TITLE SEGREGATIONAL STABILITY OF PLASMIDS IN BACILLUS SUBTILIS

AUTHOR RHIANNON SANDERS

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SEGREGATIONAL STABILITY OF PLASMIDS IN *BACILLUS SUBTILIS*

By

RHIANNON SANDERS

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RESULTS AND DISCUSSION CHAPTERS

SUMMARY

The stability of plasmids in Bacillus subtilis in the absence of selective pressure under long term chemostat culture and under different conditions of nutrient limitation was investigated.

Culture and sampling conditions were established to allow the detection of plasmid-free cells and samples were routinely screened in order to monitor the structural integrity of the resident plasmid.

Initially the stability of the plasmid pHV14-F (carrying the repressor gene of phage O105) in the Bacillus subtilis strain 3G18 was investigated. This plasmid was found to be segregationally and structurally highly unstable.

The stability of the Staphylococcus aureus plasmids pC194 and pUB110 along with the Bacillus cereus plasmid pBC16 was examined in the Bacillus subtilis strain 168 trp in chemostat culture under carbon, magnesium and phosphate limitation. All three plasmids were found to be remarkably stably maintained through up to 100 culture generations of their host strain under all three of the nutrient limitations.

Under enforced competition from plasmid-free cells at 1% of the chemostat inoculum all three plasmids were retained in the culture but in the presence of 50% plasmid-free cells in the inoculum plasmid-carrying cells were rapidly displaced from the culture.

Phosphate limitation was found to exert a slightly greater stringency of selection for plasmid-free cells than either carbon or magnesium limitations (which had similar effects).

A derivative of pC194 was isolated, pC194-K1 which was segregationally unstable, the Bacillus subtilis culture losing its plasmid within 30 generations. On restriction endonuclease digestion analysis the plasmid was revealed to be physically very closely related to pHV14 (pC194 and pBR322 ligated at their HindIII sites).

Under magnesium limitation all Bacillus subtilis cultures after 2-3 days were predominantly composed of cells with an unusual helical morphology. The development of this morphology under the conditions employed and the actual form of the helical cells themselves was found to be different from previously reported instances of the appearance of helical cells in cultures of Bacillus subtilis.

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DECLARATION

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The experimental work described is entirely my own and has not been submitted for any other degree at any other university. All sources of information have been specifically acknowledged by way of reference.

TO MY MOTHER AND FATHER

AND TO ROB

for always being there

CHAPTER 1

Preview

Bacterial plasmids have commanded a considerable amount of interest since their discovery over 35 years ago and their study represents a very broad area of biological research. Nevertheless, the mechanisms responsible for the control of their autonomous replication in bacteria and their stable inheritance through numerous generations of their host cells are only recently beginning to be understood. By far the greatest proportion of the work has been carried out on the plasmids of the well-characterised Gram-negative organism Escherichia coli with very little reference to plasmids in other organisms, in particular Gram-positive species. The work described in this thesis therefore seeks to put right this deficit and, in addition, to point the way to the valuable part that continuous culture can play in the study of plasmid biology.

The rest of this chapter is a review of the literature on the subject of bacterial plasmid biology. It begins with a brief introduction to plasmids, in particular those found in the bacilli, and then deals predominantly with plasmids in Escherichia coli of which we have a far superior knowledge. Different plasmids employ different means for controlling their replication and some representative

examples of these are described in section 2.1. The following section covers the systems which ensure the stable inheritance or partitioning of certain E.coli plasmids. Some plasmids, particularly those with a low copy number, seem to require further control over their stability and the systems they employ for doing so are described in sections 2.4 and 2.5. There follows an outline of the extent of the information available to date on the biology of three plasmids in particular, pE194, pC194 and pUB110, which have been studied in Bacillus subtilis along with the only plasmid from a Gram-positive organism, pT181, which has been looked at in any detail with respect to its control over initiation of its replication. Finally the principles and theory of the technique of chemostat culture are introduced in section 4 of this chapter.

INTRODUCTION

1. PLASMIDS

1.1 The discovery of plasmids

In 1963 Watanabe reviewed research carried out in Japan which demonstrated that species of Shigella (the causative agent of dysentry) were simultaneously resistant to two or more drugs and furthermore that their resistance could be transferred to other bacteria. In the latter respect an analogy was drawn to the earlier report by Lederberg et al. (1952) concerning the postulated involvement of an extra-chromosomal hereditary determinant in the transfer of genetic information between strains of Escherichia coli (Mitsuhashi et al., 1960). The suggestion that extra-chromosomal genetic elements were present in multiply drug resistant Shigella strains was therefore not the first indication of the existence of plasmids. Nevertheless, it was the realisation of the medical importance of plasmids that confer drug resistance on pathogenic bacteria that provided the impetus for the first considerable expansion of interest in their study. These plasmids (later termed R factors) have since claimed an even more significant place in biological research simply because their host cells can be easily identified and enriched for by virtue of their drug resistance.

Plasmids were first studied therefore for their involvement in the transfer of genetic information in E.coli

(the process of conjugation) and for their medical importance, but bacterial plasmids have since been found to confer a wide variety of other phenotypes on their hosts including plant tumour induction, biological nitrogen fixation and metabolism of organic compounds and are therefore also of agricultural, environmental and industrial interest (see Hardy 1981 for the supportive literature).

1.2 The general properties of plasmids

Lederberg (1952) originally defined plasmids as extra-chromosomal hereditary determinants. In view of the tremendous variety of physical properties now known to be shown by plasmids any general description that may be attempted will not be particularly limiting. Thus the majority of bacterial plasmids are covalently closed circular molecules of DNA, but recently linear plasmids have been identified, for example, in Bacillus thuringiensis (Gonzales and Carlton, 1984) and in Streptomyces rimosus (Chardon-Loriaux et al., 1986). Plasmids range in size from 1×10^6 to 200×10^6 daltons and inhabit a large number of different bacterial species of both Gram-negative and Gram-positive genera. Certain broad host range plasmids are, in fact, able to exist in several different species (For example, RK2 and RSF1010, Stalker et al., 1981 ; Scholz et al., 1985).

A feature characteristic of each plasmid in a given host is its copy number, defined as the average number of plasmid copies per genome and determined by plasmid-borne maintenance functions or factors encoded by the host bacterium

(Macrina et al., 1974 ; Cress and Kline, 1976) and by the growth conditions of the host (Engberg and Nordstrom, 1975).

An attempt to classify plasmids based on their behaviour when present in the same bacterial cell has developed into an extensive system of incompatibility groups. Plasmid incompatibility is defined as the inability of two plasmids to co-exist in the descendants of a bacterial cell and has its origins in basic similarities between the maintenance control systems of incompatible plasmids (see section 2.3, page 33 for a fuller explanation).

The establishment of a methodology for the manipulation of DNA sequences and the application of the other techniques of recombinant DNA technology in conjunction with the easily selectable phenotype of antibiotic resistance conferred by the R factors has had a very significant impact on biological science. Not only has information been obtained on the plasmids themselves, but an opportunity has been created to obtain the propagation and expression of a variety of foreign genes in bacteria. Despite the fact that bacterial plasmids have been widely exploited as cloning vectors with far reaching implications for both basic and applied research, it is only recently that significant progress has been made in understanding the fundamental processes involved in their maintenance in bacterial cells. These processes as they are understood at the present time are reviewed in the rest of this chapter.

Since the main body of this thesis is concerned with the biology of the plasmids of Gram-positive bacteria and Staphylococcus aureus in particular there follows a brief description of plasmids in these bacteria. In the course of

the next two sections (1.3.1 and 1.3.2) the reasons for studying Bacillus subtilis are outlined along with the motives behind the development of a cloning system in this bacterium and the problems that have been encountered.

1.3 Plasmids in the bacilli

Since the detection of plasmid DNA in Bacillus megaterium (Carlton and Smith, 1974) there have been a number of reports of naturally occurring plasmid DNA in this and other Bacillus species. Studies on these plasmids have suffered somewhat from the fact that the great majority of naturally occurring Bacillus plasmids are cryptic (that is they do not specify any detectable genetic markers). Nevertheless, some plasmids have been isolated which specify easily selectable phenotypes - see Table 1.1.

1.3.1 Cloning in Bacillus subtilis using plasmid vectors

Cloning systems which use E.coli as the host cell have now been developed to such a degree that the techniques are well characterised and the methods routine in many laboratories. There are, however, important limitations on using E.coli as a cloning host, some of which may be overcome by the use of alternative hosts.

As an alternative to E.coli, Bacillus subtilis has been the natural choice for a variety of reasons. It is the most widely studied prokaryote other than E.coli with a well characterised genetic map (Piggot and Hoch, 1985). It is non-pathogenic, unlike certain E.coli strains and since

Table 1.1 Naturally occurring Bacillus plasmids with selectable markers

Strain	Plasmid	Size Copy Number	Marker	Reference
B.pumilus ATCC 12140	pPL10	4.4×10^6 / 20	Bacteriocin Production	(1)
B.cereus GP7	pBC16	2.8×10^6 / 20	Tet^R	(2)
B.cereus DSM336	pBC7	2.8×10^6 / 1-2	Bacteriocin production	(2)
Bacillus sp.[a] TB124	pAB124	2.9×10^6 / n.d.	Tet^R	(3)
B.subtilis RC242	pIM13	1.5×10^6 / 15	Em^R	(4)
Bacillus sp.[a] TR123	pTB19	17.2×10^6 / 1	Kam^R and Tet^R	(5)
Bacillus sp.[a] TR128	pTB20	2.8×10^6 / 24	Tet^R	(5)
Bacillus sp.[b] K-1 (kurstaki)	-	44×10^6 or 110×10^6 / n.d.	Crystal Protein production	(6)
B.subtilis	-	-	Tet^R	(7)

Table 1.1 (continued)

Key:

sp.[a] = B.stearothermophilus

sp.[b] = B.thuringiensis

Tet^R = tetracycline resistance

Em^R = erythromycin resistance

Kan^R = kanamycin resistance

References: (1) Lovett, Duvall and Keggins (1976)

(2) Bernhard, Schrempf and Goebel (1978)

(3) Bingham, Bruton and Atkinson (1979)

(4) Mahler and Halvorson (1980)

(5) Imanaka, Fujii and Aiba (1981)

(6) Gonzales and Carlton (1984)

(7) Polack and Novick (1982)

the bacilli are already used in a number of industrial fermentations the fermentation technology is well developed.

Perhaps the most important advantage of a Bacillus subtilis cloning system over that of E.coli, particularly from an industrial point of view, is that Bacillus subtilis is known to secrete a large number of proteins (Priest, 1977). Because it has a cell wall composed of only peptidoglycan and teichoic acid, any secretory proteins made in this bacterium by recombinant DNA technology would not be contaminated by cell envelope endotoxin, a potential problem in E.coli systems.

Small, high copy number plasmids carrying easily selectable genetic markers are excellent potential cloning vectors for any transformable bacterial host. As indicated earlier, the common laboratory strains of Bacillus subtilis are devoid of such plasmids. This has, however, not proved an insurmountable problem because Ehrlich reported in 1977 that several Staphylococcus aureus plasmids could replicate and express antibiotic resistance in Bacillus subtilis.

Since then a number of Staphylococcus aureus plasmids have been introduced into Bacillus subtilis and, of these, three in particular, pUB110, pE194 and pC194, have been the subject of extensive investigations (for example, Keggins et al., 1978 ; Horinouchi and Weisblum, 1982a, 1982b ; Alonso and Trautner, 1985a). In Table 1.2 some Staphylococcus aureus plasmids that have been found to replicate in Bacillus subtilis are listed. There are an ever-increasing number of Bacillus subtiiis plasmid vectors available, including shuttle vectors (Gryczan and Dubnau, 1978 ; Ehrlich, 1978) and secretion vectors (Palva et al., 1982), a

Table 1.2 Plasmids from Staphylococcus aureus which replicate in Bacillus subtilis.

Plasmid	Size (k.b.)	Genetic Marker	Reference
pC194	2.0	Cam^R	Ehrlich (1977)
pC221	2.0	Cam^R	Ehrlich (1977)
pC223	2.0	Cam^R	Ehrlich (1977)
pUB112	2.0	Cam^R	Ehrlich (1977)
pT127	2.9	Tet^R	Ehrlich (1977)
pE194	2.3	Em^R	Horinouchi and Weisblum (1982a)
pUB110	3.0	Kan^R	Gryczan et al. (1978)
pSA0501	2.8	Sm^R	Gryczan et al. (1978)
pSA2100	4.8	Cam^R Sm^R	Gryczan et al. (1978)

Key: k.b. = kilobases

Cam^R = chloramphenicol resistance

Tet^R = tetracycline resistance

Em^R = erythromycin resistance

Sm^R = streptomycin resistance

Kan^R = kanamycin resistance

large proportion of which are based on Staphylococcus aureus antibiotic resistance plasmids.

1.3.2. A Bacillus subtilis cloning system - the problems

Despite the fact that suitable technology is available and there are many examples of the successful construction and use of plasmid cloning vectors in B.subtilis the potential offered by a B.subtilis cloning system has not been realised to the extent suggested in the previous section.

Compared with the E.coli system, direct cloning of DNA sequences into B.subtilis plasmid vectors is very inefficient. The central problem seems to be that plasmid multimers are required for transformation of Bacillus subtilis (Canosi et al., 1978 ; Gryczan et al., 1980). One way in which this requirement can be satisfied is through the use of an intermediate host such as E.coli strain SK2267 to generate a high proportion of multimers of a shuttle vector which can then be used to transform B.subtilis (Ostroff and Pene, 1984).

The principle of Marker Rescue, on the other hand, can be employed to achieve transformation of B.subtilis with monomeric plasmids. By this method a plasmid vector which itself has some homology with the chromosome, or carries as an insert a gene which is homologous to part of the chromosome, is used as a cloning vector (Bensi et al., 1981). Either the whole plasmid or the region of homology becomes integrated into the chromosome as a result of the action of the host recombination system (Duncan et al 1978 ;

Rapoport et al., 1979 ; Ostroff and Pene, 1984). An alternative method of transformation is also available which offers the possibility of circumventing the requirement for plasmid multimers, namely the protoplast transformation system of Chang and Cohen (1979) which allows transformation by linear DNA molecules, but which is more tedious to perform than the competent cell method.

Cloning in B.subtilis is further restricted by the fact that DNA inserts purified from B.subtilis after shotgun cloning are generally small (Rapoport et al., 1979 ; Hutchinson and Halvorson, 1981 ; Ostroff and Pene, 1984). For instance, Michel et al. (1980) showed that the average size of inserts found in their recombinant plasmids was only one third of the average size of the original endonuclease digestion products of the donor DNA. The cause of this phenomenon is not yet clear, it could be the result of preferential transformation of recombinant plasmids with small inserts or post-transformational deletion of large plasmids (Kreft and Hughes, 1982).

There are not only restrictions in the transformation system, however. There are also many, as yet poorly understood instances of lack of expression of cloned genes and instability of recombinant plasmids in B.subtilis which occur on a scale not encountered in E.coli.

Whereas E.coli is able to recognise transcription and translation signals from other bacterial genera, B.subtilis. in contrast, is extremely restricted in its ability to recognise these sequences from Gram-negative species (Kreft et al., 1978 ; Kreft and Hughes, 1982 ; Kreft et al., 1982 ; Kreft et al., 1983).

The lack of gene expression in B.subtilis is the result of a block either at the level of transcription or translation (see below) and can be seen to be the result of important differences between the transcription and translation machinery of the two bacteria.

The block at the level of transcription hinges on lack of binding of the B.subtilis RNA polymerase to the promoters of E.coli genes. E.coli has one major RNA polymerase which recognises one type of promoter sequence (it also has a minor one which transcribes from heat shock gene promoters). The consensus sequence for the -10 (Pribnow Box) region in E.coli is TATAAT. The -35 promoter region shows variations in as many as three positions, but generally corresponds to the TTGACA sequence suggested by Rosenberg and Court (1979). The spacer region between the -10 and -35 sequences is 16-17 nucleotides long.

The situation in B.subtilis is more complex since B.subtilis contains multiple forms of RNA polymerases which recognise different promoter sequences. The enzyme found in B.subtilis consists of an RNA polymerase core associated with one of a number of sigma factors along with other polypeptide factors. Although the core polymerase is present throughout development, several core-associated polypeptides occur only in cells at specific stages of sporulation and may represent a mechanism by which B.subtilis regulates the differentiation process (Doi, 1982 ; Johnson et al., 1983).

B.subtilis vegetative promoters conform to the E.coli promoter sequences (Moran et al., 1982) but they do so more closely and frequently than the E.coli promoters themselves. Moran et al (1982) have therefore suggested that the

B.subtilis RNA polymerase is more rigorous in its requirement for a consensus sequence than that of *E.coli*. So *E.coli* promoter sequences that tend not always to conform to consensus may not be recognised by *B.subtilis* RNA polymerase. However, the *E.coli* tms promoter is not recognised in *B.subtilis* and this has the correct spacing and consensus sequence information (Moran *et al*., 1982). So the *B.subtilis* RNA polymerase must have some further requirement of the DNA sequence.

Moran *et al*. (1982) put forward the suggestion that an A-T rich sequence found upstream of the -35 region (for example in the *spoVG* gene and *Bacillus* phage SP01 promoters) was an additional requirement but more information is needed to confirm this. The same authors have tentatively suggested that *B.subtilis* RNA polymerase may also have some specific requirement for a particular spacer sequence or particular features of the spacer sequence but they provided no evidence in support of this theory.

As regards a block at the level of translation, complex formation between mRNAs of Gram-positive bacteria and the 3' terminal of their 16s rRNA seems to be consistently more stable that in Gram-negative bacteria (manifest in a greater negative free energy of interaction) with more extensive sequence complementarity (Moran *et al*., 1983). So, although the Shine-Dalgarno consensus sequence is very similar to that of Gram-negative species (McLaughlin *et al*., 1981) the Gram-positive bacterial ribosome binding sites that have been sequenced appear to conform more often and more exactly to consensus (Gold *et al*., 1981). In *E.coli*, on the other hand, correspondence to three or four bases of the

consensus sequence is relatively rare. Again, the requirements for recognition of RNA sequence by Gram-positive bacterial ribosomes are probably more stringent than in E.coli.

There are now a large number of reports in the literature of instances of disruption of cloned DNA sequences or recombinant plasmids by deletion of genetic material in B.subtilis and there are no doubt many others which were not reported before the widespread nature of the phenomenon was recognised. The occurrence of deletions seems to stem from some feature of a particular combination of vector plasmid and inserted DNA (Kreft and Hughes, 1982). Although deletions do occur in E.coli they are far more pronounced in B.subtilis and as such present a significant barrier to cloning in this bacterium.

Details of stuctural instability in B.subtilis are discussed in more detail in section 3.1.

Plasmid instability does of course pose a major problem for the future development of fermentation processes based on cloned genes. If such processes are to be considered then the long term stability of both vector and cloned DNA sequences must be assured. So there is something to be gained from an industrial as well as an academic point of view in pursuing a detailed study of plasmid biology in B.subtilis.

2. PLASMID MAINTENANCE AND STABILITY

In recent years major advances have been made in our understanding of the processes which ensure the continued replication and stable inheritance of bacterial plasmids.

The stable maintenance of plasmids is achieved through the efficient functioning of two distinct processes. These are i) the co-ordination of replication with cell growth so that at least one replication cycle takes place per cell division and ii) the distribution of plasmid copies to daughter cells in order that each daughter cell receives at least one copy of the plasmid. Partition is the name given to the process whereby plasmids are actively segregated between daughter cells. Replication control and partitioning have been shown to be functionally independent processes and as such they will be dealt with seperately in the review that follows.

Plasmids are extremely useful models for studies on the mechanisms and control of replication since they are usually dispensable to their host cells whereas interference with chromosomal replication inevitably disrupts cell growth. The replication properties of many different plasmids from both Gram-negative and Gram-positive bacteria have now been studied.

Generally speaking, plasmids fall into either of two classes based on the prominent features of their replication

Thus, the small, non-conjugative plasmids generally have one origin, and usually exhibit uni-directional replication whereas the large, conjugative plasmids in some cases have two or even three origins of replication, (for example R6K, Kolter, 1981).

All plasmids are dependent to some extent on host replication functions although individual plasmids have different requirements. For the most part the small non-conjugative plasmids for instance ColE1 (Khan and Helinski, 1978) depend entirely on host enzymes whereas the large conjugative plasmids, for example the F factor (Kline, 1985), specify at least one protein essential for their replication.

Plasmid replication can either be described as "stringent" or "relaxed". Large conjugative plasmids tend to exhibit a stringent mode of replication, that is to say that plasmid replication is obligatorily linked to that of the host chromosome. If initiation of replication of the chromosome is prevented then plasmid replication also stops. Small non-conjugative plasmids on the other hand generally show a relaxed mode of replication. So, for example, in the case of ColE1 and its derivatives, when initiation of chromosomal replication is halted, for instance by the action of chloramphenicol, the plasmid molecules continue to replicate becoming considerably amplified in copy number (Clewell, 1972).

There are, inevitably, exceptions to the above classifications. For example, the plasmid R6K. Although it is a multi-antibiotic resistance plasmid with the ability to promote conjugation it is intermediate in size with a high

copy number and relaxed mode of replication (Kontomichalou et al., 1970). pSC101 is a small plasmid which specifies tetracycline resistance and is non-conjugative, but it exists at an intermediate copy number (Armstrong et al., 1984).

It should be stressed that the classifications stringent and relaxed do not imply that replication control of plasmids categorised in this way is either tight or loose respectively. Neither do these terms provide any information on the control of replication of these plasmids. They refer simply to the fact that one group of plasmids require no plasmid-encoded proteins for their replication and the host proteins they require are stable (relaxed control), whereas the other group (stringently controlled) code for a protein or proteins essential for their replication and in the absence of protein synthesis they are unable to be replicated.

2.1 Regulation of replication

Not surprisingly, most of the available information on plasmid replication comes from studies of plasmids in E.coli. either endogenous or derived from other Gram-negative bacterium. An extrapolation of these results to describe the replication control of plasmids in Gram-positive bacteria may not be wise considering the differences between the two groups of bacteria. Nevertheless, in at least one well studied case, that of S.aureus plasmid pT181, such an extrapolation seems justified in view of the similarities that have been

revealed between this plasmid and certain plasmids of Gram-negative bacteria (Novick et al., 1984a). Therefore a description of plasmid maintenance could usefully begin with a survey of the pertinent details of the more extensively studied plasmids of Gram-negative species, particularly as the literature reveals a number of common features of their maintenance systems. As the field develops it should become apparent whether other plasmids of Gram-positive bacteria employ novel maintenance systems.

Any plasmid in a particular host under a defined set of conditions is present at a characteristic average number of copies per genome equivalent - the copy number. Obviously some mechanism exists to accurately maintain the copy number of a plasmid. The system primarily responsible for this is the control of the initiation of replication. It should also be noted that the copy number can be affected by the host (Macrina, 1974 ; Cress and Kline, 1976) and by the growth conditions (Engberg and Nordstrom, 1975) but the genetic information for the control of initiation of plasmid replication is always entirely plasmid-encoded.

There appear, on the face of it, to be many diverse mechanisms of plasmid replication control but at the same time some basic principles can be identified which exhibit some striking common features. The similarity lies in the fact that control of plasmid replication involves either recognition of RNA sequence or titration by repeated DNA sequences of a protein required for replication. Examples of each type of control will be discussed next with particular reference to the common features shared by different

plasmids.

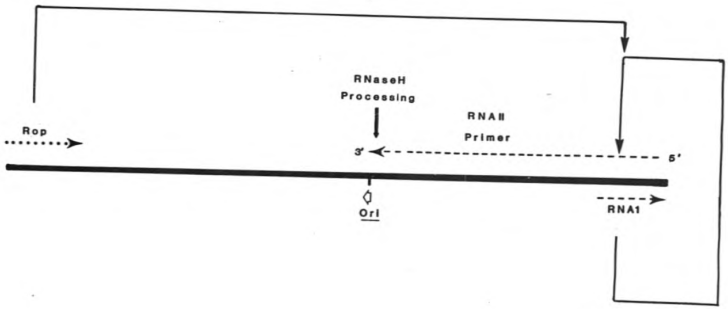
Of the plasmids of Gram-negative bacteria that have been studied, the non-conjugative plasmids are perhaps the least complex and best understood with respect to their regulation of replication. An example of this type of plasmid is ColE1.

ColE1 is the parent of many of the E.coli plasmid cloning vectors currently in use and has proved to be a valuable model for studies of DNA replication control. It is a small, naturally occurring, non-conjugative plasmid of E.coli which codes for the production of the colicinogenic protein colicin E1 as well as a protein conferring host immunity to the colicin. It exists at a copy number of 20-30 and is amplifiable by as much as 50 times in the presence of chloramphenicol (Clewell, 1972). ColE1 is entirely dependent for its replication on host enzymes (Khan and Helinski, 1978).

The minimal replicon of ColE1, as of all plasmids, is defined as the minimum portion of the plasmid replicon which is capable of autonomous replication and in the case of ColE1, codes for, as well as the origin of replication, two gene products involved in control of initiation of replication. One is a small RNA species about 108 nucleotides long called RNAI and the other is a 63 amino acid polypeptide called Rop. The RNA transcript which serves as a primer for the start of DNA replication, designated RNAII, is transcribed from a promoter located about 550 base pairs upstream (with respect to the direction of replication) from the origin of replication - see Figure 1.1.

Figure 1.1: Schematic Representation of the Region of ColE1 Involved in Replication and its Control

The direction of replication from the origin is indicated by the open arrow. Transcripts and protein coding sequences are represented by broken lines (---------- and ..... respectively). The small transcript, RNAI inhibits primer formation by binding to its complementary sequence in the pre-primer (RNAII) transcript. The protein Rop, coded for downstream of the origin of replication, co-operates with RNAI to inhibit primer formation by an unknown mechanism. The fine, unbroken lines indicate the sites of action of RNAI and Rop. After Lacatena and Cesareni (1983) and Cesareni et al. (1984) (not drawn to scale).


RNaseH
Processing
RNAII
Primer
Rop
3'
5'
Ori
RNA1

The small RNA, RNAI, is transcribed from the strand opposite to that encoding the pre-primer, RNAII, and is therefore entirely complementary to the terminal 108 nucleotides of RNAII. (Morita and Oka, 1979 ; Tomizawa et al., 1981).

Tomizawa and Itoh (1981) showed that RNAI forms hybrids with the pre-primer, RNAII, in vitro and suggested that RNAI inhibited initiation of DNA replication by preventing the formation of a DNA-RNA hybrid between the pre-primer and the DNA template.

The effect of RNAI on initiation of replication seems to be mediated largely through its secondary and tertiary structural features and those of its target, RNAII (Tomizawa and Itoh 1981 ; Lacatena and Cesareni 1983). The sequences of RNAI and RNAII show a high degree of internal symmetry and each have the potential of forming a secondary structure similar to a tRNA structure, that is three stems and loops arranged in a cloverleaf shape. The three stem and loop structures of RNAI are designated I', II' and III' to distinguish them from the complementary hairpin loops, I, II and III of RNAII (Tomizawa and Itoh 1981).

A model to explain the interactions between RNAI-RNAII was presented by Tomizawa in 1984. Tomizawa (1984) had demonstrated that contact between RNAI and RNAII occurred initially and transiently at the single stranded loops of their secondary structures. Binding then proceeded in a stepwise manner beginning at the 5' end of RNAI. The interaction at the loops was therefore proposed to facilitate binding by bringing the 5' end of RNAI close to its complementary region on RNAII. As pairing proceeded

the loop contacts were thought to be broken and the secondary structure "dissolved" so that RNAI and RNAII hybridised along their entire length.

The second regulatory element, the Rop protein, was mapped by Twigg and Sherratt (1980) to a region downstream from the replication origin. It was named Rop (for Repressor Of Primer) by Cesareni et al., (1982) who demonstrated that it repressed transcription from the RNAII promoter but did not appear to affect the transcription of RNAI. Its inhibitory effect on RNAII transcription required the presence of RNAI and Cesareni et al (1984) later suggested that Rop may enhance hybrid formation between RNAI and RNAII. Lacatena et al., (1984) added the suggestion that interaction of Rop and RNAI with RNAII could affect the stability of the pre-primer with respect to ribonuclease digestion.

The molecular mechanism of Rop action has yet to be clarified. The Rop protein could interact directly with the RNAI resulting in a complex that has a more potent inhibiting effect than either alone but the results of Tomizawa and Som (1984) would seem to refute this idea. These authors offerred the alternative explanation that Rop could increase either the rate of occurrence of, or the stability of, the initial contact between RNAI and RNAII. It could, alternatively, increase the rate of transition from this temporary contact to the stable binding which begins at the 5' end of RNAI (Tomizawa 1984). Since RNAI can clearly function in the absence of Rop (Twigg and Sherratt 1980) the possibility remains that the polypeptide may act as a fine tuning mechanism for copy number control.

There are a number of small plasmids which share certain replication features with ColE1. These plasmids specify transcripts analogous in form and function to the two ColE1 transcripts RNAI and RNAII. They include p15A, RSF1030 and CloDF13.

Plasmids of the FII incompatibility group, for example NR1 (Dong et al., 1985) and R1 (Riise and Molin, 1986) also use RNA as an inhibitor of replication. This group of plasmids have a copy number of two and carry tra genes which encode functions required for conjugal transfer of DNA. Their replication control presents a further degree of complexity over that of ColE1 and related plasmids in that the region of their genome involved in replication control also encodes a protein required for replication as well as a small, untranslated, antisense RNA which inhibits its translation.

The majority of E.coli plasmids analysed to date have an alternative system for replication control based not on RNA as an inhibitor but on direct nucleotide repeats at their origin of replication. Each plasmid also encodes a protein that is required for replication (a Rep protein) and the start of the Rep protein structural gene is generally a short distance from one end of the repeats. The highly conserved hexanucleotide TGAGPuG is often present in the direct repeats (Filutowicz et al., 1985) suggesting a possible important function for this part of the repeated sequence although its actual role remains to be clarified.

Within the group of plasmids which carry direct repeats at their origin, which includes P1 (Abeles et al., 1984), F (Kline, 1985), pSC101 (Churchward et al., 1983), R6K

(Stalker et al., 1983), RK2 (Stalker et al., 1981) and RSF1010 (Scholz et al., 1985) there are a number of striking common features worthy of mention. Firstly, the direct repeats themselves are of a similar size (17 to 22 base pairs), are similarly spaced on the plasmid genome either joined end to end or separated by one or more nucleotides and are generally located near to the origin of replication. Secondly, the Rep proteins of the plasmids (E of F, RepA of P1, Rep101 of pSC101 and pi of R6K) are of similar size (29-37 kilodaltons), are generally located near both the origin and the direct repeats and are all autoregulated (Rothman-Scott, 1984 and see the references above). So negative control of the frequency of initiation of replication in these plasmids is provided by the Rep protein itself through autorepression. Binding of the Rep proteins to the direct repeats has now been demonstrated in the cases of R6K and pSC101 (Bastia et al., 1985), P1 (Chattoraj et al., 1984) and F (Kline, 1985). The pi protein of R6K differs from the others in so far as it is probably not rate limiting for replication (Kolter, 1981 ; Rothman-Scott, 1984)

P1 and F in particular have a very similar genetic organisation even though there is only relatively little significant sequence homology between the two plasmids (Abeles et al., 1984). In both plasmids the direct repeats upstream from the initiator gene are less numerous than those downstream and appear to play a different role in replication regulation, that is, the upstream repeats are essential components of the origin whereas the downstream repeats are dispensable.

Finally it should be emphasised that there is no conclusive proof as yet that control of replication is brought about directly as a result of the binding of the Rep proteins to the direct repeats (P1 is perhaps the best characterised case, Chattoraj et al., 1984) neither is the autoregulation of the Rep proteins fully understood; as Nordstrom (1985) pointed out, autoregulation of a titrated protein seems to be a contradiction. Perhaps as suggested by Molin (unpublished, in Nordstrom 1985) one or all of the Rep proteins is bifunctional, that is, has two distinct domains, one responsible for the function essential for replication and the other for the autorepression. In this respect studies on the E protein of the F plasmid (which has been shown to be bi-functional, Rothman-Scott, 1984) may prove to be particularly fruitful.

Broad host range plasmids present extra problems for their replication control systems. Only two of these plasmids (RK2 or RP4 and RSF1010 or R1162), have been studied with a view to understanding how their replication is controlled in such a wide range of hosts which includes, in the case of RK2, almost all the Gram-negative genera that have been tested (Figurski et al., 1982).

RK2 codes for two determinants which are absolutely required for replication, oriV and trfA. Together these constitute a minimal replicon in E.coli. At least some broad host range capability is encoded within these functions since the minimal replicon is able to replicate in other Gram-negative hosts (Figurski et al., 1982).

RSF1010 is a small, multicopy plasmid which confers resistance to streptomycin and sulphonamide on its host

cells (Scholtz et al., 1985). About 50% of the genome of RSF1010 is taken up by essential genes (Scholz et al., 1985) and it seems to be a general principle that the number of essential genes in broad host range plasmids is greater than in plasmids with narrow host specificity.

Again, RSF1010 carries rep genes which code for proteins essential for replication. These Rep proteins are specific for the RSF1010 template and Scholz et al. (1985) have proposed that they might replace the host dna functions or may act as a functional link between host and plasmid replication systems. The dna functions are involved in DNA replication and repair and differ in their properties between different Gram-negative species. So perhaps this plasmid and others closely related to it overcome host range restrictions by becoming independent of the dna functions of the host.

Direct repeats are also involved in the replication control of RSF1010 and are also associated with the expression of incompatibility (Meyer et al., 1985 ; Persson and Nordstrom, 1986).

The evidence accumulated so far indicates, then, that plasmid replication control systems seem to fall into one of two categories; those that rely on inhibition of either primer formation or translation of an essential replication protein by a small antisense RNA and those whose replication is mediated by the binding of a protein essential for replication to a series of direct repeats at the origin. It is still possible, however that a small RNA molecule may be found to be responsible for replication inhibition of a member of the latter group of plasmids. The RNA primer for

initiation of replication has so far only been identified in the case of ColE1 so the primers of other plasmids may be regulated either by a means similar to that of ColE1 or by another, as yet unknown, mechanism.

2.2 Partition

In the previous section the ways in which different plasmids have evolved a number of different mechanisms for controlling their replication were described. These replication control circuits, however, merely set and maintain the copy number of a particular plasmid and are not sufficient to ensure its stable inheritance over a number of cell divisions. Obviously, low or single copy plasmids need very precise control mechanisms to ensure not only that at least one replication cycle takes place per cell division but also that the plasmid copies are accurately distributed or partitioned between daughter cells.

High copy number plasmids, on the other hand, could be stably inherited in the absence of an active partitioning process, that is, if selection of plasmids for distribution to daughter cells were a random process. The result of a random assortment of plasmid copies can be demonstrated by a simple consideration of the probability of plasmid-free cells arising in such a situation. Assuming that distribution of plasmid copies is entirely random and that each cell contains the same number of copies of the plasmid, then for a given copy number the probability of a cell failing to receive a copy of the plasmid is given by the

binomial distribution:

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

Where P(0) is the probability of a plasmid free cell arising and n is the number of copies of the plasmid in the cell at division.

So, for instance, a cell carrying a single copy of a plasmid has a probability of 1 of giving rise to a plasmid-free daughter cell (that is, 100% probability). A cell with two copies of a plasmid has a probability of 0.5 of producing plasmid-free progeny, but a cell, for example, with 15 copies gives rise to plasmid-free progeny at a rate of only 1 in 6×10^5 daughter cells per generation.

A partitioning, or *par*, locus responsible for the stable maintenance of plasmids in exponentially growing cell populations was first identified and characterised in the *Salmonella* plasmid pSC101.

Meacock and Cohen (1980) found the pSC101 *par* function to be independent of replication even though the *par* locus was adjacent to the origin of replication and they suggested that duplication of the *par* locus either at initiation or termination of plasmid DNA replication may be involved in activation of the *par* mechanism, hence its proximity to the origin of replication. However, the *par* locus was found to be able to stabilise the plasmid when placed elsewhere on the replicon and to function even when its orientation was reversed with respect to the origin (Meacock and Cohen, 1980). So its location adjacent to the origin was obviously not essential for its normal function.

The prominent features of the *par* locus reported by

Meacock and Cohen (1980) included its ability to stabilise a functionally different replicon (pACYC184) and the fact that it could only act in *cis*. The former indicated that the *par* system was not replicon-specific nor was it specific for replicons of a particular type (Meacock and Cohen, 1980) and the latter that it probably did not code for a diffusable gene product, either RNA or protein. The DNA sequence of the *par* region, as published by Miller *et al*. (1983) encoded no obvious polypeptides.

When the region containing the *par* locus was sequenced by Miller *et al*. (1983) it was found to contain three repeated sequences. These sequences were designated a, b and a' by Tucker *et al*. (1984) and were shown to have the potential of forming a hairpin loop structure, either between a and b or between a' and b (8 of 9 and 9 of 12 bases are paired). Thus a and a' are direct repeats and b is an inverted repeat of both a and a'. Partial deletion of the direct repeats was found to result in segregational instability but this type of deleted plasmid was not as unstable as those that had all three repeats deleted. The phenotype of these latter type of plasmids was termed "super-*par*- " (Tucker *et al*.. 1984).

Certain partially deleted plasmids were found to have a reduced ability to compete with a co-existing, wild-type plasmid of the same incompatibility type, the *cmp*- phenotype, but were totally stable in the absence of another plasmid. These plasmids expressed normal incompatibility against other *cmp*- plasmids and had a normal copy number (Cohen *et al*., 1985). At least some degree of functional linkage is therefore suggested between the pSC101

partitioning and replication systems.

Association of the *par* region of a plasmid with a particular membrane site or protein, first suggested by Jacob *et al*. (1963), has been put forward in many instances as a possible means of achieving the actual physical separation of plasmids into daughter cells. In support of this hypothesis, plasmid replicons carrying the pSC101 *par* locus have been found to co-purify with *E.coli* outer membrane material *in vitro* (Gustafsson *et al*., 1983). The *par* region also carries a transcription terminator which has been tentatively suggested as a possible candidate for a protein or membrane binding site (Miller *et al*., 1983).

Two plasmids which are maintained with great fidelity in *E.coli* and whose maintenance systems have received a great deal of attention are the F factor and the prophage form of phage P1, both of *E.coli*. F is a 94.5kb conjugative plasmid which exists at a copy number of one. Similarly, P1 in the prophage state is a single copy plasmid of about 90kb. The two plasmids are strikingly similar in size, copy number and in genetic organisation. They also have some sequence similarity in a region upstream of the coding sequences responsible for their autonomous replication (Abeles *et al*., 1984).

Despite their low copy numbers, both these plasmids are extremely stably maintained so that, in the case of P1, plasmid-free daughter cells arise at a frequency of less than 10^{-4} per generation (Sternberg *et al*., 1981). This property would suggest that both plasmids possess an active partitioning mechanism and, in fact, such a mechanism has been located adjacent to the origin of replication on each

plasmid.

These *par* regions are functionally distinct from the replication functions of the plasmids and are each able to stabilise the other's replication functions, (Ogura and Hiraga, 1983 ; Austin and Wierzbicki, 1983). They do not, however, exert incompatibility against one another, suggesting that the two plasmids must be sufficiently different for the *par* apparatus to be able to distinguish between them.

The *par* region of each plasmid contains a region which is required in *cis* to bind two *par* proteins (SopA and SopB of F and ParA and ParB of P1) which function in *trans* (Austin and Abeles, 1983). Presumably this protein-DNA interaction is essential for the association of the plasmid with the putative host cell partition apparatus. In the case of F, the *cis* acting *sopC* region not only interacts with the two *par* proteins but also with at least two host proteins (Ogura and Hiraga, 1983). The sequences encoding the *par* proteins are located between the *cis* acting loci and the replication region in both plasmids.

The complete sequence of the *par* region of P1 has been determined and found to contain what appears to be an operon comprising an open reading frame which codes for the essential *parA* protein, another open reading frame which has the capacity to code for a 38 kilodalton protein and an adjacent site containing blocks of extremely A-T rich sequences. Downstream of this site is another site rich in A-T, *incB*, also involved in partitioning, which exerts incompatibility against parent plasmids. Both the *incB* and upstream A-T rich sites are postulated to be binding sites

for par proteins and contain 20-base-pair imperfect inverted repeats. Another region essential for partitioning lies 172 base pairs downstream from the incB sequence (Abeles et al., 1985).

The suggestion has been made that the par operon is autoregulated and that both the parA and parB products are necessary for this autoregulation. The autoregulation is relieved when parB is lacking on the plasmid (Austin and Abeles, 1985). A similar type of regulation has been proposed for the F par proteins (Austin and Wierzbicki, 1983).

By analogy with the stabilisation of an unrelated replicon by the pSC101 par region, the par region of P1 has been shown to stabilise pBR322 at low copy numbers (Austin and Abeles, 1985).

Austin and Abeles (1983) have put forward a model for the interaction between the P1 par factors. They propose that monomeric par proteins have DNA binding activity and bind specifically to the inc site in the par region. Once bound they form dimers and it is this property that brings about daughter plasmid pairing. The DNA-dimer complexes are presumed to associate with a host factor or factors that defines the cell division plane. Subsequent septum formation at this plane would result in the separation of the daughter plasmids to either side of the division septum.

In the case of the F plasmid it may be that the SopB protein, through its interaction with sopC, acts to associate plasmid molecules with the cellular components of the partitioning apparatus. Alternatively, the SopB protein may associate with the cellular component, which alone is

not able to bind to sopC, before it binds to sopC (Ogura and Hiraga, 1983). The function of the SopA protein is less clear. It is not only possible that it could interact with sopC or indirectly with the cellular component but it could also regulate the expression of the sopB gene (Ogura and Hiraga, 1983). The model put forward by Austin and Abeles for P1 could also apply to F, especially with respect to the interaction of SopB and sopC.

Unlike the partitioning function of pSC101, P1 par and F par express incompatibility; via sopC in F and incB in P1. The incompatibility determinant in both cases appears to be the target site for the par proteins, located within the essential plasmid par sequences, (Austin and Wierzbiki, 1983). The incompatibility properties of these sequences has been proposed to be due to their ability to compete either with another plasmid for par proteins or for binding to the host partition apparatus (Abeles et al., 1985).

Another difference between the par systems of F and pSC101 is that F par, unlike pSC101 par, is able to stabilise oriC plasmids, (Hinchliffe et al., 1983 ; Ogura and Hiraga, 1983). Perhaps the supposition that the partition mechanisms of unit copy plasmids bear the closest functional relationship to that of the host chromosome is justified by this observation.

2.3 Incompatibility

Incompatibility was defined earlier as the inability of two closely related plasmids to coexist in the same

bacterial cell and its progeny. One or other of the plasmids is prevented from replicating and cannot therefore attain a copy number sufficient to ensure its stable maintenance. It would seem likely then, that incompatibility is the expression of the specificity of either the replication control mechanism or partitioning or both. The involvement of both systems in incompatibility determination has been largely borne out by observation. Generally a single plasmid expresses incompatibility in association with either its replication control functions or its partitioning determinants but not both.

In the past a number of models and extensions thereof have been put forward to explain plasmid incompatibility. In their Maintenance Site Model, Jacob et al. (1963) proposed that incompatibility was the result of competition between plasmids for binding to a common cellular membrane site. The binding of a resident plasmid to this site was envisaged to be a pre-requisite for plasmid replication. An incoming plasmid would therefore be at a distinct replicative disadvantage and would be lost during subsequent cell divisions.

The Maintenance Site Model, however, only allows for incompatibility between single copy plasmids. As most plasmids exist in considerably more than unit copy and still exhibit incompatibility, it would seem that this model cannot satisfactorily account for the phenomenon.

Pritchard et al. (1969) proposed an alternative model, the Inhibitor Dilution Model, which states that control of plasmid replication is effected by a trans-acting, freely

diffusable inhibitor. Plasmid incompatibility would result from the mutual inhibition of two related plasmids which produce cross reacting repressor molecules. Disproportions are therefore created that are amplified during subsequent cell divisions eventually leading to the loss of one of the plasmids.

Whereas evidence for the Maintenance Site Model has been largely refuted or reinterpreted (for example Hashimotoh-Gotoh and Timmis, 1981), the Inhibitor Dilution Model predicts some testable features of plasmid replication that have stood up to experimental investigation (Uhlin and Nordstrom, 1975 ; Cabello et al., 1978 ; Hashimoto-Gotoh and Inselberg, 1979 ; Molin and Nordstrom, 1980).

The results of certain density-shift experiments (Rownd, 1969 ; Bazaral and Helinski, 1970 ; Gustafsson and Nordstrom, 1975 ; Gustafsson et al., 1978) cannot be explained by the Inhibitor Dilution Model in its simplest form. Therefore Rownd (1969) proposed an additional model, the Random Replication Model, to account for these results.

The Random Replication Model extends the Inhibitor Dilution Model by proposing that there is a random choice of template for each new round of replication from the cellular pool of plasmids.

This model explains why one member of a pair of incompatible plasmids is lost during cell division. A resident plasmid would be present at a high copy number and is therefore much more likely to be chosen for each round of replication than the single copy of an incoming plasmid. So the challenging plasmid is continually diluted out during subsequent cell growth.

Novick and Schwesinger (1976) have proposed a model to explain plasmid incompatibility which does not involve replication. They suggested that a specific cellular site exists by means of which attached plasmids are randomly partitioned to daughter cells. Inequalities arise when related, and therefore incompatible, plasmids are partitioned since the cell site cannot distinguish between them. The amplification of these inequalities over a number of cell divisions would eventually result in the loss of one of the plasmids (see also Novick and Hoppenstead, 1978 and Ishii et al., 1978).

The involvement of a membrane-attached protein or protein complex in plasmid partitioning was suggested by Jacob et al. (1963). The plasmid pSC101 which is actively partitioned and the plasmid R1 have been shown to co-purify with the outer cell membrane (Gustafsson et al., 1983). So in cases where incompatibility is associated with distribution of plasmid copies to daughter cells it may be that a cell membrane site is involved but at the present time there is no direct evidence in support of the theory.

2.4 Control of cell division

In addition to par, certain plasmids, notably those which have very low copy numbers, have other systems for ensuring their stable maintenance. In the case of the F plasmid evidence indicates that it may replicate independently of the host cell cycle (Andresdottir and Masters, 1978) which suggests a necessity for plasmid control over host cell division in order to prevent the

appearance of plasmid-free cells.

Miki et al. (1984) found that if replication of the F plasmid was prevented, the host cells went through only one more division before they formed non-septate filaments. This effect was assumed to be a consequence of a reduction in the number of F copies to one per cell. The system of F responsible for this cell division inhibition is known as ccd for Control of Cell Division and comprises two gene products, LetA and LetD. These two products are coded for within an operon whose expression is controlled by termination of plasmid DNA replication. Lack of suppression of the two genes results in cell division inhibition and filamentation eventually leading to cell death (Miki et al. 1984 a and b).

LetA is only able to act in cis (Miki et al. 1984a) therefore it probably codes for a non-diffusable protein. It is thought to function in a similar way to the termination protein proposed to be involved in the coupling of host cell replication to cell division (Jones and Donachie 1974).

Ccd is functionally independent of replication and partitioning since the coding sequences involved can be translocated or inverted within the F genome without loss of function, (Miki et al., 1984a).

A model for the control of cell division by the F plasmid has been proposed (Miki et al., 1984b) wherein it was suggested that host processes leading to cell division are coupled to completion of F replication and LetA and LetD are responsible for this coupling. This model assumes that the Let D polypeptide is the factor which prevents cell

division. When F replication is completed the LetA and LetD polypeptides are produced. The LetA gene supresses the activity of the LetD polypeptide and prepares the cell for the next division. This would only take place when both chromosomal and plasmid DNA replication are completed and the postulated chromosomal termination protein and the LetA polypeptide are present. The LetA polypeptide must be inactivated somehow during cell division in order for reactivation of LetD polypeptide to occur upon distribution of plasmid DNA copies to daughter cells and this would prevent more cell division until the next round of plasmid replication was completed.

In E.coli inhibition of cell division is controlled by at least two pathways termed sfi-dependent and sfi-independent. Very little is known about the sfi-independent pathway, only that it operates during the C period of the growth cycle (Burton and Holland, 1983). F-encoded inhibition of cell division is closely related to the sfi-dependent pathway which is a component of the SOS system in E.coli (Miki et al., 1984b).

The F plasmid carries a further locus for cell division control, letB, but this is in the par region so perhaps there is some interaction between ccd and partitioning (Miki et al., 1984b). This may in fact be necessary to ensure co-segregation of F and the LetD polypeptide. Otherwise it may be possible for a plasmid free cell to arise which still carries the LetD polypeptide. Such a cell would suffer division inhibition and possibly death. An excessive amount of LetD polypeptide per plasmid replicon would have the same effect. So perhaps this is why the intermediate copy number

plasmid pSC101 has not been reported to code for a ccd function.

It has not yet been properly determined whether P1 has a ccd system although, by inference, from the results of MacQueen and Donachie (1977), it would seem that it does.

Beyond the relatively well studied ccd system of the F plasmid there are examples of less well characterised cases in which a low copy number plasmid appears to ensure its stable inheritance by a means other than partitioning. A case in point is the plasmid R1. This plasmids codes for production of a toxic factor which is capable of bringing about collapse of the membrane potential of the host if its manufacture is not prevented by the presence of a small antisense inhibitor RNA also coded for by the plasmid (K.Nordström, personal communication and Gerdes et al., 1985).

The plasmid RK2 codes for a series of genes designated kil genes A, B, C and D which are regulated by their corresponding kor genes and which are either lethal to their host or interfere with plasmid maintenance (Figurski et al., 1982).

These systems differ therefore from the ccd system of the F plasmid in that they employ more extreme tactics for controlling replication of their host since if the appropriate plasmid encoded inhibitor is not present the host cell is killed.

2.5 Site-Specific Recombination

If two daughter copies of a unit copy plasmid were to

recombine with each other producing a dimer then they would no longer be equally partitioned between daughter cells at cell division and plasmid free cells would arise at a relatively high frequency in the population.

Wild type P1 is maintained very efficiently in host cells whereas a miniplasmid derivative was found to be unstable and asymmetrically partitioned. Austin et al. (1981) showed that it was dimer formation which caused the segregational instability of this particular miniplasmid and that this was due to the fact that it lacked a locus of wild type P1 which was involved in site-specific recombination. This locus on P1 has two components, loxP and cre. loxP being the site at which recombination takes place and cre being the gene coding for a potent recombinase. Recombination therefore takes place between the two loxP sites on a dimer and is catalysed by the cre recombinase.

The loxP site and the cre gene are in separate but adjacent segments of DNA. Abremski and Hoess (1984) have shown that the cre gene product is a 35kd polypeptide which forms a dimer in the presence of magnesium chloride. The dimer is capable of binding specifically to a single loxP site. Each cre protein binding site is composed of a 13 base pair inverted repeat together with 14 base pairs of adjacent DNA which contributes to an 8 base pair spacer region between the inverted repeats (Abremski and Hoess, 1984). The organisation of the loxP site, interestingly, bears a striking similarity to the Lambda attP site and the yeast 2-micron FLP (for "flip-flop" interconversion between two relative orientations of unique regions on the plasmid) sites which perform different physiological roles (Hoess and Abremski, 1984).

The efficiency with which intraplasmidic recombination

carried out by the loxP-cre system occurs is independent of the orientation of the loxP sites and the conformation of the P1 genome. The loxP-cre system is also capable of catalysing interplasmidic recombination (Abremski et al., 1983).

Although it would seem that in the case of unit copy plasmids maintenance of monomeric conformation is vital for efficient partitioning, a system for resolution of dimers has not yet been demonstrated on the F plasmid.

The high copy number plasmid ColE1 has been shown to be randomly partitioned and for such plasmids the major cause of instability is reduced copy number. Multimerisation would be particularly effective in reducing the copy number of the plasmid and Summers and Sherratt (1984) have demonstrated a direct relationship between multimerisation and instability by showing that supplying the Resolvase protein in trans to pACYC184 carrying a transposon-encoded site-specific recombination system promoted simultaneous monomerisation and stabilisation of the plasmid.

ColE1 has also been found to code for a site-specific recombination system. In this case the plasmid carries a 380 base pair sequence designated cer which acts only in cis and which is independent of host recA, recF and recE functions (Summers and Sherratt, 1984).

Electron microscopic observations of intrastrand base-paired, single stranded ColE1 have revealed the presence of an inverted repeat within the cer region by virtue of the observation of a stem and loop structure. Such sequences have been shown to catalyse plasmidic recombination events (Stahl 1979 ; Edlind and Ihler 1981).

Since the DNA sequence is too small to encode a protein the size of the other known site-specific recombinases, it is unlikely that cer codes for such a protein. Summers and Sherratt (1984) suggested that perhaps cer encodes only a recombination site and a specific host encoded recombination protein is used.

It is also worth noting that recA-independent recombination may occur at any time between two plasmids with identical or related mobilisation functions (Broome-Smith, 1980).

It has been suggested that CloDF13 codes for a site-specific recombination system although recombination seems to be important only in certain copy number mutants (Hakkaart et al., 1982). CloDF13 has two loci designated parA and parB involved in stable maintenance of the plasmid. The available evidence points to the fact that parB, a 328 base pair non-coding region, promotes recA-independent, site-specific recombination and that host-encoded recombination proteins are used (Hakkaart et al., 1983). The function of the parA locus is uncertain, it appears to make only a minimal contribution to stability (Hakkaart et al., 1985).

2.6 Recombination and structural instability

A problem often encountered but seldom investigated directly is that many newly constructed recombinant plasmids are structurally unstable. So although the entire plasmid is not lost from the cell, it nevertheless suffers structural rearrangements, most often in the form of deletion of

genetic material.

In general the cause of deletions of plasmid DNA is badly understood because deletion often seems to occur irrespective of such influences as the rec character of the host cell, the source of the DNA (Uhlen et al., 1981 ; Kreft and Hughes, 1982) and any homology with other plasmid-borne sequences or sequences on the chromosome (Uhlen et al., 1981) (For details of structural instability in B.subtilis see section 3.1). Nevertheless some general causes can be identified which are classified below according to whether they are independent of, or dependent on, the recombination (rec) system of the host.

2.6.1 Rec-Dependent Rearrangements

In E.coli the chromosome and small ColE1-like plasmids recombine via different pathways. The chromosome follows mainly the recBC pathway which depends on exonuclease I as well as exonuclease V, the product of the recBrecC gene. In the absence of exonuclease I or exonuclease V the chromosome can recombine via the recF pathway which depends on the recF and recJ activities as well as a LexA-controlled function. An additional pathway, recE, can be activated in E.coli strains carrying the rac prophage. The recE pathway depends on the product of the recE gene, exonuclease VIII and on recF gene activity. All three pathways have an absolute dependence on the recA gene product (Fishel et al., 1981).

Plasmids in E.coli, on the other hand, recombine predominantly by the recF and not the recBC pathway. They

also engage very efficiently in the recE pathway in abcA cells. In this situation intraplasmidic recombination continues totally independently of the RecA protein but interplasmidic recombination proceeds at a 40 times lower rate that in the presence of the RecA protein (James et al., 1982). Although plasmid recombination in wild type cells depends on a functional recA product, this dependence is not absolute since a residual plasmid recombination activity of 1 to 4% of that of the wild type is observed in a recA mutant (Laban and Cohen, 1981 ; Fishel et al., 1981).

Plasmids and chromosomes appear to follow different recombination pathways in B.subtilis as well. Prozorov et al. (1982) found that, of twelve rec genes tested three had different effects on plasmid and chromosome recombination. In addition Weinrauch and Dubnau (1983) found that recB and recC mutations inhibited recombination of plasmid DNA to a greater extent than the chromosome and the recE mutation was shown to have the opposite effect (see also Dubnau et al., 1973 and Michel et al., 1983).

In a plasmid-carrying, recombination-proficient host rec-dependent rearrangements can occur between regions of DNA with sequence homology. Recombination can therefore take place in three different ways: it can take place between plasmid and chromosome where some homology exists between the two, for example, if the plasmid carries a cloned chromosomal sequence or gene. In this case recombination results either in integration of the whole plasmid into the chromosome including the non-homologous regions of plasmid DNA (Dubnau and Cirigliano, 1974 ; Duncan et al., 1978 ; Rapoport et al., 1979) or integration of only the region

having homology with the chromosome (Ostroff and Pene, 1984 ; Uhlen et al,. 1981). Dubnau and Cirigliano (1974) have suggested that the recE gene product of B.subtilis is involved in integration of plasmid DNA into the chromosome.

Recombination can take place between two plasmids in the same cell which carry homologous sequences, that is, interplasmidic recombination. Dubnau and Cirigliano (1974) and Tanaka (1979) have suggested that, again, the recE4 function of B.subtilis is involved, as is the recA function in E.coli (James et al., 1982).

Finally recombination can occur between regions of homology on the same plasmid, that is, intra-plasmidic recombination (Brutlag et al., 1977 ; Cohen et al., 1978 ; Puhler et al., 1979).

Systems of DNA repeats have been artificially created in order to study rec-dependent recombination between homologous sequences. It has been shown that the frequency of occurrence of recombination events depends on the length of the repeated sequence and the degree of homology. So frequencies of 1 in 10^7 to 1 in 10^8 cells have been observed when very short direct repeats were involved (Farabaugh et al., 1978 ; Edlund and Normark, 1981 ; McCorkle and Altman, 1982).

For these reasons it has been general practice to employ rec deficient bacterial hosts for cloning experiments. However, the problems do not end here because plasmid recombination reactions are at least partially rec-independent and rec-independent recombination events actually pose more of a barrier to the stable maintenance of recombinant plasmids.

2.6.2 Rec-independent Rearrangements

Until recently reports of rec-independent recombination events were restricted to two types; i) site-specific events, that is recognition of a specific DNA sequence by a recombinase as in the ColE1 cer/xer or the P1 loxP/cre systems (see section 2.5) and, ii) recombination events associated with transposable elements catalysed by transposases (Nisen et al.. 1977 ; Ross et al.. 1979 ; Bernardi and Bernardi 1981). These systems by-pass the need for the host rec gene products by supplying other enzymes capable of performing a specific recombination function and are involved in creating deletions independently of extensive base pair homology or generalised recombination.

More recently, however, rec-independent deletions have been found to occur between short direct repeats, for example, in the lac operon (Albertini et al., 1982) and in pHV14, a hybrid plasmid, in E.coli (Jones et al.. 1982)

The deletion of some transposable elements has been shown to take place in the absence of transposition functions (Foster et al., 1981 ; Egner and Berg, 1981). In fact, Collins (1982) found that excision of repeated sequences, or palindrome excision, occurred at inverted repeats within the Tn5 sequence at a frequency of four to five orders of magnitude higher than so-called precise excision of the transposon.

A mechanism for the excision of direct repeats was put forward by Farabaugh et al., (1978) and a similar model was proposed by Efstratiadis et al. (1980) which suggests, in

essence, that two elements of homology will form a stem-loop structure which is missed out by the DNA polymerase during replication. These authors suggested that the RecA protein was responsible for catalysing this so-called slipped mispairing but because deletions occurred in *recA* hosts, then at least one other enzyme must have been capable of bringing about the deletions if, indeed, the deletions were enzyme mediated at all.

Another way in which *rec*-independent genome rearrangements may come about is as a result of site-specific recombinases acting at sequences which bear only partial homology to the sequences at which they normally act. For example, recombination at the *lox*B site by the P1 *cre* recombinase (Hoess *et al.*, 1982), intermolecular recombination mediated by the *cin* gene of P1 (Kennedy *et al.*, 1983) or site-specific recombination in the *oriV* region of the F factor (O'Connor *et al.*, 1986).

So to summarise, deletions can occur independently of the source of the DNA and the host restriction-modification system, in either *E.coli* or *B.subtilis* but far more frequently in *B.subtilis* (for example, Alonso and Trautner, 1985b). They occur both in *rec*-proficient or *rec* deficient cells and can be either site-specific (between repeats in *rec*-proficient cells or transposon-mediated in *rec*-deficient cells) or have random end points (caused by an unknown mechanism but probably again often involving base pair homology - for example, Alonso and Trautner, 1985b).

3. REPLICATION AND MAINTENANCE OF PLASMIDS IN BACILLUS SUBTILIS

Compared with the volume of information now available on the replication and maintenance of the plasmids of Gram-negative bacteria, very little is known of the biology of the majority of plasmids of Gram-positive species. Recently, however, there has been rapid progress in the study of the maintenance of four plasmids in particular; pE194, pC194, pUB110 and pT181 all originating from S.aureus. pE194, pC194 and pUB110 will be described in some detail as the information is relevant to the rest of this thesis.

The 3.5kb plasmid pE194 encodes erythromycin-inducible resistance to macrolide antibiotics, lincosamide and streptogramin type B (MLS) (Weisblum et al., 1979 ; Iordanescu, 1980). This plasmid was originally isolated from S.aureus (Iordanescu, 1980) and has been introduced into B.subtilis (Weisblum et al.,1979). It has a wild type copy number of 10-20 in S.aureus and about 10 in B.subtilis (Weisblum et al.. 1979).

By deletion analysis, Gryczan et al. (1982) have not only identified a replication region on pE194 which contains the replication origin, but they have found that this region also specifies a trans-acting function, rep. a cop gene and an incompatibility determinant, incA. The rep function is a positively acting factor required for fidelity of plasmid

replication and the product of the *cop* gene is a negatively acting copy control function.

The location of the pE194 replication region with respect to other markers is shown in figure 1.2. Since a mutation (*cop6*) which inactivates inhibition of initiation of replication by the *cop* gene product also eliminates expression of *incA*, it was concluded that *incA* and *cop* are the same (Gryczan *et al*., 1982).

Another incompatibility determinant, *incB* has been located on the MboI C fragment (see figure 1.2). This DNA sequence appears to be unnecessary at least for partitioning of pE194 since plasmids lacking this sequence segregate plasmid-free daughter cells no more often than do the parents (Gryczan *et al*., 1982).

With the completion of the nucleotide sequencing of pE194, its two biological functions, antibiotic resistance and replication, were assigned to specific sequences on the plasmid by Horinouchi and Weisblum (1982a) - see Figure 1.3.

The inducible MLS resistance determinant was thus localised to the TaqI fragment A. The TaqI fragment B was found to carry a replication determinant comprising two sets of inverted, complementary repeat sequences, one of which spans 124 base pairs and is adjacent to a second smaller set of 24 bases rich in guanine and cytosine. Although the inverted repeats may be involved in some aspect of replication control (Horinouchi and Weisblum, 1982a), they are not all required for replication (Gryczan *et al*., 1982).

Three open reading frames were also found on TaqI fragment B (Horinouchi and Weisblum, 1982a) each with the potential of encoding proteins of greater than 100 amino

Figure 1.2: Physical map of pE194 showing the replication determinants

The region essential for replication, as determined by Gryczan et al. (1982), is shown by a broken line. The location of the origin of replication, the direction of replication and the erythromycin resistance gene are also indicated inside the map. Restriction sites for the enzymes MboI. MspI, PstI and BclI are shown to aid comparison with the map of Horinouchi and Weisblum (1982a) - see Figure 1.3.

After Gryczan et al. (1982).

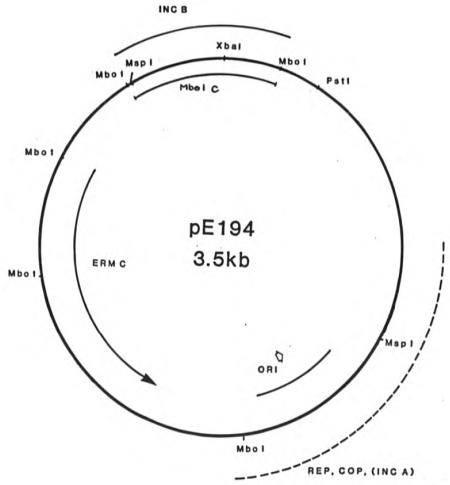
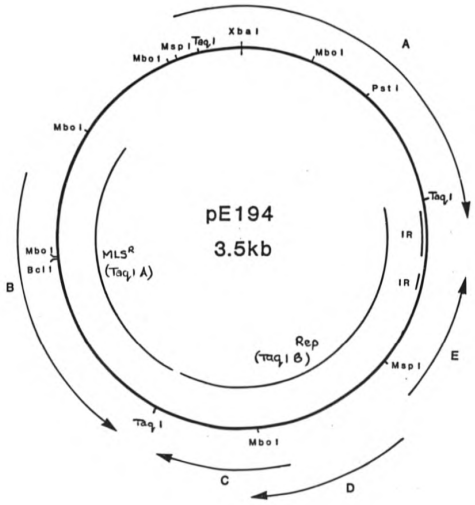

INC B
Xba I
Msp I
Mbo I
Mbo I
Pst I
Mbo I C
Mbo I
pE194
3.5kb
ERM C
Mbo I
Msp I
ORI
Mbo I
REP, COP, (INC A)

Figure 1.3: Physical map of pE194

The location of the open reading frames as determined by Horinouchi and Weisblum (1982a) are shown outside the map (A to E). The origin of replication and restriction sites for the enzymes MboI. MspI. BclI and PstI are indicated for comparison with the map of Gryczan et al. (1982) - see Figure 1.2. The location of the inverted repeat sequences determined by Horinouchi and Weisblum (1982a) are indicated by lines inside the map itself, each labelled IR.

After Horinouchi and Weisblum (1982a).


pE194
3.5kb
MLS^R
(Taq I A)
Rep
(Taq I B)
IR
IR
Xba I
Mbo I
Msp I
Taq I
Mbo I
Pst I
Taq I
Msp I
Mbo I
Taq I
Mbo I
Bcl I
Mbo I
A
B
C
D
E

acids. Because no ribosome binding sites were found associated with the open reading frames, the existence of translational products of this region is questionable.

Shivakumar et al., (1980) detected several polypeptides synthesised in minicell extracts and have located the DNA sequences specifying these polypeptides. None seems to be specified solely by the TaqI B region of pE194, although the coding sequence for a polypeptide, E1, overlaps the TaqI B fragment. None of the five major polypeptides identified in minicells was specified by the replication region. This region is, nevertheless, actively transcribed and contains an RNA polymerase binding site. Two major transcripts were identified by Gryczan et al (1982) which were strikingly more abundant in extracts of cells carrying a cop6 mutant than in those carrying pE194.

Finally, a mutant of pE194 was isolated by S.Gruss whose replication is temperature sensitive (Gryczan et al., 1982) so that at 42°C initiation of replication from the origin of the mutant pE194 does not take place. This feature has proved to be extremely valuable in analysing the replication properties of this plasmid and other plasmids replicated under the control of the pE194 replication origin.

The plasmid pC194, another small R plasmid originating from S.aureus shows some interesting similarities to pE194 and has been of value as a cloning vector in the analysis of pE194-encoded functions.

pC194 specifies inducible resistance to chloramphenicol mediated by the enzyme chloramphenicol acetyl transferase

(Iordanescu *et al.*, 1978). It has a relatively wide host range and has been reported to replicate, for example, in *Bacillus thuringiensis* (Martin *et al.*, 1981) and in *E.coli* (Goze and Ehrlich 1980). It has also been widely used as a cloning vector particularly as part of *E.coli-B.subtilis* shuttle vectors (Ehrlich, 1978). Its copy number is about 15 per cell in *S.aureus* and in *B.subtilis* (Alonso and Trautner, 1985a).

As in the case of pE194, the locations of a number of pC194 genes have been determined from the sequence and their products have been identified (Horinouchi and Weisblum, 1982b ; Shivakumar *et al.*, 1979).

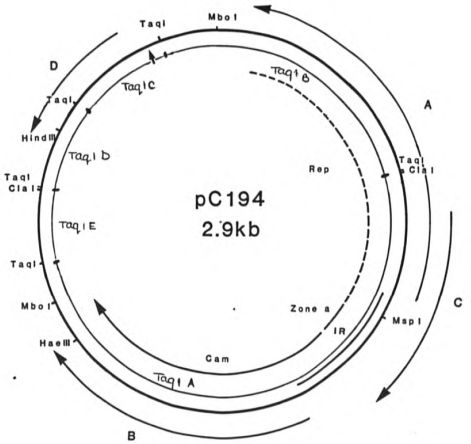
The open reading frames revealed by sequence analysis are shown on the restriction site map of pC194 - see Figure 1.4. Open reading frame B was shown to code for the chloramphenicol acetyl transferase (CAT). In fact, all of the information necessary for inducible CAT production lies in the sequence between the unique MspI site and the MboI site which includes open reading frame B.

The boundaries of the pC194 functional replication unit were determined by Horinouchi and Weisblum (1982b) by sub-cloning pC194 fragments of various sizes into pBR322 followed by transformation into *B.subtilis*. The sequence between the two ClaI sites from residues 616 to 2284 was found to confer ability to replicate in *B.subtilis*. The downstream portion of this fragment contains the CAT gene and its controlling sequences and extends 300 nucleotides back from the MspI site, so the replication functions could be localised to a region corresponding approximately to open reading frame C.

Figure 1.4: Physical map of pC194

The location of the open reading frames determined by Horinouchi and Weisblum (1982b) are shown along with the region of the genome containing the inverted repeat sequences (labelled IR). The approximate location of the region essential for replication as determined by Dagert et al. (1985), including zone a, is represented by the dotted line inside the map. Horinouchi and Weisblum (1985b) reported that the region of the pC194 genome labelled Cam here contained all the information necessary for the inducible expression of chloramphenicol resistance. Restriction sites for the enzymes MboI, ClaI, MspI, HaeIII TaqI and HindIII are marked around the periphery of the map. The location of the sequence altered in the mutant plasmid described by Alonso and Trautner (1985b) is indicated by an arrow on the inside of the map at the 5' end of open reading frame D.

Adapted from Horinouchi and Weisblum (1982b) and updated according to Dagert et al. (1985).


TaqI
MboI
D
TaqI
TaqI C
TaqI δ
A
HindIII
TaqI b
Rep
TaqI ClaI
TaqI ClaI
pC194
2.9kb
TaqI E
TaqI
MboI
Zone a
IR
MapI
C
HaeIII
Cam
TaqI A
B

The sequence of pC194 published by Horinouchi and Weisblum (1982b) was later amended by Dagert et al. (1984) with the result that the region reported to code for open reading frames A and C by Horinouchi and Weisblum (1982b) was redefined as a single open reading frame of 855 bases read in the direction of open reading frame A and extending in the 5' direction to include a sequence designated zone a by Dagert et al. (1984).

The most striking similarity between plasmids pC194 and pE194 lies in their sequence organisation. The pC194 sequence in the immediate proximity of the MspI site (see Figure 1.4) contains a 121 nucleotide inverted repeat region with an organisation similar to that of the pE194 TaqI B fragment. Like the pE194 sequence, the pC194 repeated sequence is next to a guanine-cytosine rich inverted complementary repeat which in this case contains the MspI site (Horinouchi and Weisblum, 1982b). In fact, the TaqI fragment B from pE194 was found by Horinouchi and Weisblum (1982b) to replace the replication functions of pC194. This region corresponds to zone a as delineated by Dagert et al. (1984). The palindromes in this region bear a resemblance to other replication origins (Dagert et al., 1984).

Dagert et al. (1984) presented evidence which suggested that it is the actual sequence of zone a in pC194 which is necessary for replication rather than any other gene product. They therefore suggested, by implication, that this could be the replication origin of pC194.

In 1982 Canosi et al. described a mutant plasmid derived from pC194, designated pC194-1 which was segregationally unstable in B.subtilis. Cells growing on

chloramphenicol-containing medium were rendered extremely sensitive to methly methane sulphonate (MMS) or UV light by the presence of the plasmid. Since the sensitivity of pC194-1-carrying cells to MMS or UV irradiation was only apparent in the presence of chloramphenicol (when expression of the CAT gene of the plasmid was required for cell survival) it is possible that the pC194 mutation sensitises a plasmid function required for plasmid maintenance against MMS or UV light (Alonso and Trautner, 1985a).

Alonso and Trautner (1985a) reported the identification of the base changes distinguishing pC194 and pC194-1. pC194-1 carries an additional two A:T base pairs at positions 2711 and 2713 in the TaqI fragment C (see figure 1.4). The DNA sequence of this part of the pC194 genome must, therefore, also be indispensable for stable maintenance of the plasmid. The region of pC194 affected by this mutation corresponds to the open reading frame D described by Horinouchi and Weisblum (1982b) - Figure 1.4.

The mutation in pC194-1 was given the name *seg* by Alonso and Trautner (1985a). The presence of the related plasmid, pE194 in the same cell was sufficient to stabilise pC194-1 independently of the *rec*E4 function of the cell (Alonso and Trautner 1985a) presumably by virtue of the production of a trans-acting gene product of pE194 involved in either replication or partitioning. The gene of pE194 that provided the stabilising product has not been identified but attempts are currently being made to do so. Present speculation is that the *inc*B function of pE194 may be involved (Gryczan *et al.*, 1982).

A diffusable product of gene D of pC194 was suggested as a candidate for the mediator of stable plasmid maintenance that pC194-1 is lacking, but there is no evidence for the predicted protein encoded by this open reading frame D being produced in minicell extracts (Shivakumar et al., 1979). So it is possible that the product of the gene D region is RNA and not protein. Dagert et al. (1984) noted that a plasmid lacking open reading frame D of pC194 was stably maintained at the wild type copy number in B.subtilis. therefore the function of this coding region is not clear.

The presence of a par or par-related region on pC194 is suggested by the fact that a 30 base pair region of pC194 bears significant homology to the par region of pSC101, (Novick et al., 1984b) but a plasmid, pAT30, which carries this region was found to be unstable (Alonso and Trautner, 1985a) so this observation is of dubious significance.

Finally, te Reile et al. (1986a) have reported that replication of pE194 and pC194 results in the production of single stranded copies of plasmid DNA in the proportion of one third of the total number of plasmid copies and in a later publication they propose that the single stranded copies are replication intermediates which are later converted to double stranded DNA (te Riele et al., 1986b).

pUB110 is a 4.5kb plasmid originally isolated from S.aureus which codes for kanamycin resistance and is maintained at a copy number of about 40 in B.subtilis (Shivakumar and Dubnau, 1978). Plasmid copies have been shown to be selected randomly for replication (Winston et al., 1980) and to replicate

unidirectionally from a fixed origin. The origin has been localised with respect to other genetic markers, (Scheer-Abramovitz *et al*., 1981 ; Tanaka and Sueoka, 1983).

The origin of chromosomal replication has been reported to be associated with the membrane in both *B.subtilis* (Winston and Sueoka, 1980) and *E.coli* (Kusano *et al*., 1984) and there is strong evidence for a critical involvement of membrane association of the replication origin in the initiation of DNA replication in *B.subtilis* (Winston and Sueoka, 1980). In order to evaluate the role of origin-membrane complexes in initiation of DNA replication Korn *et al*. (1983) have investigated the membrane binding properties of the plasmid chimera pSL103 composed of pUB110 and a *trpC*-containing fragment from *B.pumilus*. They demonstrated that pSL103 could specifically bind to an isolated membrane fraction from *B.subtilis* *in vitro* and that the specificity of the binding was determined by the origin-carrying half of pSL103, that is, pUB110.

Tanaka and Sueoka (1983) later showed that this *in vitro* binding was different to that occurring *in vivo*. The *in vitro* binding, or type I binding, occurred at undefined sites on the plasmid, was high salt resistant and dependent on the product of the *dnaI* gene. The *in vivo* , or type II binding, was high salt sensitive and independent of the *dnaI* gene product. The fact that the type II binding occurred at specific regions on pUB110 implies that it may have a functional role *in vivo*, perhaps to keep the plasmid bound to the membrane (Korn *et al*., 1983).

Tanaka and Sueoka (1983) identified four regions of pUB110 that bound to the membrane and showed that initiation of pUB110 replication occurred at a site within the TaqI fragment C which was flanked by two membrane binding regions (BA1 and BA2 in Figure 1.5, as named by McKenzie *et al*., 1986). The presence of the third and the fourth binding sites (BA3 and BA4) seemed not to be essential for plasmid replication, (Tanaka and Sueoka, 1983 ; Scheer-Abramovitz *et al*., 1981) although this does not mean that they did not contribute to the efficiency of replication.

Tanaka and Sueoka (1983) have suggested that the type II binding may have been involved in the partitioning of pUB110 copies into daughter cells but there is as yet no further evidence that this is the case.

Muller *et al*. (1986) constructed deleted derivatives of pUB110 in order to study the effects of the deletions on the replication functions of the plasmid. They also constructed deletions of the kanamycin resistance plasmid pTB913 isolated from a thermophilic *Bacillus* (Imanaka *et al*., 1981). Both pTB913 and pUB110 are able to replicate in *B.subtilis* which was used as the host for these experiments

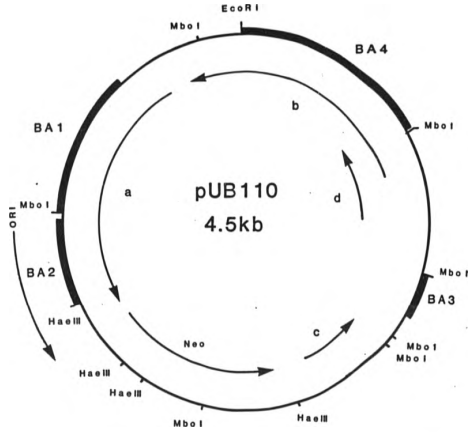
Two deletion plasmids were studied in detail; pRBH1 from pTB913 and pUB110dB from pUB110. The complete nucleotide sequences of the deletion derivatives were determined and found to be identical apart from two bases (Matsumura *et al*.,1984 ; Muller *et al*..1986).

The replication origins and the flanking regions of pRBH1 and pUB110dB were found to contain four promoters, two surrounding a 63 base pair inverted repeat, a Shine-Dalgarno

Figure 1.5: Physical map of pUB110

The locations of the membrane binding sites determined by Winston and Sueoka (1980) are shown along with the modified position of BA1 reported by McKenzie et al. (1986). The origin and direction of replication as determined by Scheer-Abramowitz et al. (1980) are indicated by the arrow outside the map itself. The open reading frames a, b, c and d correspond to the open reading frames α, ß, δ, and ó determined from the sequence by McKenzie et al. (1986). The location of the gene coding for neomycin (kanamycin) resistance, marked Neo here, was reported by Matsumura et al. (1984). The positions of sequences recognised by the restriction endonucleases EcoRI, MboI and HaeIII are shown around the periphery of the map.

After McKenzie et al. (1986)


EcoR I
Mbo I
BA4
b
Mbo I
BA1
a
pUB110
4.5kb
d
Mbo I
ORI
BA2
Mbo I
BA3
Hae III
c
Neo
Mbo I
Mbo I
Hae III
Hae III
Mbo I
Hae III

sequence and a large open reading frame capable of encoding a 235 amino acid protein (27 kilodaltons). This potential protein had previously been designated RepB by Imanaka et al., (1984). A protein of this size was reported by Shivakumar et al. (1979) as being synthesised in vivo from pUB110 in B subtilis minicells.

McKenzie et al. (1986) also reported an open reading frame in this region capable of encoding a 334 amino acid protein whose sequence would contain a consensus sequence found in several other proteins known to bind to DNA. Two hydrophobic regions flank the consensus sequence, therefore McKenzie et al. (1986) suggested that this putative protein could bind to the cell membrane as well as to pUB110.

In two high copy number mutants of pRBH1 a single base change within their coding sequences was found to result in a single amino acid substitution in each case. So if alteration of a single amino acid in the RepB protein sequence was sufficient to cause an increase in plasmid copy number, then this strongly suggests that the RepB protein is involved in plasmid replication control. The effect of a single base change in the coding region of a replication control protein is reminiscent of similar phenomena reported for R6K (Stalker et al., 1983) and pSC101 (Armstrong et al., 1984).

With the information available at the present time it can be said that pUB110 does not seem to conform to many of the general features of plasmid replication noted to date. According to McKenzie et al. (1986) there is no long inverted repeat near to the pUB110 origin of replication as has been found near the origins of pE194 and pC194

(Horinouchi and Weisblum, 1982a and b). There is thus no obvious region of internal symmetry which might give rise to a secondary structure similar to that reported for RNAI and RNAII at the origin of ColE1 (Tomizawa and Itoh, 1981). No direct repeats of the type found at the origins of plasmids such as F, P1 and pSC101 are in evidence nor is the 9 base pair sequence (TTAT(C/A)CACA) which has been found repeated several times in the vicinity of the replication origins of both E.coli and B.subtilis chromosomes (McKenzie et al 1986).

The other two plasmids, pE194 and pC194 were found by Te Reile et al. (1986a) to be present in single stranded form . Despite the original failure to detect single stranded copies of pUB110 reported by Te Reile et al. (1986a), Ehrlich et al. (personal communication) now believe that pUB110 is also replicated in this way.

Shivakumar and Dubnau (1978) have carried out an extensive survey of the host functions required for pUB110 replication. They studied pUB110 replication in a number of temperature sensitive dna mutants of B.subtilis. The dna genes encode host functions known to be involved in synthesis and repair of DNA. In summary, of the nine genes tested, only three were required - dnaG, dnaH and dnaF. So, in common with the E.coli plasmids and R6K, pUB110 can replicate in a host cell deficient in certain dna functions. The replication of all these plasmids is thus not linked obligatorily to that of the host chromosome. Several other E.coli and S.typhimurium plasmids, on the other hand, cannot replicate when chromosomal replication ceases (for example P1, R1, F, ColV, R28K, Hly and Col1b : Goebel and Schrempf,

1972 ; Spratt, 1972 ; Arai and Clowes, 1975). In addition, Winston and Sueoka (1980) found that the dnaB1 gene product (a host replication initiation factor) was necessary for both membrane binding and replication of pUB110.

pUB110 is also unable to replicate in a polC26 strain which is deficient in DNA polymerase III but it can replicate in a DNA polymerase I-deficient host, a property very different from several multicopy E.coli plasmids, for example ColE1 and CloDF13, which require DNA polymerase I but not DNA polymerase III. These plasmids can also replicate in the presence of chloramphenicol whereas pUB110 cannot (Shivakumar and Dubnau, 1978).

Another S.aureus plasmid whose replication control system has been well studied is the 4.4 kilobase, tetracycline resistance plasmid pT181. The regulatory system of pT181 is functionally similar to that of ColE1 and the IncFII plasmids of E.coli in that control of replication is brought about by two RNA molecules, RNAI and RNAII. In this case their separate roles remain unclear. RNAI and RNAII are co-transcribed from a region which specifies the 5' end of a 35 kilodalton protein, RepC, which is absolutely required for replication and acts in trans. The replication origin is, in fact, contained within the repC coding sequence. So regulation of pT181 replication is probably brought about by interaction of RNAI and RNAII with the repC mRNA leader in such a way as to interfere with its translation (Novick et al., 1984a).

In summary, not very much can be said on the subject of

how the maintenance of plasmids in Gram-positive bacteria is controlled in comparison with what is known about plasmids in Gram-negative bacteria. Work on plasmids of Gram-positive bacteria has some way to go before their maintenance functions can be compared directly with those of plasmids of Gram-negative bacteria. There are indications that their replication control may be similar in that RNA species are involved, particularly in the case of pT181. Differences are certainly apparent between these and certain E.coli plasmids, for example in the host requirements of pUB110 replication and the production of a single stranded intermediate in the replication of pE194, pC194 and pUB110. A partitioning system has not yet been reported on a plasmid in a Gram-positive bacterium but in this respect it would be more fruitful to investigate large conjugative plasmids which usually exist at low copy numbers and which would have more need for such a system. Plasmids of this kind exist in B.thuringiensis but are uncharacterised with respect to their replication (see, for example, Gonzales and Carlton, 1984).

3.1 Instability of plasmids in Bacillus subtilis

A number of plasmids have been reported to be either segregationally or structurally unstable in B.subtilis and, although very little attention has been directed towards the causes of segregational instability in B.subtilis (see, for example, Bron and Luxen, 1985) certain plasmids which exhibit a high level of structural instability in B.subtilis have been extensively studied.

Deletions occur in both E.coli and B.subtilis but are far more pronounced in B.subtilis. Very often plasmids containing a particular insert will be stably maintained in an E.coli host, but on extraction and re-transformation into a B.subtilis strain will undergo extensive deletion (for example, Grandi et al., 1981).

Alonso and Trautner (1985b) noted a profound species difference in the recognition of a sequence of the mutant plasmid pC194-1 which appeared to act as a deletion generator and which was proposed to have a stem-loop type of secondary structure. Deletions occurred in this plasmid a thousand times more often in B.subtilis than in E.coli and Alonso and Trautner (1985c) suggested that this phenomenon indicated the presence of a very potent recombination system in B.subtilis. more efficient at recognising stem-loop structures as deletion hot spots than the corresponding system in E.coli. Structural instability therefore constitutes a considerable barrier to cloning in B.subtilis.

The features of deletions in the hybrid plasmid pHV14 in particular have been extensively characterised. pHV14 was constructed by Ehrlich (1978) and is composed of the E.coli plasmid pBR322 and the S.aureus plasmid pC194 joined at their HindIII sites. This plasmid is highly structurally unstable in both E.coli (Primrose and Ehrlich, 1981 ; Jones et al., 1982) and in B.subtilis (Alonso and Trautner, 1985a). Details of this particular plasmid are reviewed in detail in a later chapter (Chapter 3).

Deletions have often been reported in hybrid plasmids composed in part of the genomes of plasmids pC194, pUB110 and to a lesser extent pBC16 (For example Alonso and

Trautner, 1985a, b and c ; Hahn and Dubnau, 1985 ; Kreft et al., 1982).

In the cases which have been described deletions have been found to occur between regions of homology, as is also the case in E.coli (Albertini et al., 1982 ; Jones et al., 1982). The results of Alonso and Trautner (1985b) and Hahn and Dubnau (1985) indicate that base pair homology is not sufficient to cause deletion. They found that only particular orientations of subsequently deleted sequences were effective in inducing deletion formation. Because the homologous seuences lay in the vicinity of the origins of replication of pUB110 and pC194 Hahn and Dubnau (1985) suggested that replication from these origins could have been involved in the recombination event.

In this context, pE194, pC194 (Te Riele et al., 1986a) and probably pUB110 (S.D.Ehrlich, personal communication) have been shown to produce single stranded copies during their replication. Single stranded DNA has been implicated in initiation of recombination (Hotchkiss 1974 ; Meselson and Radding 1975) and so in the cases reported in which plasmids carrying functional pE194, pC194 or pUB110 origins of replication are found to be structurally unstable it may be that the production of single stranded DNA is involved in stimulating recombination leading to deletion (S.D. Ehlich, personal communication).

There are indications that the DNA uptake system of B.subtilis is involved in deleting regions of plasmids used to transform competent cells (Michel et al., 1982). On contact with competent cells, plasmid molecules are probably linearised in order to be transported into the cell when

they must be re-circularised in order to become viable. Recircularisation requires homology and since plasmids bearing homology to the chromosome are active in transformation of B.subtilis (Contente and Dubnau, 1979 ; Canosi et al., 1981) this requirement can be satisfied by intermolecular recombination. Plasmid monomers are not active in transforming B.subtilis , oligomers are required (Canosi et al., 1978) but monomers can be rendered active by the construction of internal repeats (Michel et al., 1982). So recircularisation can probably also occur by intraplasmidic recombination. In this situation sub-parental plasmids have been found to be generated, presumably by recombination between the repeated regions (Michel et al., 1982).

4. CHEMOSTAT CULTURE

4.1 Description of the apparatus

The development of the technique of continuous culture was based on the realisation that the nutrient requirements of most bacterial strains are very simple. Their minimal requirement is for essential inorganic elements (for example, phosphorous, sodium, potassium, sulphur, iron and magnesium), an energy source in the form of an oxidisable substrate (glucose or other sugar) and a reducible substrate to "mop up" the electrons that have provided the energy. In circumstances where a bacterial strain is deficient in the synthesis of, for example, a particular amino acid, then that amino acid constitutes part of the minimal growth requirements of the strain.

Thus when all the required nutrients are available except one, growth will be suspended until the missing nutrient is supplied. When it is once more available growth will proceed at a rate dependent on the concentration of the so called limiting nutrient until either the supply is exhausted or the concentration of another nutrient becomes limiting. Clearly, if one nutrient can be accurately supplied at a limiting concentration while all others are present in excess a means is provided whereby a bacterial culture can be maintained at a constant and controllable growth rate. This is the basis of the technique of continuous culture and the underlying principle behind the operation of the chemostat.

The first chemostat was no more than a simple culture

vessel with a means of controlling a constant addition of growth medium and an overflow for waste products, excess medium and bacteria (Monod, 1950). A functionally similar apparatus, the Bactogene, was developed at the same time (Novick and Szilard, 1950). Further development of the apparatus over the years has produced the sophisticated chemostats available today which have accurate systems for monitoring and maintaining the pH, temperature and the concentration of dissolved gases along with a means of preventing foaming of the culture by the controlled addition of antifoaming agents.

4.2 Theory of bacterial growth in continuous culture

An organism growing in the culture vessel of a chemostat will be subject to being washed out of the vessel by the continuous input of fresh growth medium. The rate at which this occurs is known as the dilution rate and is expressed as a fraction of the fermenter vessel volume per hour (with the units h^{-1}).

At any given dilution rate, the growth and maximum density achieved by the organisms in the culture vessel will not exceed a maximum value determined by the concentration of the limiting nutrient. Since there is a continuous replacement of the chemostat vessel contents with fresh medium, growth of the organisms cannot cease as they would be washed out. The result of this situation would be a steady state in which the growth rate, designated μ exactly matches the rate at which the vessel contents are replaced, D, the dilution rate. In other words, under steady state

conditions, $\mu = D$, and the culture maintains a constant density.

In practice, however, the situation is complicated by the fact that, in the first place, due to incomplete mixing of incoming nutrients with the culture contents, not all cells would be exposed to identical conditions and, secondly, mutational changes in the culture would lead to selection of bacteria with different growth properties.

Despite these unavoidable complications it has still proved possible to describe accurately the kinetics of steady state bacterial growth using mathematical formulae.

In 1942, Monod expressed the dependence of growth rate, on limiting nutrient concentration in mathematical terms and, furthermore, showed that the relationship held true for a variety of real cultures.

Monod's equation resembles in form the Michaelis-Menten equation for enzyme rates:

$$\mu = \mu max \left(\frac{S}{K_s + S} \right)$$

Where μ = specific growth rate of a nutrient limited culture

S = concentration of the limiting substrate

K_s = the saturation constant equal to the concentration of the limiting substrate that permits growth at half its maximal rate, that is when $\mu = 1/2\ \mu$ max.

The resemblance between Monod's equation and first order enzyme kinetics is not entirely coincidental since nutrient

uptake by bacterial cells involves enzyme catalysed reactions.

Herbert *et al.* in 1956 and Kubitshek in 1970 have expanded on this equation. They describe in mathematical terms how the dilution rate determines the concentration of limiting substrate in the growth medium and how this in turn controls the growth rate of the organisms, verifying the conclusions reached by Monod.

The generation time or doubling time of a chemostat culture can be determined directly from the dilution rate. Consider a batch culture of bacteria. Throughout the growth cycle of the organism in batch culture the properties of a single cell can be expected to change considerably as it goes through lag phase, exponential phase and then stationary phase. During exponential phase, however, the properties of the average cell would be expected to remain relatively constant as the density of the culture doubles at a relatively constant rate.

For an exponential culture in mathematical terms, it can be shown that

$$1/x \cdot dx/dt = d(\ln 2.x)/dt = \mu = \ln 2/t_d$$

where x = the concentration of organisms

t = time

μ = the specific growth rate or rate of increase per unit of organism with time

t_d = the doubling time

At steady state, therefore, when, as already shown, $D = \mu$, the doubling time, t_d, is equal to $\ln 2/D$ or $0.693/D$.

So at steady state the culture maintains a constant doubling time, dependent on the dilution rate, and thus a constant cell density. Determination of the number of organisms in the culture, measured either as the optical density or the viable count, can therefore be used as an indicator of steady state since, by definition, if the cell density is constant a steady state exists in the culture. This is an important point to bear in mind since, in the work described in this thesis, culture density (as measured by the viable count) was taken as the primary indicator of steady state.

4.3 Competition and selection in the chemostat

When either two different bacterial species or two strains of the same species co-exist in the same chemostat the result is often that one species or strain comes to predominate after a number of generations. This feature of mixed cultures is due to competition for the limiting nutrient. Any cell which has an increased growth rate over another cell (higher μ), an improved ability to take up the limiting nutrient (lower K_s) or the best combination of both will be at a competitive advantage. That is to say that it will be fitter in terms of survival in the chemostat.

Mathematical analyses of the parameters of such competition have been published by Powell (1958), Taylor and Williams (1975), Freidrickson (1977) and Dykhuizen and Hartl (1983). The accuracy of these predictions does, however, depend on, in the first place, there being no interaction between competing populations that would affect free competition and secondly, on the growth environment remaining constant throughout.

The chemostat provides the ideal means for maintaining a constant growth environment making it an extremely useful apparatus in which to carry out competition experiments. Furthermore, because cultures can be maintained in the chemostat over a large number of generations, even a slight difference in the fitness of two simultaneously cultured strains will eventually be detected in the population.

Competition in the chemostat has been studied either by deliberately introducing two strains simultaneously into the chemostat or by observing the effect on the population of

mutants which arise during the course of the culture of an initially pure strain.

The former approach can be particularly useful for studying selection in the chemostat. For example, Zamenhof and Eichorn (1967) demonstrated that auxotrophic mutants, in this case his and trp B.subtilis strains, had a marked growth rate advantage over isogenic protoprophic strains probably as a result of their having to carry out fewer biosynthetic steps.

Conversely, when a mutant which over-produces a particular metabolite (especially one not required for growth) is grown simultaneously with the wild-type strain in the chemostat, it has been shown to be at an energetic disadvantage (Baich and Johnson, 1968)

Mutants can arise during chemostat culture which are either selected for or against but neutral mutations may occur which have no effect on the fitness of a strain.

The normal rate of mutation in any one gene is about 1 in 10^7 to 1 in 10^8 per generation. The appearance of neutral mutations, however, has been shown to increase linearily with time for periods of 50 to 100 generations since not only does each mutant continue to grow and divide in the chemostat population but the same mutation could arise elsewhere in the culture (Novick and Szilard, 1950 ; Cox and Gibson, 1974). Eventually a mutation will arise which confers a selective advantage on the cell carrying it. Such a cell will grow to occupy a large proportion of the culture at the expense of neutral or disadvantaged mutations. Neutral mutations will then accumulate in this population.

This cycle of events will repeat itself many times, particularly during long term chemostat culture, with a frequency of recurrence depending on how often fitter variants arise in the population. The result is an erratic and unpredictable fluctuation in the proportions of dominant, neutral and disadvantaged mutants within a chemostat culture and this phenomenon has been given the name "periodic selection" by Atwood et al. (1951).

The phenomenon of periodic selection is a difficult one to study. Mutations which affect the fitness of a strain could arise in a great number of genes involved in intermediary metabolism and, as already stated, the process is entirely unpredictable.

Nevertheless, there are some cases where a spontaneously arising, fitter mutation has been identified. For example, mutants which constitutively produce beta-galactosidase regularly arise in lactose limited chemostat cultures of E.coli (Novick and Horiuchi, 1961). The genetic basis for the periodic selection of certain E.coli strains known as "mutator" strains in the chemostat is well characterised (Gibson et al., 1970 ; Cox and Gibson, 1974). It would seem that such strains are fitter because they have a greater chance of undergoing a favourable mutation (Nestman and Hill, 1973 ; Chao and Cox, 1983). At least four mutator genes have been characterised in E.coli, all of which confer a selective advantage over an otherwise isogenic counterpart in glucose limited chemostat culture.

The technique of chemostat culture has been widely exploited in the study of plasmid stability since it offers the opportunity of monitoring the maintenance of plasmids

over a large number of host cell generations in a constant growth environment.

The opportunity of varying the growth rate and the nature of the limiting nutrient during chemostat culture allows investigation of the parameters involved in selection of plasmid free cells.

In the work that follows the technique of continuous culture has been used to investigate the stability of three plasmids in chemostat cultures of Bacillus subtilis and the effect of competition from plasmid-free cells and changes in the nutrient limitation on their maintenance. The work therefore illustrates many of the uses of continuous culture described in the previous section of the introduction.

The practical difficulties inherent in the technique are also highlighted (for instance see Chapters 4 and 6) and, in this context, the importance of the maintenance of a steady state and the process of achieving a steady state at the beginning of the chemostat experiment are paid particular attention to in the discussion sections of the experimental chapters (again, see Chapters 4 and 6).

CHAPTER 2

MATERIALS AND METHODS

2.1. Bacterial strains, plasmids and phage.

The bacteria and plasmids used in this work are listed in Table 2.1.

B.subtilis strains 3G18 and 168 Ø105 were obtained from Dr.J-I Flock (Searle Research and Development). B.subtilis 168 and B.subtilis 168 trp came from the National Collection of Industrial Bacteria and E.coli HB101 was one of the laboratory frozen stock strains.

Plasmids pUB110, pBC16 and pHV14-F were kindly donated by Dr.J-I Flock and pC194 by David Barstow, C.A.M.R., Porton Down. pBR322 was extracted from a laboratory strain of E.coli HB101 carrying this plasmid.

Phage Ø105 DII:6C was also supplied by Dr.J-I Flock. This phage is deficient in repressor production (Flock, 1977).

2.2 Storage and batch culture of bacterial strains

General purpose culture of bacteria on solid medium for storage or for selection of transformants was carried out using nutrient agar (Oxoid) in the case of B.subtilis or L-agar in the case of E.coli.

Filter-sterilised antibiotics were added where appropriate to the following final concentrations:

Table 2.1: Bacterial Strains and Plasmids

Bacterial Strains and Plasmids	Phenotype conferred	Source or Reference
Plasmids		
pC194	Cam^R	*S. aureus* Horinouchi and Weisblum (1982b)
pUB110	Kan^R	*S. aureus* Winston and Sueoka (1980)
pBC16	Tet^R	*B. cereus* Bernhard *et al.* (1978)
Bacterial Strains	**Genetic markers**	
B. subtilis 3G18	auxotrophic for ade, met and trp	Uhlen *et al.* (1981)
B. subtilis 168 (marburg)	trp	National Collection of Industrial Bacteria
E. coli HB101	pro leu bio recA hsdM hsdR rpsL	Maniatis *et al.* (1982)

B.subtilis:	chloramphenicol	5μg/ml (for strain 3G18 carrying pC194)
		40μ/ml (for strain 168 *trp* carrying pC194)
	kanamycin	5μg/ml
	tetracycline	10μg/ml
E.coli:	chloramphenicol	100μg/ml
	ampicillin	40μg/ml
	tetracycline	20μg/ml

Antibiotics were obtained from Sigma (tetracycline, kanamycin and ampicillin) and from Boehringer Mannheim (chloramphenicol).

Batch culture of *B.subtilis* was carried out either in Penassay Broth (Difco) or Spizizen minimal salts (Anagnostopoulos and Spizizen, 1961) with the addition of tryptophan to a final concentration of 50μg/ml to allow for growth of *B.subtilis* 168 *trp*. *E.coli* HB101 was grown in Luria broth (L. broth). All cultures were grown at 37°C. Strains were stored in the short term on plates at 4-5°C or in the longer term in 50% glycerol (v/v) and rich liquid medium at -20°C.

All media were routinely sterilised by autoclaving at 15psi (121°C) for 15 to 20 minutes.

2.3 The operation of the chemostat

2.3.1 Apparatus

The chemostat used for the majority of the magnesium and glucose-limited runs was a New Brunswick Scientific

BioFlo Model C30 with a working volume of 235ml under the conditions of aeration and agitation employed.

The phosphate-limited runs were carried out in an LH 500, Series 3 with a working culture volume of 450ml.

Growth medium was supplied by means of a peristaltic pump from a reservoir of 12 or 20 litres. The culture vessels were aerated with 0.7 litres of air per minute and the relative concentration of dissolved oxygen monitored by means of an oxygen electrode immersed in the culture. Temperature was maintained via an immersion heater with thermostatic control.

Cell size (volume) in chemostat samples was determined using the Coulter Counter. Samples were diluted in Isoton (Coulter Electronics) and counted using a 30μm aperture.

2.3.2 Media

Three defined minimal media were used for the chemostat culture each providing a different nutrient limitation. All three media were based on the recipe for Spizizen minimal salts, that is;

ammonium sulphate	2.0 gl^{-1}
magnesium sulphate	0.2 gl^{-1}
sodium citrate	1.0 gl^{-1}
dipotassium hydrogen orthphosphate	14.0 gl^{-1}
potassium dihydrogen orthophosphate	6.0 gl^{-1}

For glucose limitation the concentration of glucose was 0.5 gl^{-1}

For magnesium limitation the 0.2 gl^{-1} of $MgSO_4.7H_2O$ was reduced to 0.016 gl^{-1} and the amount of glucose increased to 2 gl^{-1}.

A phosphate-limited medium was obtained when the amounts of K_2HPO_4 and KH_2PO_4 were reduced to 0.0348 gl^{-1} and 0.0136 gl^{-1} respectively and 1.18 gl^{-1} of potassium chloride was added to provide potassium ions.

Phosphates were autoclaved seperately from the basal salts and added after cooling. Glucose and tryptophan were filter sterilised together and added to the phosphates and salts after autoclaving and cooling. The final concentration of tryptophan was 50μgml^{-1}.

2.3.3 Growth conditions

The pH of the culture was stable at 6.8 without addition of alkali under conditions of glucose or magnesium limitation. When the phosphate concentration was limiting, however, it was necessary to maintain the pH at 6.8 by the automatically controlled addition of 1M NaOH. The temperature in the culture vessel was held at 37°C.

Aeration and agitation were provided by an air pump which delivered a steady stream of sterile, humidified air at a rate of 0.7 $lmin^{-1}$ and a bladed magnetic stirrer seated in the culture vessel. An arrangement of baffles maximised the effects of the agitation.

The flow rate of growth medium was set by means of a graduated pipette set into the medium delivery line before the pump. In this way the dilution rate was set to the desired values as specified in the relevant results section.

2.3.4 Inoculation procedure

A single colony of the strain to be used to inoculate the chemostat was first grown overnight in Penassay broth at 37°C. A small sample was then transferred to 25ml of Spizizen salts and grown to early stationary phase (determined by reference to the growth curve of B.subtilis in Spizizen salts) prior to inoculation of the chemostat. 5ml of this culture was then used to inoculate the chemostat. Batching up of the culture was allowed to take place for approximately 16 hours overnight. The inoculum was sampled at the time of inoculation and the chemostat culture sampled after batching up, immediately before dilution of the culture was started. All samples were looked at under the phase contrast microscope, primarily to check for the presence of contaminants.

2.3.5 Sampling procedure

Approximatly 2ml samples were drawn into sterile McCartney bottles via a sampling port by displacement of air using a 50ml syringe.

Samples were either diluted immediately into cold (4°C) broth (100μl into 900μl in 2ml bijou bottles), serial dilutions made and 100μl from each dilution plated on nutrient agar and on selective agar, or mixed with an equal volume of glycerol and stored frozen at -20 C for future use.

Determination of the relative numbers of antibiotic resistant and antibiotic sensitive cells in the samples was carried out, in the first place, by comparing series of serial dilutions, one series on nutrient agar, the other on antibiotic-containing nutrient agar, and in the second place by transferring 100 colonies from the nutrient agar serial

dilution plates, after overnight incubation, onto 50-square grid plates containing antibiotic (and also onto a nutrient agar reference plate).

2.3.6 Determination of limiting state

The increase in OD_{500} of a culture to which the suspected limiting nutrient was added in excess and a decrease in OD_{500} when the concentration of this nutrient is returned to the original level was taken as qualitative proof of the existence of a limited state.

The growth medium reservoir was replaced during a chemostat run with one containing the same medium but with an excess of the presumptive limiting nutrient. After allowing about 12 hours for the culture to adjust to the new conditions a sample was taken, the OD_{500} measured and the medium reservoir replaced with the original. After another 12 hours a second OD_{500} was measured to confirm that the density of bacteria had decreased.

2.4 DNA manipulations

2.4.1 Preparation of plasmid DNA

When a large amount of DNA was required, plasmid DNA was extracted from E.coli by the cleared lysate procedure of Clewell and Helinski (1969) and from B.subtilis by a scaled-up* version of the alkaline denaturation method of Birnboim and Doly (1979). In both cases the DNA was purified on caesium chloride-ethidium bromide density gradients.

* scaled up in exact proportion with the original method.

For small scale plasmid preparation the alkaline denaturation method was used for both E.coli and B.subtilis alike.

Preparation of pBR322 from E.coli was preceeded by amplification of the plasmid by the addition of chloramphenicol to the culture to a final concentration of $150 \mu g ml^{-1}$.

2.4.2 Physical analysis of plasmid DNA

Plasmid DNA was analysed by electrophoresis through agarose gels. Minigels for rapid screening of DNA samples were prepared from 0.7% (w/v) agarose dissolved in Tris-borate buffer and run at 100mA for 35 minutes in the same buffer. Larger, horizontal gels for determination of molecular weights were prepared and run at 15 mA overnight in Tris-acetate buffer. The agarose concentration of these gels was 0.7% (w/v) unless otherwise stated.

Gels were stained with ethidium bromide (Sigma) ($5 \mu g ml^{-1}$) and photographed under UV-illumination with a Polaroid Land camera fitted with an orange filter.

Analysis of plasmids by restriction endonuclease digestion was carried out using buffers and conditions recommended by the manufacturers (Amersham or BDH). Generally $1 \mu g$ of DNA was digested in a $50 \mu l$ digestion mix for one to two hours at 37°C. The reaction was halted by precipitation of the DNA. This was achieved by the addition of 4M sodium chloride to a final concentration of 0.4M, glycogen to a final concentration of $1 mg ml^{-1}$ followed by 2.5 volumes of cold ethanol. The mixture was held at -20°C for

at least 30 minutes and the DNA recovered by centrifugation at 1200 rpm in an Eppendorf microcentrifuge for 10 minutes. The resulting pellet was washed in 70% ethanol, dried in a vacuum dessicator and redissolved in 10mM Tris pH 8.0, 1mM EDTA (T.E. buffer).

DNA was also precipitated prior to digestion and in order to concentrate it.

DNA molecules were ligated when required by the use of T4 DNA Ligase (Amersham) using a buffer recommended by the manufacturer. Ligation was carried out at 5°C for 16 hours.

When it was necessary to extract a particular DNA species from an agarose gel the DNA was reversibly bound to DE81 paper (Whatman) by the method of Dretzen et al. (1981).

A strip of DE81 paper was inserted in a slot in the gel in front of the required band so that as the gel continued running, the DNA, on coming into contact with the paper, was tightly bound. The DNA was extracted from the paper in a high salt elution buffer, the ethidium bromide removed by extraction with water saturated butan-1-ol and the DNA precipitated by the addition of ethanol.

2.4.3 Preparation of E.coli competent cells and transformation

After induction of competence by successive treatments with 10mM $MgCl_2$ and 100mM $CaCl_2$, 200µl of the cells were incubated with the DNA (usually 10µl) for 30 minutes. The mixture was heat shocked at 42°C for two minutes then incubated at 37°C for 90 minutes after the addition of 1ml of L-broth. Transformants were selected for by spreading

aliquots of the transformation mixture onto nutrient agar plates containing the relevant antibiotics followed by overnight incubation at 37°C

2.4.4 Preparation of Bacillus subtilis competent cells and transformation

B.subtilis competent cells were prepared by the method of Bott and Wilson (1968) using Spizizen minimal medium with the addition of 50μgml^{-1} histidine and 50μgml^{-1} tryptophan and extra magnesium (to a final concentration of 0.072% (w/v) anhydrous $MgSO_4$). This medium was inoculated to a starting OD_{500} of 0.1. Competence was attained three hours after the culture left exponential phase.

Transformation of B.subtilis was carried out in sterile 2 ml Bijou bottles. The DNA sample in 100μl final volume was added to 900μl of competent cells. The mixture was incubated at 37°C in a shaking water bath for 45 minutes. After this time 1ml of double concentration Penassay broth (Difco) was added and a further 60 to 90 minutes of incubation allowed. Transformants were again selected for by spreading samples on antibiotic plates.

2.5. Phage preparations:

2.5.1 from broth culture of a lysogen

B.subtilis 168 0105 was grown in nutrient broth (8 gl^{-1}) supplemented with yeast extract (5 gl^{-1}) to an optical density at 500nm of 0.2-0.3. Freshly dissolved mitomycin C

(Sigma) was added to a final concentration of 0.4μgml^{-1}. Three to four hours later, when the cells had lysed, solid NaCl was added to a final concentration of 1M and the cell debris removed by centrifugation. The supernatant containing the phage was stored at 4°C over chloroform.

2.5.2 from a plate lysate

0.3ml of a 5 hour broth culture of strain 3G18 was mixed with 2ml of top agar and 5ml of 0105 DII:6C (containing approximately $3.5x10^7$ pfu) and the mixture poured onto a nutrient agar plate. After overnight incubation at 37°C, 4ml of phage buffer (NaCl 5.3 gl^{-1}, $MgSO_4.7H_2O$ 2.0 gl^{-1}, Tris 1M pH 7.5, Gelatin 2% (w/v)) were placed over the clear lawn and this was removed after some hours at 4°C into a sterile bottle over chloroform.

2.6. Tests for immunity to phage

Colonies of bacteria under test were either, i) streaked horizontally against vertical streaks of phage on chloramphenicol-containing plates and regions of lysis looked for where the two streaks met or, ii) colonies were transferred onto a 50-square grid on chloramphenicol-containing plates with phage in top agar. In the latter case colonies were also transferred onto chloramphenicol plates without phage as a reference.

RESULTS AND DISCUSSION CHAPTERS

CHAPTER 3
CONTINUOUS CULTURE OF BACILLUS SUBTILIS HOST STRAINS

Introduction

Most of the research on how B.subtilis behaves during chemostat culture has centred around the effects of different nutrient limitations on such features as its cell wall composition, the relative proportions of its cellular components and its morphology. A number of studies have also been carried out on sporulation during continuous culture.

Studies on the effects of phosphate limitation on the wall content of B.subtilis grown in the chemostat have revealed considerable alterations in the major components (for example, Ellwood and Tempest, 1972).

B.subtilis cell walls are composed basically of teichoic acids and peptidoglycan (Archibald and Baddiley, 1966). Teichoic acids are anionic phosphorylated polymers and occur in probably all Gram-positive bacteria, not only in the cell wall but also in the membrane of the cell. Several different types of teichoic acid have been identified, those associated with the cell wall showing a greater diversity than those associated with the membrane (Archibald and Baddiley, 1966).

Under phosphate limitation the cell wall composition is considerably altered, teichoic acid being replaced by the non-phosphorylated anionic polymer, teichuronic acid (Ellwood and Tempest 1972). Rosenberger (1976) found that in B.subtilis (Marburg) 168 two teichoic acids which together

accounted for more than half the dry weight of phosphate-grown cells were replaced under phosphate limitation by a teichuronic acid previously not formed.

Only the teichoic acid content of the cell wall has been shown to change under phosphate limitation. The peptidoglycan content of the cell wall remains constant under various conditions (Schleifer et al., 1976 ; Kruyssen et al., 1981). The teichoic acid content of the cell membrane has not been reported to be altered under different growth conditions.

Different carbon limitations (glucose, glycerol, malate and galactose) were shown to have no effect on the teichoic acid content of B.subtilis var. niger cell walls regardless of the growth rate and provided that phosphate was present in the medium (Kruyssen et al.. 1980). A correlation was noted, however, between growth rate and cell wall composition. As the dilution rate of the culture was raised from 0.35 h^{-1} to 0.7 h^{-1}, with glucose as carbon source and under limiting phosphate, the level of teichuronic acid fell and the level of teichoic acid rose.

Tempest et al.(1967) have also looked at the effects of magnesium limitation on B.subtilis var. niger and found that under magnesium limitation the magnesium content of this organism varied with the growth rate, increasing as the growth rate (dilution rate) increased. At dilution rates below 0.5 h^{-1} over 90% of magnesium added to the culture was present in the organisms. Cellular magnesium was tightly bound and when magnesium limited B.subtilis cells were suspended in medium containing magnesium the ion was rapidly

adsorbed.

On analysing the cellular composition of B.subtilis var. niger at different growth rates Tempest et al (1967) also demonstrated that cellular RNA and carbohydrate content increased with increasing growth rate but DNA and protein contents either decreased or varied irregularly. The molar ratio of RNA to magnesium was relatively constant.

Magnesium is an essential component of micro-organisms, being an integral part of ribosome structure (Tissieres and Watson, 1958) and an essential requirement for various stages of translation and transcription. In fact, in most enzymatic reactions in which ATP participates as phosphate donor its active form is a $MgATP^{2-}$ complex (Lehninger, 1982).

B.subtilis has an active uptake system for magnesium, first described by Scribner et al. in 1974, which is energy dependent and highly specific for magnesium.

A number of studies have been carried out on sporulation of B.subtilis in continuous culture. Summarising the important results: Dawes and Mandelstam (1970) showed that the frequency of sporulation was higher under glucose or nitrogen limitation than either magnesium or phosphate limitation. Sporulation frequency also increased under glucose or nitrogen limitation with decreasing growth rate and below pH 6.0 (it remained constant at a given growth rate of 0.12 h^{-1} between pH 6.0 and pH 6.8).

Adherence of bacteria to the chemostat vessel walls, or "wall-growth" as it is commonly known, has been shown to be the cause of many departures from theoretical predictions of bacterial behaviour during chemostat culture (for example Herbert et al., 1956). Wall growth of bacteria represents a

reservoir of cells subject to quite different environmental effects than organisms in free culture.

Adherence of organisms to chemostat walls can be the result of inherent or inherited (as a result of mutation) ability to stick to glass and is more prevalent in some cultures than others. Larsen and Dimmick (1964) have shown that B.subtilis demonstrates very little propensity for adhering to chemostat vessel walls, as, in fact, do most Bacillus species.

Results and Discussion

B.subtilis 3G18 was chosen as the original host for this work because it had previously been shown to yield more transformants than other commonly used B.subtilis strains on transformation of competent cells with plasmid DNA (J-I Flock, personal communication). It also has more than one selectable genetic marker facilitating detection of contaminants and verification of the nature of cultures. The strain is auxotrophic for adenine, methionine and tryptophan and at least partially recombination deficient. This uncertainty as to its genotype is obviously unsatisfactory if the organism is to be used in any genetic studies and for this reason the host strain was later changed to B.subtilis 168 trp. Nevertheless the initial experiments carried out using 3G18 illustrate some important features of B.subtilis grown in chemostat culture and are included here for this reason.

The genotype of B.subtilis 3G18 was confirmed by

plating on Spizizen minimal medium plates of the same composition as liquid minimal salts medium containing one, two or all three of the required factors adenine, methionine and tryptophan. The organism was unable to grow unless all three factors were supplied.

In preparation for chemostat inoculation a single colony was picked from an agar plate and transferred to an overnight 25ml batch culture of Penassay broth. Thereafter the culture was treated in the manner described in the Materials and Methods, section 2.3.4 prior to inoculation of the chemostat. The culture was sampled after batching up and after a suitable equilibration time (about 24 hours) once dilution of the culture was begun. Samples of 2ml or less were removed at approximately 12 generation intervals for plating, microscopic examination and measurements of culture density and cell size distribution using the Coulter Counter.

It is now a well established fact that bacteria growing at high growth rates with short generation times have an increased size over their slower growing counterparts (Kubitshek, 1970). During the initial chemostat culture of B.subtilis 3G18 under carbon limitation the dilution rate was changed periodically and, after a reasonable interval (about 24 hours) in which a new steady state was achieved, samples were withdrawn, diluted appropriately with Isoton and the Coulter Counter used to determine the average cell size. A typical profile of cell size showed two peaks. An example is shown in Figure 3.1. The difference in the size of cells maintained at different growth rates is illustrated by the profiles in Figure 3.2.

Figure 3.1: Coulter Counter profile of B.subtilis strain 3G18.

Samples were taken from a carbon-limited chemostat culture and diluted directly into Isoton to bring the cell count onto the scale of the graph. In this case the final cell concentration was 1.8×10^6 ml^{-1} in Isoton. The two peaks illustrate the distribution of single and double cells (joined end to end) in the culture.

Instrument settings were:

Window width	100
Amplification	0.50
Current	0.50mA
Orifice	30um
Count range	4000
Base channel threshold	15

Culture conditions are described in the text.

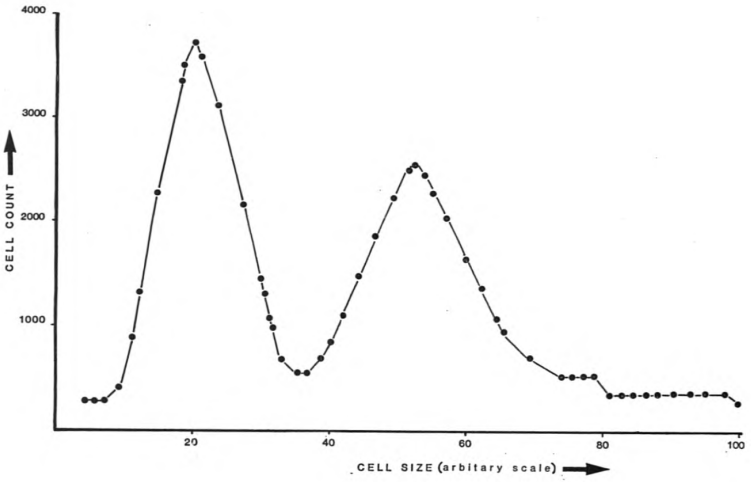
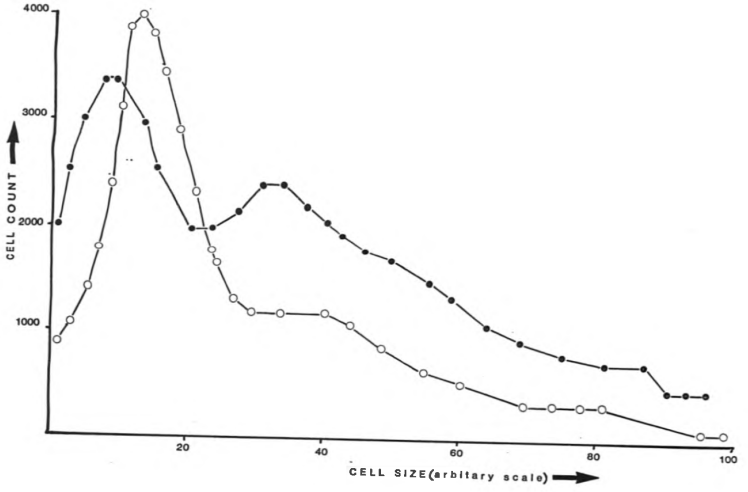

4000
3000
2000
1000
CELL COUNT
20
40
60
80
100
CELL SIZE (arbitary scale)

Figure 3.2: Coulter Counter profile from samples of two different chemostat cultures of B.subtilis grown at different dilution rates.

Continuous culture conditions are described in the text. One culture was maintained at a dilution rate of 0.12 h^{-1} (●) and the other at a dilution rate of 0.30 h^{-1} (O). Samples were removed from the chemostat and diluted directly into Isoton to the cell density required to bring the cell count onto the scale of the graph. In this case the final cell concentrations in Isoton were 2.42 x 10^{5} ml^{-1} (●) and 1.24 x 10^{6} ml^{-1} (O).

Instrument settings were:

Window width	100
Amplification	0.50
Current	0.50mA
Orifice	30μm
Count range	4000
Base channel threshold	15


4000
3000
2000
1000
CELL COUNT
20
40
60
80
100
CELL SIZE(arbitary scale)

As seen under the microscope such a culture contained both single and double cells, that is, two cells of normal size joined end to end with a clearly visible division septum.

Using a facility in the BBC Coulter Counter program the cell volume corresponding to each of the peaks was calculated and it was found that cells represented by the right hand peak were in all cases close to or exactly twice the size of the cells represented by the other peak - see Table 3.1. The peaks in the Coulter Counter profile therefore corresponded to the single and double cells seen under the microscope.

A feature of the Coulter Counter profile shown in Figure 3.1 which is notable for its absence is the shoulder between the peaks which can be seen in the Coulter Counter profiles of E.coli cultures. The absence of a shoulder in profiles of these B.subtilis cultures may simply reflect the existence of a very long cell division time and therefore short cell cycle between each division relative to the E.coli cell cycle, otherwise no explanation is available for this phenomenon.

The presence of single and double cells in B.subtilis cultures has been noted on many previous occasions (for example Dawes and Thornley, 1970 ; Sargent, 1975) and in fact Coulter Counter profiles of cells from carbon-limited cultures at a number of dilution rates consistently showed this feature.

This feature of B.subtilis cultures can therefore be extremely useful in the context of the work described here, in that the Coulter Counter profile of a culture at any given dilution rate is characteristic of that particular dilution rate (see Figure 3.2) and, as such, can be used as a reliable indicator of steady state. In practice, however, the density of the culture in terms of the viable count was used routinely as the primary marker of steady state (see later chapters).

The number of bacteria in the culture, as determined by the Coulter Counter, was found to increase with dilution rate (see Table 3.2).

Under carbon limitation B.subtilis 168 trp behaved very similarly to strain 3G18 but with one difference. Cultures of this organism consistently

Table 3.1: Sizes of B.subtilis 3G18 cells at different growth rates as determined by the Coulter Counter

Dilution Rate	Volume of Small Cells (μm^3)	Volume of Large Cells (μm^3)
0.17	0.62	1.24
0.20	0.68	1.34
0.23	0.78	1.44
0.30	0.92	1.70

The sizes of cells corresponding to the two peaks in Coulter Counter profiles of B subtilis 3G18 from chemostat culture are listed with respect to the dilution rate of the culture. The culture was maintained under carbon limitation. Note that the larger cells are close to, or exactly, twice the size of the smaller cells. Each figure is an average of 3 readings with a mean deviation of ±0.02.

Table 3.2: Cell numbers in chemostat culture of B.subtilis 3G18 as a function of the dilution rate

Dilution Rate h^{-1}	Coulter Count Cells ml^{-1}
0.12	2.42×10^8
0.23	8.8×10^8
0.30	1.24×10^9
0.43	1.78×10^9
0.50	1.13×10^9

The culture was maintained under carbon limitation and the dilution rate altered periodically allowing time between dilution rate increases and sampling for the culture to reach a new steady state. Each figure is an average of 6 readings from the same sample.

grew to higher density in minimal medium than strain 3G18. So at a dilution rate of 0.3 h^{-1} *B.subtilis* 168 *trp* grew to a concentration of 1.60×10^8 colony forming units per ml on average whereas 3G18 attained an average concentration of only 1.24×10^7 colony forming units per ml.

The ability to sporulate is of course a prominent feature of *B.subtilis* strains and it may be the case that the presence of spores in the chemostat culture, particularly during the later stages of batching up may be a significant problem when investigating plasmid maintenance or simply when trying to maintain as stable as possible an environment in the chemostat. However, no spores were ever seen in chemostat samples when looked at under the phase contrast microscope and furthermore, when a single colony of *B.subtilis* 168 was used to inoculate 25 ml of minimal salts and the culture incubated for 4 days at 37°C, although a large number of spores could be seen using the phase contrast microscope, there were also a significant number of single and double cells present. It may be expected that, after this time, the great majority of cells would have sporulated. So it may be that this *B.subtilis* strain is not able to sporulate to any great extent in the minimal medium used here.

During chemostat culture of both *B.subtilis* strains, asporogeneous (*spo*$^-$) mutants arose after between 12 and 20 generations. These mutants could be easily identified as grey, slightly translucent colonies on nutrient agar plates. Despite the fact that after this time the *spo*$^-$ bacteria were predominant in the culture, the *spo*$^+$ bacteria were not eliminated but remained at a very low density in the culture. The appearance of *spo*$^-$ mutants in chemostat culture was also noted by Dawes and Mandelstam (1970) although under their culture conditions *spo*$^-$ mutants took about 10 days to appear. The relative speed at which *spo*$^-$ mutants appeared in the chemostat could be taken as further support for the idea that this

Figure 3.3:

Photograph of B. subtilis 168 trp cells

Cells in a chemostat sample taken shortly after batching up were examined and photographed using a Leitz Dialux 22 phase contrast microscope fitted with a Leitz Vario-Orthomat 2 camera. Note the presence of both single and double cells (joined end to end) in the sample.

Bar = 1μm.

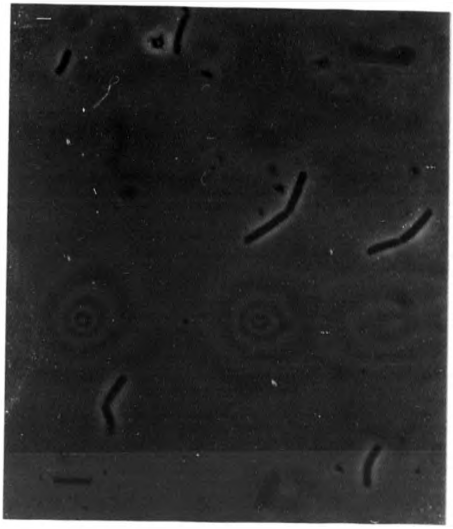


Figure 3.4: Photograph of chains of B.subtilis cells

A sample was removed from a carbon limited chemostat culture after 3 days and examined and photographed using an Olympus PM6 phase contrast microscope with an integral camera.
Bar = 5μm.

B.subtilis strain may not sporulate particularly effectively in the medium used here.

In carbon-limited chemostat cultures after about one and a half days, or about 15 generations, long chains of cells were observed in the culture. B.subtilis strains growing exponentially have frequently been reported to consist of long chains of cells (Sargent, 1975 ; Zaritsky and Macnab, 1981). In addition cell length has been reported to increase with increasing growth rate but cell width remained constant (Sargent, 1975) - see Figures 3.3 and 3.4.

During chemostat culture of B.subtilis 168 trp cell length was observed to increase considerably for the first 10 to 20 generations until long chains of cells were formed. After another 20 to 30 generations the chains were replaced by normal length cells. At slower growth rates, particularly on nutrient agar plates, only short, motile cells were observed as also reported by Zaritsky and Macnab (1981).

On varying the dilution rate of a culture of B.subtilis 3G18 it was found that a constant culture density was maintained and dilution rates up to and including 0.43 h^{-1} whereas, at a higher dilution rate (0.50 h^{-1}) the culture density began to steadily decline. In other words the organisms began to wash out of the culture. A dilution rate of 0.23 h^{-1} was therefore chosen for subsequent experiments using strain 3G18 as this was well within the range of growth rates which the organism could maintain.

CHAPTER 4

CONTINUOUS CULTURE OF B.SUBTILIS 3G18 CARRYING pHV14-F

Introduction

The plasmid pHV14

The plasmid pHV14 was constructed by Ehrlich (1978) from the E.coli plasmid pBR322 (derived from ColE1) and the S.aureus plasmid pC194, each in their entirety, joined at their unique HindIII sites - see Figure 4.1. The resulting plasmid was 7.27 kilobases with unique EcoR1. Pst1 and BamH1 sites and 2 HindIII sites (see Figure 4.1). pHV14 will replicate in both E.coli and B.subtilis. It confers ampicillin and chloramphenicol resistance on E.coli but only chloramphenicol resistance on the B.subtilis host (Ehrlich 1978). Ehrlich (1978) also reported that insertion of foreign DNA into either the EcoR1. BamH1 or Pst1 site (all in the pBR322 DNA) did not interfere with the replication of the parental plasmids in E.coli. Nevertheless Goze and Ehrlich (1980) reported that pHV14 was segregationally unstable in B.subtilis. 30 to 70% of the cells losing the plasmid within 15 generations of growth in the absence of selection for chloramphenicol resistance.

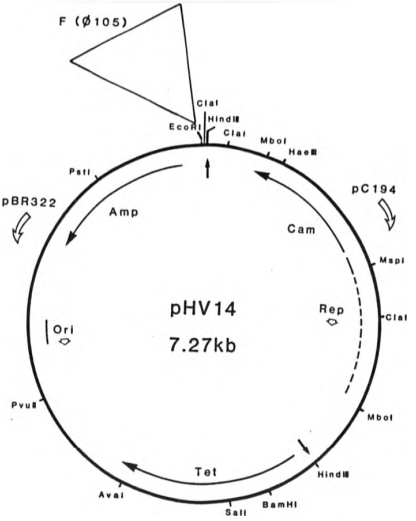
During batch culture in a rich medium the plasmid itself has since been found to be structurally unstable in a Rec-deficient E.coli strain and to undergo one specific deletion (see below) at a frequency of about 1-3 x 10^{-7} (Jones et al., 1982) or 1 x 10^{-6} in a rec^{+} E.coli strain (Primrose and Ehrlich, 1981).

Deletion events were detected by virtue of the fact

Figure 4.1: Map of pHV14

The relative orientations and locations of the origins of replication of both pC194 and pBR322 are shown. The direction of replication in each case is indicated by an open arrow outside the map. Restriction sites for the enzymes HindIII, EcoRI. MboI and ClaI are shown on both pC194 and pBR322 genomes. The enzymes which cut at single sites on pBR322 and the single HaeIII site on pC194 are also indicated (the HaeIII sites on pBR322 are omitted for the sake of clarity). The antibiotic resistance determinants of the two component plasmids are represented by arrows outside the map itself (Sutcliffe, 1978 ; Horinouchi and Weisblum, 1982b). The broken line inside the pC194 portion of the map indicates the approximate location of the origin of replication of pC194. Two small arrows pointing to the HindIII sites show the junctions of the pBR322 and pC194 genomes.

The position of insertion of the cloned EcoR1 F fragment (2.9 kb) of phage Ø105 is shown at the unique EcoR1 site of pHV14 (Uhlen et al., 1981).


F (Ø105)
ClaI
HindIII
EcoRI
ClaI
MboI
HaeIII
PstI
pBR322
Amp
pC194
Cam
MspI
pHV14
7.27kb
Rep
ClaI
Ori
PvuII
MboI
Tet
HindIII
AvaI
BamHI
SalI

that they allowed the expression of the pBR322 tetracycline resistance gene to be resumed (tetracycline resistance was inactivated by insertion of pC194 into the HindIII site of pBR322). Accurate location of the deleted sequences revealed that sequences normally involved in pBR322 replication and its control had been lost (Jones et al 1982).

Jones et al., (1982) analysed the products of deletion of pHV14 and found short direct repeats of about 7 base pairs at the deletion end points. Two of the end points occurred in the inverted repeat adjacent to the pBR322 beta-lactamase gene (originating from Tn1) and two within the coding sequence of the RNAI copy control element of pBR322 (see the description of ColE1 replication control in Chapter 1, section 2.1). The RNAI coding sequence also contains repeated regions (Tomizawa and Som, 1981) so in view of the fact that recombination has been commonly found to occur between repeated sequences (for example Albertini et al., 1982 ; Edlund and Normark, 1981) it is perhaps not surprising that deletion junctions occur at these sites on pHV14. Deletion end points in pC194 were, in all cases determined, close to the junction with pBR322, that is, close to the HindIII site of pC194. In this region Alonso and Trautner (1985b) reported the presence of a sequence with at least the potential of forming a stem-loop type of secondary structure. With the addition of two A-T base pairs in this sequence a mutant of pC194 (pC194-1) was created whose mutated sequence was implicated in the generation of deletions in the hybrid plasmid pHV15-1 (pC194-1 and pBR322 joined at their HindIII sites with the reverse relative orientation to that in pHV14).

Stem-loop structures have previously been reported to be involved in generation of deletions (Stahl, 1979), but this sequence in the wild type plasmid obviously does not cause any structural instability. Alonso and Trautner (1985b) therefore suggested that the addition of two A-T base pairs stabilised a stem-loop structure in this region.

Alonso and Trautner (1985b) found that when the orientation of pC194-1 with respect to pBR322 was reversed (in pHV14-1) far fewer deleted plasmids were isolated from B.subtilis transformants. However, when pC194-1 was joined to a derivative of pBR322 lacking the tet gene deletions occurred at the same frequency in either relative orientation of the two replicons (Alonso and Trautner 1985b). Alonso and Trautner (1985b) therefore suggested that generation of deletions depended on a particular combination of pBR322 and pC194 as well as on some feature of the deletion generating sequence.

The deletion end points in pBR322 were also not random. Generally deletions fell into unique size classes with termini in pBR322 at regions having some homology with the deletion generator of pC194-1. Alonso and Trautner (1985b) thus suggested that recognition of homology may have been involved in deletion generation.

Despite this detailed characterisation of a deletion process the actual mechanism of deletion formation remains a mystery. The size of the deletions (up to 4 kilobases) would seem to rule out the possibility of their having occurred by slipped mispairing (see Albertini et al., 1982), but the fact that Alonso and Trautner (1985b) found that deletion in hybrid plasmids constructed from pC194-1 and a plasmid other

than pBR322 also had non-random end points implies that at least recognition of homology was involved.

The involvement of a cis-acting process in generation of deletions was suggested by the fact that the presence of pE194 in the same cell as pHV15-1 could abolish appearance of deletions if the host was recombination proficient but not in a recombination deficient host. In a recE4 host deletions were encountered only in pHV15-1 (Alonso and Trautner, 1985b).

The plasmid pHV14-F

Uhlen et al., (1981) reported the sub-cloning of the repressor gene of phage Ø105 into the EcoRI site of pHV14. The phage repressor gene had been identified on a 2.9 kilobase, EcoR1-generated fragment, the F fragment, of the Ø105 genome by Cully and Garo (1980) - see Figure 4.1. The presence of the functional phage repressor allows a positive selection procedure to be applied to the detection of plasmids carrying this particular insert. Bacteria carrying the sub-cloned F fragment in the vector pUB110 were shown by Cully and Garo (1980) to be resistant to infection by the clear plaque mutant phage Ø105DII:6c which produces no repressor (Flock, 1977).

Uhlen et al., (1981) found that after 200 generations, in the absence of selection for plasmid markers, pHV14-F DNA extracted from either rec$^+$ or recE4$^-$ cells was smaller than the original. The vector plasmid itself was unaltered. The same deletion in pHV14-F was observed in a host free of integrated phage. Therefore deletions took place

irrespective of the rec nature of the cells and irrespective of the presence of homology with the chromosome. The same effect was observed with the cloned EcoR1 E fragment of the Ø105 genome. Uhlen et al., (1981) also showed that if selective pressure was applied to the Ø105 insert as well as to the antibiotic resistance markers of the vector plasmid then no deletion of Ø105 DNA was observed.

The plasmid pHV14 carrying the EcoR1 F fragment of Ø105 DNA was therefore chosen for an investigation of plasmid segregational and structural instability in the B.subtilis strain 3G18 and, although the selection pressure for the phage repressor phenotype eventually proved unreliable, information was, nevertheless gained on the segregational stability of the recombinant plasmid as a whole.

Results

To begin with the plasmid DNA was checked by digestion with EcoR1 (see Figure 4.2) and its size confirmed including the size of the insert DNA.

B.subtilis strain 3G18 was then transformed with pHV14-F DNA by the competent cell method. The plasmid content of the transformants obtained was confirmed by agarose gel electrophoresis (not shown).

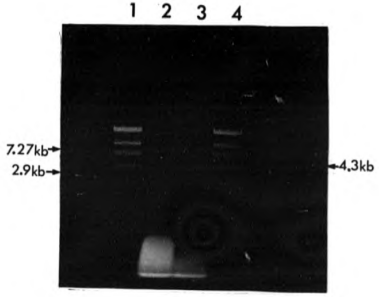
A single colony was chosen for the chemostat inoculum and grown overnight in 25ml of Penassay broth containing chloramphenicol. A small volume of this culture was then used to inoculate 25ml of Spizizen minimal salts, also containing chloramphenicol. After about 7 hours of growth

Figure 4.2: EcoRI digestion of pHV14-F

Plasmid DNA was extracted from B.subtilis 3G18 (pHV14-F) by the small scale, alkali denaturation method of Birnboim and Doly (1979) and digested with EcoRI according to the procedure described in the Materials and Methods, section 2.4.2. Samples were then run on a 0.7% (w/v) agarose gel.

Track: 1 HindIII-digested DNA, molecular size standard
2 EcoRI-digested pHV14-F
3 EcoRI-digested pBR322 from E.coli HB101
4 HindIII-digested DNA, molecular size standard

HindIII fragment sizes are: 23.7 kb
9.46 kb
6.61 kb
4.26 kb
2.26 kb
1.98 kb
0.58 kb


1 2 3 4
7.27kb
2.9kb
4.3kb

(corresponding to late exponential phase) 5ml of the minimal medium culture was used to inoculate the chemostat. Batching up then required about 15 hours. The medium in the chemostat contained no chloramphenicol.

Thereafter samples were taken at approximately 12 generation intervals and treated as described in the Materials and Methods section, chapter 2.

Colonies which came up on chloramphenicol-containing plates after overnight incubation were transferred to nutrient agar containing phage Ø105DII:6c in an overlay of top agar to test for resistance to infection. The results given in Table 4.1 and Figure 4.3 were obtained using this method.

An alternative method involved picking single colonies from the chloramphenicol-containing serial dilution plates and streaking these across streaks of phage Ø105 on the surface of nutrient agar plates (again containing chloramphenicol). Each plate carried positive and negative controls (3G18 untransformed and B.subtilis 168 lysogenic for Ø105 respectively). This method generally gave clear, reproducible results but required a large number of plates to screen a reasonable number of colonies and used large amounts of phage. So it was generally used to check single colonies for phage sensitivity.

The results obtained from a single chemostat run with B.subtilis 3G18 (pHV14-F) are set out in Table 4.1 and represented in graphical form in Figure 4.3.

The inoculum was checked and found to contain only chloramphenicol resistant cells immediately prior to inoculation. The inoculum was also tested for resistance to

Table 4.1: Results of Chemostat Culture of 3G18 (pHV14-F)

Number of generations after inoculation	% of Cells Cam^R	% of Cam^R cells also ∅105 sensitive
0.27	5.9	19
12.46	0.45	25
28.86	0.22	27
44.14	0.39	26
56.84	0.10	31
72.18	0.16	n.d.
87.38	0.12	n.d.

Key: n.d. = not determined

Cam = chloramphenicol

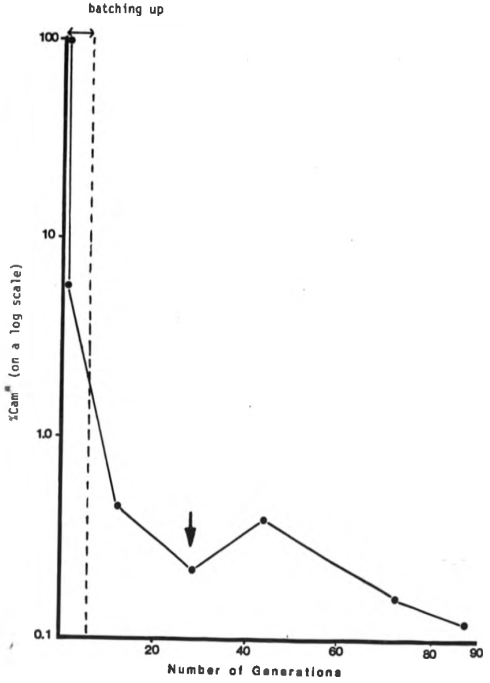
Cam^R = chloramphenicol resistant

B.subtilis carrying the plasmid pHV14-F was grown in the chemostat under carbon limitation at a dilution rate of 0.23 h^{-1} (generation time 3 h.). Approximately 2ml samples were taken at 12 to 16 generation intervals and tested for resistance to chloramphenicol and at the same time for sensitivity to infection by the phage ∅105 (in the presence of chloramphenicol). Chemostat inoculation and sampling procedures are described in the text and the Materials and Methods, chapter 2, sections 2.3.4 and 2.3.5, with the exception that the inoculum culture was grown overnight and the chemostat culture was allowed to batch up during the day. Note that the number of generations is expressed in terms of the number of generations elapsed since inoculation of the chemostat. (see the discussion, chapter 4).

Figure 4.3: Loss of the chloramphenicol resistance phenotype from chemostat culture of Bacillus subtilis 3G18 (pHV14-F).

Graphical representation of results set out in Table 4.1. Note the log scale on the vertical axis. B.subtilis 3G18 carrying the plasmid pHV14-F was grown under carbon limitation at a dilution rate of 0.23 h^{-1} (doubling time 3 hours) for 90 generations. Inoculation and sampling procedures are described in the text and in the Materials and Methods chapter, sections 2.3.4 and 2.3.5.

The first sample point by which the culture was in steady state is indicated by an arrow. The end of the batching up period is shown by a vertical line parallel to the y-axis.


batching up
100
10
1.0
0.1
±Cam^R (on a log scale)
20
40
60
80
90
Number of Generations

ø105DII:6c and all colonies tested were found to be resistant.

All colonies which appeared as chloramphenicol sensitive were checked and all tests were carried out in duplicate.

Micro-colonies about a quarter of the size of normal colonies appeared on sample plates containing chloramphenicol after batching up of the chemostat culture but were not in evidence on nutrient agar or in later samples. This was taken as indicative of a reduced copy number of the plasmid in these cells and hence reduced resistance to chloramphenicol. After a second cycle of growth on chloramphenicol-containing nutrient agar the colonies grew to normal size. See Chapter 6 for a further discussion of this observation.

Discussion

It is now a well established fact that pHV14 represents an example of a highly unstable plasmid, prone to both structural and segregational instability (Goze and Ehrlich, 1980 ; Primrose and Ehrlich, 1981 ; Jones et al., 1982). The structural instability of the plasmid has been well characterised by two different groups (Jones et al., 1982 ; Alonso and Trautner, 1985b) who come to essentially similar conclusions regarding the features of this instability. As yet the process of deletion formation remains to be understood but investigations involving this plasmid would seem to offer an excellent opportunity for analysing the mechanisms involved.

The studies described in the introduction to this

chapter (Primrose and Ehrlich 1981 ; Uhlen et al., 1981 ; Jones et al., 1982 ; Alonso and Trautner 1985b) were carried out in batch culture using a rich medium and there have been no reports to date of the use of continuous culture and a minimal medium to look at the stability of either pHV14 or pHV14 carrying an insert of foreign DNA.

The results described here, although only the product of an initial investigation into the behaviour of pHV14-F carrying cells in continuous culture, show that pHV14-F is lost very rapidly from the chemostat culture. Without further work the cause of the instability can only be guessed at. It may have been that plasmid replication ceased completely on removal of selective pressure since no lag period was observed before loss of the plasmid from the chemostat culture. The instability could, alternatively, have been the result of the failure of the plasmid to be segregated to daughter cells, either because of the loss or disruption of a system responsible for accurate partitioning of the plasmid or because of lowering of its copy number due to the generation of multimers (see, for example, section 2.5 of the introduction, chapter 1).

The protocol for this and other early chemostat culture experiments differed from the later experiments, described in chapters 5, 6, and 7, in that the chemostat culture was allowed to batch up during the day and could therefore be watched constantly (although no samples were taken during this time). From these early experiments with both strain 3G18 and 168 trp it was determined that at least 12 hours were needed before there was any visible growth in the chemostat and by 16 hours the culture had reached a density at which the medium flow could be started

without washout of the culture resulting (a problem in the case of strain 3G18, which is the reason this strain was not used further).

An important point which should be made at this stage is that the results illustrated in Figure 4.3 show that, at least in the case of this highly unstable plasmid, considerable changes in the proportions of plasmid-carrying and plasmid-free cells can occur during the batching up period alone and prior to steady state being attained.

During batch culture significant changes take place in the composition of the medium, particularly as the organism reaches stationary phase, and in the case of B.subtilis it might also be expected that spores will begin to appear in the medium when stationary phase is reached (but see the discussion section of chapter 3).

Since the very purpose of chemostat culture is to avoid these problems associated with batch culture, then, in retrospect, it would have been advisable to begin the flow of medium to the culture as the organisms began to grow exponentially. This might also have reduced the time needed for the culture to achieve steady state density. During this time the organisms are of course also subject to environmental changes and no real conclusions can be drawn from the samples taken at this stage of the culture. These problems are discussed further in Chapter 6 but should be kept in mind during the chapter that follows.

In retrospect it is surprising that the plasmid pHV14-F, when checked on agarose gels after transformation and immediately prior to cultivation of its host strain in the chemostat appeared to be structurally stable, in other words all colonies tested contained a plasmid of the correct size. As described in the introduction to this chapter pHV14 itself has been shown on a number of occasions to be structurally unstable. It is not known why in this case the plasmid was invariably found to be of the correct size but it may be that the frequency of deletions was sufficiently low to preclude the detection of altered plasmids after a single cultivation step (of about 20 generations) on rich medium.

The use of continuous culture offers an ideal means of studying the segregational stability of plasmids because it provides the opportunity for observing changes in the plasmid content of a strain over a large number of generations and for investigating the effects of different growth conditions of the host on plasmid maintenance. The advantage of long term cultivation is not illustrated most effectively by this study of pHV14-F since the plasmid is lost from the culture after only very few generations. Experiments described later, however, (see chapters 5 and 6) put this aspect of the technique to better use.

CHAPTER 5
CONTINUOUS CULTURE OF PLASMID-CARRYING *BACILLUS SUBTILIS* STRAINS

Introduction

Although some attention has been given to features of plasmid maintenance in *E.coli* grown in chemostat culture there are no published reports to date on plasmid maintenance in chemostat cultures of *B.subtilis*. In fact, considering the advantage to be gained from the use of continuous culture in studying plasmid stability, particularly the means by which to characterise the growth parameters affecting plasmid maintenance, it is surprising that more use has not been made of the technique in this respect.

One aspect of the biology of plasmid-carrying *E.coli* strains in chemostat culture which has been consistently noted is the effect of different nutrient limitations either on plasmid stability or the rate of loss of an unstable plasmid. The commonly studied nutrient limitations have been carbon (usually in the form of glucose) and phosphate and to a lesser degree nitrogen and magnesium. Most of the publications referring to plasmid maintenance in chemostat cultured *E.coli* strains contain some observation regarding differential effects of nutrient limitations.

Thus, Melling *et al*. (1977) reported that *E.coli* cells containing the otherwise very stable plasmid RP1 could be displaced from a chemostat culture by the introduction of an

isogenic, but plasmid-free strain present at a density of 1% of the resident chemostat population. Displacement of the RP1-carrying strain occurred only under phosphate limitation and no effect was observed under carbon or magnesium limitation. Under carbon limitation an inoculum of plasmid-free cells at a level of 50% of the chemostat population was required in order for the plasmid-free cells to become predominant in the culture.

Godwin and Slater (1979) reported that markers other that the tetracycline resistance gene on the plasmid TP120 (sulphonamide, streptomycin and ampicillin) were lost under phosphate limitation but not under carbon limitation. Tetracycline resistance was lost under both nutrient limitations. Whan-Lee and Edlin (1985) ,however, showed that expression of the tetracycline resistance gene of pBR322 reduced the reproductive fitness of E.coli cells so this may have been the basis for the greater instability of the tetracycline resistance marker of TP120.

Jones et al., (1980) found that pBR322-carrying E.coli cells were lost from chemostat culture under either carbon or phosphate limitation and at a similar rate under each limitation (Jones, 1984). Wouters et al. (1980) also reported the loss of cells carrying pBR322 from both glucose- and phosphate-limited chemostat culture.

Noack et al. (1981), on the other hand, found that pBR322 was stably maintained under their particular conditions of glucose and nitrogen limitation and put the difference down to unidentified differences in the genotype of the plasmids and the medium and host strains employed. In contrast, however, they found that pBR325, which is a direct

descendent of pBR322, was lost under identical conditions.

If any generalisation is to be made it should therefore be to point out that, if there is a difference, it is usually phosphate that exerts a greater stringency of selection for plasmid-free cells than any other nutrient limitation.

Another factor which has been reported to affect plasmid loss is the dilution rate of the culture. Godwin and Slater (1979) showed that the degree of competition between a plasmid-free and a plasmid-carrying strain of E.coli K12 increased with decreasing growth rate. Both Roth et al. (1980) and Wouters et al. (1980) have found that segregation of plasmid-free cells was faster at lower dilution rates.

Host genotype can also be important as evidenced by the different rates of plasmid loss in E.coli GY2354 and E.coli GM31. Alternatively, the results of Jones et al. (1980 and 1984) as compared with Wouters et al. (1980) indicated that plasmid stability was independent of the host genotype.

An effect of temperature was noted by Wouters et al. (1980) who found that the lag period preceeding appearance of plasmid-free cells was longer at a lower temperature (30°C) and decreased at a high temperature (42°C).

A feature of plasmids which seems to be of great importance in determining their stable maintenance in chemostat culture is their copy number. Both Jones (1984) and, by inference, Wouters et al. (1980) reported a drop in the copy number of an unstable plasmid over a number of generations. Jones (1984) found that over a period of 82 generations of growth of the host strain the copy number of pDS1109 (ColE1 with an insert of Tn1 DNA) fell 4 to 5 fold.

After a single cycle of growth on plates containing antibiotic the copy number of these plasmids returned to the original value.

Since there have been a number of reports of the effects of various nutrient limitations on plasmid maintenance in chemostat cultures of E.coli K12, analogous experiments were carried out to determine the effect of nutrient limitations on plasmid-carrying B.subtilis in the chemostat. At the same time the stability of the three plasmids pC194, pUB110 and pBC16 under long term continuous culture was investigated.

The small, antibiotic resistance S.aureus plasmids most often used as cloning vectors in B.subtilis have also been the subject of the majority of investigations into plasmid maintenance in B.subtilis, presenting as they do the most accessible system for such studies. The two S.aureus plasmids pC194 and pUB110 were used in this work and recently a number of publications have described their nucleotide sequences, the location of their essential replication determinants and the detection of the protein products encoded by either replicon. In the case of pUB110 the host factor requirements of its replication have also been determined. Most of the features of these two plasmids that are understood to date are reviewed in section 3 of the Introduction, chapter 1, but their important features are repeated for convenience in Table 5.1.

The plasmid pBC16 was included as a representative of naturally occurring Bacillus plasmids. This plasmid was originally isolated from Bacillus cereus by Bernhard et al in 1978. pBC16 was reported by Bernhard et al., (1978) to be

Table 5.1: Features of pC194, pUB110 and pBC16

Plasmid	Size (kb)	Copy Number	Detectable Markers	Original Host and Reference
pC194	2.9	15	Cam^R	S. aureus Iordanescu (1975)
pUB110	4.5	30-50	Kan^R	S. aureus Winston and Sueoka (1980)
pBC16	4.25	20-30	Tet^R	B. cereus Bernhard et al. (1978)

Key: Cam^R = chloramphenicol resistance, (inducible)

Kan^R = kanamycin resistance

Tet^R = tetracycline resistance

For further details of the physical properties of these plasmids see the text of chapter 1, section 3.

stable in B.subtilis 168 and to retain its size and copy number after more than 100 generations of growth (on plates of rich medium). It is approximately 4.25 kilobases and confers tetracycline resistance on its host to a level of $200 \mu g ml^{-1}$, whether the host be B.cereus or B.subtilis 168. Interestingly, the transformation frequencies obtained using B.subtilis 168 as the recipient of pBC16 were low (2.3×10^6 to 8×10^6 transformants per μg of DNA). Whatever the reason for this, it was not due to differences in the restriction-modification systems of the two hosts (Bernhard et al., 1978). The plasmid pBC16 has a unique BamH1 restriction site and two EcoR1 sites which, along with its small size, has allowed its exploitation as a cloning vector in B.subtilis (Kreft et al., 1982).

Of particular relevance is the fact that Polack and Novick (1982) reported that pBC16 and pUB110 have extensive sequence homology in the region of their replication origins including the BA1, BA2 and BA4 membrane binding sites of pUB110 (see Figure 1.5 and Tanaka and Sueoka, 1983).

Results

Three nutrient limitations were chosen for the experiments on the maintenance of pC194, pUB110 and pBC16 in chemostat culture; carbon (glucose), magnesium and phosphate. The composition of the basal medium and the concentrations of the components required to achieve the different limiting conditions are laid out in the Materials and Methods section, Chapter 2.

Once the plasmid content of the B.subtilis

transformants had been checked by small scale extraction of plasmid DNA and comparison with stock plasmid DNA (also extracted by the small scale procedure) see figure 5.1a, a single colony was selected for the chemostat inoculum. Each chemostat run was carried out by a procedure similar to that described earlier. Chemostat cultures were maintained in the absence of any selection for plasmid antibiotic resistance markers. Any antibiotic transferred with the inoculum would have been diluted by a factor of 47 and thereby rendered ineffective. The dilution rate was set at 0.34 h^{-1} and each culture maintained for at least 100 generations. Sampling and plasmid preparations were carried out as outlined in the Materials and Methods, chapter 2, sections 2.4.1 and 2.3.5.

5.1 Carbon limitation

All three plasmids were found to behave the same way when their host strain was grown for 100 generations in the chemostat under carbon limitation. They were each extremely stable in that antibiotic sensitive bacteria were detected only very occasionally as single colonies that failed to grow on antibiotic-containing nutrient agar.

Sensitive cells, when they arose, were found to contain no trace of plasmid DNA within the limits of detection of the method used. These cells are discussed later in section 5.4. On the other hand, analysis of the antibiotic resistant cells in each chemostat sample revealed the presence in all cases of a plasmid the same size as the original - see Figure 5.1.

Figure 5.1: Plasmid DNA before and after chemostat culture

pC194 DNA was extracted from B.subtilis 168 trp by the small scale alkali denaturation method of Birnboim and Doly (1979) from the inoculum and from the chemostat culture after 97 generations. Representative samples are shown on this 1% w/v agarose gel.

Track: 1 pC194 DNA from chemostat inoculum, colony from sample plate

2 pC194 DNA from chemostat inoculum colony from sample plate

3 pC194 DNA from chemostat inoculum, colony from sample plate

4 pC194 DNA from chemostat inoculum, colony from sample plate

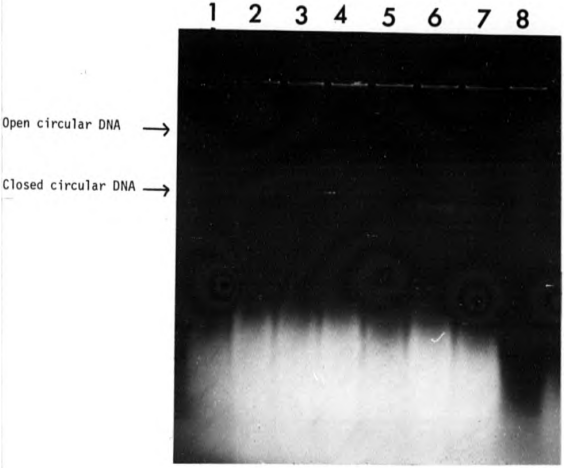
5 pC194 DNA from chemostat sample after 97 culture generations

6 pC194 DNA from chemostat sample after 97 culture generations

7 pC194 DNA from chemostat sample after 97 culture generations

8 pC194 DNA from chemostat sample after 97 culture generations

The progressive displacement of the sample bands on this gel was an artefact of the gel system used and was also reflected in the dye front on the gel.


1 2 3 4 5 6 7 8
Open circular DNA
Closed circular DNA

5.2 Magnesium limitation

The chemostat experiments carried out under carbon limitation were next repeated with magnesium as the limiting component in the medium. All other conditions were the same as before.

Again, all three plasmids were remarkably stable over a period of at least 100 generations. Occasionally single colonies appeared on nutrient agar sample plates which failed to grow when transferred to antibiotic-containing medium and which contained no detectable plasmid DNA. There was no significant difference in the numbers of these cells detected in carbon and in magnesium limited cultures (for their further treatment refer to section 5.4 of this chapter). All antibiotic resistant cells tested contained plasmid DNA of the same size as the original plasmid extracted from the inoculum at the beginning of the experiment.

Magnesium was, therefore, no more selective for plasmid-free cells than carbon limitation under these conditions.

There was, nevertheless, one noticeable difference between cells growing under magnesium limitation and those growing under carbon or phosphate limitation. Magnesium limited cells after 1 or 2 days of chemostat culture developed an unusual morphology. Slightly curved or hook-shaped cells were first observed in samples of the culture by phase contrast microscopy. At about the same time that chains of cells appeared in carbon limited cultures (after about 15 generations or one and a half days) chains also formed in magnesium limited cultures. Very shortly afterwards, however, the magnesium-limited cells appeared

twisted and eventually after 25 to 35 generations of growth most of the chemostat population comprised tightly coiled helical cells or helical chains of cells. The longest helices consisted of 24 turns. Only right-handed helices were observed in each culture - see figures 5.

Interestingly, these helical cells did not appear to be particularly well adapted to growth in the chemostat because after two days or so they were always slowly replaced by normal sized cells.

In the culture at the start of the chemostat run cells were either single or double, joined end to end (with a clearly visible septum), but all were highly motile. Under magnesium limitation their motility was lost as the cells became progressively more twisted and helical in form. Any degree of motility shown by these cells was usually self-defeating. Under carbon limitation motility was lost at approximately the same age of the culture and long chains of cells were formed but no helical cells were ever observed.

When a sample of the culture containing a large proportion of helical cells was grown on nutrient agar, the cells, after overnight incubation, were the normal size and shape of cells maintained on a rich medium. In fact, helical cells were never observed in conditions other than after about 25 generations of growth under magnesium limitation in the chemostat. Long chains of cells, on the other hand, were occasionally observed in samples of colonies from nutrient agar plates and in both rich medium and minimal medium batch culture.

Colonies which arose on sample plates during the period

Figure 5.2: Photograph of B subtilis cells from early stages of magnesium limited chemostat culture

A sample was taken from the chemostat culture after approximately 8 culture generations had elapsed and examined and photographed using an Olympus PM6 phase contrast microscope and camera.
Bar = 1μm

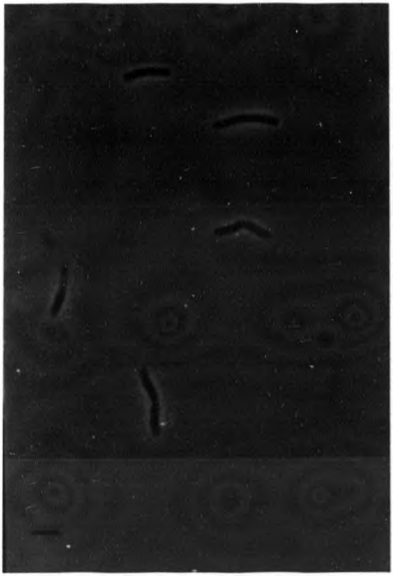


Figure 5.3: Photographs of B.subtilis cells from a magnesium-limited culture

(A) A sample was taken from a culture of B.subtilis 168 trp (pUB110) after approximately 19 generations, about 6-8 generations prior to the appearance of tightly-coiled, helical cells in the culture. Note the twisted appearance of the short chains of cells.
Bar = 2μm

(B) A helical cell from a magnesium-limited culture after 27 generations of growth. This particular cell was still incompletely coiled. After 30 generations the majority of cells in this culture formed more tightly-coiled helices of up to 24 turns.
Bar = 2μm

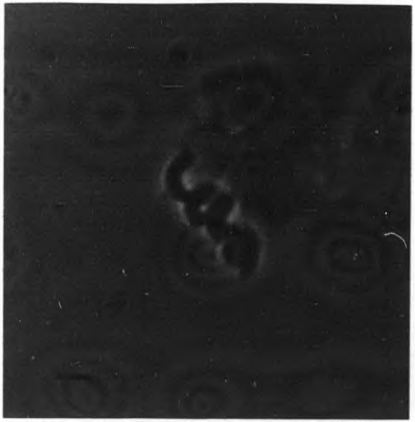


Figure 5.3 (C): Photograph of helical cell from magnesium-limited culture

The B.subtilis 168 trp host strain was grown under magnesium-limited culture and helical cells such as the one pictured here were observed in large numbers. After about 30 generations of growth the great majority of cells in the culture had a helical morphology. The culture sample was examined and photographed using an Olympus PM6 phase contrast microscope and integral camera.
Bar = 2μm

when most of the cells in the culture were helical, despite being composed of cells of normal morphology, had a characteristic appearance. A large number of thin projections arose from the periphery of each colony giving it an appearance similar to the "pinwheel" colonies described by Mendelson and Karamata (1982). The projections in this case had a clockwise orientation.

5.3 Phosphate Limitation

Perhaps predictably, the plasmid-carrying strains which had proved to be so stable in chemostat culture under carbon and magnesium limitation showed the same degree of stability under phosphate limitation. A discussion of the effects of different nutrient limitation regimes will therefore be postponed until their effects can be better observed (see chapter 6 and chapter 7).

5.4 Loss of stable plasmids

Earlier (in section 5.1) it was stated that, despite the fact that pC194, pUB110 and pBC16 were very stably maintained throughout 100 generations of growth of their host cells in chemostat culture, plasmid-free cells nevertheless occasionally arose. The loss of the entire plasmid genome from these cells was confirmed by a second transferral to antibiotic-containing plates and an attempt to extract plasmid DNA with the appropriate positive and negative controls.

These cells were re-transformed with the same plasmid

as they had originally carried and, when their plasmid content had been checked, they were grown up in the usual way prior to inoculation of the chemostat. They were maintained in the chemostat for 100 generations under the same nutrient limitation as the original culture from which they had been isolated.

In this way a single colony of cells which had lost pUB110 under magnesium limitation were again grown under magnesium limitation as were cells which had lost pBC16. Cells which had lost pC194 under carbon limitation were grown again under carbon limitation. Each of the three plasmids was stable during 100 generations of chemostat culture in their re-transformed host cells.

Discussion

It is apparent from the results just described that the three plasmids pC194, pUB110 and pBC16 are remarkably stably maintained in B.subtilis during chemostat culture. It may be, however that this observed stability is simply a reflection of the fact that plasmid-free cells, should they arise, would be at a considerable disadvantage in the chemostat. This possibility is examined further in chapter 6. For the moment though the degree of stability of these plasmids deserves some thought.

As stated earlier, these three plasmids all have high copy numbers. The chances of plasmid-free cells arising on division of their host cells can be calculated using the binomial distribution, assuming that distribution of plasmid

copies to daughter cells is a random process. The results of this type of calculation for several different copy numbers are listed in Table 5.2 (see also section 2.2 of the Introduction, chapter 1).

The instability is very low for each of the plasmids used so it is conceivable that random distribution of plasmid copies to daughter cells is sufficient to account for the level of stability observed. For example, take the plasmid with the lowest copy number of the three, pC194, with a copy number of 15. The probability of cells carrying this plasmid giving rise to plasmid-free daughter cells is about 1 in 6×10^5 which, with a cell density of about 1×10^8 cellsml^{-1} in the chemostat, means that by chance only 1000 plasmid-free cells would be expected to be found in 1ml of chemostat culture or 100 in the 100μls taken for plating (which was serially diluted to 10^{-7}). So the limits of detection of the sampling procedure used here were not sufficient for a quantitative analysis of the appearance of plasmid-free cells should they arise at this frequency.

It would seem then that these plasmids have no need for any specific control over their distribution to daughter cells at cell division and that random assortment alone would be enough to ensure their stable inheritance. This of course relies on their copy numbers remaining high. If their copy numbers should drop then the probability of plasmid-free cells arising would increase dramatically (see Table 5.2).

A high copy number *E.coli* plasmid, ColE1, has a specific mechanism to ensure that its copy number remains

Table 5.2: The effect of random distribution of plasmid copies to daughter cells on cell division

Plasmid Copy Number At Cell Division	Probability of Plasmid Free Cells Arising	Example
1	1	F or P1[a]
2	0.5	
3	0.25	R1[b]
4	0.125	
5	0.0625	
10	1.95×10^{-3}	
15	6.10×10^{-5}	pC194[c]
20	1.91×10^{-6}	pBC16[d]
30	1.86×10^{-9}	
40	1.82×10^{-12}	
50	1.78×10^{-15}	pUB110[e]

References: a = Abeles et al. (1984)
b = Nordstrom et al. (1980)
c = Iordanescu et al. (1975)
d = Bernhard et al. (1978)
e = Winston et al. (1980)

Table 5.2: The probabilities listed were calculated according to the binomial distribution,

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

Where P(0) is the probability of plasmid-free cells arising and n is the number of copies of the plasmid in the cell at cell division. The calculation assumes that distribution of plasmid copies to daughter cells is entirely random and that each cell carries the same number of copies of the plasmid at cell division.

high. It codes for a site specific recombination system able to resolve plasmid multimers should they arise. Such a system would seem vital to a high copy number plasmid whose major cause of instability would probably be a lowering of its copy number, for example as a result of multimerisation. So although no such system has been detected on either pC194, pUB110 or pBC16 it is something to bear in mind when considering the maintenance functions of these plasmids.

At this stage no conclusions can be drawn as to the differential effects of the three nutrient limitations on plasmid stability in B.subtilis. Certainly under these conditions no differences can be detected.

The morphological features of cells grown under magnesium limitation are particularly interesting. Similar features have been noted before. A B.subtilis mutant which had an unusual double helix morphology was described by Mendelson in 1976. This organism was isolated from a nitrosoguanidine-treated culture of B.subtilis 168 and a high proportion of helical cells were present in colonies of this strain growing on tryptose blood agar.

Mendelson (1976) made use of the helical growth property of the mutant B.subtilis in showing that normal cells elongate in a helical manner, that is, new cell surface components are incorporated along a helical path but whether or not this could be a common feature of all rod shaped cells was not determined.

Under specific growth conditions Mendelson (1978) found that certain B.subtilis mutants produced thread-like helical macrofibres as long as 1cm in liquid culture. The morphology

of these structures depended on the genotype of the cells, the culture conditions and the composition of the medium (Mendelson, 1978).

Mendelson and Karamata (1982) also stated that the handedness of helix structure could be reversed by altering the temperature and ionic environments in certain B.subtilis mutants defective in cell separation. The helix orientation of these strains was also influenced by the concentration of divalent cations, particularly magnesium. Conditions generally used for cultivation of B.subtilis were given as favouring right handed structures.

Cells forming right handed helices produced colonies with projections which arose from several points on their periphery and which always grew out from the centre of the colony in a clockwise direction. In at least one case of a strain which formed left-handed helices, the projections from its colonies grew out anticlockwise relative to the centre of the colony (Mendelson and Karamata, 1982).

Mendelson and Karamata (1982) therefore proposed that, in general, the characteristic arrangements of projections from the colonies of helix producing B.subtilis strains depended on the helix hand of the strain.

So how do the observations of Mendelson (1978) and of Mendelson and Karamata (1982) compare with the morphology of B.subtilis 168 trp reported here?

The strains used by Mendelson (1978) and by Mendelson and Karamata (1982) differed from the helical cells described in this work in being obviously mutated and in producing helical cells on rich medium. Whereas the B.subtilis 168 trp cells observed under magnesium limitation

carrying a plasmid reported here could be mutants which arose during chemostat culture, it is unlikely that the same mutant should arise in a number of different experiments (eleven in all) (although such an occurrence is not entirely without precedent, see Dykhuizen and Hartl, 1983). By the same token, it is unlikely that the resident plasmid was involved in the helical phenotype because helical cells were observed in cultures of bacteria containing each one of the plasmids studied and in cultures of the host strain alone under magnesium limitation. In the latter case no differences were observed between helices formed in this culture and in cultures of the plasmid-carrying strains - see Figures 5.3 B and C.

The appearance of helical macrofibres of the sort reported by Mendelson (for example, 1978) has also been observed in cultures of wild type B.subtilis strain OI1, but only on inoculation of a rich medium with a very small number of cells (Zaritsky and Macnab, 1981).

Mendelson and Karamata (1982) have stated that the normal culture conditions employed for B.subtilis would give rise to right hand helices and, indeed, only right hand helices were observed during magnesium-limited chemostat culture of B.subtilis 168 trp with, or without, a resident plasmid. Similarly, these cultures gave rise to colonies on nutrient agar plates with a clockwise arrangement of projections (cf. Mendelson and Karamata, 1982).

So this is the first report of the routine observation of helical cells in a culture of presumably wild-type cells (and also having a slightly different morphology from previously described helical cells,

that is, the pitch of the helices was different) under commonly used

culture conditions. It seems unlikely that this observation has not been made by other workers but it might be that small differences in media composition are sufficient to prevent the appearance of helical cells.

The re-transformation of cells which had lost otherwise stable plasmids revealed that, at least in the cases of the cells which were tested loss of the plasmid was not the result of a host mutation, for instance mutation in a chromosomal gene essential for plasmid replication. So plasmid loss was possibly caused either by a daughter cell failing to receive a copy of the plasmid purely by chance or it was the result of a mutation in the plasmid which prevented its replication. Unfortunately the number of chemostat runs which could be carried out was strictly limited so it was not possible to check a larger number of cells which had lost an otherwise stable plasmid.

Despite the fact that plasmid-free cells obviously arose in these cultures from time to time, they never rose to dominate the culture as they might have been predicted to do if they had had a growth advantage over the plasmid-carrying cells. This could be taken as an indication that the plasmid-carrying cells were favoured for some reason over plasmid-free cells. Indeed it could be that an active selection process was operating against plasmid-free cells. In order to test this possibility experiments were carried out to investigate the effect of the presence of a known quantity of plasmid-free cells on the persistence of plasmid carrying cells in chemostat culture. These experiments are described in the following chapter.

CHAPTER 6
COMPETITION BETWEEN PLASMID-CARRYING AND PLASMID-FREE STRAINS

Introduction

Experiments designed to provide an indication of the stability of plasmids pE194, pUB110 and pBC16 in *B.subtilis* and to identify any effects that different nutrient limitations may have on their maintenance were described in the previous chapter. The results pointed to a high level of stability of all three plasmids and an extremely low rate of appearance of plasmid-free cells.

Despite the fact that plasmid-free cells did occasionally arise, on no occassion did they take over the chemostat culture, presumably because they had no significant growth advantage over the plasmid-carrying cells. It could be the case, however, as indicated in Chapter 5, that the plasmid-free cells were actively discriminated against in some way. For instance, if plasmid-carrying cells produced a toxic factor encoded by the plasmid (such as, for example colicin produced by cells carrying ColE1) and if at the same time the plasmid conferred immunity to this factor on its host, then plasmid-free cells would be killed as they arose in the culture (see for example Adams *et al.*, 1979). Plasmid-free cells may also grow slower for some reason, particularly if the presence of the plasmid affects the uptake of the limiting nutrient in a beneficial way.

In order to determine whether plasmid-free cells, if present in the culture, could displace plasmid-carrying cells and at the same time to better determine the extent of the stability of these three plasmids, a number of enforced competition experiments were carried out. If cells which have lost their plasmid do indeed have a growth advantage then even if they are present in relatively low proportion in the culture they should eventually displace the plasmid-carrying cells.

Results

Cultures for inoculation were set up in essentially the same way as for previous experiments. B.subtilis strain 168 trp transformed with either pC194, pUB110 or pBC16 was used as before and the same strain without any plasmid DNA used as the plasmid-free inoculum. Both strains (168 trp with and without plasmid DNA) were grown to early stationary phase in minimal medium prior to inoculation to ensure they were at approximately the same stage of growth and culture density (confirmed by sampling the inoculum for viable counts at the time of inoculation). The inoculum was then composed by simply taking 1% by volume of plasmid-free cells (R^- cells) and 99% by volume plasmid-carrying cells (R^+ cells), mixing them and using this whole mixture to inoculate the chemostat. So for 50% R^- competition experiments the inoculum was composed of 50% by volume of each strain. The chemostat inoculum was 10ml for each of the experiments.

Samples were taken from the inoculum on inoculation of the chemostat, from the chemostat culture after batching up

and thereafter at approximately 24 hour intervals. The temperature, pH, rate of aeration, agitation and dilution of the culture were as for previous experiments.

6.1 Carbon limitation

One run was carried out with each of the plasmids under carbon limitation. The different chemostat cultures described here took varying times to reach steady state, determined from the viable counts of the samples, (as indicated on each individual figure). Some variation is therefore to be expected between experiments and the sample points prior to steady state being reached should be treated with some caution.

For each of the plasmids tested the results were very similar. Plasmid-free cells, present at a level of 1% of the inoculum failed to displace the plasmid-carrying cells in subsequent continuous culture (see Figures 6.1, 6.2 and 6.3) even though measures were taken to ensure that each type of cell had an equal opportunity to establish itself in the chemostat.

When the experiments were repeated with an inoculum composed of 50% R^- and 50% R^+ cells the R^+ cells were displaced from the culture in each case (see Figures 6.4 to 6.6 inclusive).

This result indicates that the R^+ strain is at a disadvantage in mixed populations when in competition with an equal number of R^- cells and the extent of the disadvantage is illustrated by the fact that a lower proportion of R^- cells in the inoculum failed to displace the R^+ cells.

One important feature of these results which should be noted is that, in each case when the R^+ cells were displaced from the chemostat culture by R^- cells, a small proportion, about 1%, remained in the culture. This continued maintenance of a basal level of R^+ cells was observed in all

Figure 8.1: Chemostat culture of pC194-carrying *B.subtilis* under carbon limitation in competition with 1% (◆) and 50% (●) plasmid-free cells.

Plasmid-free cells were mixed with pC194-carrying cells before inoculation as described in the text and the culture maintained at a dilution rate of 0.34 h^{-1}. The number of generations elapsed was calculated using the time that dilution of the culture began as the starting point. In the course of the 1% competition experiment, a steady state concentration of 1.31 (+ or - 0.4) x 10^8 cells ml^{-1} was reached after 22.3 generations, indicated by an arrow on the graph. In the course of the 50% competition experiment a steady state cell density of 3.2 (+ or - 0.4) x 10^8 was reached at the time indicated by an arrow on the graph.

For chemostat culture conditions see Materials and Methods section, chapter 2.

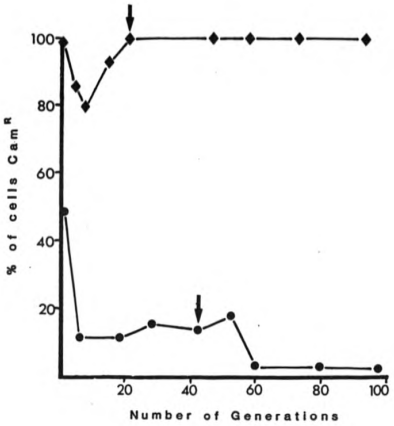

100
80
60
40
20
% of cells Cam^R
20
40
60
80
100
Number of Generations

Figure 6.2:

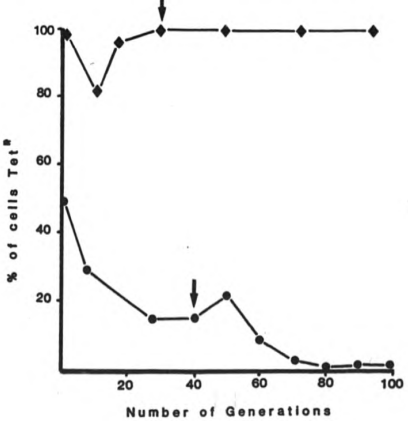
Chemostat culture of pUB110-carrying B.subtilis under carbon limitation in competition with 1% (◆) and 50% (●) plasmid-free cells

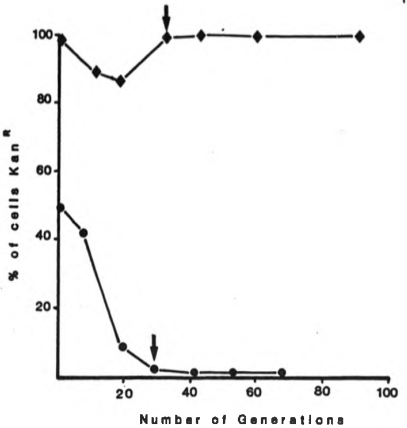
Plasmid-free cells were mixed with plasmid-carrying cells before chemostat inoculation as described in the text and the culture maintained at a dilution rate of 0.34 h^{-1}. The number of generations was calculated using the time that dilution of the culture began as the starting point. A steady state viable count of 2.56 (+ or - 0.13) x 10^7 colony forming units ml^{-1} was achieved during the 1% competition experiment at the stage of the culture indicated by an arrow on the graph (32.14 generations). In the case of the 50% competition experiment a steady state viable count of 2.22 (+ or - 0.43) x 10^7 was reached by 28.47 generations, the point indicated by the arrow. For chemostat culture conditions see Materials and Methods section, chapter 2.

Figure 6.3:

Chemostat culture of pBC16-carrying B.subtilis under carbon limitation in competition with 1% (◆) and 50% (●) plasmid-free cells

Plasmid-free cells were mixed with plasmid-carrying cells before chemostat inoculation as described in the text and the culture maintained at a dilution rate of 0.34 h^{-1}. The number of generations elapsed was calculated using the time that dilution of the culture began as the starting point. A steady state viable count of 8.97 x 10^7 colony forming units ml^{-1} was reached, during the 1% competition experiment, by the 29.73 generations sampling point, indicated by an arrow on the graph. By 39.66 generations in the 50% competition experiment, a steady state of 1.08 x 10^8 colony forming units ml^{-1} was reached. For chemostat culture conditions see Materials and Methods section, chapter 2.


100
80
60
40
20
% of cells Tet^R
20
40
60
80
100
Number of Generations


100
80
60
40
20
% of cells Kan^R
20
40
60
80
100
Number of Generations

the chemostat runs carried out in the course of this work in which a R^+ strain was lost from the chemostat culture.

The residual level of R^+ cells varied, but generally was close to 1%. Samples of at least 12 antibiotic resistant colonies were checked for the presence of plasmid DNA at the end of each chemostat run and in all cases found to contain plasmid molecules of the same size as the plasmid in the inoculum culture.

Although R^- cells did not displace R^+ cells when the former were present at 1% of the population, there was an initial increase in the proportion of R^- cells in samples taken before 12 generations. Thereafter the proportion of R^+ cells once again rose to predominate in the culture and remained the only type observed up to at least 100 generations later. These samples, however, were taken before the culture reached steady state and are probably a reflection of the changes in the culture during batching up when plasmid free cells may have been favoured to a greater extent than during dilution of the culture.

6.2 Magnesium limitation

Similar experiments were carried out under magnesium limitation. The protocol was the same as employed for competition experiments under carbon limitation; a mixture of either 1% R^- and 99% R^+ cells or 50% of each was used to inoculate the chemostat and the culture was thereafter maintained under the same conditions as before, the only difference being that magnesium was limiting in the medium. The results are shown in Figures 6.4 to 6.6 inclusive.

Again, the R^+ cells were not displaced by R^- cells when

Figure 6.4: Chemostat culture of pC194-carrying *B.subtilis* under magnesium limitation in competition with 1% (◆) and 50% (●) plasmid-free cells

Plasmid-free cells were mixed with pC194-carrying cells before inoculation as described in the text and the culture maintained at a dilution rate of 0.34 h^{-1}. The number of generations elapsed was calculated using the time that dilution of the culture began as the starting point. A steady state viable count of 9.89 (+ or- 0.07) x 10^7 colony forming units ml^{-1} was reached by the 37 generations sampling point in the 1% competition experiment (indicated by an arrow on the graph) and a steady state viable count of 1.1 (+ or - 0.2) x 10^8 colony forming units ml^{-1} was achieved by the 34 generations sampling point in the 50% competition experiment (indicated by the arrow). For chemostat culture conditions see Materials and Methods section, chapter 2.

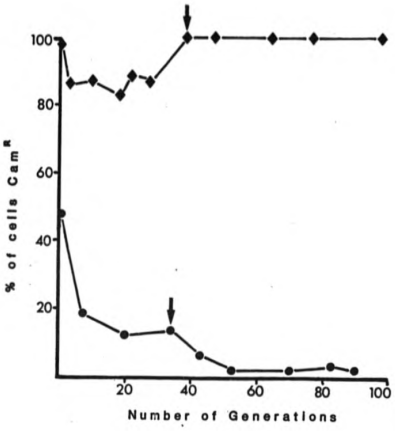
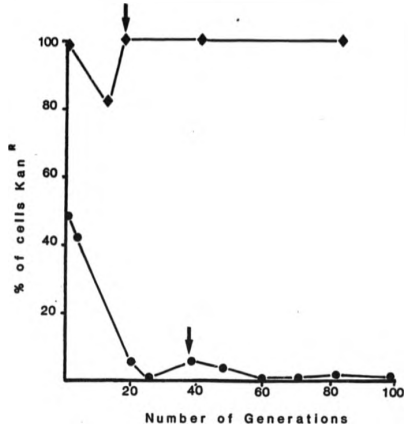

100
80
60
40
20
% of cells Cam^R
20
40
60
80
100
Number of Generations

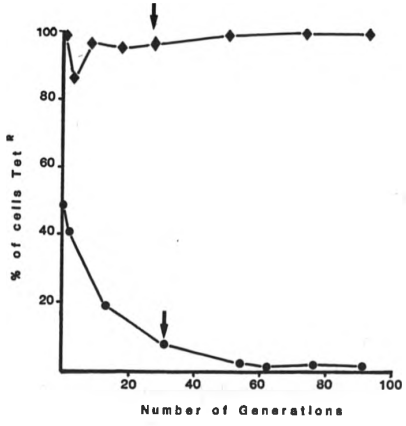
Figure 6.5: (upper figure) Chemostat culture of pUB110-carrying B.subtilis under magnesium limitation in competition with 1% (◆) and 50% (●) plasmid-free cells.

Plasmid-free cells were mixed with pUB110-carrying cells before chemostat inoculation as described in the text and the culture maintained at a dilution rate of 0.34 h^{-1}. The number of generations elapsed was calculated from the time that dilution of the culture began. A steady state viable count of 2.72 (+ or - 0.23) x 10^7 colony forming units ml^{-1} was reached by the 16.53 generations sampling point (indicated by the arrow on the graph) in the 1% competition experiment and a steady state viable count of 1.565 (+ or - 0.30) x 10^7 was achieved by the 36.99 generations sampling point, indicated by the arrow in the 50% competition experiment. For chemostat culture conditions see the Materials and Methods section, chapter 2. Figure 6.5 is the upper of the two figures, two pages on, on page 142.

Figure 6.6: (lower figure) Chemostat culture of pBC16-carrying B.subtilis under magnesium limitation in competition with 50% (●) and 1% (◆) plasmid-free cells.

Plasmid-free cells were mixed with pBC16-carrying cells before inoculation as described in the text and the culture maintained at a dilution rate of 0.34 h^{-1}. The number of generations was calculated using the time that dilution of the culture began as the starting point. A steady state viable count of 6.60 (+ or -0.05) x 10^7 colony forming units ml^{-1} was achieved by the 26.69 generations sample point, as indicated by the arrow, in the 1% competition experiment and a steady state viable count of 5.60 (+ or - 0.16) x 10^7 by the 30.90 generations sampling point in the 50% competition experiment (indicated by the arrow). For chemostat culture conditions see the Materials and Methods, chapter 2.


100
80
60
40
20
% of cells Kan R
20
40
60
80
100
Number of Generations


100
80
60
40
20
% of cells Tet R
20
40
60
80
100
Number of Generations

the latter were present at a level of 1% of the population on inoculation. When the proportion of R^- cells was increased to 50% of the inoculum R^+ cells were rapidly displaced from the culture.

The reason for repeating these experiments with a magnesium limited culture was to determine whether magnesium represented a more stringent selection environment for the appearance of plasmid-free cells than did carbon limitation.

In Figure 6.7 the results of one experiment using 50% B.subtilis 168 (pC194) and 50% B.subtilis 168 under carbon limitation are compared with the results of the corresponding experiment carried out under magnesium limitation. It should be evident from a comparison of (i) the immediate decline in the ratio of R^+ to R^- cells, (ii) the rate at which R^+ cells subsequently disappeared from the culture (represented by the slope of the graphs) and (iii) the time taken for the plasmid-containing cells to be reduced to a very small percentage of the population that there was very little difference in the effect of these two selection regimes on maintenance of pC194.

A similar treatment of the results obtained for pUB110 and pBC16 are illustrated in Figure 6.8 and 6.9 and the same can be said in these cases.

It could be argued that magnesium limitation results in a slightly faster rate of loss of plasmid-carrying cells, but since duplicate experiments were not performed an estimate of the reproducability of these results is not available and therefore no conclusion can be drawn in this respect.

Figure 6.7: Chemostat culture of pC194-carrying B.subtilis under magnesium (○) and under carbon (●) limitation in competition with 50% plasmid-free cells.

In each case the pC194-carrying and plasmid-free cells were mixed before chemostat inoculation as described in the text and the culture maintained at a dilution rate of 0.34 h^{-1}. The number of generations elapsed was calculated from the time that dilution of the culture began. Under magnesium limitation a steady state viable count of 1.10 (+ or - 0.20) x 10^8 colony forming units ml^{-1} was reached by the 34 generations sampling point, as indicated by the arrow on the graph, and under carbon limitation a steady state of 3.20 (+ or - 0.40) x 10^8 colony forming units ml^{-1} was achieved by the 40 generations sampling point, indicated by the arrow. For chemostat culture conditions see the Materials and Methods, chapter 2.

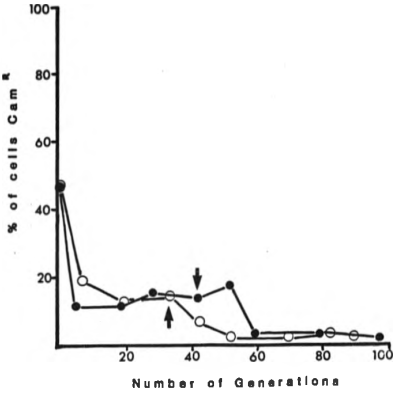
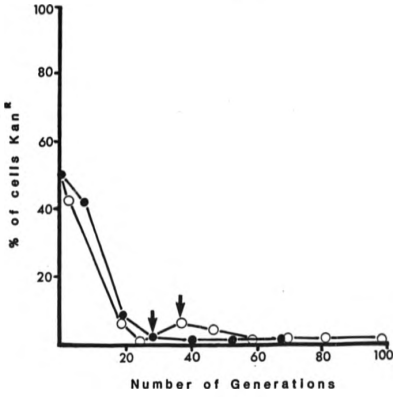

100
80
60
40
20
% of cells Cam^R
20
40
60
80
100
Number of Generations

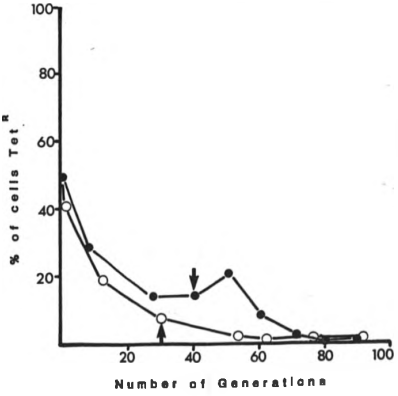
Figure 6.8: Chemostat culture of pUB110-carrying B.subtilis under magnesium limitation (○) and under carbon limitation (●) in competition with 50% plasmid-free cells.

In each case plasmid-free cells and plasmid-carrying cells were mixed before inoculation as described in the text. Both cultures were maintained at a dilution rate of 0.34 h^{-1}. The number of generations was calculated using the time that dilution of the culture began as the starting point. Under magnesium limitation a steady state viable count of 1.56 (+ or - 0.30) x 10^7 colony forming units ml^{-1} was achieved by the 36.99 generations sampling point as indicated by the arrow on the graph. In the carbon limited culture a steady state of 2.22 (+ or - 0.43) x 10^7 was reached by 28.47 generations as indicated by the arrow. For the chemostat culture conditions see the Materials and Methods, chapter 2. See two pages on for the figure - top figure is 6.8, lower figure 6.9.

Figure 6.9: (lower figure) Chemostat culture of pBC16-carrying B.subtilis under magnesium limitation (○) and under carbon (●) limitation in competition with 50% plasmid-free cells.

In each case plasmid-free cells were mixed with pBC16-carrying cells before inoculation, as described in the text. Both cultures were maintained at a dilution rate of 0.34 h^{-1}. The number of generations elapsed was calculated using the time that dilution of the culture began as the starting point. Under magnesium limitation a steady state viable count of 5.6 (+ or - 0.16) x 10^7 colony forming units ml^{-1} was achieved by the 30.90 sampling point as indicated by the arrow on the graph. In the carbon-limited culture a steady state of 1.08 x 10^8 colony forming units was achieved by 39.66 generations as indicated by the arrow. For the culture conditions see the Materials and Methods section, chapter 2.


100
80
60
40
20
% of cells Kan^R
20
40
60
80
100
Number of Generations


100
80
60
40
20
% of cells Tet^R
20
40
60
80
100
Number of Generations

6.3 Phosphate limitation

Figures 6.10 shows the effect of competition from R- cells at 1% and 50% of the inoculum on the retention of cells carrying pBC16 in the chemostat under phosphate limitation. The number of phosphate limited runs which could be performed was unfortunately restricted by the length of time for which the LH chemostat was available, so competition runs with pC194 and pUB110 were not carried out.

The results show again that plasmid-free cells were unable to displace plasmid-carrying cells when present at 1% of the inoculum. When present at a level of 50%, however, they successfully out-grew the plasmid-carrying cells.

Phosphate limitation was not a significantly more selective regime, in the case of pBC16 at least, than carbon or magnesium limitation under these conditions. Nevertheless, some differences could be seen in the rate at which plasmid-carrying cells were displaced from the culture and the degree to which the plasmid-free cells had taken over the culture within 40 generations.

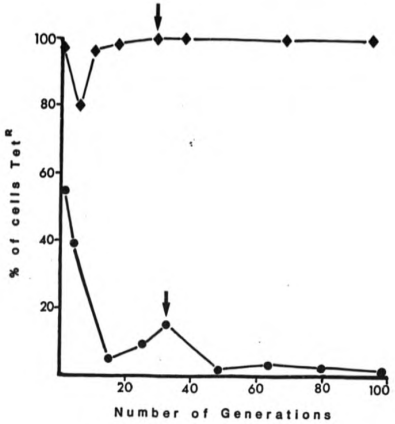
Once again, it should be made clear that it was not possible to carry out duplicate experiments, so these are the results of single chemostat runs and as such carry an undetermined degree of variation.

Discussion

It has often been suggested that the maintenance of a plasmid is a significant matabolic burden to its host cell and this theory has often been invoked to explain the

Figure 6.10: Chemostat culture of pBC16-carrying *B.subtilis* under phosphate limitation in competition with 1% (◆) and 50% (●) plasmid-free cells.

Plasmid-free cells were mixed with pBC16-carrying cells before inoculation as described in the text and the culture maintained at a dilution rate of 0.34 h^{-1}. The number of generations elapsed was calculated using the time that dilution of the culture began as the starting point. A steady state viable count of 3.06 (+ or - 0.35) x 10^7 colony forming units ml^{-1} was reached by the 29 generations sampling point in the 1% competition experiment (indicated by an arrow on the graph) and a steady state viable count of 4.76 (+ or - 0.17) x 10^7 colony forming units ml^{-1} was achieved by 32.51 generations in the 50% competition experiment (indicated by an arrow). For chemostat culture conditions see the Materials and Methods section, chapter 2.


100
80
60
40
20
% of cells Tet^R
20
40
60
80
100
Number of Generations

replacement of plasmid-carrying cells in chemostat culture by their plasmid-free counterparts. In certain cases strains which have lost a large plasmid have been shown to have a reduced generation time over otherwise isogenic plasmid-carrying cells (Zund and Lebek, 1980) and auxotrophic mutants have been shown to grow faster than heterotrophs (Zamenhof and Eichorn, 1967). So there is some evidence in support of this idea.

Notwithstanding these observations the situation is not as straightforward as it may seem because Zund and Lebek (1980) also measured an increased generation time in some plasmid-free populations over isogenic plasmid-carrying cells.

The plasmid could, alternatively, confer a positive advantage on the host cell so that its loss would prove to be disadvantageous and vice versa, if as, for example, in the case of the deleterious effects of the expression of the tet gene of pBR322, the loss of a particular plasmid proves to be of some benefit to the host.

So what this goes to say is that much depends on the particular plasmid-host combination in question and the situation is complicated by any type of interference with the metabolism of the host by the plasmid, be it production of a toxic factor (Adams et al., 1979), interference with nutrient uptake (Whan-Lee and Edlin, 1985) or cell wall (Villarejo and Ping, 1978 ; Gilbert and Brown, 1978 ; Moyed and Bertrand, 1983).

The examples of the effect of the presence of plasmid-free cells on the maintenance of pC194, pUB110 and pBC16 described here point to the fact that, in these cases,

plasmid-free cells have no real advantage over plasmid-carrying cells since in order to eventually dominate the culture they must initially be present at 50% of the population. Take over by plasmid-free cells could of course occur from a lower initial proportion of the culture than 50%. Whether this was the case was not determined.

It may be that lack of susceptibility to competition by only 1% plasmid-free cells is a general feature of very stable plasmids. The fact that this is probably not the case is indicated by the observation made by Jones et al. (1980 and 1984) and by Melling et al. (1977) that the otherwise very stable plasmid RP1 is rapidly displaced from chemostat culture following the introduction of a sample, at 1% of the culture volume, of plasmid-free cells.

The sudden halt in the initial take-over by plasmid cells observed when cells carrying all three plasmids, pC194, pUB110 and pBC16 were challenged with 1% plasmid-free cells begs further attention.

On the face of it, it would seem that the plasmid-carrying cells gained an advantage over plasmid-free cells, for instance, by virtue of a favourable mutation which allowed them once again to predominate in the culture.

However, in every case the change in composition of the culture with respect to plasmid-carrying and plasmid-free cells occurred during the period immediately following batching up when steady state had yet to be reached. It was seen in the results of the chemostat culture of a pHV14-F-carrying strain (reported in Chapter 4) that a large decrease in the numbers of plasmid-carrying bacteria occurred during batching up and it was noted that large changes in the culture composition could be expected to occur during this time (see Chapter 4 discussion, p. 113). It is equally important to

consider the time following batching up, before steady state is reached, when the culture environment is not only still changing but is also conceivably different from that which persists during steady state.

If the results of the experiments described in this chapter are interpreted with this in mind the most likely explanation for the sudden recovery of the plasmid-carrying bacteria is that, as steady state is reached, they gain an advantage over the plasmid-free bacteria which they did not have during the batching up and the period following. One possibility is that the culture sporulated during the late stage of batching up and that the plasmid is lost on sporulation of its host bacterium. Sporulation of the B.subtilis host strain used here was discussed in Chapter 3 and it was thought to be unlikely that sporulation of this strain occurs in minimal medium in the chemostat to any significant degree. Nothing is known about the effect of sporulation of the host strain on plasmid maintenance in B.subtilis. The same phenomenon occurred in each of the 1% competition experiments so it seems to be a feature of the chemostat conditions rather than the plasmids themselves. No simple explanation is therefore available for the halt in the take-over by the plasmid-free cells which was observed when these cells were present at only 1% of the inoculum.

A reproducible feature of experiments in which a plasmid-bearing strain is displaced from a chemostat culture is the persistence of a small proportion (around 0.1%) of plasmid-carrying cells after the majority have been washed out (see also Jones et al., 1980 ; Godwin and Slater, 1979).

When a sub-population of cells is present at this low a proportion of the total population they would be expected to have a lower probability of being washed out of the culture, assuming washout to be a totally random process and assuming that these cells compete on an equal standing with the larger population. This is probably not the case, at least in the experiments reported here, because instead of the sub-population of plasmid-carrying cells being slowly

further reduced in number they remained at a constant proportion of the population for up to 150 generations. (because in 100 colonies no antibiotic resistant colonies were detected, the results in question come from a comparison of viable counts on nutrient agar and nutrient agar containing the relevant antibiotic).

Some theories have been put forward to explain this phenomenon, but to date no satisfactory explanation has been found. Jones (1984) suggested that, in the case of RP1, the source of the plasmid-carrying cells in the culture could have been the presence of a population of cells adhered to the chemostat walls which discharged plasmid-carrying progeny into free culture at a rate sufficient to account for the numbers detected. This was, however, tested and found not to be the case, at least not for RP1

Since wall growth of B.subtilis has been reported to be negligible (Larsen and Dimmick, 1964) and no visible wall growth was observed in any of the chemostat runs reported here, it is unlikely that such a cause is behind the persistence of cells carrying pC194, pUB110 or pBC16. On the other hand, the plasmid could confer on its host an increased ability to adhere to the chemostat walls, so it would not be wise to eliminate this possibility without direct testing.

The stability of pBC16-carrying cells in chemostat culture is particularly interesting in view of the fact that this plasmid encodes tetracycline resistance. The tetracycline resistance marker of pBR322 (derived from the plasmid pSC101) has been revealed to be unstable in E.coli in the absence of selection for tetracycline resistance (Nosok et al., 1981), as has the tetracycline resistance

marker of pTP120 (Godwin and Slater, 1979). Whan-Lee and Edlin (1985) reported that expression of the tetracycline resistance gene of pBR322 reduced the reproductive fitness of the E.coli cells carrying this plasmid. Cells which carried a plasmid whose tetracycline resistance gene had been deleted or inactivated had a growth advantage over cells carrying the wild type plasmid (Whan-Lee and Edlin, 1985). It has been proposed that the adverse effects of expression of this tetracycline resistance gene in E.coli are mediated via alterations in the cell membrane (Villarejo and Ping, 1978 ; Moyed and Bertrand, 1983).

pSC101 also codes for tetracycline resistance but in this case the gene is inducible (Tait et al., 1977) and perhaps therefore has a less significant effect on the host cell under conditions in which its expression is not selected for.

CHAPTER 7

THE PLASMID pC194-K1

Introduction

The study of plasmids such as pC194 pUB110 and pBC16 under conditions in which they are extremely stable has its limitations. Often an understanding of what happens when plasmid maintenance control systems fail to function properly can reveal more about their normal behaviour than observations of wild-type plasmids under stable conditions.

In this context a comparison of mutant plasmids (most often copy number control mutants) with their wild-type counterparts has been particularly fruitful in dissecting the control systems of a number of plasmids (pE194 for instance, Gryczan et al., 1982).

This chapter is concerned with a plasmid which was extracted from a single colony obtained after transformation of B.subtilis 168 trp with a sample of pC194 DNA.

The plasmid was originally obtained as a DNA preparation and used to transform B.subtilis 168 trp to chloramphenicol resistance. Before transformation the DNA was checked on a gel and was found to be the correct size. When plasmid DNA was extracted from one particular colony after the transformation, however, it was found to contain a plasmid of about 7 kilobases, considerably larger than pC194. Other colonies were checked but all other colonies tested which grew on chloramphenicol contained plasmid DNA identical to pC194, as far as could be determined by digestion of the DNA and comparison with molecular weight markers on an agarose gel -

see Figure 7.1.

So what was the reason for the difference in size in only one colony picked after the transformation? Experiments were carried out to determine the phenotype of bacteria carrying this plasmid, particularly with respect to their behaviour in chemostat culture, and the nature of the plasmid itself.

For convenience this plasmid was designated pC194-K1, K1 referring simply to its location on the original master plate used to screen the transformants.

Results

To determine the stability of pC194-K1 during chemostat culture of its host a single colony was grown up from the transformation plate in the usual way in preparation for inoculation of the chemostat (see the Materials and Methods section, chapter 2). After about 20 generations under carbon limitation a significant number of chloramphenicol resistant cells began to appear in the culture - see Figure 7.2. Plasmid extractions were carried out on both the chloramphenicol-resistant and chloramphenicol-sensitive bacteria on the sample plates. Colonies which did not grow on chloramphenicol-containing plates were invariably found to contain no detectable plasmid DNA, but the chloramphenicol-resistant bacteria in all cases determined, contained a plasmid the same size as the pC194-K1 in the chemostat inoculum.

The plasmid was therefore segregationally unstable, but, as far as could be determined, structurally stable.

Figure 7.1: Agarose gel of pC194 extracted from B. subtilis transformants

Plasmid DNA was extracted from B. subtilis transformants by the alkali denaturation method of Birnboim and Doly (1979) and purified on caesium chloride-ethidium bromide gradients. Representative samples are shown here. The DNA was linearised by digestion with HindIII and subjected to agarose gel electrophoresis on a 0.7% (w/v) agarose gel as described in the Materials and Methods, section 2.4.1.

Track: 1 HindIII-digested λ DNA, molecular weight standard

2 HindIII-digested pC194 from colony 1

3 HindIII-digested pC194 from colony 2

4 HindIII-digested pC194 from colony 3

5 HindIII-digested pC194 from colony 4

6 HindIII-digested λ DNA, molecular weight standard

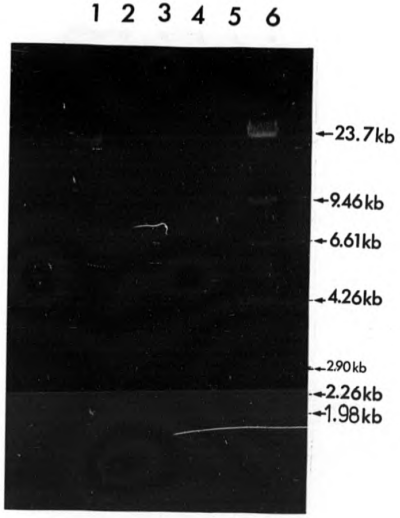
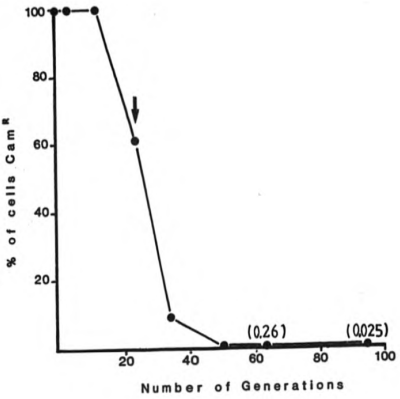

1 2 3 4 5 6
23.7kb
9.46kb
6.61kb
4.26kb
2.90kb
2.26kb
1.98kb

Figure 7.2: Chemostat culture of pC194-K1-carrying *B.subtilis* under carbon limitation

The culture was maintained at a dilution rate of 0.34 h^{-1}. The number of generations was calculated using the time that dilution of the culture began as the starting point. A steady state viable count of 6.4 (+ or - 0.61) x 10^8 was achieved at 24.11 generations, indicated by the arrow on the graph. For chemostat culture conditions see the Materials and Methods section, chapter 2.


100
80
60
40
20
% of cells Cam^R
(0.26)
(0.025)
20
40
60
80
100
Number of Generations

Provided that chloramphenicol was present in the medium, even after numerous successive sub-cultures (of about 20 generations each) no significant loss of the plasmid or alteration of its size or restriction endonuclease digestion pattern was detected.

The effects of different nutrient limitations on maintenance of pC194-K1 were next investigated. The results illustrate the same trend as implied in earlier competition experiments (see chapter 5 discussion). Magnesium limitation was perhaps slightly more selective for plasmid-free cells than carbon limitation (see Figure 7.3), but phosphate limitation was significantly more stringent a selection regime (see Figure 7.4).

In competition with 1% plasmid-free cells, cells harbouring pC194-K1 were rapidly displaced from chemostat culture under each of the three nutrient limitations (see Figures 7.5, 7.6 and 7.7). Plasmid-free cells therefore have a significant growth advantage over pC194-K1-carrying cells so if they did arise in the culture as the result of loss of pC194-K1 they would be expected to take over rapidly. Again, under phosphate limitation plasmid-free cells appear in the culture at a faster rate than under magnesium or carbon limitation.

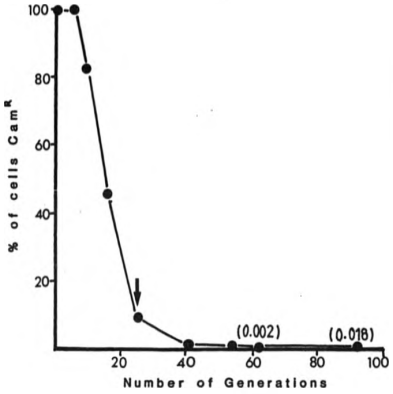
As had been observed before, a basal level of about 2% of chloramphenicol resistant cells persisted in the culture and even after 150 generations were not lost, nor did they increase in number. As a result of competition from plasmid-free cells the pC194-K1-carryng cells were reduced to approximately 0.025% of the population under carbon limitation, 0.018% under magnesium and to 1% under phosphate

Figure 7.3: Chemostat culture of pC194-K1-carrying B.subtilis under magnesium limitation.

The culture was maintained at a dilution rate of 0.34 h^{-1} and the number of generations calculated using the time when dilution of the culture began as the starting point. A steady state viable count of 1.21 (+ or - 0.31) x 10^8 colony forming units ml^{-1} was achieved by 23 generations, indicated by the arrow on the graph. For chemostat culture conditions and sampling procedure see the Materials and Methods section, chapter 2.

Figure 7.4: Chemostat culture of pC194-K1-carrying B.subtilis under phosphate limitation

The culture was maintained at a dilution rate of 0.34 h^{-1} and the number of generations calculated using the time that dilution of the culture began as the starting point. A steady state viable count of 2.5 (+ or - 0.10) x 10^8 colony forming units ml^{-1} was achieved by 19.25 generations, indicated by the arrow on the graph. For chemostat culture conditions and the sampling procedure see the Materials and Methods section, chapter 2.


100
80
60
40
20
% of cells Cam^R
(0.002)
(0.018)
20
40
60
80
100
Number of Generations

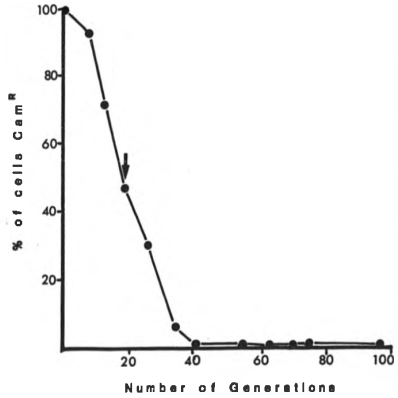

100
80
60
40
20
% of cells Cam^R
20
40
60
80
100
Number of Generations

Figure 7.5: Chemostat culture of pC194-K1-carrying *B.subtilis* under carbon limitation and in competition with 1% plasmid-free cells

Plasmid-free cells were mixed with plasmid-carrying cells before inoculation as described in the text. The culture was maintained at a dilution rate of 0.34 h^{-1} and the number of generations calculated using the time that dilution of the culture began as the starting point. A steady state viable count of 6.01 (+ or - 0.07) x 10^8 colony forming units ml^{-1} was achieved by 17.09 generations, indicated by the arrow on the graph. For chemostat culture conditions and the sampling procedure see the Materials and Methods section, chapter 2.

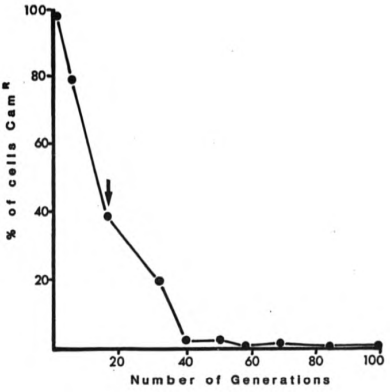
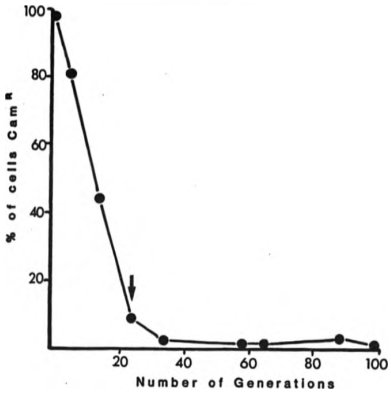

100
80
60
40
20
% of cells Cam^R
20
40
60
80
100
Number of Generations

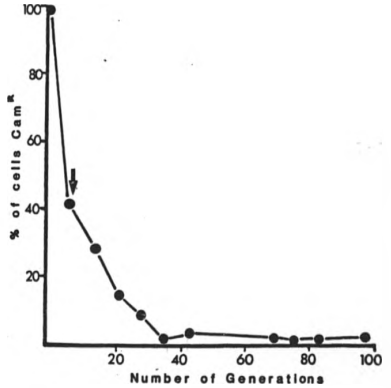
Figure 7.6: Chemostat culture of pC194-K_r-carrying B.subtilis under magnesium limitation and in competition with 1% plasmid-free cells

Plasmid-free and plasmid-carrying cells were mixed before inoculation of the chemostat as described in the text. The culture was maintained at a dilution rate of 0.34 h^{-1} and the number of generations calculated using the time that dilution of the culture began as the starting point. A steady state viable count of 5.70 (+ or - 0.60) x 10^7 colony forming units ml^{-1} was achieved by 20.93 generations, indicated by the arrow on the graph. For chemostat culture conditions and sampling procedure see the Materials and Methods section, chapter 2.

Figure 7.7: Chemostat culture of pC194-K_r-carrying B.subtilis under phosphate limitation and in competition with 1% plasmid-free cells

Plasmid-free and plasmid-carrying cells were mixed before inoculation of the chemostat as described in the text and the culture maintained at a dilution rate of 0.34 h^{-1}. The number of generations elapsed was calculated using the time that dilution of the culture began as the starting point. A steady state viable count of 2.42 (+ or - 0.04) x 10^8 colony forming units ml^{-1} was achieved by 13.78 generations, indicated by the arrow on the graph. For chemostat culture conditions and sampling procedure see the Materials and Methods section, chapter 2.


100
80
60
40
20
% of cells Cam^R
20
40
60
80
100
Number of Generations


100
80
60
40
20
% of cells Cam^R
20
40
60
80
100
Number of Generations

limitation. The nature of these cells was of particular interest in this case since pC194-k1 was otherwise extremely unstable under these conditions and would be expected to be eventually completely lost from the culture.

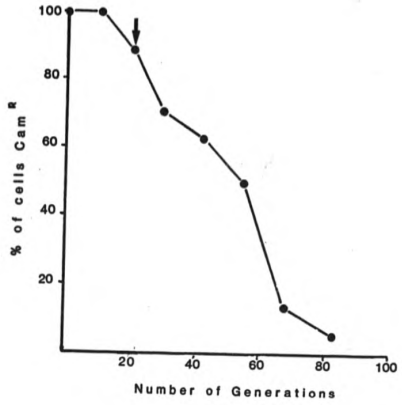
In order to determine whether a specific host-plasmid relationship was responsible for the maintenance of pC194-K1 in these cells a single colony was taken from a sample plate at the end of the chemostat run under carbon limitation (see Figure 7.2) and used to inoculate a new chemostat culture, again under carbon limitation. As can be seen from the graph in Figure 7.8, the chloramphenicol resistance phenotype was again lost rapidly from the chemostat culture although not quite as fast as in the earlier experiment. So, on the whole, retention of pC194-K1 by these cells seemed to be a feature of their existence in the first culture along with plasmid-free cells comprising 99.09% of the culture. Possible reasons for this will be discussed later in this chapter.

The strain carrying pC194-K1 originated from a single colony which arose after transformation of B.subtilis 168 trp with pC194 DNA. It may be, therefore, that the host clone carrying this plasmid was somehow involved either in the instability of an otherwise stable plasmid or in the generation of an unstable derivative of this plasmid. In order to examine the involvement of the host strain the following experiment was carried out:

Several chloramphenicol-resistant colonies from a culture of B.subtilis 168 trp (pC194-K1) which had undergone 100 generations of growth under carbon limitation were transferred to fresh nutrient agar plates and checked once

Figure 7.8: Repeat chemostat culture of pC194-K1 -carrying B.subtilis

A colony still chloramphenicol resistant after 100 generations of chemostat culture of B.subtilis 168 trp (pC194-K1) was used to inoculate a new chemostat run, again under carbon limitation and at a dilution rate of 0.34 h^{-1}. The number of generations was calculated using the time that dilution of the culture began as the starting point. A steady state viable count of 1.70 (+ or - 0.13) x 10^8 colony forming units ml^{-1} was achieved by 21.60 generations, indicated by the arrow on the graph. For the culture conditions and the sampling procedure see the Materials and Methods section, chapter 2.


100
80
60
40
20
% of cells Cam R
20
40
60
80
100
Number of Generations

again for chloramphenicol resistance and for their plasmid content. One colony grew on chloramphenicol this time and contained plasmid DNA but the other colonies were again found to be chloramphenicol sensitive and to contain no detectable plasmid DNA. Competent cells were then prepared using two of these latter colonies. One competent cell prepartion was used as a negative control to which no DNA was added and the other was transformed with wild-type pC194 (as described in section 2.4.4 of the Materials and Methods chapter). The negative control to which no DNA had been added produced an unexpected result when plated on chloramphenicol-containing nutrient agar. A large number of colonies grew up which were then found to contain plasmid DNA identical in size and MboI digestion pattern to pC194-K1.

The culture, therefore, despite being chloramphenicol-sensitive and containing no detectable plasmid DNA now contained a large number of chloramphenicol-resistant, pC194-K1-carrying cells (almost a complete lawn of cells grew up after 100µls of the competent cells were spread on nutrient agar containing chloramphenicol).

The original colony which was used to inoculate the competence medium was then purified through six cycles of growth on nutrient agar plates (approximately 120 generations) in the absence of chloramphenicol. A single colony was used to inoculate a Penassay broth culture and after incubation samples of this culture were, i) streaked for single colonies on nutrient agar and, ii) spread on chloramphenicol-containing nutrient agar. No growth was observed on nutrient agar which contained chloramphenicol.

Nevertheless, a single colony from this culture (from i) which did not grow in the presence of chloramphenicol, when grown in competence medium with no addition of plasmid DNA and then plated on chloramphenicol-containing nutrient agar, grew to form a confluent lawn of chloramphenicol-resistant cells which contained plasmid DNA identical, as far as could be determined, to pC194-K1.

This procedure was repeated with five other colonies and the same results were obtained. It therefore seems that a small number of colonies in the culture always retained plasmid DNA and when the cells in the culture became competent either plasmid DNA was released into the culture and was taken up by competent cells or the plasmid-carrying cells were greatly favoured in some way.

The cause of the increase in the size of pC194-K1 was next investigated by analysis of the restriction endonuclease digestion products of the plasmid. pC194-K1 DNA was digested with a number of restriction endonucleases known to cut pC194 and the sizes of the fragments generated were compared with their expected sizes. A map of the restriction endonuclease sites on pC194, published by Horinouchi and Weisblum (1982b), was used as reference. The results are summarised for convenience in Table 7.1 and the digestion products themselves as they appeared on agarose gels are shown in Figures 7.9 to 7.11 inclusive.

Table 7.1: pC194-K1 digestion products.

Enzyme	Expected Products	Products Obtained	Gel
		7.60 kb	
HindIII	2.90kb	2.70kb 4.10kb	Fig 7.9
ClaI	1.68kb 1.20kb	1.67kb 5.80kb*	Fig 7.10
MboI	2.00kb 0.87kb	1.98kb 1.34kb 0.78kb	Fig 7.11
HaeIII	2.90kb	2.55kb & Small Fragments	Fig 7.11

Key: kb = kilobases

* = Not accurate

Samples of pC194-K1 from large scale plasmid preparations from B.subtilis 168 trp were digested according to the protocol outlined in section 2.4.2 and separated on 2.5%W/V agarose gels. Photographs of the appropriate gels are presented in Figures 7.9 to 7.11 inclusive.

Figure 7.9: HindIII digestion of pC194-K1

Plasmid DNA was extracted from B.subtilis 168 trp (pC194-K1) by the method of Birnboim and Doly (1979) and purified on a caesium chloride-ethidium bromide gradient. The lower band from the gradient (see Figure 7.12) was digested with HindIII by the procedure described in the Materials and Methods, section 2.4.1 and run on a 0.7%(w/v) agarose gel.

Track 1 HindIII-digested λDNA molecular weight standard
2 pC194-K1 undigested
3 HindIII-digested pC194-K1
4 pC194-K1 undigested
5 HindIII-digested λDNA molecular weight standard

The sizes of the λ HindIII molecular weight standard bands are given in kilobases

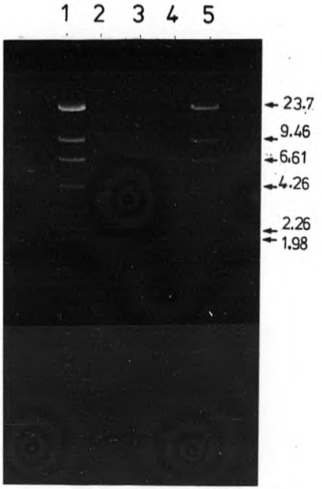

1 2 3 4 5
23.7
9.46
6.61
4.26
2.26
1.98

Figure 7.10: ClaI digestion of pC194-K1

Plasmid DNA extracted from B.subtilis 168 trp (pC194-K1) by the large scale alkali extraction method of Birnboim and Doly (1979) and purified on a caesium chloride gradient was digested with ClaI by the procedure described in the Materials and Methods, section 2.4.1 and run on a 2.5% (w/v) agarose gel.

Tracks: 1 HindIII-digested λ DNA, molecular weight standard
2 pC194-K1 undigested
3 ClaI-digested pC194-K1
4 Sau3A-digested pC194-K1
5 HaeIII digest of pBR322 DNA, molecular weight standard
6 Sau3A-digested pC194
7 CalI-digested pC194
8 undigested pC194
9 HindIII-digested λ DNA, molecular weight standard

The sizes of the λ HindIII molecular weight standard bands are given in kilobases

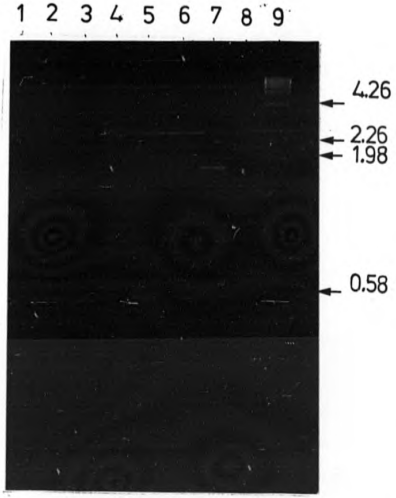
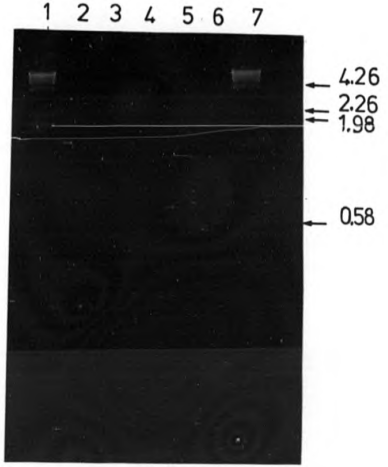

1 2 3 4 5 6 7 8 9
4.26
2.26
1.98
0.58

Figure 7.11: MboI and HaeIII digestions of pC194-K1

Plasmid DNA was extracted from B.subtilis 168 trp (pC194-K1) by the large scale alkali denaturation method of Birnboim and Doly (1979) and purified on a caesium chloride-ethidium bromide gradient. Samples of the DNA were digested with MboI and with HaeIII and run on a 2.5% (w/v) agarose gel.

Tracks: 1 HindIII digest of λ DNA, molecular weight standard
2 MboI digested pC194-K1
3 undigested pC194-K1
4 Sau3A digested pC194-K1
5 HaeIII digested pC194-K1
6 HaeIII digested pBR322 (degraded)
7 HindIII digest of λ DNA, molecular weight standard

The sizes of the λ Hind III molecular weight standard bands are given in kilobases


1 2 3 4 5 6 7
4.26
2.26
1.98
0.58

It should be noted that, when pC194-K_1 was purified on caesium chloride-ethidium bromide gradients, three distinct bands were obtained but when each band was extracted and digested separately with Hind III the same digestion products were obtained in each case (although most of the digestions seem to be partial despite extended incubation and for no apparent reason) - see Figure 7.12. The bands do not therefore represent three different plasmids.

The generation of digestion products by these enzymes was consistent with an insertion of 4.1-4.5 kilobases at, or near to, the HindIII site on pC194 which did not remove the HindIII site but which created another HindIII site. The HaeIII digestion products are particularly interesting since the pattern of smaller fragments generated is very similar to that generated by digestion of pBR322 with HaeIII (compare Figure 7.11 track 5 with Figure 7.10 track 5). In fact when the digestion products generated by the other restriction endonucleases used were examined they could be seen to correspond closely to the expected products of digestion of pHV14 (pBR322 and pC194 joined at their HindIII sites - see Figure 7.13) - see Table 7.2.

Discussion

The highly unstable plasmid pC194-K1 described in this chapter presents a number of problems. The analysis of the restriction endonuclease digestion products indicates that this plasmid is at least very closely related to pHV14 in its physical properties. It was isolated from what should have been a pure sample of pC194 DNA so two explanations can be proposed: either the original DNA sample was contaminated with pHV14 DNA or a sample of pHV14 was mislabelled at some stage and had become uniformly deleted leaving only the pC194 moiety. In either event, if pC194-K1 originated from a

Figure 7.12: HindIII digestion of sub-fractions of pC194-K1 DNA from a caesium chloride gradient

The three bands obtained on purification of pC194-K1 on a caesium chloride-ethidium bromide gradient were digested seperately with HindIII and the products separated on a 0.7% (w/v) agarose gel. The sizes of the λ HindIII fragments are indicated alongside the photograph.

Track: 1 HindIII digested λ DNA, molecular weight standard

2 Lower band from gradient, HindIII digested

3 Lower band from gradient, undigested

4 Middle band from gradient undigested

5 Middle band from gradient, digested with Hind III

6 Upper band from gradient, undigested

7 Upper band from gradient digested with HindIII

8 HindIII digested λ DNA, molecular weight standard

The sizes of the λ Hind III molecular weight standard bands are given in kilobases

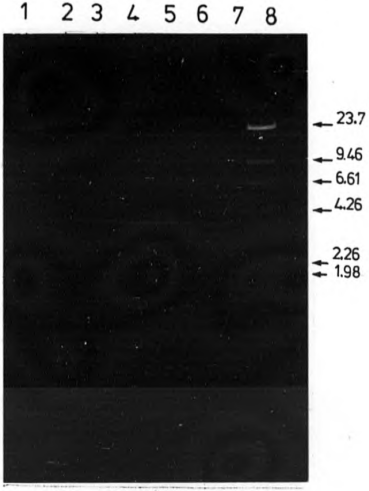

1 2 3 4 5 6 7 8
23.7
9.46
6.61
4.26
2.26
1.98

Table 7.2: pHV14 - expected digestion products

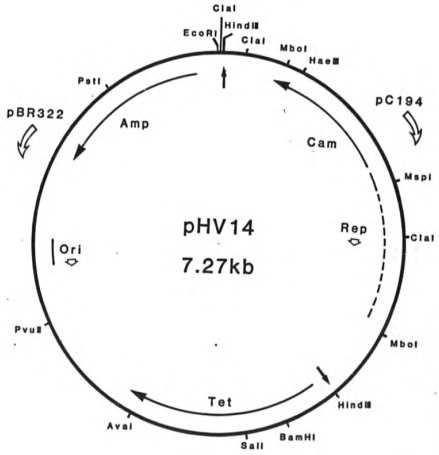
Enzyme	Expected Digestion Products
HindIII	2.80kb 4.36kb
ClaI	6.07kb 1.20kb
MboI	2.00kb 1.40kb 0.58kb 0.60kb
HaeIII	2.4kb & Small Fragments

Key: kb = kilobases

The sizes of the digestion products expected from restriction endonuclease digestion of pHV14 were calculated from the maps of pC194 and pBR322 published by Horinouchi and Weisblum (1982b) and Sutcliffe (1978) respectively and from the map of pHV14 shown in Figure 4.1.

Figure 7.13: Map of pHV14

The relative orientations and locations of the origins of replication of both pC194 and pBR322 are shown. The direction of replication in each case is indicated by an arrow. Restriction sites for the enzymes HindIII, EcoRI, MboI and ClaI are shown on both pC194 and pBR322 genomes. The enzymes which cut at single sites on pBR322 and the single HaeIII site on pC194 are also indicated (the HaeIII sites on pBR322 are omitted for the sake of clarity). The antibiotic resistance determinants of the two component plasmids are represented by arrows outside the map itself (Sutcliffe, 1978 ; Horinouchi and Weisblum, 1982b).


ClaI
HindIII
EcoRI
ClaI
MboI
HaeIII
PstI
pBR322
Amp
pC194
Cam
MspI
pHV14
7.27kb
Rep
ClaI
Ori
PvuII
MboI
Tet
HindIII
AvaI
SalI
BamHI

sample of pHV14 DNA it represents a plasmid which remains highly segregationally unstable but which is no longer structurally unstable. It would have been of interest to determine whether this plasmid has perhaps suffered a small structural alteration which precludes the occurrence of further, major structural alterations.

There is, however, an alternative explanation for the appearance of this unidentified plasmid which can be proposed in the light of a report by Shishido et al. which appeared in 1983. They found that a tetracycline resistance plasmid of 4.9 kb was generated in protoplasts of a Bacillus subtilis strain when infected with another, Staphylococcus aureus tetracycline resistance plasmid (pNS1) which itself was incapable of replication and did not carry a functional tetracycline resistance gene.

The 4.9 kb plasmid (designated pNS1981) which they isolated was completely unrelated to pNS1 and was shown to be identical to pBC16. So it was proposed that B.subtilis carries an integrated copy of pNS1981 (pBC16) at a single site in its chromosome and this sequence can be excised by introduction of a certain segment of pNS1 which lacks the property of autonomous replication. It is conceivable that something similar may have occurred in the B.subtilis strain used here when it was transformed with pC194-K_1 lacking an intact replication function.

Shishido et al. detected excised pNS1981 (when infecting with replication-deficient pNS1) at a frequency of 10^{-3} fold lower that they detected normal transformants using pNS1. The frequency of appearance of the unidentified plasmid designated pC194-K_1 cannot of course be estimated but it does seem to be likely that it was a rare event.

The reference to the work of Shishido et al. was only obtained after this experimental work was completed so it is not possible to determine by restriction mapping whether any part of pC194-K_1 bears resemblance to pBC16. There is a possibility of course that part of pC194-K_1 may have derived from excision of a plasmid sequence other than that of pBC16 in the host chromosome, or even a chromosomal sequence.

Regardless of the nature of the plasmid DNA the fact remains that what is assumed to be a derivative of pC194 is highly unstable in B.subtilis. In the absence of further work to characterise this instability it is difficult to speculate on its cause. Presumably the plasmid loss observed could be brought about either by the failure of the plasmid to be replicated resulting in a drop in its copy number or by the failure of plasmid copies to be distributed to daughter cells on cell division.

If the loss of pC194 is explained by either occurrence it should be possible to account for the lag period observed before its loss from carbon-limited culture (see Figure 7.2) by assuming this to be the time taken for the copy number to drop sufficiently to result in loss of resistance to chloramphenicol.

If pC194-K_1 copies are randomly distributed to daughter cells then if all plasmid replication stopped, after only two cell divisions the wild type copy number of 15 would be reduced to 3 or 4. The lag period, however, including the number of generations elapsed during batching up amounted in each case to about 25 generations, too long to account for an immediate precipitous drop in the copy number of pC194-K_1 on removal of selection for chloramphenicol resistance, even from a far higher copy number.

So it seems rather more likely that some event occurred soon before the observation of loss of pC194-K_1 from the culture, in magnesium- and phosphate-limited cultures as well as under carbon limitation, which was responsible for the instability of the plasmid. Once again one can only speculate as to its nature.

An explanation for the sudden loss of pC194-K_1 from the chemostat culture originally suggested by Kurt Nordström (Biomedical Centre, Uppsala, Sweden) is that the plasmid became integrated into the host chromosome. The sudden reduction in the number of chloramphenicol resistant cells in the culture would in this case be the result of the very low or negligible chloramphenicol resistance of a cell carrying a single integrated copy of the plasmid.

There is some support for the idea that pC194 may become integrated into the host chromosome. In S.aureus pC194 may be involved in illegitimate recombination events (Iordanescu, 1975 ; Iordanescu et al., 1978). Martin et al. (1981) referred to a personal communication from R. Novick who apparently found that pC194 was able to be integrated into specific sites in the S.aureus genome.

So perhaps pC194-K_1 was replicated stably for several generations in the absence of selective pressure without loss of the plasmid or significant lowering of its copy number but was then lost rapidly from the culture while a sub-population of plasmid copies became integrated into the chromosome.

The continued detection of small numbers of chloramphenicol-resistant, plasmid-carrying cells might then be explained by a residual number of plasmid copies recombining out of the chromosome and, freed of copy number restraints, achieving a copy number sufficient to confer chloramphenicol resistance on their host cells at the level selected for.

A note of caution should be introduced at this point in the analysis of the results of experiments using pC194. As was stated in the introduction, Chapter 1 (pages 52 and 53) the gene on pC194 coding for chloramphenicol acetyltransferase (CAT) is inducible and induction is at the level of translation, an unusual feature of bacterial inducible genes (Duvall and Lovett, 1986 - reference added in proof).

Bacteria removed from the chemostat have been grown in the absence of selection for the plasmid which they carry and the plasmid CAT gene is in the uninduced state. Depending of course on the level of CAT mRNA (which may, in turn, depend on the copy number of the plasmid) and the stability of the mRNA, there will be a delay between the time when selection is imposed on the bacteria and the time when the level of CAT becomes sufficient to confer chloramphenicol resistance.

When selecting for chloramphenicol-resistant bacteria from the chemostat using a high concentration of chloramphenicol there is a danger that the bacteria will be killed before sufficient CAT is available. The chances of this occurring when the plasmid copy number is low (as it may be

after long term culture in the absence of selective pressure) might be higher because, although the initial time for the response to chloramphenicol to occur may not be much slower, the final level of CAT would be lower.

So it would have been advisable during these experiments to plate the bacteria out initially on a lower concentration of chloramphenicol and then to reculture them on a higher concentration, that is, the 40 ug/ml used routinely for these experiments. The level of chloramphenicol required to induce translation of the CAT mRNA is sub-inhibitory (2 ug/ml) so only very low concentrations need be used for the induction.

In the case of the experiments using the "wild type" pC194 the time required for induction of the CAT gene did not seem to create any problem for the development of chloramphenicol resistance because there was always very little or no difference between the viable counts on nutrient agar and on nutrient agar containing chloramphenicol, and the 100 colonies growing on nutrient agar which were transferred to chloramphenicol plates invariably grew as well on chloramphenicol as on nutrient agar.

During the culture of the pHV14-F-carrying strain described in Chapter 4 the appearance of micro-colonies (see page 111) indicates that at least a proportion of the bacteria in the culture contained plasmid at a lower than normal copy number because these colonies, when transferred to chloramphenicol-containing nutrient agar for a second time grew to a normal size. Despite their lowered copy number these bacteria were still able to grow in the presence of chloramphenicol so the fact that their resistance required to be induced may not have been a significant factor in impairing their growth.

In fact, induction of the CAT gene at the level of translation possibly allows faster reaction to inducing levels of the antibiotic than induction at the level of transcription because translation induction, in the case of pC194 CAT, is not dependent on concurrent transcription. pE194 uses a very similar means of induction of its erythromycin-resistance gene

If integration of sub-populations of plasmids into the B.subtilis chromosome is a common phenomenon this explanation may also apply to the low level of continued maintenance in the cases of pUB110 and pBC16 (see Chapter 6) and in the case of pBC16 there is some evidence that integration could be a common occurrence (Shishido et al., 1983 and see page 173).

The persistence of a small number of plasmid-carrying cells in the chemostat culture appears to be mirrored by the results of the attempt to cure a pC194-K_1-carrying strain on a rich medium both on solid and in liquid batch medium.

Insertion at the Hind III site therefore considerably disrupts replication of pC194 in B.subtilis whether it be due to the actual nature or size of the inserted DNA or the position on the pC194 replicon at which it is inserted. Of relevance to the involvement of the sequence in the vicinity of the Hind III site in the instability of pC194-K_1 is the

observation by Canosi et al., (1981) that a derivative of pC194 with foreign DNA inserted at the HindIII site was structurally and segregationally stable.

Some experiments were, in fact, set up to determine to what extent this phenomenon was dependent on the nature of the inserted DNA and to examine the possibility that loss of the integrity of this region in itself was sufficient to cause pC194 to become segregationally unstable. The following manipulations were carried out:

(i) pC194 (wild type) was digested with MboI which cuts at two sites (see Table 7.1). The digestion products were separated on an agarose gel and the larger of the two fragments removed from the gel using DE81 paper (see Materials and Methods, Chapter 2). This DNA was first diluted 100 fold to discourage production of oligomers and then ligated to create a plasmid of 2.0 kilobases lacking the MboI B fragment

(ii) pC194 (wild type) was cut with HindIII and then mixed with an excess of DNA from a HindIII digestion of phage lambda DNA and the mixture ligated with the intention of producing a number of pC194 replicons with DNA insertions of different sizes at their HindIII sites.

These experiments were, however, unsuccessful since no transformants were obtained even after several repetitions. The positive controls also failed to produce transformants and it was later realised that this was not a fault of the competent cell preparation method but was due to the fact that the B.subtilis strain used was no longer capable of becoming competent. The instability of pC194-K1 therefore remains something of a mystery.

CHAPTER 8

OVERALL CONCLUSIONS AND OUTLOOK

In Chapters 3 to 7 of this thesis a large number of chemostat experiments have been described and in the great majority of these, particularly in Chapters 4, 5 and 6 there was a clear illustration of one of the major problems inherent in this type of chemostat work, namely, the difficulty in rapidly achieving a stable, steady state. When the long term stability of the culture environment is particularly important then the period of batching up and the time taken for the culture to reach steady state which follows should be given particular attention. During this time the culture conditions are constantly changing in an unknown and uncontrolled way and, as was noted in Chapter 4, changes can also take place within the plasmid-carrying portion of the bacterial population. In Chapter 6 a considerable change was recorded in the proportions of plasmid-carrying and plasmid-free cells during the period immediately following batching up which was suggested to be primarily due to perturbation of the culture before steady state was reached. It is therefore of importance that the culture should be sampled throughout the period of batching up as well as throughout the remaining culture time.

There is a considerable variation inherent in the results of such experiments - variation between results of different runs in the same chemostat as well as variation between results obtained in different chemostats which is more to be expected. This is a problem which would be difficult to overcome completely but which can be controlled to some extent by use of reliable chemostat equipment with sensitive control systems and by sampling in duplicate etc.

If these points are borne in mind and measures taken to reduce their effect then the method of chemostat culture is a powerful tool in the study of plasmid biology.

Although there is still little of real significance understood of the maintenance system of plasmids in Gram positive bacteria and less, before this work, of their behaviour in chemostat cultures, we are perhaps now approaching a situation where it becomes possible to draw parallels between replication control of these plasmids and what is already known of plasmids in E.coli, the archetypal Gram-negative bacterium.

Recently a model for pUB110 replication control was proposed by Ano et al. (1986) which was complex and far reaching in its predictions but seriously lacking in hard evidence as support. A system for inhibition of initiation of replication at the levels of both transcription and translation mediated by small RNA species was envisaged, analagous to the replication control systems of ColE1 and the IncFII plasmids in E.coli.

In the light of recent reports of the involvement of single stranded intermediates in the replication of pE194, pC194 (te Reile et al., 1986) and possibly pUB110 (S.D.Ehrlich, personal communication), it is perhaps appropriate that this type of model which relies heavily on the analogy with E.coli plasmids should be re-thought to take into account the likely existence of quite different mechanisms of replication and its control in these plasmids in Gram-positive bacteria.

Investigation of the involvement of the production of single stranded DNA in recombination of plasmids in B.subtilis and the implication for the structural stability of these plasmids when linked to foreign DNA may point the way to a better understanding of the mechanism of recombination which leads to the structural alterations which are so much more common in B.subtilis than in E.coli

The three plasmids, PC194, PUB110 and pBC16 show a remarkable degree of stability in B.subtilis 168 trp in comparison with, for instance, the plasmid RP1 which, unlike the above plasmids, is displaced from chemostat culture by plasmid-free cells at 1% of the inoculum (Jones et al., 1980 and 1984 , Melling et al., 1977). The mechanism(s) behind this stable maintenance does indeed warrant further investigation. Indeed, the fact that plasmid-free cells at 1% of the inoculum failed to displace the cells carrying either pC194, pUB110 or pBC16 (whereas plasmid-free cells at 50% of the inoculum were successful) implies that additional processes other than control of replication are involved in their maintenance.

Two distinct phenomena are involved in the loss of an unstable plasmid from a chemostat culture which should each be given consideration. Firstly the molecular events leading to loss of the plasmid need to be understood but also the take-over of the culture by plasmid-free cells when they arise can contribute significantly to the disappearance of the plasmid from the culture. So, for instance, in the experiments described in Chapter 6 the loss of pC194, pUB110 and pBC16 from a mixed culture of plasmid-carrying and plasmid-free cells was probably due to the growth advantage of the plasmid-free cells as opposed to any inherent instability of either of these plasmids whereas both factors

were no doubt involved in the loss of pC194-K1 from *B subtilis*.

The rate at which take-over of a chemostat culture by plasmid-free cells occurs has yet to be explained satisfactorily, particularly if the concept of energetic demand is invoked to account for their growth advantage. At the present time there are conflicting reports of the growth difference between plasmid-free and plasmid-carrying cells (Dykhuizen and Hartl, 1983).

If plasmid-free cells out grow plasmid-carrying cells as readily and as often as is implied by a number of chemostat studies (for example, Jones, 1984 ; Godwin and Slater, 1979), the implications for plasmid maintenance *in vivo* are serious. It may be that this phenomenon is an artefact of chemostat culture but an analogy does exist in nature in the persistence of antibiotic-resistant organisms in the faeces of patients who have been treated with antibiotics. Richmond (1977) reported that after antibiotic therapy ceased the level of antibiotic-resistant organisms, although considerably reduced, never fell to zero.

In the discussion of Chapter 7 an integrated copy of the plasmid in the host chromosome was suggested as the source of the low level of plasmid-carrying cells detected at the end of a chemostat run when all other cells in the culture had lost their plasmid. In the absence of another explanation for this phenomenon (such as wall growth of the host strain or a breakdown of steady state conditions in the chemostat culture) integration of the plasmid is offerred as a simple explanation for the basal maintenance of plasmid-carrying, antibiotic resistant cells in the culture.

It is difficult to imagine otherwise how a sub-population of plasmid-carrying cells are maintained so stably in the culture. Transfer of plasmid molecules between cells may occur, particularly if a number of the cells in the culture are competent. Frequencies of transfer of plasmids in chemostat cultures have been reported (Dykhuizin and Hartl, 1983) but these are in general too low to account for the number of plasmid-carrying cells detected.

The involvement of binding of plasmids to the cell membrane as a means of partitioning or as a necessary part of replication and the concommitant expression of incompatibility has attracted considerable interest, particularly recently.

In this respect the membrane binding of pUB110 represents a relatively well characterised model system for the involvement of the cell membrane in initiation of replication, although its range of application remains to be determined. The origin of replication of pUB110, on initial inspection (McKenzie *et al.*, 1986), lacks the common features of other replication origins that have been characterised.

So whether it be for their unusual stability, their mode of replication or their membrane binding properties the plasmids described in this thesis represent very interesting subjects whose study should greatly advance our knowledge of the biology of plasmids in Gram-positive species.

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AUTHOR RHIANNON SANDERS

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