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6 Recombinant plasmids

Caroline Hussey

1. Introduction

There can have been few scientific advances where potential risks were as conscientiously anticipated, as in the development of DNA manipulation technology and genetic engineering. The 'moratorium' on certain types of experimentation in genetic manipulation, instigated by Berg *et al.* (1974) and endorsed by the Asilomar Conference (Berg *et al.*, 1975) was a unique incident in the history of modern science, where scientists adopted a self-denying ordinance to retard progress in recombinant DNA (r-DNA) experimentation until the inherent risks had been evaluated.

Yet, ironically, 18 years later, although most scientists are intellectually satisfied that the majority of DNA manipulations fall into a zero- or low-risk category, the fears of the public have not been allayed and the scientific community has little to offer, in the way of concrete data, to support the conviction that the use of genetically engineered microorganisms is 'safe'.

In this chapter, some of the properties of recombinant plasmids will be considered and an attempt will be made to summarize how these characteristics may determine the safeguards required for their use, particularly in industrial fermentations. An attempt will also be made to review our knowledge of the behaviour of microorganisms containing recombinant plasmids under fermentation conditions and discuss how these traits might be manipulated to optimize productivity and limit risk.

2. Recombinant plasmids

A 'genetically engineered microorganism' (GEM), now usually known as a 'genetically modified microorganism' (GMO), is defined by the European Commission (EC) as 'a microorganism in which the genetic material has been altered in a way that does not occur naturally by mating or natural recombination', (EC, 1990a). An organism may be altered by rearranging or deleting native genetic material or by introducing heterologous, or 'foreign', DNA from another species. A foreign gene may be introduced into the chromosome of the recipient, or 'host', strain where it can be stably maintained; that is, the gene is replicated as an integral part of the host chromosome so that one copy of the gene is inherited by both daughter cells at each round of cell division. However, for a number of reasons, foreign genes are, more usually, introduced to the host as components of multicopy plasmids.

Plasmids are autonomously replicating, extrachromosomal genetic elements, (i.e. DNA molecules containing an origin of replication) which occur naturally in most, if not all, microbial species. Naturally occurring plasmids can range in size from 2 kb to

more than 100 kb. The number of copies of the plasmid molecule in each cell (the copy number) is characteristic of the plasmid, although it can vary from host to host and be modified by changes in the environment (see section 5.1.2), and ranges from one to several hundred. In general, large plasmids have low copy numbers while small plasmids have high copy numbers (Sherratt, 1986). A host strain can harbour more than one type of plasmid, provided that the plasmids are compatible. Plasmids that have very similar structures can usually not be accommodated in the same host and they are then referred to as belonging to the same incompatibility group (Novick, 1987).

Little is known about the functions or evolution of plasmids in nature, except that many of the plasmids that have been isolated and characterized from a variety of species contain genes for enzymes that inactivate, or otherwise neutralize, antibiotics (Davies and Smith, 1978; Foster, 1983) or protect the host from the toxic effects of heavy metals (Silver and Misra, 1988). These plasmids confer an advantage on the host strain, permitting growth in adverse environments. However, whatever advantage they may confer on their natural hosts, plasmids have proved to be of enormous benefit to scientists and biotechnologists as vectors for introducing foreign DNA into bacteria and other microorganisms.

2.1 Construction of recombinant plasmids

In 1973, Cohen *et al.* published their now famous paper in which they described linking DNA fragments to a bacterial plasmid replicon and subsequently introducing the composite, chimeric, molecule into *Escherichia coli* by transformation. Although the intervening 19 years have seen a large number of developments in DNA manipulation technology, the methods which Cohen *et al.* describe in this paper are still essentially the standard techniques used for gene transfer (Maniatis *et al.*, 1982; Rodriguez and Tait, 1983).

DNA from a plasmid-containing strain is extracted and the plasmid DNA is separated from chromosomal DNA and purified. Like the bacterial chromosome, plasmids of bacterial origin are usually covalently closed circular double-stranded DNA molecules but they can be linearized by cutting with a Type II restriction endonuclease (Roberts, 1982) if the plasmid contains a single, unique target site for that enzyme (the cloning site). Restriction endonucleases usually cut the double-stranded DNA asymmetrically, thereby generating linear plasmid molecules with 'sticky ends', i.e. short complementary single-stranded sequences at each end of the molecule. The DNA to be inserted is also treated with an endonuclease which generates fragments with similar sticky ends, so that when plasmid and insert DNA are mixed under suitable conditions, annealing takes place between complementary sequences. The enzyme ligase (Weiss *et al.*, 1968) is used to form covalent bonding between the insert and the vector DNA.

2.1.1 Genomic DNA and cDNA cloning

If the foreign DNA is of bacterial origin, preparation of the insert fragment is relatively simple. Total bacterial DNA is purified, cleaved with a restriction endonuclease and

the fragment containing the gene of interest is identified, isolated and ligated into the plasmid vector. However, the arrangement of genes on eukaryotic chromosomes is more complex. Within genes, coding sequences are frequently interspersed with non-coding sequences (Breathnach and Chambon, 1981). Chromosomal DNA from higher organisms is rarely transferred directly into a bacterial host. Instead, the RNA transcript of the gene is used as a template for reverse transcriptase (Verma, 1977) to synthesize a DNA molecule (cDNA) containing only the coding sequences.

2.1.2 Genetic exchange

Many bacterial species have a natural capacity to absorb DNA and incorporate it into the genome. Hence, over billions of years, bacteria have evolved through a process akin to genetic engineering. This natural capacity for DNA uptake is exploited, in various ways, in order to establish recombinant plasmids in host cell lines (clones). Transformation, the uptake of DNA through the cell membrane, is the method most commonly used to introduce recombinant plasmids into bacteria. Some bacteria have a natural competence for DNA uptake (Tomasz, 1969; Hotchkiss and Gabor, 1970). For example, the transformation of *Bacillus subtilis* with recombinant plasmids is relatively simple (Dubnau and Davidoff-Abelson, 1971; Van Randen and Venema, 1984). Some Gram-negative bacteria, such as *E. coli*, *Pseudomomas putida* and *Azotobacter vinelandii*, can take up plasmid DNA if the cells are treated with divalent cations (Cohen *et al.*, 1972; Chakrabarty *et al.*, 1975; David *et al.*, 1981). In some bacteria and in yeasts, the cell wall must be removed before transformation is possible (Hinnen, *et al.*, 1978; deVos and Venema, 1981; Hopwood and Chater, 1982; Katsumata *et al*, 1984). Electroporation, where DNA uptake is facilitated by an electrochemical gradient, enhances transformation efficiency in several species (Hashimoto *et al.*, 1985; Somkuti and Steinburg, 1987; Wolf *et al.*, 1989; Conchas and Carniel, 1990).

Conjugation is an alternative method of introducing plasmid DNA to the host, which permits vectors based on broad host range conjugative plasmids, such as RP4 (Datta and Hedges, 1972), to be introduced into almost all species of Gram-negative bacteria (Thomas and Smith, 1987). Conjugation is also possible with some *Streptomyces* species (Lydiate *et al.*, 1985) and *Streptococcus* plasmids (Oultram and Young, 1985). Vectors derived from bacteriophage DNA can be introduced to the host by infection.

The fortuitous occurrence of antibiotic resistance genes on most plasmids can be exploited to isolate transformants. Clones harbouring a plasmid can be positively selected by plating on antibiotic-containing medium. The antibiotic-resistant phenotype is also exploited for the maintenance of plasmid-containing strains in the laboratory: maintaining strains on antibiotic-containing slopes imposes selective pressure, which prevents reversion to the plasmid-free host.

2.1.3 Selection

Isolating the gene of interest is frequently the most difficult step in a cloning experiment. It is usually more practicable to carry out a 'shotgun' cloning where the

DNA fragments generated by endonuclease digestion, or cDNA molecules prepared by reverse transcription of total messenger RNA, are not separated, and the heterogeneous mixture is ligated into a population of plasmids which are then used to transform the host. This gives rise to a mixed population of transformants, only a small fraction of which contains the required gene. Selecting the correct transformant can be approached in a variety of ways. If the cloned gene causes an obvious alteration in the phenotype of the host, then selection may be relatively simple. For example, if the cloned gene confers antibiotic resistance on the cell, clones can be selected by plating on medium containing the antibiotic. However, most gene products do not have such a conspicuous effect on phenotype and alternative strategies must be adopted. Antibody to the cloned gene product can be used to detect colonies producing the antigen but this is possible only when relatively large amounts of protein are available to raise the antibody. The most universally applicable technique is colony hybridization (Grunstein and Hogness, 1975) where a single-stranded DNA molecule, complementary in sequence to the cloned gene, is labelled, radioactively or chromogenically, and used as a probe to detect colonies containing the gene. The use of DNA probes is discussed in greater detail in section 4.4.1.

2.1.4 Expression of cloned genes

Insertion of a gene into a microbial host does not automatically guarantee expression of the gene, i.e. synthesis of the coded protein. Initiation of transcription and translation requires the presence of control elements in the DNA upstream of the coding sequence which are, to some extent, species specific. This specificity erects phylogenetic barriers to the expression of cloned genes.

Even among prokaryotes there are significant differences between the control elements in Gram-positive and Gram-negative bacteria. For example, although the promoter sequences that initiate transcription in *E. coli* and *B. subtilis* are similar (Moran *et al.*, 1982; Reznikoff and McClure, 1986) and *B. subtilis* genes are expressed in *E. coli* (Rapapórt *et al.*, 1979), *E. coli* genes can be expressed in *B. subtilis* only when cloned downstream of Gram-positive control elements, probably because *B. subtilis* ribosomes have very stringent requirements for binding sites on the DNA (Ehrlich and Sgaramella, 1978; Kreft *et al.*, 1978; Hager and Rabinowitz, 1985). By contrast, most *Streptomyces* signals cannot function in *E. coli* (Jaurin and Cohen, 1985), although many Gram-negative promoters are active in *Streptomyces lividans* (Bibb and Cohen, 1982).

To achieve efficient expression of cloned genes from diverse sources, most cloning vectors in common use contain control elements, including strong transcriptional promoters, immediately beside the cloning site (Remaut *et al.*, 1981; Williams *et al.*, 1981; Leemans *et al.*, 1987; Amman *et al.*, 1988). In these 'expression vectors', transcription, initiated at the plasmid-coded promoter, reads through into the inserted sequence. To ensure efficient initiation of translation, the 5′, plasmid-coded, sequence of the transcript frequently also contains a ribosome binding site and translation start signal (Stanssens *et al.*, 1985; Grandi *et al.*, 1986; Botterman *et al.*, 1987).

2.2 Host/vector systems

The first vector tailor-made for cloning heterologous genes, pBR322, was constructed for use in *E. coli* (Bolivar *et al.*, 1977). This 4363 bp circular plasmid is still widely used as a cloning vector, because of its simplicity and the availability of the complete nucleotide sequence, and many other vectors have been derived from it (Viera and Messing, 1982; Balbas *et al.*, 1986). Many *E. coli* plasmids are host specific and will replicate only in enteric bacteria. Some conjugative plasmids, such as RP4, have a broad host range (Datta, 1985) and these replicons have been used as the basis for vectors which will replicate in most Gram-negative bacteria (Ditta *et al.*, 1980; 1985). Recently, Harayama and Rekik (1989) have developed a procedure which permits transfer and maintenance of non-conjugative plasmids, such as pBR322, to other Gram-negative species.

Escherichia coli is still the most popular host for laboratory studies, because of the wealth of information available on its physiology and molecular biology. However, for industrial development, *E. coli* has limitations, including pyrogen production, protein insolubility, incorrect protein folding, limited secretion abilities and the inability to make certain post-translational modifications (Klotz, 1983). Efficient cloning systems have also been developed for other Gram-negative bacteria, particularly *Pseudomonas* spp. Because of their extensive degradative capacities (Reineke and Knackmuss, 1979; Werneke *et al.*, 1985; Ramos *et al.*, 1986; Rojo *et al.*, 1987), and broad host-range plasmids (Farinha and Kropinski, 1989), and phasmids (Moroni *et al.*, 1987) have been developed for cloning in *Pseudomonas* species. Cloning systems are now available for several other Gram-negative bacteria of industrial interest, including *Zymomonas mobilis* (Su and Goodman, 1988) and *Myxococcus xanthus* (Breton *et al.*, 1986). Throughout the past decade, intensive research has focused on developing cloning systems for two Gram-negative species of agricultural interest, *Rhizobium* spp. (Selvaraj and Iyer, 1981; Beringer and Hirsch, 1984), and *Agrobacterium tumifaciens* (Chilton *et al.*, 1977; Hernalsteens *et al.*, 1980; Zambryski *et al.*, 1983).

There appears to be a lower level of host specificity in plasmids which replicate in Gram-positive bacteria (Novick, 1989). Many of the plasmids which have been used to construct cloning vectors for *B. subtilis* were originally isolated from *Staphylococcus aureus* (Ehrlich, 1977; Gryczan *et al.*, 1978; Ehrlich *et al.*, 1982) and they can also replicate in other *Bacillus* species.

There has been considerable interest in the development of cloning systems for Gram-positive bacteria. Initially, attention was focused on *B. subtilis* (Keggins *et al.*, 1978; Dean and Kaelbling, 1981; Debebov, 1982; Ortlepp *et al.*, 1983; Doi, 1984). As a host, *B. subtilis* has the advantage of a long tradition of industrial use and natural systems for protein secretion (Pugsley and Schwarz, 1985; Nicaud *et al.*, 1986), but suffers from the disadvantages of plasmid instability (Michel *et al.*, 1980), especially when plasmids contain heterologous inserts (Bron and Luxen, 1985; McLoughlin *et al.*, 1987), and of protease digestion of excreted proteins (Ulmanen *et al.*, 1985).

A large number of naturally occurring plasmids have been isolated from *Streptomyces* strains and used to construct cloning vectors which can be used for different species of *Streptomyces* (Hopwood and Chater, 1982). Host vector systems have been

developed for many other Gram-positive bacteria such as the corynebacteria, because of their biosynthetic potential, particularly for amino acid production (Santamaria, *et al.*, 1987; Trautwetter *et al.*, 1987; Wolf *et al.*, 1989: Patek *et al.*, 1989). A comprehensive list of host vector systems for Gram-positive bacteria of industrial interest has been compiled by Schwab (1988).

Host range can be expanded by constructing vectors containing origins of replication of two plasmids with different host specificities. These co-integrates can replicate in a wide range of hosts. For example, a number of 'shuttle vectors', which will replicate in both Gram-positive and Gram-negative hosts have been constructed using this strategy (Kreft, Bernhard and Goebel, 1978; Gryczan *et al.*, 1980; Dhaese *et al.*, 1984a; Ostroff and Pene, 1984; Larson and Hershberger, 1984; Lalonde and O'Hanley, 1989) and a shuttle system has been developed for cloning in the photosynthetic cyanobacteria (Ferino and Chauvat, 1989).

Despite the enormous range of bacteria now available as hosts for recombinant plasmids, eukaryotic microorganisms, such as *Saccharomyces cerevisiae*, may prove to be better hosts for the cloning of genes from higher organisms (Gunge, 1983; Kingsman *et al.*, 1985; Carty *et al.*, 1987). Autonomously replicating plasmid systems are rare among the fungi, with the exception of *S. cerevisiae* and a few other yeasts, although linear plasmids have been found in the mitochondria of some filamentous species (Kim, Whitmore and Klassen, 1990). The autonomous replicating sequences (ARS) of the *S. cerevisiae* 2-μm plasmid have been used to construct vectors for cloning in *S. cerevisiae* and other yeasts (Old and Primrose, 1985). Similar KARS sequences have been used to construct cloning vectors for *Kluyveromyces fragilis* (Das *et al.*, 1984) and *Kluveromyces lactis* (de Louvencourt *et al.*, 1983) and ARS have been isolated from *Candida maltosa* (Kawamura *et al.*, 1983) and *Schizosaccharomyces pombe* (Beach and Nurse, 1981). Other yeasts, such as the methylotrophs *Hansuela polymorpha* (Ledeboer *et al.*, 1987) and *Pichia pastoris* (Cregg and Madden, 1988) and the filamentous yeast *Trichospora cutaneum* (Glumoff *et al.*, 1989) offer interesting possibilities for industrial use.

Recently, considerable success has been achieved in developing vectors which give rise to stable transformants of filamentous fungi, such as *Aspergillus nidulans* (Ballance and Turner, 1985; Picknett *et al.*, 1987; Punt *et al.*, 1987; Malardier *et al.*, 1989), *Aspergillus niger* (Buxton *et al.*, 1985; Unkles *et al.*, 1989), *Penicillium chrysogenum* (Cantoral *et al.*, 1987; Carramolino *et al.*, 1989), *Cephalosporium acremonium* (Chapman *et al.*, 1987), *Ustilago violacea* (Bej and Perlin, 1989) and *Podospora anserina* (Razanamparany and Begueret, 1988). However, in most of these transformants the cloned gene has integrated into a chromosome and the vector does not replicate autonomously. The use of fungal plasmids as a basis for vector development has been successful in only a few species, including *Neurospora crassa* (Stohl and Labowitz, 1983) and *Podospora anserina* (Stahl *et al.*, 1982). Perrot *et al.* (1987) have achieved non-integrative transformation of *P. anserina* by a linear vector stabilized by incorporation of the chromosomal ends of *Tetrahymena thermophila*. Schwab (1988) summarizes the properties of cloning systems for the filamentous fungi.

The development of a cloning system for the slime mold, *Dictyostelium discoideum* offers an interesting opportunity to study the expression of cloned genes in a simple, differentiating system (Dingerman *et al.*, 1989).

3. Uses of recombinant plasmids

An exhaustive list of potential uses for recombinant microorganisms is beyond the scope of this chapter. The subject has been discussed imaginatively in a number of reviews, including Imanaka (1986), OECD (1986), ECRAB (1986), Hutter (1987), Driesel (1988), Schwab (1988), and Malik (1989).

3.1 Research and development

To date, the greatest benefits from recombinant DNA (r-DNA) have accrued in research and development. By using cosmids, plasmids containing bacteriophage DNA (Collins and Hohn, 1978; Davison *et al.*, 1987; Yankovsky *et al.*, 1989), which can maintain very large DNA inserts, it is now possible to clone the entire genome of complex organisms in a single population of bacteria. The establishment of such 'gene libraries' for a number of organisms has already permitted the sequencing of the entire genome of several plasmids, viruses and organelles (Moores, 1987) and sequencing the chromosomes of *E. coli* (Smith *et al.*, 1987), yeast and more complex organisms, including humans, is under way.

Gene cloning provides a relatively simple approach to establishing the amino acid sequence of virtually any protein. The polymerase chain reaction (PCR, see section 4.4.1) makes it possible to make a cDNA copy of any gene, even one expressed at the very lowest level in its native tissue (Akowitz and Manuelidis, 1989; Parfett *et al.*, 1989) or to 'walk' along the chromosome from one gene to the next (Shyamala and Ferro-Luzzi Ames, 1989). Once a gene is cloned on a multicopy plasmid, it can be sequenced and the base sequence unambiguously establishes the amino acid sequence of the translation product. In addition, the facility to produce relatively large amounts of virtually any protein, of prokaryotic or eukaryotic origin, has made possible rapid progress in our knowledge of the structure and function of proteins and paved the way for adventures in protein engineering (Wodak, 1987; Seno *et al.*, 1988; Dodson, 1989). Conversely, gene cloning, in combination with the powerful techniques of immunochemistry, peptide sequencing and oligonucleotide synthesis, now permits the isolation and identification of the gene for any protein which can be prepared in a relatively pure form (Malik, 1989).

The ability to sequence genes and their adjacent control elements has also contributed greatly to our understanding of the mechanisms which regulate gene expression, and cloning vectors have been designed specifically to study the characteristics of control elements such as promoters (Williams *et al.*, 1981; Farinha and Kropinski, 1989; Robbins *et al.*, 1989), translation initiation signals (Schauder and McCarthy, 1989), transcription terminators (Kupsch, 1989; Madden and Landy, 1989; Peabody *et al.*, 1989) and other modulator binding sites (Aiba *et al.*, 1989, Kim *et al.*, 1989).

Site-directed mutagenesis (Smith, 1985; Bautista and Graham, 1989; Hofer and Kuhlein, 1989) of cloned DNA sequences allows study of the alteration in phenotype resulting from the introduction of a mutant gene into an organism. This reverses the protocols of classic genetics and exploits what Malik (1989) refers to as 'the predictive power of recombinant DNA technology'.

3.2 The fermentation industry

The potential industrial uses of GMOs are virtually unlimited. DNA manipulation technology affects manufacturing industry in two major ways, particularly in the food and beverage, pharmaceutical and health-care sectors. Firstly, it offers the possibility of genetic improvement of traditional industrial strains at a rate that may be orders of magnitude faster than conventional selection approaches. Schwab (1988) discusses the potential for altering traits which affect the productivity and cost-effectiveness of industrial fermentations, including specific production rate, yield, product quality, product recovery and substrate utilization. Examples of characteristics which might be amenable to genetic manipulation are the ethanol tolerance (D'Amore and Stewart, 1987) and carbohydrase activities (Cantwell *et al.*, 1986) of brewing yeasts; regulation in amino acid producing strains (Beppu, 1986; Niederberger, 1989); efficiency of single cell protein-producing strains (Schwab, 1988); antibiotic synthesis in *Streptomyces* (Epp *et al.*, 1989; Hunter and Baumberg, 1989).

Secondly, and perhaps more dramatically, new technologies now make possible the production, by large-scale microbial fermentation, of substances previously available, if at all, only in microgram quantities. Therapeutic agents, such as insulin, human somatotropin and interferons are already in production using GMOs. The benefit of using GMOs in these syntheses is not primarily the economics of the process but the fact that such agents of human origin would be otherwise virtually unobtainable. Other products of human genes, such as clotting (Malik, 1989) and anti-clotting agents (Gan *et al.*, 1989), are currently at the development stage. Another major advance has been in the preparation of vaccines against human and animal diseases (Winther and Dougan, 1984; Kaper and Tacket, 1988). The currently available anti-hepatitis vaccine, produced from recombinant yeast (McAleer *et al.*, 1984) has the advantage of low cost and high purity, being free from the real or perceived risk of contamination with adventitious agents, such as HIV.

Another economically attractive use for GMOs is in manufacturing industrially important enzymes such as amylase (Sato *et al*, 1989; Vehmaanpera and Korhala, 1986; Reinikainen *et al.*, 1988; Kim *et al.*, 1990), and glucanase (Lee and Pack, 1987)). The use of GMOs as the source of commercial enzymes permits increased yield (Vehmaanpera *et al.*, 1987) and product stability (Oriel and Schwacha, 1988; Ishiawata *et al.*, 1990).

3.3 Environmental management

Ironically, in view of public unease about adverse environmental impacts of genetic engineering, many of the most promising developments in r-DNA technology are in the area of environmental protection and control. The development of microorganisms with new or improved degradative capacities offers new approaches to toxic waste disposal (Dwyer *et al.*, 1988: Liaw and Srinivasan, 1990), oil pollution (Reddy *et al.*, 1989) and effluent treatment (Sojka and Ying, 1987). Enhanced recovery of metals (Romeyer *et al.*, 1988) and oil (Springham, 1984) is a potential use for GMOs which has environmental, as well as economic implications.

3.4 Agricultural applications

Recombinant plasmids have a crucial role in the genetic manipulation of plants. *Agrobacterium tumefaciens* infects plants by transferring the T_i plasmid into plant tissue (Chilton *et al.*, 1977) and integrating a portion of the plasmid DNA into the plant genome (Zambriski, 1988). By constructing expression vectors derived from the T_i plasmid, where the cloned gene is under the control of plant-specific promoters, it has proved possible to achieve stable expression of foreign genes in plants (Herrera-Estrella *et al.*, 1983; 1984). The potential uses of T_i-based vectors, which include improving the nutritional qualities of crop plant proteins, modifying the growth requirements of plants, and increasing their resistance to pests, diseases and adverse environments (Flavell *et al.*, 1984; Larkins, 1987; Beachy *et al.*, 1987) seem limited only by the host-range of *A. tumifaciens*. The conventional wisdom postulated that, since only certain dicotyledonous plants were susceptible to infection by *A. tumifaciens*, other vector systems would be required for the transfer of genes to monocotyledons. However, it has transpired that T_i-based vectors transfer genes to some monocotyledons (Hooykass-Van Slogteren *et al.*, 1984; Byteber *et al.*, 1987; Schafer *et al.*, 1987). The development of *A. tumifaciens* mutants with expanded host range (Bucholtz and Thomashow, 1984) and new detection methods for integrated genes (Vancanneyt *et al.*, 1990) promise virtually unlimited potential for this system.

The genetics of the symbiotic nitrogen-fixing *Rhizobium* spp. have been the subject of intensive study with a view to improving efficiency and transferring nitrogen-fixing systems to other organisms (Beringer and Hirsch, 1984; 1988). Recent advances in the molecular biology of photosynthetic bacteria also suggest the possibility of transferring their nitrogen-fixing and photosyntheic systems to higher organisms (Scolnik and Marrs, 1987).

Two agriculturally significant projects which have proved successful involve the development of GMOs which enable plants to withstand adverse circumstances. The now famous 'ice-minus' recombinants of *Pseudomonas syringae* protect potatoes and other broad-leaved plants from frost injury (Lindow and Panopulos, 1988). The *Bacillus thuringensis* insecticidal endotoxin gene cloned in root-colonizing pseudomonads (Dean, 1984; Obukowicz *et al.*, 1987) protects crops from soil-borne pests.

4. Safety considerations

Obviously, to talk of 'safety', implies the existence of risk, real or perceived. Berg *et al.* (1974; 1975), when calling for a moratorium on certain types of DNA manipulation experiment, listed a number of potential risks of recombinant organisms. Since then, many thousands of words have been devoted to the subject (for example, Beers and Bassett, 1977; Sussman *et al.*, 1988). The OECD report, *Recombinant DNA Safety Considerations* (OECD, 1986), predicts that the potential hazards of using GMOs in industrial processes are qualitatively the same as those for other biological agents: (i) pathogenicity to man, animals or plants; (ii) toxicity, allergenicity or other biological effects of the non-viable cell or its products, and (iii) adverse effects on the environment

in the event of deliberate or accidental release. More succinctly, Beringer and Bale (1988) summarize the hazards of GMOs as pathogenicity and colonization of the environment.

4.1 The regulatory framework

Because of scientific and public concern about possible hazards in the use of GMOs, national and international agencies have seen a need to regulate their use. The subject of regulation is dealt with in detail in Chapter 2, so this chapter will outline only those provisions which have a direct bearing on the construction and use of plasmid vectors.

In the western world, three agencies dominate our thinking on the use of GMOs: the US National Institutes of Health (NIH), the EC and the OECD. Despite a period of incipient confusion in the early 1980s, when national and international agencies appeared to be acting independently of each other, a consensus view is now emerging from these agencies with respect to terminology, the classification of organisms, hazard assessment and conditions of use.

The OECD (1986) report, which set benchmarks for international harmonization of the regulatory framework, reviews national positions on safety of recombinant (r-DNA) organisms for industrial, agricultural and environmental use, and existing or proposed regulations for r-DNA organisms. It also identifies criteria for authorization of the use of r-DNA organisms and explores ways of monitoring production and use in these sectors, in the belief that 'a common understanding of these safety issues will provide the basis for taking initial steps toward international accord, the protection of health and of the environment, the promotion of international commerce, and the reduction of national barriers to trade in the field of biotechnology'.

The survey of national legislation revealed an array of legislation relating to health, safety and environmental protection which could be applied to manage risks which might arise from GMOs. In addition, specific provisions for r-DNA technology exist in most countries in the form of voluntary guidelines or recommendations.

All regulatory systems and guidelines now distinguish between contained use of GMOs, in laboratory studies or industrial fermentations, and deliberate release to the environment. The EC Commission has perceived a need for directives in both areas for several years. Within the past decade, three directives specifically related to recombinant organisms were mooted but these have now been rationalized to two directives for regulation of contained use and deliberate release, respectively, which were adopted in April 1990 (EC, 1990a,b). Looking at the amendments and revisions which have been made to successive drafts of the directives during the past 3 years, it is possible to detect the growth of two opposing influences: (a) the demands of technical progress for increased deregulation (or self-regulation) of industrial uses of GMOs, and (b) a greater political sensitivity to environmental issues resulting in more stringent requirements for reassurance on potential risk to the ecosystem.

The directives will give regulatory effect to the *de facto* situation in most member states. A Commission document published in 1988 (EC, 1988) contains, in addition to contemporary versions of the draft directives and their annexes, an explanatory

memorandum for each directive which includes a summary of the objectives and a review of the contemporary regulatory position in each member state. In the UK, regulations under the *Health and Safety at Work Act, 1974* (SI 1810 of 1989) provide for authorization and monitoring of the use of GMOs by the HSE, whereas in Ireland similar provisions are contained in guidelines monitored by a national RDNA Committee, which represents industry, government interests, the universities and the trades unions, under the aegis of the National Board for Science and Technology (NBST, 1987). As in most member states, this procedure is voluntary but serious breaches of the guidelines could be prosecuted under environmental and worker protection legislation.

In addition to the two EC directives on GMOs, the Council has also adopted a directive on the protection of workers from risks arising from exposure to biological agents, including GMOs (EC, 1987). There are also a number of directives and proposed directives relating to worker protection and to the environment which impinge indirectly on the use of GMOs. There is a useful summary of these related instruments in a Commission inventory (EC, 1986).

4.2 Risk assessment

Risks to the health of workers and others in the immediate vicinity of the workplace are the main concern in assessing the hazards associated with the contained use of GMOs. These risks are considered proportional to the scale of the operation and all regulatory systems distinguish small-scale use for research and development, defined rather arbitrarily as volumes of less than 10 litres, from large-scale industrial use. The EC directive does not require notification or authorization for small-scale use of GMOs for research and development, unless they are pathogenic or contain genes for toxin production.

For large-scale industrial use, the risks to health and possible risks to the environment in the event of escape of the organism from the production area must be evaluated and an appropriate level of containment applied. Containment may be physical, i.e. barriers limiting escape of the organism, or biological, i.e. physiological limitations to the survival and replication of the organism outside the process environment (Curtiss *et al.*, 1977; Curtiss, 1988).

4.2.1 Risks to health

Uncertainty about the safety of GMOs has been ameliorated by three 'compelling lines of evidence' (OECD, 1986): (i) experimental risk assessment studies, designed specifically to test the hypothesis that host organisms can acquire unexpected hazardous properties from DNA donor cells, have failed to demonstrate the existence of such conjectural hazards; (ii) more rigorous evaluation of existing information regarding basic immunology, pathogenicity and infectious disease processes has resulted in the relaxation of containment specifications recommended by national authorities; and (iii) experimentation conducted in recent years has elicited no observable novel hazard.

It is now accepted that the level of risk from GMOs may be reliably evaluated from the known characteristics of the components used in their construction. The properties of the host, the vector and the insert which are relevant to human health in an industrial context, include pathogenicity, communicability, host range, infective dose, survival outside the host and antibiotic resistance. Possible toxic or allergenic effects of the non-viable organism or its products, particularly the product of the cloned gene, should also be taken into consideration.

4.2.2 Risks to the environment

GMOs may be accidentally released into the environment during contained use in normal operating conditions, e.g. in waste or as air-borne emissions, or in abnormal operating conditions, where release into the environment results from the process getting out of control. In some cases such releases will pose risks to man or the environment. Therefore the assessment should identify these hazards, working practices and containment measures to minimize the risk of accidental release should be adopted and emergency plans to limit the consequences of accidents drawn up.

The EC directive on contained use of GMOs lists possible hazards to the environment which should be evaluated before deciding on the appropriate level of containment, in order to give a 'legal framework which will both provide adequate protection and, at the same time, allow society to benefit from this rapidly evolving technology', (EC, 1988).

An assessment of the environmental impact of accidental release involves consideration of the host microorganism and the GMO. Relevant characteristics of the host include reproductive cycles, survival, stability of genetic traits, pathogenicity, virulence, infectivity, toxicity, host range, natural habitat and geographical distribution, involvement in environmental processes and interactions with other organisms. Characteristics of the GMO which should be evaluated include the nature of the genetic modification, function of the cloned gene(s), the nature and source of the vector, the amount of non-native DNA in the final construct, genetic stability, frequency of plasmid mobilization or other forms of genetic exchange, the level of expression of inserted genes and the activity of expressed protein, survival and multiplication in the environment, and techniques for monitoring persistence and detecting gene transfer. Risk assessment should also take into consideration the site where accidental release might occur, and details of the ecosystem at risk, including geographical and climatic features, social factors and possible methods for decontamination of the area in the event of accident.

4.3 Appropriate levels of containment

The objective in selecting containment strategies is to match an appropriate level of safety measures to the conclusions of the risk assessment. Most GMOs used in industry will be low-risk and require only minimal controls and containment. The EC directive defines criteria for classifying low-risk, or Group 1, GMOs. These are similar to those

suggested by the OECD (1986) and by the European Federation of Biotechnology (EFB) (Küenzi *et al.*, 1987). The host should be non-pathogenic, not contain adventitous agents, have an extended history of safe industrial use or built-in limitations to survival in the environment. The vector/insert should be well characterized and free from known harmful sequences; should be limited in size to the required sequence; should not increase the stability of the construct; should be poorly mobilizable; should not transfer resistance markers to species not known to acquire them naturally. The GMOs should be non-pathogenic, as safe industrially as the host and without adverse consequences to the environment. Group 1 organisms can be managed similarly to other industrial microorganisms but other, more potentially hazardous, GMOs (Group 2) require a degree of containment.

The purpose of containment is to reduce exposure of workers and other persons within the plant (primary containment) and to prevent release of potentially hazardous agents into the environment (secondary containment).

4.3.1 Good industrial large-scale practice (GILSP)

The vast majority of microorganisms used in conventional processes are considered safe because, in long periods of industrial use (sometimes centuries), they have rarely given rise to safety problems. 'Industries that use biotechnology processes in OECD countries have maintained safety through adherence to good industrial practices that favour the use of low-risk microorganisms' (OECD, 1986).

The concept of GILSP for GMOs which can be handled at a minimal control level, which was developed by the OECD (1986) and the EFB (Frommer *et al.*, 1989a), has been endorsed by the EC directive. For GILSP, only fundamental principles of good occupational safety and hygiene are required, including: (i) keeping workplace exposure to any physical, chemical or biological agent to the lowest practicable level; (ii) exercising engineering control measures at source, supplemented with protective clothing and equipment, if necessary; (iii) testing and maintaining control measures and equipment; (iv) monitoring viable process organisms outside the area of primary containment; (v) providing information and training for personnel; (vi) establishing a biosafety committee; and (vii) formulating and implementing codes of practice for worker safety.

An OECD Working Party is currently drafting recommendations to harmonize the use of GILSP in member countries and another Working Party has recently issued similar recommendations for good development practice (GDP), applicable to pilot-scale research with GMOs (OECD, 1990).

4.3.2 Matching physical containment to assessment of risk

The EC (1990a) Directive for Contained Use has the objective of ensuring that the level of control is commensurate with possible risks. There will be cases where the potential pathogenicity of the GMO demands special measures for containment and for accident response. Group 2 organisms require containment, waste control and, in some cases, emergency response plans.

An evaluation of the characteristics of the GMO may indicate that the containment level used in the laboratory for the construction of the GMO is not appropriate to the large-scale process. For example, the laboratory level of containment may be high if the donor is a pathogen, but the GMO may be non-pathogenic, because it contains only sequences not associated with the pathogenic phenotype, e.g. an *E. coli* host/vector system expressing a viral surface antigen. Therefore, the GMO should be re-evaluated for appropriate containment level before transfer to a large-scale process. Disabled host strains are recommended (biological containment), provided they are efficient under process conditions.

The industrial process should be considered in unit process steps, as this allows flexible selection of procedures and design best fitted to assure adequate containment. Physical containment includes three elements: (i) Equipment is the principal means for achieving containment, but optimum design will vary according to the nature and scale of the process. (ii) Operating procedures should ensure that personnel are aware of risks and are trained and proficient in practices and techniques for safe handling of hazardous material. Elements of operating procedure include: (a) a biosafety or operations manual specifying practices and procedures to minimize or eliminate risks; (b) mechanisms to advise of hazards and to require personnel to follow correct operating procedures; (c) direction of activities by an individual trained and knowledgable in operating procedures, safety measures and potential hazards in the workplace. (iii) Facility design contributes to protection of the environment and persons outside the production area. The degree of sophistication required is determined by the risk and is not independent of practices and procedures or equipment design.

The EC directive (EC, 1990a) lists appropriate containment measures which are very similar to those proposed by the EFB (Frommer *et al.*, 1989, 1991a and b; see also Chapter 3).

4.4 Deliberate release

Many of the most exciting and potentially rewarding (financially and socially) projects in r-DNA technology involve the use of GMOs in open systems. This has proved to be a much more sensitive issue to scientists, regulatory agencies and the public than contained use. Such projects are classified as *deliberate* (or intentional) *release*.

An explanatory memorandum for the EC directive on Deliberate Release (EC, 1988) refers to anxieties about the impact of novel organisms on the environment: 'Not only known or predicted traits of the organism (such as pathogenicity) may raise questions, but also the possibility of displacement of natural populations, alteration of ecological cycles and interactions, and undesired transference of novel genetic traits to other species (i.e. pesticide resistance of a crop-plant passed on to weeds).' However, the memorandum also acknowledges that 'the use of genetically modified organisms could lead to improvements in health and the environment, by permitting the development of more precise agricultural inputs for protection and nutrition and more effective treatment of waste'.

The EC directive on deliberate release (EC, 1990b) requires that a case-by-case notification and endorsement procedure for experimental releases be adopted by a competent authority in each member state. The notifier will be required to present a *dossier* containing details of genetic, physiological and ecological characteristics of the organism, a geographical, ecological and socioeconomic profile of the site of release, monitoring programmes and emergency response plans. A similar, but more extensive, notification is required before placing any product on the market which consists of, or contains, viable recombinant organisms; the notification must take into account the diversity of sites of use of the product, include an assessment of any risks to man or the environment and specify labelling, packaging and instructions for use.

The perception of a greater level of risk associated with deliberate release is based, not only on the obvious factor of environmental exposure, but also on the assumption that organisms whose effect is dependent on interaction with one or more components of an ecosystem, must, by their nature, have a finite probability of survival in the wild. However, despite the much more rigorous conditions that national regulatory agencies and the EC directive apply to proposals for deliberate release, the central question in risk assessment is the same as that pertaining to contained use: what is the potential for damage to man and the environment? Most national regulatory agencies, and the EC, have adopted the 'step-by-step' approach to deliberate release advocated by the OECD (1986). The European Committee on Regulatory Aspects of Biotechnology have proposed a checklist (ECRAB, 1986) of consecutive steps, during each of which information can be collected which would facilitate the estimation of risk in proceeding to the next step: (i) project initiation; (ii) contained laboratory experiments; (iii) small-scale, closely monitored, non-contained experiments; (iv) field application; (v) commercial application.

The elements of a risk assessment for the deliberate release of a GMO are: (i) an estimate of the probability of survival in competition with other components of the ecosystem; (ii) effective methods for monitoring persistence of the test organism in the ecosystem; (iii) methods for 'recall' of the test organism; and (iv) potential hazards to humans and the ecosystem if the test organism becomes established.

Domsch *et al.* (1988) suggest that the assumption should be made, as a matter of principle, that an ecological disturbance will take place when a GMO is released and that proof is required that such effects will not occur. Colwell *et al.* (1988) point to serious gaps in our knowledge that must be plugged by research before we can confidently assess the impact of deliberate release. 'If the major ecological issues that arise from introduction of GEMs to the environment are reduced to their essential points, it can be concluded that the following represent information gaps, that is, areas of research needed: (i) detection and monitoring, (ii) horizontal transfer of the genetic information of the GEM, (iii) fate of the GEMs after release into the environment, e.g. survival and dispersion, and (iv) effects of GEMs on the environment. Subsidiary to these, but equally important, are containment and recovery, i.e. 'recall', if adverse effects occur after release of GEMs.'

The most controversial aspect of the EC directive is the provision that the Commission and all member states must be consulted before a competent authority may endorse a product. This is proposed in order to promote free circulation of the

product within the Community and for the harmonization of the internal market. This approach has been criticized (Young and Millar, 1989) as being over-bureaucratic and cumbersome and as an invitation for the erection of non-tariff trade barriers. The wisdom of including products in a deliberate release directive, which is aimed primarily at research and development projects, has also been questioned. The USA regulates products rather than the process involved, as the regulatory agencies insist that whether an organism is 'unmodified' or 'genetically modified' is not, in itself, a useful determinant of safety or risk. New products must undergo adequate and appropriate testing to ensure safety and efficacy, regardless of the technology employed (Grant, 1986; Fordham and Block, 1987). The European National Biotechnology Associations, which link diverse industry interests in the food, agricultural and pharmaceutical sectors have sought a similar approach by the EC, so that products would be subject to appropriate product sector legislation, which would require safety evaluations of products, including an environmental impact assessment.

4.4.1 Detection and monitoring

At the first International Conference on the Release of Genetically Engineered Microorganisms (Regem) in 1988, Sayler laid down basic criteria for the monitoring methods to be used for the detection of GMOs in the environment (Sayler, 1988). He defined five factors which must be considered when evaluating the method to be used in particular circumstances: *sensitivity*, the ability to detect small numbers of organisms; *specificity*, unambiguous detection of specific genotypes; *in situ analysis*, direct or indirect environmental analysis, avoiding cultivation bias; *speed/efficiency*, many or large samples processed with short turn around; *cost* must be in proportion to value of product or process.

The limitation of many methods currently available is that they depend, either directly or indirectly, on cultivation of the recombinant strain. The standard method for monitoring the stability of recombinant plasmids in populations grown under laboratory conditions, is to assess the percentage of viable cells expressing the plasmid-coded phenotype. This type of approach makes two basic assumptions: (a) that the recombinant will be viable; and (b) its phenotype will be expressed under the test conditions. Even for recombinants grown under laboratory conditions, these assumptions are not wholly justified (see section 5.1.1) and it would be rash to assume their validity for the recovery of recombinant strains from samples of soil, water or air.

An additional drawback of cultivation-dependent methods is that they may not adequately track the gene of interest, thus not detecting horizontal transfer of the cloned gene to other species. Colwell (1988), discussing the inadequacy of cultivation-dependent methods, draws on her experience of enumerating enteric pathogens in water samples (Colwell *et al.*, 1985; Brayton and Colwell, 1987), to argue that, 'a well-studied organism such as *E. coli* is recovered with variable success by culture methods', and that, if such methods are inefficient for monitoring non-engineered bacteria, more efficient and specific methods must be developed for the detection of GMOs. The low plating efficiency of most microorganisms (Postgate, 1989) is seen as a serious weakness of any monitoring methodology which includes a plating step (Ford and Olson, 1987; Roszak and Colwell, 1987).

Direct microscopy and fluorescent monoclonal antibodies can be used with great precision to detect specific organisms in environmental samples (Colwell *et al.*, 1988). The fluorescent antisera stain cells brightly so that they can be detected in samples containing suspended solids. Muyzer (cited by Sayler, 1988) reportedly used fluorescent antibodies successfully to estimate the relative abundance of *Thiobacillus ferro-oxidans* in coal-water slurries. Trevors *et al.* (1990) have used a similar method to detect non-culturable *Pseudomonas fluorescens* cells in soil and water samples. With this method it is even possible to distinguish between dead and living cells, by using nalidixic acid and yeast extract to enlarge viable cells (Kogure *et al.*, 1979; Brayton and Colwell, 1987). Zoltick *et al.* (1989) have developed a strategy for the specific generation of antibody directed against any protein encoded in a cDNA molecule.

However, the limitations of antibody-based methods are cross-reactivity between closely related species, the difficulty of distinguishing between plasmid-bearing strains and their 'cured' isogens and their unsuitability for detecting horizontal transfer.

Methods for monitoring and detection of GMOs, particularly those containing recombinant plasmids, must ultimately rely on gene-specific probes. Sayler (1988) reporting a roundtable discussion of detection and monitoring at the Regem Conference, singles out nucleic acid hybridization as having the merits of being both cultivation-independent and gene-targeted, and discusses the relative merits of using DNA probing in colony hybridization techniques and the alternative, more tedious but possibly more effective, method of using DNA probes to detect small amounts of target DNA in total DNA samples extracted from environmental populations of organisms.

Colony hybridization has been used successfully to enumerate and identify conventional and recombinant bacteria in a variety of environments. Hodgson and Roberts (1983) reported a high level of strain specificity when they used colony hybridization to determine nodule occupancy by strains of *Rhizobium*. Similar methods have been used to detect *E. coli* and *Salmonella* spp. in food samples (Hill *et al.*, 1983; Fitts *et al.*, 1983), to distinguish closely related species of *Bacteroides* (Saylers *et al.*, 1983) and *Thiobacillus* (Yates *et al.*, 1986), to identify *P. fluorescens* strains (Festl *et al.*, 1986), to monitor mercury resistance (Barkay *et al.*, 1985) and catabolic genotypes in environmental samples (Sayler *et al.*, 1985). The method achieves a high level of sensitivity and specificity: one target colony can be detected in 10^6 non-homologous background colonies (Sayler *et al.*, 1987).

Holben *et al.* (1988) developed a protocol to yield purified bacterial DNA from a soil bacterial community which they digested and analysed by hybridization. With this method they were abe to detect *Bradyrhizobium japonicum* at densities as low as 4.3×10^4 g^{-1} (dry weight) of soil.

However, although these methods are highly specific for detecting the gene of interest, even if transferred to indigenous species, they do not distinguish between living and dead cells and they require enrichment to develop sufficient DNA for hybridization (Colwell *et al.*, 1988). If the DNA sequences in GMOs are to be detected by DNA probes, a high concentration of these sequences must be present in the sample, otherwise the target sequence concentration will be below the detection threshold of conventional probes. Somerville *et al.* (1988) developed a method for enhancing sensitivity, based on chain elongation. In this method, specificity is determined by probe hybridization, while sensitivity is enhanced by polymerization into adjacent

genes. Steffan and Atlas (1988) also used target DNA amplification to enhance the detection of genetically engineered bacteria. They used the polymerase chain reaction (PCR) technique of Saiki *et al.* (1988) which involves melting the DNA, annealing short oligomer primers to regions flanking a target sequence and using *taq* polymerase to extend the DNA from the primers across the target region. The new duplexes are melted by heating and the process is repeated. The result is an exponential increase of the target sequence, such that the it can be amplified by a factor of more than 10^7 within a few hours. Applying PCR to bacterial DNA isolated from sediment samples permitted the detection of as few as 100 cells of *Pseudomonas cepacia* per 100 g of sediment against a background of 10^{11} diverse non-target organisms. Bej *et al.* (1990) have used a similar technique to detect *E. coli* and other coliform bacteria in water samples.

The use of DNA probes, in conjunction with PCR amplification of the target DNA, appears to be the single most promising technology for the monitoring of GMOs and until such time as the scientific community has accumulated sufficient data on the impact of GMOs on the environment, it is likely that national and international regulatory agencies will insist on the use of similar strategies to monitor the fate of GMOs during pilot stages of release projects. However, at the current stage of its development, this technique is labour intensive and costly, and alternative, simpler approaches to detection are still a focus for research.

Another possible strategy for monitoring is the introduction of a 'reporter gene' into recombinant plasmids to facilitate monitoring. Barry reported at the Regem Conference on the successful use of the *lac* gene to track *P. fluorescens* (in Sayler, 1988) and there is an intriguing suggestion of using the *lux* genes of bioluminescent bacteria (Meighen, 1988) to construct a reporter strain which would give off visible light.

It is clear that ability to detect and monitor genetically engineered strains is a major consideration in the success of deliberate release projects and, as Ford and Olson (1987) point out, it is an important feature that should be anticipated in the design and construction of recombinant strains. The discussions on this topic at the Regem Conference concluded that 'simple qualitative detection of GMOs is not a goal of current research, rather the specific quantitative relationships of GMOs to the physical, chemical and ecological parameters affecting their maintenance, stability and effects is the primary target of research in the field' (Sayler,1988).

It is worth noting that the current research initiative to develop more effective methods of bacterial identification and enumeration, although triggered by the economic imperatives of genetic engineering, is likely to pay dividends in more mundane, but perhaps more important, applications, such as the monitoring of water quality, where existing methods are less than adequate (Roszak and Colwell, 1987).

4.4.2 Survival and persistence of GMOs

Colwell *et al.* (1988) point out that 'the research needs for improved systematics in microbial ecology are most critical for species that comprise the soil, water and atmosphere populations of the natural environment'. It is true that these are areas of microbiology that, for want of commercial incentive and financial support, have been

neither 'popular or profitable' in recent decades and our knowledge of fundamental facts about the life and death of bacteria is scant (Postgate, 1989).

Bacteria of the genus *Rhizobium* have long been of commercial importance as natural soil fertilizers and much of our knowledge of bacterial persistence and inter-species competition is based on studies of this group of organisms, for example studies by Alexander (1985), Lambert *et al.* (1987), Joos *et al.* (1988). The results of such studies suggest that natural or genetically engineered rhizobia applied as root inoculants are confronted with an extremely dynamic and complex ecosystem and a number of ecological constraints that will hold them in check.

Similar conclusions can be drawn from studies of *Pseudomonas* spp. in soil. Compeau *et al.* (1988), monitoring the survival of rifampicin-resistant mutants of *P. putida* and *P. fluorescens* in soil, found that colonization of sterile soil with either the wild type or a mutant strain precluded normal colonization by the second added strain, and concluded that there are limited sites for colonization of *Pseudomonas* spp. in soils. Dijkstra *et al.* (1987) observed competition between *P. fluorescens* and *B. subtilis* for colonization of wheat seedling roots.

As yet, there are relatively few published reports on the fate of GMOs in ecosystems. Devanas and Stotsky (1988) found that the fate of plasmid-containing strains of *E. coli* was influenced by interactions of the host and vector, such as plasmid maintenance, plasmid copy number and host fitness, by biotic factors, such as competition with indigenous populations, and by environmental factors, such as soil type and humidity. In this laboratory we have also found that environmental conditions, such as temperature and nutrient availability, affect the survival of plasmid-containing strains of *B. subtilis* in a microcosm and influence competition with other *B. subtilis* strains. We also noted that the competition dynamics were very different from those observed in conventional laboratory cultures (Meyler and Hussey, unpublished).

Dwyer *et al.* (1988) studied the persistence of a genetically engineered *Pseudomonas* strain in synthetic sewage and demonstrated a survival pattern similar to the parental strain. Trevors *et al.* (1990) used vertical microcosms, through which water could be percolated, to study the survival and transport of a genetically engineered strain of *P. fluorescens* and, using DNA probes, could detect non-culturable cells in soil and water up to 10 days after inoculation.

Winkler (1987) reported that the survival of Group 1 (GILSP) organisms in the event of accidental release was improbable because they would arrive as single organisms and would have to adapt to a temperature difference of at least 15°C and to a low level of unusual substrate.

4.4.3 Horizontal gene transfer

Significant sequence homologies in plasmid-coded genes from diverse bacterial genera is evidence that transfer of plasmid DNA takes place in natural ecosystems. Levy and Marshall (1988) summarize the evidence for transfer of antibiotic resistance in natural populations and conclude that a genetic trait, once introduced into a bacterium, has the potential to be transferred, and that the possible spread of genes borne on plasmid vectors must be assessed before release to the biosphere. Stotsky and Babich (1986), in

a comprehensive review of genetic transfer between species, point out the hazards of *E. coli* as a host for recombinant plasmids because it is 'sexually promiscuous' and can transfer genetic information to more than 40 other Gram-negative genera. Although most published data on genetic exchange relate to conjugative plasmids (Trevors and Oddie, 1986; Khalil and Gealt, 1987; Trevors, 1987), other mechanisms, such as transformation, transduction and cell fusion, can transfer genes through natural populations and require further study (Trevors *et al.*, 1987).

Stotsky and Babich (1986) conclude that there has not been adequate research on the perturbations which anthropogenic microorganisms might occasion to the homeostasis of the biosphere which they regard as the fundamental concern regarding deliberate release.

5. Optimizing production from recombinant plasmids

Much of the emphasis in current research on GMOs is on designing recombinant plasmids which will allow maximum product recovery, at minimum cost, without risk to health or the environment. A variety of factors must be taken into account when designing a fermentation system for yield optimization, not only the characteristics of the host/vector system, but also reactor design and process control.

Laboratory studies indicate that a number of intrinsic, genetically-controlled, properties of the organism affect its behaviour and yield potential under fermentation conditions. Productivity is influenced by characteristics of the plasmid such as stability, gene dosage and gene expression, as well as characteristics of the host and of the recombinant organism, such as secretory mechanisms, growth rate and substrate utilization. Less attention has been paid to the behaviour of recombinant strains, particularly high level producers, under fermentation conditions but reactor configuration and process factors such as temperature, pH and nutrient availability, are all potential influences on product yield. However, neither the intrinsic, genetically determined, characteristics of the organism or the extrinsic, environmentally defined, properties of the system are independent variables and the interactions between them can affect, not only the productivity, but also the biosafety of the process.

5.1 Genetically-determined characteristics

Recombinant DNA techniques permit the construction and manipulation of host/vector systems to contrive optimum product yield. However, although many of the variables that determine productivity have been identified, our knowledge of the genetic determinants of these characteristics, and our understanding of the interaction between these variables, is still limited.

5.1.1 Strain stability

Imanaka and Aiba (1981) define stability as the ability of transformed cells to maintain a plasmid unchanged during growth and to manifest the phenotypic characteristics of the plasmid.

It is usual to distinguish two types of instability in plasmid-containing strains: (a) segregational (or heritable) instability, where errors in plasmid replication or partition give rise to cells that contain no plasmid; and (b) structural instability, where an alteration in the plasmid genome (usually a deletion or rearrangement of genetic material) prevents expression of a plasmid-coded function. However, if we define instability as loss of plasmid-coded phenotype, then it may be necessary to add a third type; (c) expression instability, where cells containing intact plasmid may respond to environmental conditions by failing to express a plasmid-coded function.

Stability is probably the single most important parameter in the design of recombinant strains for industrial use. Productivity will be severely reduced if the plasmid is lost or structurally damaged in the course of the fermentation. Stability also has important implications for risk assessment; whereas the success of a process may depend upon stable maintenance of the recombinant plasmid throughout the fermentation, a very stable construct poses the risk of prolonged survival in the environment in the event of accidental release.

It is usually assumed that genetic control mechanisms must function effectively in naturally-occurring host/plasmid systems if the plasmid is to be stably maintained. However, genetically engineered plasmids can be structurally and functionally very different from the parent plasmid and they are frequently introduced into a non-native host. Both characteristics contribute to a loss of control of replication and partition mechanisms and such plasmids are frequently unstable. Many recombinant plasmids have been shown to be unstable in *E. coli* (Wouters *et al.*, 1980; Jones and Melling, 1984; Noack *et al.*, 1981) and in other Gram-negative bacteria (Kim and Meyer, 1984). Grandi *et al.* (1981) found that *S. aureus* plasmids were unstable in *B. subtilis* and virtually all recombinant plasmids show some degree of segregational instability in *B. subtilis* (Ostroff and Pene, 1984; McLoughlin *et al.*, 1987; Newell *et al.*, 1988).

A number of plasmid-related characteristics affect stability including the size of the insert, the origin of the heterologous DNA and the level of expression of cloned genes (McDonald and Burke, 1982; Michel *et al.*, 1980). Warnes and Stephenson (1986) found that inserts smaller than 2 kb had no detectable effect on the stability of pAT153 in *E. coli* but that larger (8–21 kb) inserts had an increasingly destabilizing effect. Bron and Luxen (1985) saw a similar effect when shuttle plasmids were cloned in *B. subtilis* but not in *E. coli*.

The source of the insert also appears significant. Kim and Meyer (1984) found that the presence of *E. coli* chromosomal DNA on the plasmid pQSR49 in *P. putida* resulted in plasmid instability which increased with the size of the insert. When DNA fragments of similar size from the *P. putida* chromosome were inserted into the same plasmid, no instability was observed. This instability was peculiar to *P. putida*; plasmids containing *P. putida* DNA were stable in *E. coli*.

Laboratory studies of the segregational stability of recombinant plasmids usually measure the rate at which plasmid-free cells accumulate within a population of recombinant bacteria. Two components contribute to the rate of accumulation of plasmid-free cells: (a) the frequency of segregation of plasmid-free cells; and (b) population balance between plasmid-containing cells and the plasmid-free segregants.

Segregation of plasmid-free cells from a population of plasmid-containing unicellular organisms (bacteria or yeasts) results from a gradual loss of plasmid from daughter

cells over a number of generations, due to defective replication or partitioning at cell division (Nordstrom and Austin, 1989). To ensure plasmid maintenance within the cell through successive generations, plasmid replication and partition must be accurately controlled. Bacterial chromosome replication must be accompanied by a doubling of the cell's complement of plasmid molecules; at each round of cell division, the content of plasmid molecules must be equally divided between daughter cells. Some low copy number plasmids have a determinant that prevents cell division in cells containing a single plasmid copy (Ogura and Higara, 1983; Miki *et al.*, 1984).

Several genetic loci in *E. coli* have been described which appear to control the replication and partition of low copy number plasmids, such as R1 (Gerdes and Molin, 1986; Pal *et al.*, 1986; Wagner *et al.*, 1987; Nordstrom *et al.*, 1988). The replication function involves the *ori* (origin of replication) locus in the plasmid and plasmid-coded RNA and protein molecules which exercise positive and negative control of replication initiation in a mechanism which compensates for any deviation in copy number by adjusting replication frequency (Nordstrom *et al.*, 1984; Scott, 1984; Sherratt, 1986; Nordstrom and Austin, 1989).

However, most *E. coli* vector systems are based on high copy number replicons, such as ColE1. A complex regulatory system, involving host- and plasmid-coded RNA and protein molecules, RNA processing and DNA methylation control plasmid replication and also determine compatibility with other plasmids. Many of the plasmid-coded functions required have been mapped and identified (Hakkaart *et al.*, 1984; Scott, 1984; Veltkamp *et al.*, 1984; Sherratt, 1986). The deletion or rearrangement of some of these genes in the construction of cloning vectors is probably responsible for the instability of many recombinant plasmids. Some of these functions are *trans*-acting and 'par' genes cloned into vectors such as pBR322 increase their stability (Ogura and Hiraga, 1983; Austin and Abeles, 1983; Gerdes *et al.*, 1985; Saurugger *et al.*, 1986; Schwab, 1988). Conversely, Kolot *et al.* (1989) have identified a region of pBR322 DNA that destabilizes the plasmid in *cis* or in *trans*.

Plasmid-coded determinants for replication and partition have also been tentatively identified in *B. subtilis* (Gryczan *et al.*, 1982; Alonso and Trautner, 1985; Chang *et al.*, 1987; Watabe and Forough, 1987), *Streptomyces* (Kendall and Cullum, 1988) and *S. cerevisiae* (Caunt *et al.*, 1988). Association of the origin of DNA replication with the cytoplasmic membrane may also play a role in regulating the initiation of replication (Sueoka *et al.*, 1984; 1986).

Little is known about the mechanisms which control the partition of plasmid copies at cell division. All low-copy number plasmids are likely to have an active partitioning system analogous to the segregation of chromosomes by mitosis in higher cells (Nordstrom and Austin, 1989) but the segregation of high copy number plasmids may be random.

Novick (1975) proposed a model based on equipartition, where each daughter cell inherits half the total number of plasmids present at cell division. Hashimotoh-Gotoh and Ishii (1982) proposed an alternative single-site inheritance model in which only the copies of the last plasmid replication are equipartitioned, while the rest are randomly distributed. A modification of this latter model, called the pair-site model, is similar to it in all respects but does not assume a link between replication and partitioning (Nordstrom and Aagaard-Hansen, 1984).

Many high-copy plasmids exhibit greater instability than would be predicted. In *E. coli* this instability probably arises from plasmid multimerization (Summers and Sharratt, 1984). The plasmid ColE1 encodes a recombination site, 'cer', at which site-specific recombination can occur to convert plasmid multimers to monomers (Sherratt, 1986). Sherratt has identified at least three host-coded proteins, Xer A, B and C required for resolution of plasmid multimers. These exemplify the genetic parsimony and opportunism of plasmid biology, as each of these proteins has another quite different, host-related, function (Stirling *et al.*, 1988). We have found no evidence of multimerization in *B. subtilis* and the instability of recombinant plasmids in this host must have other explanations, possibly the fact that most cloning vectors are based on non-native replicons (Newell *et al.*, 1987).

Structural instability is manifest as a partial loss of plasmid-coded phenotype, due to insertions, deletions or structural rearrangement of the plasmid DNA (Primrose and Ehrlich, 1981; Kreft and Hughes, 1982; Nugent *et al.*, 1983; Kadam *et al.*, 1987; Harington *et al.*, 1988). In most systems, the incidence of structural instability is relatively low and it does not pose a major problem in an industrial context, except in recombinants where the cloned gene is expressed from a very strong promoter. In such cases, transcription appears to interfere with accurate replication of the plasmid DNA and this effect is enhanced by strong selection in favour of deleted plasmids (Nugent *et al.*, 1983; Burke and Hussey, unpublished). Janniere *et al.* (1990) found that using large plasmids as vectors decreased the incidence of structural instability.

5.1.2 Copy number

One of the reasons for using plasmid vectors for construction of GMOs, rather than the more stable configuration of chromosome integration, is the opportunity to introduce multiple copies of the gene into each cell, on the assumption that product yield will be proportional to gene dosage. This is sometimes true but the capacity of the cell to produce large quantities of a protein is limited by the nature and function of the protein. In *E. coli* production of β-lactamase is a function of plasmid copy number but, although the specific activity of chloramphenicol acetyltransferase also increases linearly with the gene dosage up to a level at least tenfold higher than the wild-type plasmid, chloramphenicol resistance reaches a plateau at a gene dosage less than twice that of the wild-type plasmid, presumably due to the high energy demand on the cells (Uhlin and Nordstrom, 1977). Similarly, we have observed that, while chloramphenicol acetyltransferase activity in *B. subtilis* is a fairly accurate estimate of the copy number of plasmid pPGV138, levels of kanamycin nucleotidyltransferase, which is encoded by the same plasmid, do not increase proportionately with gene dosage (Newell *et al.*, 1987).

A number of factors limit the cell's ability to synthesize a protein. The supply of energy and raw materials is critical and, if the synthesis of a single protein sequesters too large a share of metabolic resources, the viability of the host is threatened. The replication and maintenance of high copy number plasmids, high level expression of a cloned gene and the accumulation of large quantities of an unnecessary protein all adversely affect the cell. However, even though high copy number may not produce a large amount of protein and may have adverse effects on growth rate and substrate

utilization, it can contribute to increased stability (Futcher and Cox, 1984; Newell *et al.*, 1988).

If the segregation of high copy number plasmid molecules at cell division is random, then the probability of either daughter cell failing to inherit a plasmid is a binomial function directly related to copy number. Primrose *et al.* (1984) postulate that the probability, $P(0)$, of either daughter cell failing to inherit a plasmid is described by the equation:

$$P(0) = 2(1/2)^c$$

where c is the copy number at cell division. For plasmids with a copy number less than 10, this equation predicts a relatively high frequency of segregation of plasmid-free cells. However, most plasmids used as vectors for the construction of GMOs have higher copy numbers and, at first glance, the equation predicts very low segregation rates for such plasmids, e.g. a plasmid with a copy number of 40 should have a frequency of segregation of plasmid-free cells of 1.75×10^{-12}. However, this is a simplistic interpretation of the effects of random partitioning. If replication and partition are not controlled, each round of cell division will give rise to subpopulations of cells with copy numbers distributed around a mean for the culture. Successive rounds of cell division will increase the spread around the mean and subpopulations with low copy numbers will have increasingly higher probabilities of segregating a plasmid-free cell.

We have constructed a model, based on this assumption, which calculates the binomial distribution of single cell copy number around the population mean at each generation and predicts the time of appearance of the first plasmid-free cell in a culture of a plasmid-containing strain as a function of the initial copy number (McLoughlin *et al.*, 1990).

The upper limit of the distribution around the mean will be determined by a combination of environmental and genetic factors, and represents the maximum capacity of the cell to support the maintenance and expression of plasmid molecules. There is probably a physical limitation, such as the number of attachment sites on the host cell membrane which, in turn, is modified by extrinsic factors, such as growth rate and cell size. A competitive element will also effectively set an upper limit on copy number distribution in a population; cells with a very large complement of plasmids will be rapidly outgrown by their slimmer, fitter, low copy number sisters. A lower limit to the distribution will be imposed only if possession of a plasmid confers an advantage on the host. For example, when cells containing a plasmid carrying an antibiotic resistance gene are grown in the presence of an inhibitory concentration of the antibiotic, the lower limit of the distribution will be the minimum number of plasmids needed to confer resistance to that concentration. Under these conditions, the population mean represents the copy number giving the highest growth rate, i.e. where increased advantage due to a high level of resistance is not offset by disadvantage due to the metabolic burden imposed by high copy number.

In the absence of selective pressure, there is no lower limit to copy number and cells which are plasmid-free, or contain only a small number of plasmids, will have a growth rate advantage and will rapidly dominate the population. We found that the mean copy

number of the plasmid pPGV138 gradually decreased in the absence of selective pressure accompanied by a proliferation of plasmid-free cells in the population (Newell *et al.*, 1988). An interesting feature of this type of result was a consistent discrepancy observed between the fraction of the population which appeared sensitive to antibiotic on plating cells directly from liquid culture onto antibiotic containing plates, which was always significantly lower than that determined by replica plating colonies which had been subcultured on non-selective medium. We interpret this discrepancy as reflecting a subpopulation of cells with low copy numbers which are unable to express sufficient resistance when challenged directly with antibiotic but which can form colonies on antibiotic plates after passage on non-selective agar (probably because the colony on non-selective agar includes some high copy number segregants). This further emphasizes the unreliability of such cultivation-dependent methods for detecting the persistence of GMOs.

One of the reasons why some recombinant plasmids are unstable may be that the mean copy number is lower than that of the naturally occurring plasmids from which they were derived. If the mean copy number is a compromise by the host cell between the advantages and disadvantages of maintaining plasmid copies, then the lower mean copy number of recombinant plasmids implies a greater retarding effect on growth.

While high copy number enhances stability, the optimum copy number of a plasmid with industrial applications will not necessarily be its maximum copy number. It is known that the higher the copy number, the more product is released, up to a certain level (Uhlin and Nordstrom, 1977). However, this gene dosage relationship breaks down at high copy numbers where the rate limiting factor becomes the transcriptional or translational machinery rather than copy number (Zabriskie and Arcuri, 1986). Damage to the host cell may often result from the accumulation of plasmid gene products when the copy number is excessively high. In addition, the growth rate of high copy number cells may be severely reduced leading to the replacement of the recombinant organism with plasmid-free segregants. Thus, the determination of the optimum copy number for maximum productivity of a process employing a plasmid-carrying organism involves a compromise between the positive effects of high copy number and its deleterious consequences.

5.1.3 Gene expression

From an industrial perspective, the efficiency with which a cloned gene is expressed is one of the most important features of a GMO and the development of expression vectors is an important area of research. The most conspicuous feature controlling gene expression is the promoter and vectors have been constructed for a number of hosts where cloned gene expression is initiated from strong phage (Poth and Youngman, 1988; Davison *et al.*, 1989), chromosomal (Mellor *et al.*, 1985; Velati-Bellini *et al.*, 1986) or synthetic (Amann *et al.*, 1988) promoters. Initiation of translation may also require other plasmid-coded elements, such as Shine–Delgarno sequences, to facilitate ribosome attachment (Grandi *et al.*, 1986; Arthur *et al.*, 1990). An alternative strategy is to insert the cloned gene downstream from, and in frame with, the translation start site of another gene, so that the foreign protein is synthesized as a fusion with part, or

all, of another protein (for example, Botterman *et al.*, 1987; Hellebust *et al.*, 1988; Rimm and Pollard, 1989; Strandberg *et al.*, 1990). Maina *et al.* (1988) have developed this approach to design a vector which facilitates purification of the cloned product which is fused with the *E. coli* maltose binding protein; the hybrid protein can be recovered by affinity chromatography to amylose.

However, strong transcription and translation start sites may not, by themselves, be sufficient to achieve maximum gene expression. Other regions of the DNA, upstream (Nishi and Itoh, 1986) and downstream (Kammerer *et al.*, 1986; Deuschle *et al.*, 1986) of the classic promoter sequences may be required for optimum RNA synthesis. Other barriers to heterologous gene expression may include codon preference (Calcott *et al.*, 1988; Ernst and Kawashima, 1988; Brinkman *et al.*, 1989; Branlant *et al.*, 1989), RNA instability (Belasco and Higgins, 1988; Gross and Hollatz, 1988) and proteolysis (Kitano *et al.*, 1987; Dalboge *et al.*, 1989). Requirements for post-translational modification and secretion will be discussed below in section 5.1.4.

High level expression of cloned genes can have the same detrimental effects on the host as high levels of gene dosage. It has generally been observed that growth conditions that engender increased expression of plasmid-coded genes lead to plasmid instability (Imanaka *et al.*, 1980; Kim and Ryu, 1984; Caulcott *et al.*, 1985; Keshavarz, *et al.*, 1985; Pierce and Gutteridge, 1985) and reduce the growth rate and viability of plasmid-containing cells (Lee and Edlin, 1985; Betenbaugh *et al.*, 1989). Such effects may be the result of a greater drain on the resources of the host (Helling *et al.*, 1981; Peretti *et al.*, 1989) or high level transcription may interfere with plasmid replication. Steuber and Bujard (1982) showed that transcription from an efficient promoter (T5 early promoter) can interfere with plasmid replication by causing transcriptional readthrough into the origin of replication. This effect was reversed by the insertion of an efficient transcription terminator at the end of the reporter gene (Gentz *et al.*, 1981).

We also found that expression of plasmid-coded genes reduced copy number and decreased stability in *B. subtilis*. Induction of chloramphenicol resistance by subinhibitory concentrations of the antibiotic caused a rapid decrease in copy number and increased the rate of accumulation of plasmid-free cells (Newell *et al.*, 1988).

5.1.4 Protein secretion

Nicaud *et al.* (1986), in a review of protein secretion by GMOs, identify the complications resulting from the over-production of foreign proteins as (i) precipitation and aggregation of the polypeptide; (ii) rapid degradation of foreign proteins by proteases; (iii) toxicity for the host cell; and (iv) difficulties in purifying the protein in an active form.

Escherichia coli, by far the most popular host for cloning foreign genes, has very limited capacity for protein secretion. However, secretion of foreign proteins into the periplasm has been achieved in a number of ways. Using vectors where the coding sequence of the protein is fused to the N-terminal signal sequence of a naturally exported protein (Miyake *et al.*, 1985; Muller *et al.*, 1989), the recombinant protein can amount to as much as 50% of total cellular protein and is located in the periplasm, from which it can be recovered by osmotic shock. Leemans *et al.* (1989), used the

β-lactamase signal sequence to export a heterologous protein to the periplasm and found that export competence was correlated with the solubility of the protein and postulated that secretion proficiency depends on the ability of the mature protein to fold into a soluble conformation.

Maturation and solubility are functions of vector design (Denefle *et al.*, 1989) and characteristics of the host; in some strains eukaryotic proteins aggregate in insoluble, inactive, inclusion bodies (Kenealy *et al.*, 1987). Passage of proteins from the periplasm across the outer membrane has been achieved by using a 'leaky' (*lky*) mutant as host (Lazzaroni and Portalier, 1982; Atlan and Portalier, 1984), or by cloning a secretory system from a bacillus into the host (Kato *et al.*, 1983; Kudo *et al.*, 1983).

It has also been reported that when the genes for naturally exported proteins of Gram-positive origin are cloned in *E. coli*, they are partially excreted (Malke and Ferretti, 1984). Other Gram-negative bacteria, such as *Myxococcus xanthus* (Breton *et al.*, 1986) appear to have natural secretory systems which can be harnessed to export foreign proteins.

Bacillus spp., being Gram-positive organisms, have a naturally occurring secretory mechanism which has long been exploited for the industrial production of enzymes. Therefore, fusing the cloned gene to the signal sequence of an extracellular enzyme, such as an amylase, leads to the secretion of the product (Palva *et al.*, 1983; Mosbach *et al.*, 1983; Yamane *et al.*, 1984; Honjo *et al.*, 1986; Nakayama, *et al.*, 1988). However, there is a risk of degradation of secreted products by exoproteases (Ulmanen *et al.*, 1985).

Saccharomyces cerevisiae also has exciting possibilities for commercial production of cloned proteins. It is classified as a GRAS (generally regarded as safe) organism, it can post-translationally modify (Innis *et al.*, 1985; Zaworski *et al.*, 1989; Elliott, *et al.*, 1989) and assemble (Jacobs *et al.*, 1989) eukaryotic peptides and vectors for high level expression and secretion of recombinant proteins are available (Bitter, 1988). Several foreign proteins have been secreted by fusing the cloned gene to the N-terminal sequence of the precursor of mating factor alpha (Bitter *et al.*, 1984; Jacobson *et al.*, 1989) or of other exported proteins (Sato *et al.*, 1989; Nakamura *et al.*, 1986; Nishizawa *et al.*, 1989), but the efficiency of these systems may be limited by the size of the cloned protein. As in *E. coli*, export by yeast is dependent on the correct folding and conformation of recombinant proteins (Sato *et al.*, 1989) and on host-coded functions (Shuster *et al.*, 1989). The methylotrophic yeast *H. polymorpha* can also modify and excrete cloned protein (Shen *et al.*, 1989).

5.1.5 Growth rate

The maximum specific growth rate (μ_{max}) of recombinant strains is important for several reasons. Obviously, production will be more efficient when biomass formation is rapid but, more importantly, growth rate retardation due to plasmid maintenance appears to be a causative factor in segregational instability and also has significant implications for risk assessment.

In discussing growth rate, a distinction should be made between μ_{max}, a dependent variable which is a function of interactions between the genetic characteristics of the cell

and the growth environment, and the specific growth rate μ, an independent variable, which can be controlled in cultures by limiting the supply of nutrients, so that the cell cannot achieve its maximum growth rate. Under these conditions, μ is equal to D, the dilution rate at which nutrient is made available. The effect of dilution rate on plasmid stability, competition with plasmid-free segregants and product yield is discussed in section 5.2.1.

Strains of *E. coli* harbouring recombinant plasmids have significantly lower maximum specific growth rates than the plasmid-free isogens (Godwin and Slater, 1979; Pierce and Gutteridge, 1985; Filanov *et al.*, 1985; de Taxis du Poet *et al.*, 1986) and plasmid amplification causes decreased growth rates and lower substrate yields (Betenbaugh *et al.*, 1987). The larger the plasmid, the lower the growth rate of the plasmid-containing cell (Zund and Lebek, 1980; Noack *et al.*, 1981; Warnes and Stephenson, 1986). Betenbaugh *et al.* (1989) found that high-level expression of plasmid-coded genes also depressed growth rate and cell viability and that high copy number, resulting from increased plasmid replication, reduced the growth rate by as much as 70%. Expression of a cloned gene also reduces the growth rate of *S. cerevisiae* (Lee *et al.*, 1986).

However, plasmid maintenance does not always result in a detectable reduction in μ_{max}. For example, Wouters *et al.* (1980) found that the plasmid pBR322 did not affect the μ_{max} of *E. coli* in batch culture. Nevertheless, when the plasmid-less strain was introduced into a steady-state chemostat culture of pBR322-containing cells, it rapidly outgrew the plasmid-containing strain. In *B. subtilis*, Bron and Luxen (1985) showed that plasmid size had no effect on the growth rate. However, the rapid accumulation of plasmid-free cells in cultures of most *B. subtilis* recombinants implies a competitive advantage for plasmid-free cells and we have found that, applying the models of Jannasch (1969) or Slater and Bull (1978), differences in μ_{max} are not sufficient to explain the competitive advantage of plasmid-free cells over pPGV2- or pPGV138-containing cells (Hussey *et al.* 1990). Similarly, plasmid-free cells rapidly proliferate in cultures of *B. subtilis* containing pE194cop6 when plasmid replication is inhibited, although no significant difference in the growth rates of the two strains was detectable in batch culture. We have found that the K_s for glucose of the plasmid-containing strain is much higher than that of the plasmid-free isogen and we attribute the predominance of plasmid-free cells to this competitive advantage (Byrne *et al.*, 1991).

Competition between recombinants and the plasmid-free host is an important consideration in predicting the survival of GMOs in the environment. If it could be demonstrated that GMOs were inherently segregationally unstable and were consistently less fit than plasmid-free segregants, then the risks associated with accidental release of GMOs would be relatively minor, since any disturbance of the ecosystem would be short-lived.

5.1.6 Host effects

The characteristics of the host cell are also very important (Alonso and Trautner, 1985). The efficiency of transcription and translation, maximum specific growth rate and the relative availability of amino acyl-tRNAs all play a part in determining the

expression and stability of plasmid DNA. A significant difference in the level of gene expression was observed when a recombinant plasmid was cloned in *Bacillus megaterium* or *B. subtilis* (Shivakumar *et al.*, 1989). We have found that two strains of *B. subtilis* maintain the plasmid pPGV138 at different copy numbers and that, while plasmid maintenance causes a reduction in growth rate in both strains, the strain with the higher copy number, not surprisingly, shows the stronger effect (Hussey and McGoff, unpublished). Lee *et al.* (1986) also found that maintenance and expression of a recombinant plasmid had different effects on the growth rates of three strains of *S. cerevisiae* and that production of recombinant protein differed significantly between strains.

Specific phenotypic traits may also affect maintenance of certain plasmids. We found that an amylase-negative *B. subtilis* strain was unable to harbour plasmids containing an amylase gene, even under conditions where expression of the amylase was repressed by glucose, although it could stably maintain plasmids carrying deletions which prevented expression of the enzyme (Burke and Hussey, unpublished). Similarly, Vehmaanpera *et al.* (1986) found that sacU9 strains could not stably maintain an amylase-producing plasmid. Shuster *et al.* (1989) found mutants that had increased resistance to the adverse effects of accumulation of the heterologous product.

5.2. Process factors

Environmental factors, such as temperature, nutrient availability and pH, have been shown to affect the copy number and stability of plasmids and the yield of recombinant protein in a number of species. It is difficult to interpret these results because it is not always possible to distinguish between effects on the rate of segregation of plasmid-free cells, on growth rate and competition between plasmid-free and plasmid-containing populations, on gene expression or on protein stability.

5.2.1 Nutrient availability

'The pre-occupation of microbial geneticists and molecular biologists with batch cultures is both foolish and puzzling', (Primrose *et al.*, 1984). In batch culture, conditions alternate between nutrient excess and nutrient starvation, whereas continuous culture permits the study of recombinant strains growing in a constant environment for hundreds of generations at a growth rate controlled by the supply of nutrients. Batch culture also has the disadvantage that inhibitors, such as acetate, may accumulate at high cell densities (MacDonald and Neway, 1990).

Controlling growth rate can increase plasmid stability. Wouters *et al.* (1980) found that, in a batch culture of pBR322-containing *E. coli*, 80% of cells were plasmid-free after 20 cycles of subculturing, whereas in continuous culture at a moderate dilution rate, no plasmid-free cells were detected after nearly 200 generations. However, at lower dilution rates, the plasmid became progressively more unstable.

Most studies indicate a trend where a reduction in dilution rate results in decreased plasmid stability and product yield (Sterkenberg *et al.*, 1984; Koizumi *et al.*, 1985;

Forberg and Haggstrom, 1988; Lee *et al.*, 1988; Kryukova *et al.*, 1989). However, there have been a few reports of increasing dilution rate resulting in reduced stability, which was attributed to reduction in copy number (Warnes and Stephenson, 1986) or increased competition by plasmid-free segregants (Melling *et al.*, 1977). Marquet *et al.* (1988) found that the productivity of a recombinant strain of *S. cerevisiae* was independent of dilution rate, but in this system proliferation of plasmid-free cells was prevented by the use of an auxotrophic host. Seo and Bailey (1986) reported an optimum dilution rate for productivity and we have found an optimum dilution rate for stability of pPGV138- and pPGV138 amyT-containing strains of *B. subtilis*, which probably represents a balance between more frequent segregation of plasmid-free cells at high growth rates and a greater competitive advantage for the segregants at low growth rates (Hussey *et al.*, 1990). Stephens and Lyberatos (1988) postulate that if the recombinant has an advantage in adaptability, then transient changes in dilution rate can be used to stabilize plasmid maintenance. Similarly, Weber and San (1989) found that forced perturbations of the dilution rate prolonged the life of the plasmid-containing population of *E. coli*.

Changes in growth rate may affect plasmid stability and productivity by influencing competition between the recombinant and plasmid-free segregants, or by affecting plasmid replication. Balke and Gralla (1987) observed changes in plasmid DNA supercoiling during nutrient up- and down-shifts and several genes involved in DNA replication in *E. coli* are growth regulated (Chiaramello and Zyskind, 1990).

In continuous culture, the composition of the medium also affects stability and productivity, presumably by altering the balance between nutrients. De Taxis du Poet *et al.* (1987) comparing copy number and product synthesis in *E. coli* growing in minimal medium and complex broth, found that, whereas copy number was higher in minimal medium, product synthesis was depressed; productivity per plasmid copy was eightfold higher in complex broth. Limitation of individual nutrients can have a specific effect on plasmid-containing cells. Harder and Kuenen (1977) showed that different strains have different affinities for the limiting substrate and Klemperer *et al.* (1979) showed that plasmid-containing strains have a greater requirement for nutrients such as magnesium, iron and phosphorus. Plasmids have also been shown to be unstable under magnesium (Sayadi *et al.*, 1989) and sulphur limitation (Caulcott *et al.*, 1987). The effect of phosphorus limitation seems to vary from one system to another. In *E. coli*, phosphorus limitation had no effect on the stability of pHSG415 (Caulcott *et al.*, 1987), caused segregational instability of TP120 (Godwin and Slater, 1979) enhanced stability of pBR322 derivatives (Jones and Melling, 1984) and increased the competitiveness of plasmid-free cells (Primrose *et al.*, 1984). Ryan *et al.* (1989) found that there was an optimum phosphorus concentration for growth rate and product synthesis. In *B. subtilis* we observed a rapid loss of plasmid-coded antibiotic resistance after growth in phosphorus-limited culture. However, this effect appeared to be the result of a failure to express plasmid-coded phenotype. Culture samples could be shown, by gel electrophoresis, to contain normal levels of plasmid and these cells recovered the antibiotic resistant phenotype very rapidly when incubated in phosphorus-containing medium or when subcultured on non-selective agar (Licken *et al.* 1988).

In batch culture the composition of the medium also affects growth and gene expression. Galindo *et al.* (1990) found that hybrid protein synthesis was closely related to biomass production and was inversely related to the cell/glucose yield. Shimizu *et al.* (1987) achieved optimum productivity when the ratio of amino acids to glucose was high. Nudel *et al.* (1987) found that glucose concentration had no effect on product yield, whereas omission of yeast extract caued a sharp decrease in productivity.

5.2.2 Temperature

Temperature affects plasmid copy number in most species. In *B. subtilis*, single cell copy number increases with increasing temperature up to 50°C, but the added metabolic burden of plasmid replication and expression appears to destabilize the plasmid and the mean copy number of the population falls rapidly at high temperatures (Newell *et al.*, 1988). Peijnenburg *et al.* (1987) noted a similar inverse relationship between temperature and plasmid stability in *B. subtilis*.

In *B. stearothermophilus*, increasing temperature causes decreased plasmid stability in both batch and continuous culture (Aiba and Koizumi, 1984) and the difference in growth rate between plasmid-free and plasmid-containing cells is magnified. The yield of plasmid-coded product is a complex function of temperature and dilution rate (Koizumi *et al.*, 1985) and some plasmid-containing strains cannot grow at high temperatures (Aiba *et al.*, 1987).

Similar effects have been observed in *E. coli* (Betenbaugh *et al.*, 1987; Sayadi *et al.*, 1987; Reinikainen *et al.*, 1989). Relatively low temperatures appear to favour production from cloned genes: recombinant strains of *E. coli* achieved maximum levels of active human interferon at 20°C (Mizukami *et al.*, 1986) and optimal conversion of xylose to ethanol at 30–37°C (Ohta *et al.*, 1990). The effect of temperature on production may be attributable to promoter activity (Auger and Bennett, 1987) or to protein assembly (Cabilly, 1989) and solubility (Chalmers *et al.*, 1990) and Wallace and Holmes (1986) found that maintenance coefficients increased rapidly as the temperature increased, reflecting an increased demand for ATP.

5.2.3 Other factors

The pH of the growth environment can influence stability and gene expression. Rowbury (1986) found that plasmid-containing strains were more sensitive to alkali than the *E. coli* host and Otto *et al.* (1982) showed that the competitiveness of plasmid-containing strains of *Streptococcus cremoris* was adversely affected by high pH. Extracellular pH regulates the activity of specific promoters in *B. subtilis* (Hill *et al.*, 1990) and in *A. nidulans* (Caddick *et al.*, 1986) and has a significant effect on the production of plasmid-coded β-lactamase in *E. coli* (Ryan *et al.*, 1989).

Efficiency of aeration may also be an important parameter in process control. Hopkins *et al.* (1987) found that induction of dissolved oxygen shock destabilized recombinant cells of *E. coli*, and plasmid stability in *S. cerevisiae* was also affected by oxygen availability (Lee and Hassan, 1987).

5.3 Reactor design

Although most conventional industrial processes use batch culture for the production of biomass, there is increasing interest in the use of continuous culture systems such as chemostats or fed-batch culture. The use of continuous systems for the production of protein from GMOs presents certain advantages, allowing parameters, such as growth rate and nutrient availability, to be tightly controlled. Continuous systems may also offer advantages for the harvesting of secreted proteins and limit the deleterious effects of hydrolysis by extracellular proteases (Ulmanen *et al.*, 1985), particularly in Gram-positive hosts.

Several models have been constructed which attempt to describe the interactions between variables, such as dilution rate, plasmid replication, segregational stability and product formation in chemostat cultures (Imanaka and Aiba, 1981; Domach *et al.*, 1984; Chang and Lim, 1987; Byrne *et al.*, 1988; San and Weber, 1989; Satyagal and Agrawal, 1989a; Wei *et al.*, 1989).

Reactor configuration has important implications for plasmid stability, particularly where the plasmid-free segregant has a growth rate advantage. Low dilution rates, which may be advantageous for substrate utilization and for product formation, increase the competitiveness of plasmid-free segregants. Even at high dilution rates, any advantage for plasmid-free cells will lead to ultimate washout of the productive, plasmid-containing population.

Fed-batch cultures, where growth rate is regulated by the incremental addition of nutrient(s) to the reactor, combine the advantages of the tightly-controlled open system with those of a closed (batch) system. Although segregants from unstable recombinants may accumulate, the plasmid-containing population is not lost from the reactor. We have used fed-batch systems successfully to stabilize copy number and increase amylase production in recombinant strains of *B. subtilis* (Licken *et al.*, 1988) and it has been used successfully to increase stability (Horn *et al.*, 1990) and productivity (Mizutani *et al.*, 1986; Hsieh *et al.*, 1988) of recombinant strains of *E. coli*.

5.4 Strategies to optimize yield with maximum safety

The characteristics determining productivity of recombinant plasmids are not independent variables. Factors which contribute to high level protein synthesis, such as gene dosage and strong promoter activity, may lead to decreased viability of the host and unstable plasmid maintenance. Process factors which increase biomass may inhibit gene expression. Emborg *et al.* (1989) have devised a useful experimental design to test the interactive effects of these factors on the productivity of host/vector systems.

Two crucial problems must be addressed when constructing recombinant strains. Firstly, virtually all the studies discussed in this chapter indicate, in one way or another, that GMOs with high level productivity are inherently unstable because their growth rate is compromised. Secondly, solutions to this dilemma must not increase risks to health or the environment from the recombinant strain.

A number of recent studies have addressed the problem of instability and a variety of

imaginative strategies have been suggested. One of the most straightforward is the insertion of the cloned gene into the host chromosome. Vectors have been designed which exploit the host recombination system to insert a plasmid-coded gene into a targeted region of the chromosome of *E. coli* (Merryweather *et al.*, 1987) and other Gram-negative bacteria (Barry, 1988), *B. subtilis* (Prozorov *et al.*, 1985; Declerck *et al.*, 1988), *S. cerevisiae* (Xiao and Rank, 1989) and *A. nidulans* (van Gorcom *et al.*, 1986). Integration renders an inherently more stable construct but some of the advantages of plasmid-based systems are sacrificed. If a single gene copy is inserted, product yield may be low; multiple insertions have been achieved but these may also be unstable, particularly if expressed from a strong promoter (Declerck *et al.*, 1988). Lopes *et al.* (1989) report successful use of a vector which inserted 100–200 copies of the thaumatin gene into the ribosomal DNA of *S. cerevisiae* to construct a strain in which thaumatin represented half of the total soluble protein.

An alternative approach is to clone genes for the control of replication and partitition into the vector. Many vectors which are derived from pBR322 are segregationally unstable because control sequences are absent (Primrose *et al.*, 1984) and stability can be increased by cloning partition functions from other plasmids (for example, Yukawa *et al.*, 1988; Schwab, 1988).

The proliferation of plasmid-free segregants can, of course, be restricted by growing cultures under selective pressure. In research and development, this is usually achieved by growth in the presence of antibiotics. However, for large-scale reactors, using even minimal levels of antibiotic, as suggested by Panayotatos (1988), is frowned on by regulatory agencies as posing a risk of spreading resistance to natural microbial populations. Selective pressure may also be imposed in ways which do not involve antibiotic resistance. Discrimination against segregants can be operated by using an auxotrophic host strain which is complemented by a plasmid-coded product (Diderichsen, 1986; Marquet *et al.*, 1988; Satyagal and Agrawal, 1989b; Sakoda and Imanaka, 1990) or by incorporating a gene for resistance to metabolite inhibition into the vector (Ryder and DiBasio, 1984). Stability can also be enhanced by wiping out the competition with plasmid-coded toxins (Lauffenburger, 1986; Lee and Hassan, 1988).

All of these approaches, which increase the stability of the recombinant, while effective under laboratory conditions, undermine the concept of 'limited survivability', which is an important characteristic of GILSP organisms. Indeed, from the viewpoint of industrial safety, the most reassuring characteristic of the majority of GMOs is their very poor chance of survival in any environment in competition with plasmid-free species. Any genetic manipulation of a host/vector system which forces an increase in stability through enhanced plasmid maintenance is probably, therefore, not the best strategy.

One of the most promising tactics for increasing productivity seems to be to match genetic characteristics with reactor and process design in ways which enhance stability only for the duration of the fermentation. Optimum yield is achieved by maximizing biomass and protein production per unit biomass. Considering the two objectives separately, in terms of a growth phase and a production phase, offers a particularly satisfactory solution for yield optimization and risk management.

Regulating expression of the cloned gene, so that production is delayed until the end

of the growth phase, decreases competition by segregants and enhances stability and copy number. Host/vector systems are available for a variety of species in which any gene can be cloned under the control of strong promoters which are repressed under normal growth conditions but respond to specific environmental signals. The first such system (Remaut *et al.*, 1981; Simons *et al.*, 1984) used control elements from phage λ to construct an *E. coli* strain where cloned gene expression was turned on by temperature shift-up. Successful use of other *E. coli* systems containing λ control elements has been reported by Sayadi *et al.* (1987), Sugimoto *et al.* (1987), Reinikainen *et al.* (1988) and Petrenko *et al.* (1989). Other regulatable promoter systems, including *lac* (Sninsky *et al.*, 1981), *trp* (Mizukami *et al.*, 1986) and phage T7 (Muralikrishna and Wickstrom, 1989), have been used in *E. coli*, and similar vectors have been developed for *B. subtilis* (Dhaese *et al.*, 1984b; Wang *et al.*, 1989) and *A. nidulans* (Waring *et al.*, 1989). An ingenious construction by Chew and Tacon (1990), in which heterologous gene expression and plasmid replication are simultaneously controlled, ensures plasmid maintenance throughout the production phase. The kinetics of growth and induction in regulatable systems have been described in a number of models (Lee and Bailey, 1984; Betenbaugh *et al.*, 1987; Peretti and Bailey, 1988).

The genes for many microbial extracellular proteins are naturally regulated by catabolite repression so that production occurs only in stationary phase cells. Expression is controlled by the availability of glucose and the DNA sequences involved have been identified in a number of species (Postma, 1986; Nicholson and Chambliss, 1985; Nicholson *et al.*, 1987) and can be used to regulate expression of cloned genes (Hoffman and Winston, 1989; Hua Lin *et al.*, 1989; Gilbert and Barbe, 1990).

Another means of overcoming plasmid loss during the growth phase is to use a low copy number vector which can be amplified for the production phase. This can be achieved by inserting a strong, regulatable promoter upstream of a gene that is critical for plasmid replication or control; chemical or thermal induction can be used to turn on 'runaway replication' of the plasmid (Uhlin *et al.*, 1979; Larsen *et al.*, 1984; Giza and Huang, 1989). Janniere *et al.* (1985) also accomplished amplification of a gene inserted in the chromosome of *B. subtilis*.

Reinikainen *et al.* (1989) studied the optimal conditions for fermentation with a recombinant plasmid and found that optimal production required fermentation conditions quite different from those needed for growth and cell division. For this reason two-stage cultures which separate the growth and production phases have been adopted by some workers. Both stages can be operated in batch, fed-batch or chemostat reactors. Park *et al.* (1989) have developed models for cell growth and cloned gene expression in a two-stage continuous culture system and concluded that productivity is more sensitive to the combination of dilution rates than to the volume ratio of the two reactors. Siegal and Ryu (1985) found that growth rate deceleration and plasmid loss followed induction of cloned gene expression and devised a two-stage continuous culture system in which a higher temperature in the second stage induced gene expression and led to improved productivity. Plasmid stability could be improved by adjusting the dilution rate during the production stage (Lee *et al.*, 1988).

Immobilization of recombinant cells onto carrageenan beads or other carrier, has been found to be a practicable way to minimize plasmid loss without interfering with

secretion of an extracellular product. Immobilization of *E. coli* increases stability and productivity (Oriel, 1988; Birnbaum *et al.*, 1988) and co-immobilized plasmid-free cells do not overrun the plasmid-containing population (Nasri *et al.*, 1987; 1988). Walls and Gainer (1989) found that, because the plasmid-containing population was physically retained within the reactor, the system could be operated at a dilution rate above washout rate. Sayadi *et al.* (1989) observed that copy number and cloned gene expression increased in immobilized cells and that stability was enhanced in glucose-, nitrogen- and phosphorus-limited chemostat cultures. Sode *et al.* (1988) found that alternating minimal and complex medium increased productivity from immobilized yeast cells. Immobilization also enhances the stability of an *M. xanthus* recombinant where the cloned gene is carried in the chromosome (Jaoua *et al.*, 1986).

A theoretical analysis of immobilized cell growth has been prresented by de Taxis du Poet *et al.* (1987). Little information is available on the survival of immobilized cells. Birnbaum *et al.* (1988) report $t_{0.5}$ values, with respect to cloned gene product expression, of 6 and 10 days at 4°C and 21°C, respectively, which are probably longer than those of comparable free-growing cells. However, any increased environmental risk from enhanced persistence may be offset by a lower probability of accidental release when cells are physically retained within the reactor.

Properties of the host should not be overlooked. It should be capable of harbouring high copy number plasmids and withstanding the genetic burden of strong gene expression. To enhance safety, it should have limited survival and transferability and be free from detectable adventitious agents or toxin-producing functions.

6. Conclusion

The fundamental question remains: do GMOs pose a threat to health or the environment? Much of the evidence cited above points to the conclusion that any manipulation of the genetic material of a microorganism reduces its competitiveness and minimizes the probability of its survival. Indeed, there is a philosophical view that argues against the persistence of any anthropogenic organism in a natural environment. Current knowledge of genetic exchange and transposition within and between species suggests that microorganisms have been indulging in the pursuit of genetic engineering for billenia before the arrival of humans on this planet. If it were possible to construct a 'better', or 'fitter', microorganism, the argument goes, nature would have done so long ago and, therefore, it is impossible to envisage a GMO being more successful in a natural environment than the indigenous microflora.

However, since GMOs have originated in the late twentieth century, is it possible that they might find a niche in a contemporary environment, i.e. that it may be modern, man-made, technological environments that are at risk of colonization by GMOs, and not natural ecosystems? For example, it is conceivable that a GMO may prove a better utilizer of jet fuel than any naturally occurring organism. Is it possible that a genetically-modified ethanol-tolerant yeast may prove an intractable contaminant of modern breweries? To date, the balance of evidence argues against these eventualities.

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