteriophage.

2.1.1 Plasmid Vectors

A plasmid is a DNA element that can replicate autonomously within a bacterium. Plasmids can be linear molecules, but the ones which are most commonly exploited as vectors for genetic manipulation are circular.

Apart from anything else, circularity means that if the DNA molecule is cut once, all the plasmid functions remain together on the linear fragment formed so that only one vector segment needs to be joined to the foreign DNA segment to allow it to replicate.

Many naturally occurring plasmids are either “self-transmissible”, being able to spread to other bacteria via a plasmid-encoded mating or conjugation bridge, or “mobilisable”, being able to spread by conjugation so long as there is an appropriate self-transmissible plasmid present.

With the concern about escape of the products of genetic engineering it is generally desirable that genes promoting such transfer are deleted or inactivated before plasmids are exploited as vectors.

However, in some applications, mobilisability is important to allow the vector to be transferred back to the host to be manipulated.

For primary cloning and manipulation it is most common to use small, high copy number vectors. For such vectors the complexity of the hybrid formed is limited and therefore the molecular analysis is made easier.

Also, the yield of DNA is high, removing the necessity to grow large quantities of bacteria and also making it easier to produce good quality DNA because the ratio of plasmid DNA to other bacterial components such as membranes and RNA is high. However, the high copy number can cause problems.

First, abnormal expression, caused by separation from normal regulators or increased gene dosage, of many genes can disrupt the growth of host bacteria. So, a random gene library made from the chromosomal DNA of the organism of choice, a high copy number vector, will generally be deficient in certain sectors of the genome.

For this reason, the DNA libraries created to allow whole genomes to be sequenced use small fragments which are unlikely to contain whole genes and therefore minimise problems of such deleterious effects.

Directed cloning of specific sectors which have not been obtained in a random library can also run into similar problems resulting in the desired clones never being obtained or containing deletions or rearrangements of the normal DNA.

An extension of this problem is found with cosmid vectors which are designed to pick up large chromosomal segments by the step of packaging into lambda phage heads (see below). When the vectors are 5 to 10 kb in size, the inserted DNA is 30 to 35 kb minimum.

The insertion of this amount of DNA into a pMB1-based plasmid depresses its copy number so that any changes that cause loss of DNA will elevate copy number and thus rapidly result in loss of the plasmid with the original cloned segment.

If the vector is a plasmid that uses a replication mechanism with a single stranded intermediate like the strand-displacement mechanism of IncQ plasmids or the rolling circle replication of many Gram-positive plasmids then the instability is promoted by increasing the size of the single stranded segment which may induce repair and recombination pathways. The result is that such vectors are not currently favoured, or at least are used with considerable caution. This problem has been solved by the use of what are called BACs - bacterial artificial chromosomes - which rely on the replication and partitioning system of low copy number plasmids such as F and the prophage of P1.

Plasmid vectors have been manipulated to make it easier to detect or even select for the acquisition of an inserted fragment of DNA. The most common method is to use a gene which confers on bacteria a property that can be detected by a colour reaction when that bacteria are placed on a specific medium or are sprayed with a particular chemical.

The best example is the *lacZ* gene whose product β -galactosidase will hydrolyse Xgal (the chromogenic substrate) to give a blue compound that is easily detected. However, *lacZ* is a large gene (about 3 kb) and therefore not the best choice to insert into a small vector. Fortunately β -galactosidase can be split into two domains, the very small α fragment and the much larger β fragment neither of which on their own exhibit enzyme activity, that will associate in vivo to generate a functional enzyme. With a host strain encoding the β -fragment and the plasmid encoding the α -fragment one can develop plasmid derivatives with cloning sites within the region coding for the α fragment so that insertions will disrupt the orf and prevent functional α -fragment production and so give a plasmid-host combination which is colourless.

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Biographical Sketch

Christopher M Thomas studied Biochemistry at The Queen's College, Oxford followed by a DPhil in Microbiology at Linacre College, Oxford with a project on DNA replication in *Staphylococcus aureus*. A post-doctoral period as an MRC Travelling Fellow in the laboratory of Donald Helinski at UC San Diego in California introduced him to both genetic manipulation and broad host range plasmids which have been used extensively as tools in gene cloning. Since 1980 he has been at the University of Birmingham (UK) and has been heavily involved with Biotechnology management within the university since 1984. He was promoted to Professor of Molecular Genetics in 1991. His current research covers many aspects of plasmid biology, with particular emphasis on plasmids of the genus *Pseudomonas*, as well as genome partitioning in bacteria (as a means of identifying new potential drug targets in bacteria) and polyketide antibiotic biosynthesis in *Pseudomonas fluorescens*. He has played a leading role in organising international Plasmid Biology conferences, as well as in Europe bringing together molecular biologists and ecologists to study gene spread in bacteria.