Analysis of a Cell Division Gene Cluster in *Escherichia coli*

by

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Part Two

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Declaration

This thesis has been composed by myself and has not been used in any previous application for a degree. The results presented were obtained by myself, under the supervision of Dr. G.P.C. Salmond, with the exception of those instances where the contribution of others has been acknowledged. All sources of information have been specifically acknowledged by means of reference.

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Abbreviations

bp	Base pair
dNTP	Deoxyribonucleotide phosphates
dpm	Disintegrations per minute
DTT	Dithiothreitol
EDTA	Diaminoethane tetra-acetic acid
kb	Kilobase
moi	Multiplicity of infection
ml	Millilitre
OD	Optical density
PBP	Penicillin binding protein
pfu	Plaque forming unit
PNK	Polynucleotide kinase
PPO	2-5 diphenyl oxazole
SDS	Sodium dodecyl sulphate
TEMED	N N N' N' tetramethyl ethylenediamine
UV	Ultra-violet
PG	Peptidoglycan
PNK	Polynucleotide kinase

Summary

Several genes, essential for cell growth and division in Escherichia coli, have been mapped to the 76 minute region of the chromosome. DNA sequencing of part of this region revealed three cell division genes (ftsY, ftsE, and ftsX) in a putative operon. A fourth gene (orf4) was also identified that was transcribed in the opposite direction to the putative operon. The genes rpoH, fam, dnaM and ftsS have also been mapped to this region, but their location, relative to the putative operon, was unknown. In this study the fam and rpoH genes were independently cloned and shown to be allelic. The dnaM gene was also found to be an allele of rpoH, and the gene was found to lie immediately downstream of the putative cell division operon. The restriction map of the area was extended, and the distance between the putative operon and the nearest known gene clockwise on the chromosome map (pit), was determined. The ftsS gene was found to be an allele of ftsX. Two promoters were identified within the putative operon, one proximal to ftsY and the other proximal to ftsE. A combination of S1 and primer extension mapping, of the mRNA transcripts, identified the transcriptional start sites of these two promoters. A polycistronic message was also identified encoding all three cell division genes, suggesting at least some degree of co-ordinated expression.

In conclusion, the transcriptional organisation of the 76 minute, essential gene cluster has been determined, and evidence has been presented that there is some degree of co-ordinated expression of the component genes.

CHAPTER ONE

Introduction

Preface

The processes of cell growth and division are fundamental to all living organisms. Simpler organisms, such as bacteria, provide an amenable system for the study of cell growth and division due to their ease of manipulation, both physiologically and genetically. It can not be assumed though, that an understanding of the cell division process in bacteria will be directly applicable to other organisms. However, so many parallels between prokaryotic and eukaryotic systems have emerged in the past that we can be optimistic that knowledge gained by working with bacteria will facilitate the study of the more complex systems of higher plants and animals.

Escherichia coli has emerged as the most studied unicellular organism and many techniques have been developed for manipulating this bacterium. For these reasons E.coli has proved a most useful organism for the study of cell division, a process which appears complex even in the most primitive of organisms.

This chapter discusses much of the work which, to date, has contributed to an understanding of the cell division process in E.coli. The first section describes two major areas of research employed in the study of E.coli cell division. Firstly, the measurement of physical parameters such as cell length, cell volume and DNA content throughout the cell cycle, and secondly, the use of genetic analysis. Measurement of the cell cycle

variation of physical parameters has allowed the formation of a basic descriptive model for the cell growth and division cycle of E.coli. Genetic analysis has then allowed this basic model to be dissected into several discrete stages, characterised by a series of mutant phenotypes. The basic model and its partial characterisation by genetic analysis will be discussed in this section.

The following sections then describe how certain aspects of the cell growth and division cycle have been studied at the molecular level to provide a more detailed mechanism for the processes of cell growth and division.

PART

ONE

1.1 The Measurement of Physical Parameters

Much of the information required to form a basic model for the growth of E.coli has come from microscopic work. Using light microscopy individual cells can be easily seen and photographed. Cells appear essentially cylindrical with hemispherical poles; newly born cells elongating until they have doubled in length at which point they divide into two identical daughter cells. Accurate measurement of cells under different growth conditions and at various stages of the cell cycle has provided much detailed information about this seemingly simple cycle. Such measurements can be made directly using light or electron microscopy. A fundamental observation is that cells growing under different conditions have different dimensions; dividing cells in a rich medium are much longer than similar cells in a poor medium for example (Pritchard 1974). Indeed most cell parameters seem to vary with growth rate (number of doublings per hour), this is represented by equation 1.

$$\bar{P}_R = f(R) \qquad (1)$$

Where $\bar{P}_R$ is the mean value of a given parameter at growth rate R (Vicente 1984).

The mean cell volume of a population was found to increase

exponentially with increasing growth rate, as represented by equation 2 (Donachie 1981).

$$\bar{V}_R \propto 2^R \qquad (2)$$

Mean cell mass follows a similar relationship (Dennis and Bremer 1974).

$$\bar{M}_R \propto 2^R \qquad (3)$$

Mean cell length, however, follows a different relationship (Donachie 1981).

$$\bar{L}_R \propto 2^{R/3} \qquad (4)$$

Thus at growth rate three (R=3) the mean cell length is twice its value at growth rate zero, whereas the volume has increased eight fold over this range. The changes in cell diameter have proved more difficult to measure due to the small size of E.coli cells. However its relationship to growth rate can be approximately predicted by considering the cell as a cylinder. In this case:

$$V \propto L.D^2 \qquad (5)$$

and thus

$$\bar{D}_R \propto 2^{R/3} \qquad (6)$$

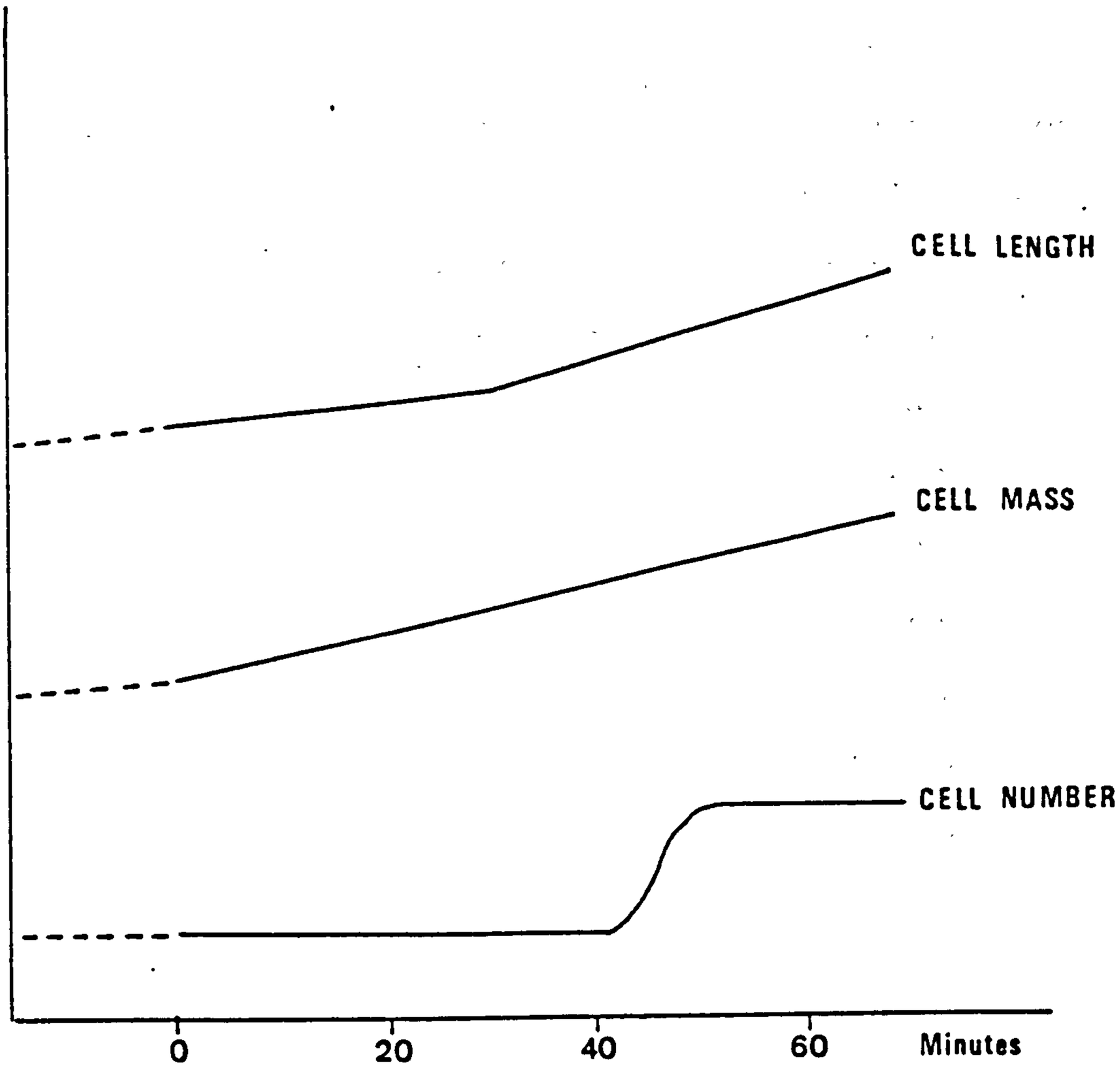
which agrees with the measurements that have been made (Pierucci 1978).

The above equations suggest the existence of several interesting relationships. One is that the ratio of cell length to cell diameter remains constant under differing growth conditions. This constant "shape" was first described by Zaritsky (1975). The ratio of surface area to volume does vary with growth rate and does so such as to decrease with increasing growth rate. This could represent the need for a relatively larger surface area under poorer growth conditions.

Of more interest, perhaps, is how the various cell parameters vary from birth to division. Length, volume and mass are all known to double and E.coli elongates continually over the cell cycle. The actual rate equation, whether it increases linearly, bilinearly or exponentially for example, is difficult to establish due to the small changes involved. In order to overcome this problem Donachie et al. (1976) attempted to magnify any possible changes by shifting a synchronously growing culture from a poor medium to a rich medium. Various methods were available for obtaining synchronously growing cultures, most relying on the selection, by size, of a discrete cell population (Helmstetter 1969). The results of such an experiment are shown in figure 1.1.

FIGURE 1.1 Changes in Cell Parameters after a Nutritional Shift

The nutritional shift-up occurred at time zero. The dotted lines represent pre-shift levels and the solid lines post-shift levels, of the various parameters. The ordinate is calibrated in arbitrary units of cell length, mass and number. The experimental procedure is described in the text.



Small cells were recovered, by sucrose gradient centrifugation, from a culture growing with a generation time of 67 minutes, and inoculated into a rich medium, in which the bacteria were expected to replicate with a generation time of 25 minutes. The cell mass immediately began to increase at the new rate and cell division occured some 40 to 60 minutes later. The elongation rate did not change immediately though, but increased to a higher rate after about 40 minutes. It was concluded that the cell continued elongating at the pre-shift rate until it reached a certain length, at whigh point a new elongation rate took over. These results led Donachie et al. (1976) to propose the existence of a "unit cell length", this being the length of a newly born cell at growth rate zero. Under constant conditions a newly born cell will elongate until it reaches a length (2A) equal to two unit lengths, the elongation rate will then double at this point. In shift-up experiments the rate will more than double in order to equilibrate with the new growth conditions. Cullum and Vicente (1978) used a more mathematical approach to arrive at a similar conclusion, and measured this length (2A) as between 3.5um and 4.5um compared with Donachie's estimate of 2.8um. The latter estimate, however, did not take into account any distribution of birth size.

In order to explain this doubling of elongation rate it was proposed that each unit cell length contained a single growth

site (Donachie et al. 1976). Upon attainment of a new unit length another growth site is activated and the elongation rate doubles. Begg and Donachie (1977) studied this by measuring the distribution of bacteriophage T6 receptor sites. They found that cells less than 2A elongate from only one pole whereas cells over 2A elongate from both poles.

Since in shift-up experiments, such as the one described above, the rate of increase in mass and volume occurs immediately whereas that of length does so only after a delay, the cell must increase in diameter during this delay period. During normal growth, under constant growth conditions, a cell elongates with little change in diameter (Trueba and Woldringh 1980).

Replication and segregation of the bacterial chromosome is obviously an essential step in the bacterial cell cycle. It has proved useful to divide this process into three stages that can relate to the cell division cycle (Helmstetter et al. 1979). The I period refers to the time required for the cell to prepare for the initiation of chromosome replication. After replication, which takes C minutes, there is a gap of D minutes before the cell divides. Helmstetter and Pierucci (1976) estimated the C and D intervals in E.coli cultures grown under various conditions, by measuring the incorporation of ^{14}C thymidine into high molecular weight DNA. Their results are shown in table 1.1.

TABLE 1.1 C and D Periods under Various Growth Conditions

Division Time	C Period	D Period
25-60 minutes	42 minutes	22 minutes
>60 minutes	~80 minutes	~40 minutes

Their data shows that over a range of growth rates the C and D periods remain fairly constant.* It can also be seen that in good growth conditions the (C+D) period is longer than the doubling time. Thus successive (C+D) periods must overlap. Indeed in very favourable conditions (doubling time of 25-40 minutes) the C periods must overlap. In this case the chromosome initiates a new replication fork before the previous round of replication has been completed. Since I is defined as the time required for a cell to prepare for initiation it can be considered as the inter-initiation time for a culture undergoing balanced growth (Campbell 1957). Figure 1.2 shows the correlation of the I,C and D periods with the cell division cycle for a cell growing with a doubling time of 30 minutes.

* Above this range of growth rates the C & D periods increase with increasing growth rate

FIGURE 1.2 Integration of the I, C and D Periods with a 30 Minute Cell Cycle

A diagrammatic representation of the cell cycle is shown, which includes the state of chromosome replication. At time zero the cell divides into two daughter cells.

Bm- birth of mother cell Bd- birth of daughter cell

Dd- division of daughter cell —ᘐ Initiation event

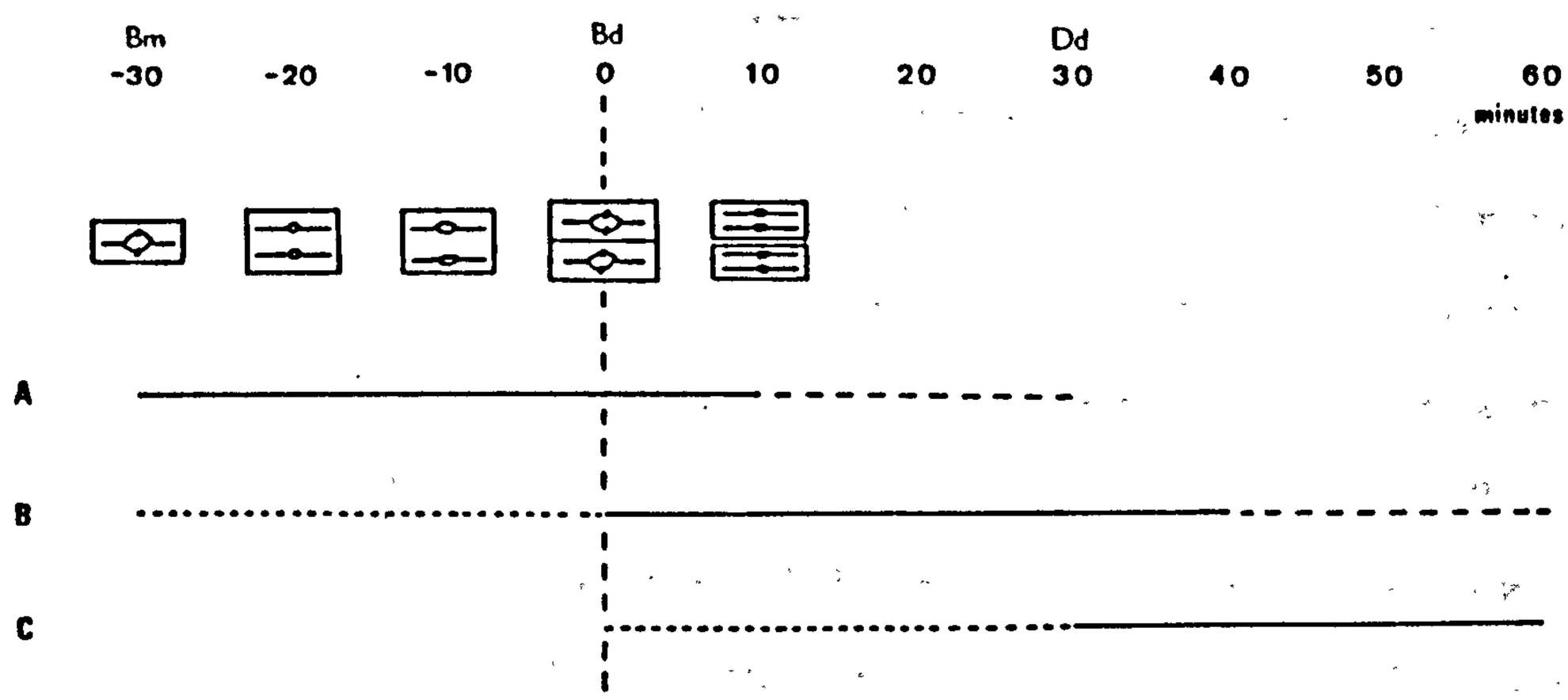
..... I period ——— C period ----- D period

A,B and C represent events occuring at time zero:

A- The eldest replication fork is 30 minutes old

B- The new replication fork is initiated

C- The cell starts to prepare for the next initiation event



Even though the cells are replicating every 30 minutes the C+D periods still take 60 minutes (40+20). Thus at birth a cells chromosome must have already spent 30 minutes replicating, that is a new round of chromosome replication must have been initiated at the birth of the mother cell. Consequently the newly born cell must immediately initiate a new round of replication and must also start preparing (the I period) for the next initiation event 30 minutes later. During the first 10 minutes after birth there are two active replication forks on the chromosome. The eldest fork terminates after 10 minutes leaving 20 minutes to prepare for division (the D period).

The relative invariance of the C and D periods with growth rate shows parallels with the eucaryotic cell cycle. Here the S (DNA synthesis), G2 (between DNA synthesis and mitosis) and M (mitosis) periods are relatively invariant, whereas the G1 period (prior to DNA synthesis) is highly variable. Events during the I period therefore seem to form the main control over chromosomal initiation. Table 1.1 shows that the D period is approximately 20 minutes long for cells growing with division times of 25 to 60 minutes. This 20 minute period is also the time taken by a cell between reaching the 2A length and dividing (Donachie et al. 1976). The termination of chromosomal replication and attainment of the two unit cell length thus seem to occur concurrently. It has been observed that the initiation of chromosome replication

occurs at a particular cell volume, the so called initiation volume (Donachie 1968). At each doubling of this initiation volume, proportionally more chromosome initiations occur. The coupling of cell division and chromosome replication will be discussed in a later section (section 1.4).

Donachie (1981) has formed a basic model for the bacterial cell cycle from the observations discussed above. The model takes the form of a series of rules which are shown below in an adapted form.

Rule 1 The ideal cell is a cylinder with hemispherical poles.

Rule 2 The ideal cell has a minimum volume V_u, and a minimum length L_u. These are the dimensions of a new born cell in a population for which the growth rate approaches zero.

Rule 3 Growth in cell volume (and mass) is exponential i.e. $dV/dt=V/T$; where T is the doubling time.

Rule 4 Growth in cell length is proportional to cell volume and to the growth rate, such that

$$\frac{dL}{dt} = \frac{K.V.2^{-40/T}}{T} \qquad \text{where } K = L_u/V_u$$

Elongation is assumed to be polar until cells reach length $2.L_u$ ($2A$), after which it is bipolar.

Rule 5 Cell division is initiated when cell length reaches $2.L_u$ ($2A$) and requires a constant period of time to complete (about 20 minutes).

Rule 6 Initiation of chromosome replication is linked to cell volume, such that initiations of rounds of DNA replication take place at each successive doubling of the unit volume V_u. To be more precise 2^n initiations occur at 2^n chromosomal origins when the cell volume reaches $2^n.2.V_u$, n being an integer. The time taken to replicate the chromosome (about 40 minutes) is constant over a large range of growth rates.

The mainly physical mode of analysis discussed in this section shows that the cell cycle of E.coli is tightly controlled, but gives little indication of the mechanism of this control. The following section describes how the use of genetic analysis has been used to further study the processes of cell growth and division.

1.2 Genetic Analysis

By the isolation of mutants carrying mutations that effect the cell cycle, it has been possible to study the cycle at a much more defined level than a purely mathematical approach. Such mutations are generally lethal to the cell and can only be isolated as conditional lethals. Mutants containing temperature (or cold) sensitive missense mutations show a normal phenotype at the permissive temperature but a mutant phenotype at the non-permissive temperature. This is generally due to a single amino acid change which results in the polypeptide having different activities at the permissive and non-permissive temperatures. The second major class of conditional lethal mutants isolated are due to amber, nonsense mutations in a temperature sensitive suppressing background. At the permissive temperature a functional suppressor produces a full-length, functional polypeptide, at the non-permissive temperature the suppressor is inactive and a truncated polypeptide is produced which results in the mutant phenotype.

The isolation of many cell cycle mutants allow them to be placed into various classes (Donachie 1984). These classes define various discrete stages within the cell cycle. In this way the respective genes can be implemented as having a role at a particular stage in the cell cycle. These classes and a brief description of the mutations which define them is given below.

1.2.1 Genes Affecting Formation of the Cell Wall

Mutations in the relevant genes affect the laying down of the peptidoglycan layer that maintains the cell's shape and integrity. Most of the genes identified code for cytoplasmic enzymes with the notable exception of the penicillin binding proteins (PBPs, Spratt 1983). These are found in the inner membrane and catalyse the final stages in the synthesis of the sacculus. Conditional mutations in these genes can cause the cells to either grow as spheres or as filaments depending on whether they affect the laying down of the cell wall at the lateral surface or at the septum. Also in this class are the genes ompA and lpp which code for the outer membrane proteins OmpA (Sonntag et al. 1978) and Brauns lipoprotein (Braun and Rehn 1969) which link the outer membrane to the cell wall and, in doing so, help maintain the cell shape.

1.2.2 Genes Affecting Elongation

E.coli, under constant conditions, elongates without much change in diameter. The β-lactam antibiotic mecillinam specifically blocks this elongation and mutants that are resistant to this

drug map to two genes pbpA and rodA (Spratt et al. 1980). Under non-permissive conditions rodA and pbpA conditional mutants grow as spheres. Three other mecilliam-resistant mutants have been isolated that are not totally defective in the ability to elongate. These mutants carry mutations in cya (adenylate cyclase, Kumar 1976), crp (cAMP binding protein, Kumar 1976)) and envB (Donachie et al. 1984). Their mutant phenotypes differ from rodA and pbpA in that they can form long filaments upon treatment with nalidixic acid (an inducer of the SOS induced cell division block) whereas rodA and pbpA mutants form large spherical cells.

1.2.3 Genes Affecting Nucleoid Segregation and Septum Localisation

The tight co-ordination between DNA replication, chromosome segregation and septum localisation is lost in mutants defective in these genes. Mutations in gyrA, gyrB (Filutowicz and Jonczyk 1983) and dnaG (Arai and Kornberg 1981) are all known to affect DNA replication directly, the resulting catenation of the DNA seems to prevent proper segregation of the chromosomes (Steck and Drlica 1984). Furthermore gyrA and gyrB mutations also affect septation, mutants laying down septa at unusual locations (Norris et al. 1986). Mutations in the parA and perhaps also the parD gene also show this defect (Hussain et al. 1987a and 1987b). Unlike the gyrase mutants, parA mutants show no defect in DNA

replication. It has been proposed that the timing and positioning of the cell septum requires complete replication of the chromosome and the involvement of the parA gene product (Donachie 1987).

1.2.4 Genes Affecting Septum Initiation

Conditional mutations in this class of genes can be characterised by the fact that on shifting to the the non-permissive temperature they no longer initiate septum formation, but can complete any septa which have already been initiated. To date only one mutant allele (ftsH, Santos and De Almeida 1975) has really been characterised that shows this phenotype, suggesting that its behaviour may be a characteristic of the particular mutation rather than its product being involved in a true initiation event (Donachie et al. 1984).

1.2.5 Genes Affecting Septum Formation

Many mutants have been isolated that define genes involved in septum formation. At the non-permissive temperature, these mutations prevent cell division whereas cell growth continues normally. This results in the formation of long filamentous cells without any visible septa. The clearest examples of these mutations lie in the following fts (filamentation temperature sensitive) genes:

ftsD (Ricard and Hirota 1973)

ftsE (Ricard and Hirota 1973)

ftsI (Walker et al. 1975)

ftsQ (Begg et al. 1980)

ftsZ (Lutkenhaus et al. 1980)

ftsA mutants (Begg and Donachie 1985) are slightly different in that visible constrictions can be seen at the septal sites, though complete septa are not formed. A characteristic of mutants defective in any of these genes is their ability to continue cell division at the permissive temperature after a period at the non-permissive temperature. Other mutants have been isolated that carry mutations which cause the formation of aseptal filaments at the non-permissive temperature, but are unable to continue division upon returning to the permissive temperature. The relevant mutations include ftsB (nrdB, Kren and Fuchs 1987) and ftsG (Ricard and Hirota 1973). Finally other mutants show a filamentation phenotype which, like ftsB is probably a consequence of their primary defect. These include azi (Yura and Wada 1968), secA (Oliver and Beckwith 1981), dnaK (Paek and Walker 1987) and rpoH (Yura et al. 1984).

1.2.6 Genes Affecting Septum Separation

Mutations in these genes appear to prevent cell division even

though the septa have been completed as defined by electron microscopy. Two genes, envA and cha, have been thus characterised. Mutations in either of these genes results in the formation of strings of cells in which the inner membrane has completely segregated, but the outer membrane remains continuous across the junction. A single ,but double thickness, peptidoglycan septum is found. A low level of N-acetylmuramyl-L-alanine amidase activity was found in the envA mutant (Wolf-Watz and Normack 1976), which since this enzyme is involved in peptidoglycan catabolism, could explain the observed phenotype. The cha gene product has been implicated in the regulation of outer membrane attachment to the inner membrane and peptidoglycan layer (Chakraborti et al. 1986).

1.2.7 Genes Affecting Inactivation of Septal Sites

As the cell elongates, potential division sites (pds) appear to be laid down at the sites where septa are to form. Once a septum has been laid down at this site no more are laid down after elongation from the new pole. Mutations in minB however, seem unable to cause this inactivation and as a result small, almost spherical, "minicells" are formed close to the new cell pole (Davie et al. 1984).

PART TWO

1.2.8 Genes Affecting Coordination of DNA Replication and Cell Division

This class describes a group of genes which affect the coupling of DNA replication and cell division. They will be discussed fully in later sections (1.4 and 1.5).

The use of basic physical/physiological and genetic analysis as discussed in the previous two sections has allowed the formation of a simple model for the E.coli cell cycle. The following sections will use this model as a basis for detailed study of particular aspects of the cell growth and division cycle.

1.3 Growth of the Cell Envelope and Septum

The essence of the cell cycle is that a newly born cell elongates until such a point that it lays down a septum and divides to form two daughter cells. An understanding of the nature of the envelope and septal components and the regulation of their synthesis is thus essential for a complete understanding of the process of cell division.

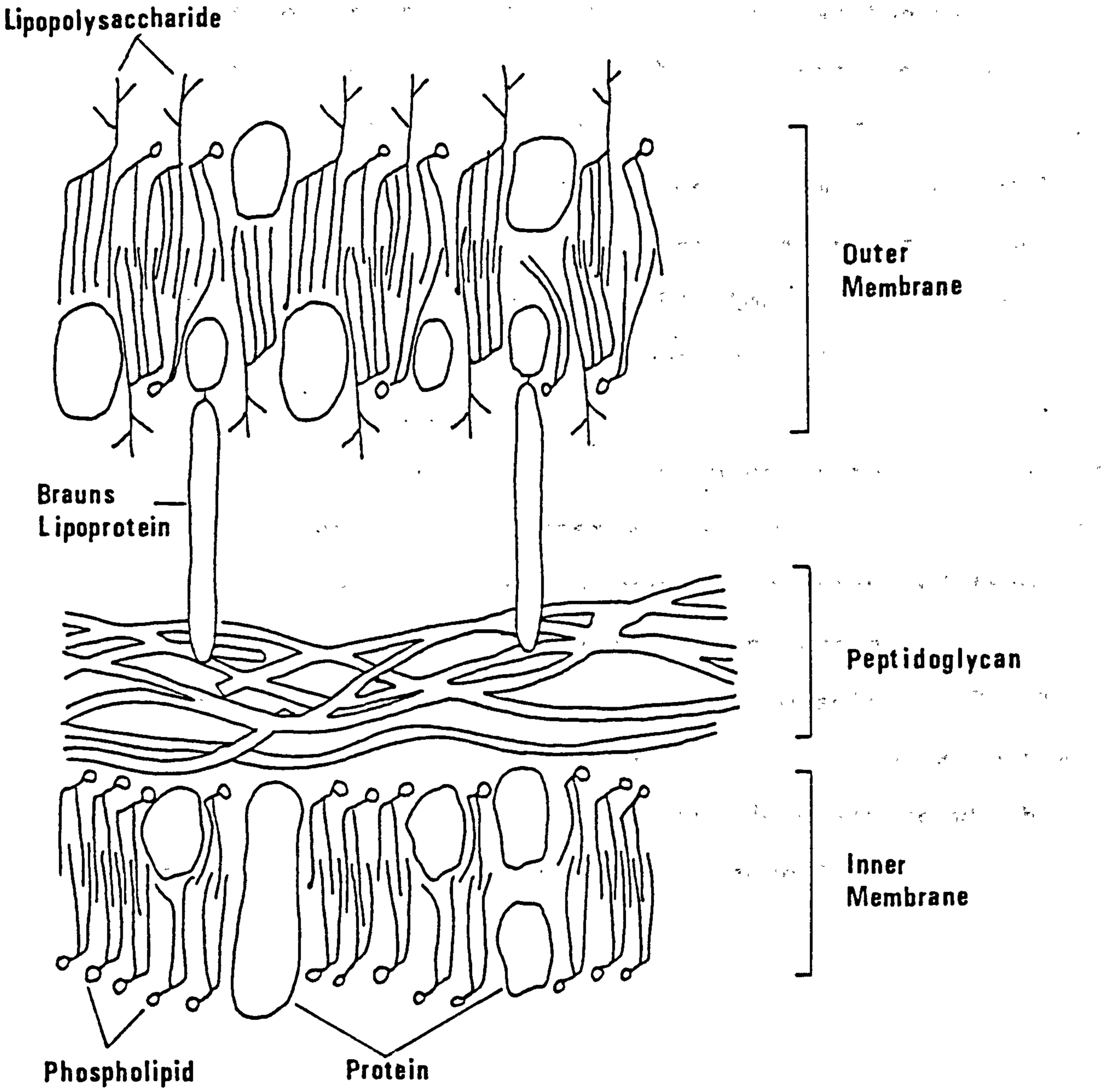
1.3.1 Structure of the Cell Envelope

The Gram negative cell envelope of E.coli can be considered to consist of three layers (Costerton et al. 1974); the outer membrane, inner membrane and periplasm. This is represented by figure 1.3. The inner, cytoplasmic membrane is the main permeability barrier and consists of a phospholipid bilayer with associated proteins in a fluid mosaic arrangement (Singer and Nicholson 1972).

The outer membrane is an asymmetric bilayer, the inner layer consisting of phospholipid whereas the outer layer contains lipopolysaccharide and less phospholipid.

FIGURE 1.3 Structure of a Gram Negative Cell Envelope

Redrawn from Costerton et al. (1974).



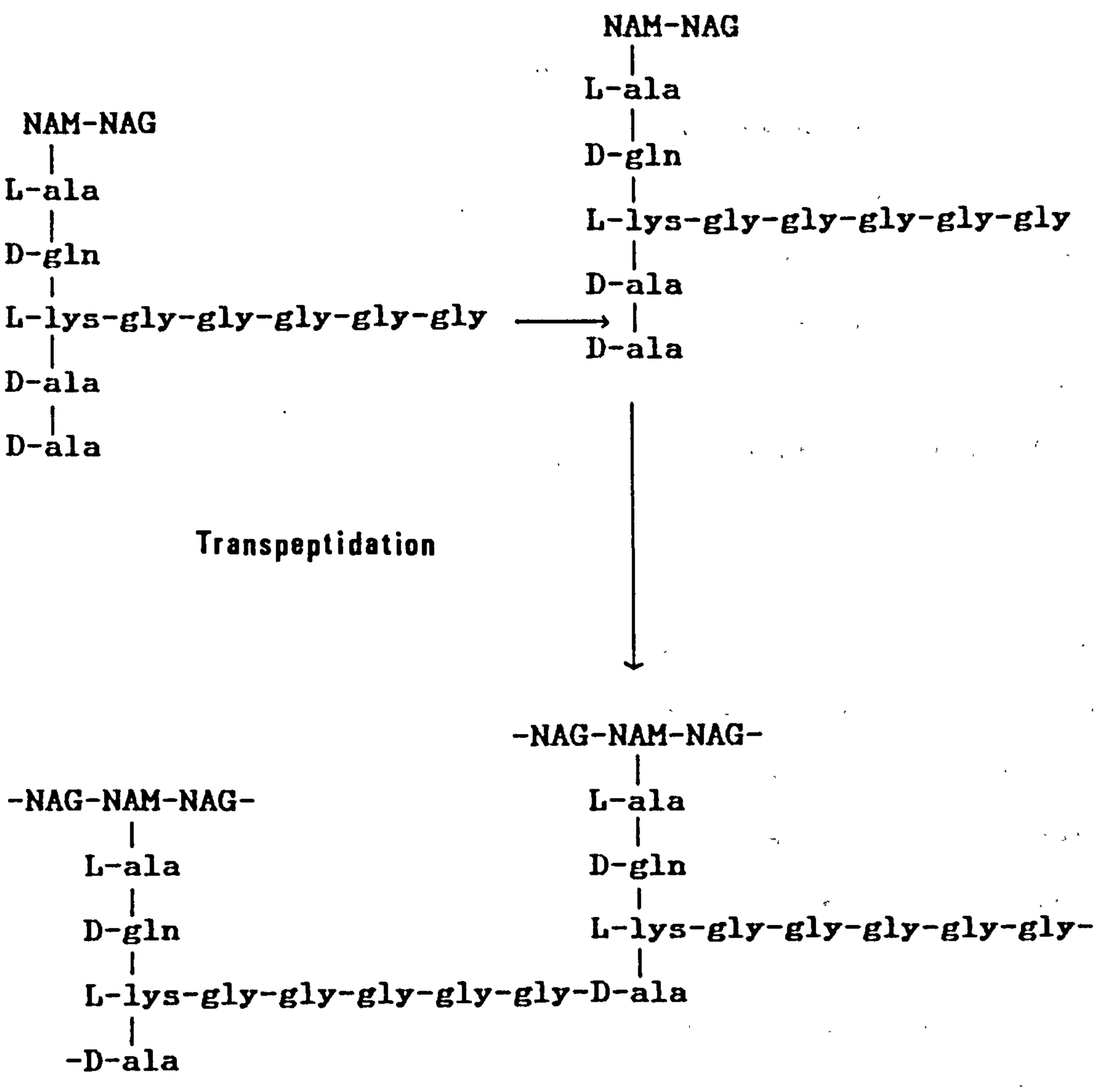
The outer membrane, too, contains proteins, two of these (Brauns lipoprotein and OmpA) are attached to the peptidoglycan sacculus found in the periplasm (Wensink et al. 1982). This peptidoglycan is a rigid polymer that maintains cell shape and integrity. Genetic studies with ompA and lpp (lipoprotein) mutants suggest that the attachment of the proteins to the cell wall is important in maintaining cell shape, although it seems that the two can substitute for each other. Only in double mutants (ompA lpp) does the cell lose its shape and grow as a sphere (Fung et al. 1978, Sonntag et al. 1978). Nevertheless the cells remain osmotically stable suggesting that the sacculus is still maintaining the cells integrity. Begg and Donachie (1984) found that the OmpA protein is largely found at the poles of the cell.

As mentioned above the peptidoglycan sacculus is responsible for maintaining the shape of the bacterial cell wall against a high internal osmotic pressure. Peptidoglycan is constructed from layers of cross-linked polymers based on the sugars N-acetyl-muramic acid (NAM) and N-acetyl-glucosamine (NAG). The basic structural unit consists of one NAG and one NAM moiety and attached to the NAM residue is a pentapeptide chain to which is linked a pentaglycine chain as shown in figure 1.4.

FIGURE 1.4 Structure of Peptidoglycan

NAG - N-acetyl-glucosamine NAM - N-acetyl-muramic acid

Standard abbreviations have been used for the amino acids.



A linear polymer is constructed by joining the NAG and NAM residues to form a repeating (..NAG-NAM-NAG-NAM..) unit. Finally the linear polymers are cross-linked via the pentapeptide and pentaglycine chains in a transpeptidase reaction also shown in figure 1.4.

1.3.2 Growth of the Cell Envelope

As a cell elongates new peptidoglycan must be laid down to maintain the rod shape. The question arises as to whether the cell wall is laid down uniformly or at particular sites. It was discussed in section 1.1 that, up to a certain length, elongation occured in only one direction but after reaching this length elongation continued in both directions. It was postulated that a single growth site exists for each unit length capable of elongating in a particular direction.

Most searches for growth sites have worked on the principle of pulse labelling either the outer membrane or the peptidoglycan layer, and then allowing growth to continue. It is assumed that any growth site would then lack the label on the basis that only new material would be incorporated into this site. Many workers concentrated on labelling outer membrane antigens (with fluorescent antibodies (Chung et al. 1964)) or bacteriophage receptors (with the respective bacteriophage (Begg and Donachie 1977)). As demonstrated by Schindler et al. (1980) these methods

suffered from the problem of lateral diffusion within the outer membrane. Davison and Garland (1983) overcame this problem by cross-linking outer membrane-bound antibodies with anti-IgG antibodies thus preventing lateral diffusion. The workers showed that zonal growth did indeed occur and the number of zones depended on the growth rate.

Labelling of the peptidoglycan layer was achieved by using ^{3}H-diaminopimelic acid (Ryter et al. 1973) and because the PG layer is tightly cross-linked there should be no diffusion of label. In rich media, growth zones were seen both in the centre of the cell and nearer the poles. It was concluded that the latter zones were involved in elongation and the former in cell division.

1.3.3 Structure and Growth of the Septum

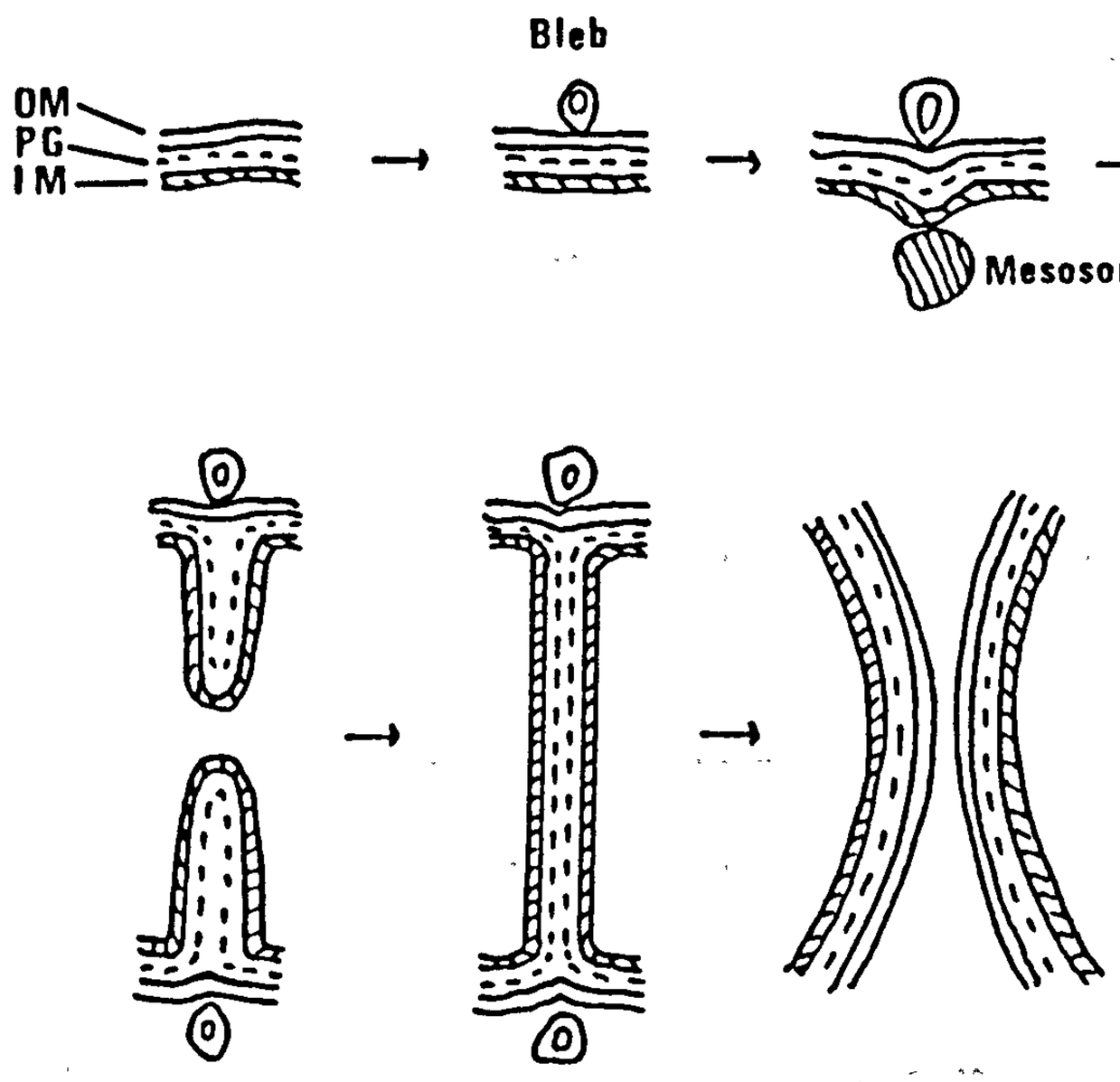
The morphological changes occuring during septation were determined in an electron microscopic study by Burdett and Murray (1974), their results are shown in figure 1.5.

The first visible sign of septum formation is the appearance of a "bleb" from the outer membrane. Invagination of the cytoplasmic membrane and the cell wall then occurs around the circumference . Only when the invaginations have fused and begun to separate does a new outer membrane form. Mesosomes are often found linked to the cytoplasmic membrane at the early stages.

FIGURE 1.5 Diagrammatic Representation of Septum Formation

Redrawn from Burdett and Murray (1974)

OM-Outer Membrane PG-Peptidoglycan IM-Inner Membrane



The role of these mesosomes, which appear to be sac-like extensions of the membrane, is unclear; they are sometimes seem at the cell poles although this may represent relics of a former division. Burdett and Murray summarised the events of septum formation as consisting of four periods.

1 Modification of the cell wall by hydrolases

2 Assembly of enzymes (involved in septation) at the septal site

3 Initiation of cell wall and membrane synthesis to form a septum (possibly with the assistance of mesosomes)

4 Ingrowth of the outer membrane followed by separation.

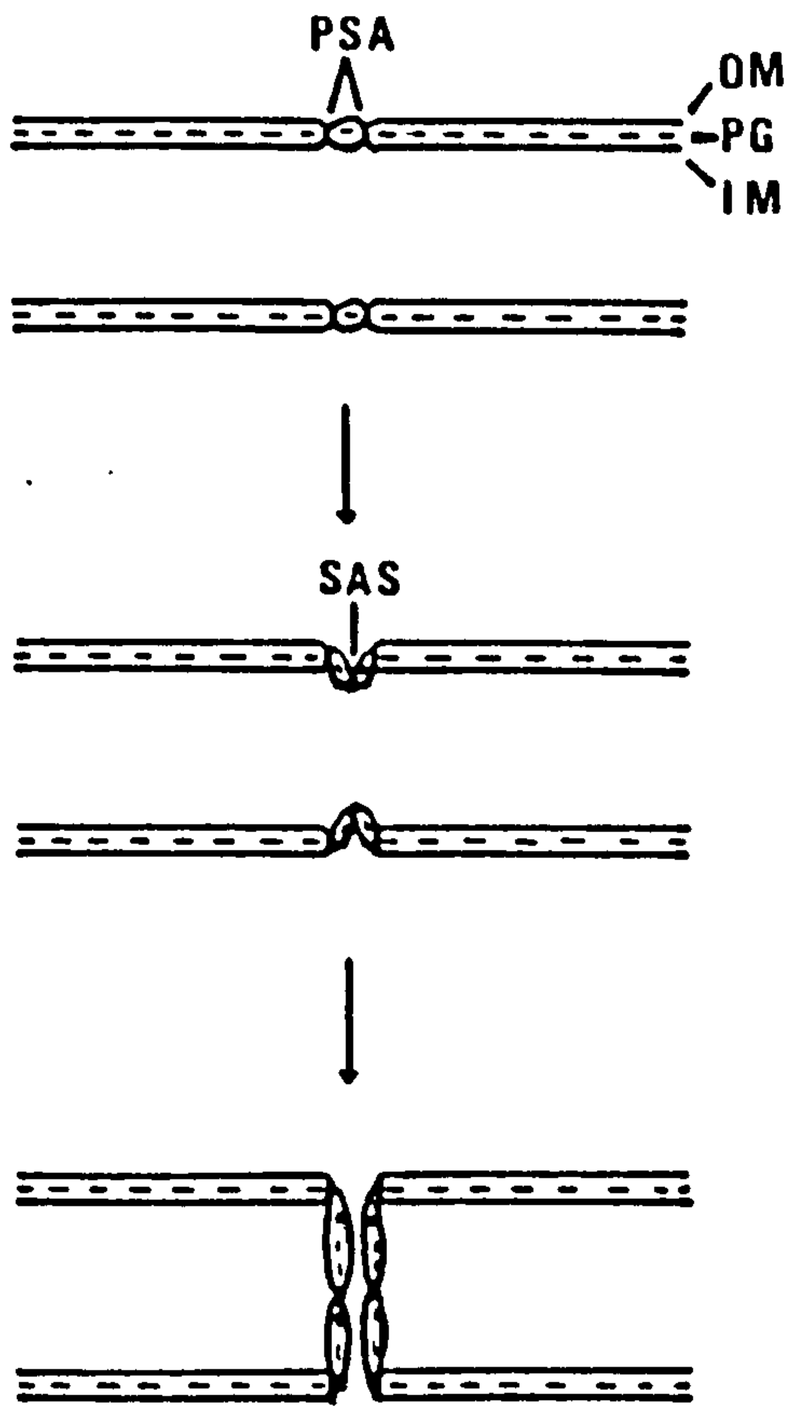
Rothfield and workers have recently studied the formation of the Salmonella typhimurium septum and have identified novel septal structures. MacAlister et al. (1983) and Cook et al. (1986) described a structure consisting of two circumferential rings, the periseptal annuli, that form in the region of the cell envelope in which septal ingrowth will occur. Each annulus is a continuous zone where inner and outer membranes are closely apposed to the murein layer (figure 1.6).

FIGURE 1.6 Diagrammatic Representation of Periseptal Annuli and Septal Attachment Sites

Redrawn from MacAlister et al. (1987)

OM-Outer Membrane PG-Peptidoglycan IM-Inner Membrane

PSA-Periseptal Annuli SAS-Septal Attachment Site



Cook et al. suggested that the paired annuli define the periseptal domain and restrict essential elements of the division machinery to this location. MacAlister et al. (1987) has more recently identified another structure located between the two periseptal annuli at the onset of invagination. This septal attachment site (SAS) was seen to consist of a bulbous enlargement of the murein-outer membrane layer, closely apposed to the inner membrane at its point of deepest penetration. The workers speculated that the function of the SAS during ingrowth of the septum may be to provide a site for insertion of septal peptidoglycan. These structures are shown diagrammatically in figure 1.6.

1.3.4 The Penicillin Binding Proteins

The transpeptidase enzymes that catalyse the cross-linking of peptidoglycan are inhibited by the penicillin (β-lactam) antibiotics (Waxman and Strominger 1983). Since the transpeptidation reaction is essential for the construction of the cell wall and septum, the β-lactam antibiotics have proved extremely useful in the study of cell growth and division.

As well as the transpeptidase activity, a second β-lactam sensitive activity has been detected; the terminal D-alanine residue of the pentapeptide chain can be removed by a D-alanine carboxypeptidase (Izaki et al. 1966). Futhermore some β-lactam

sensitive enzymes show a third ß-lactam insensitive activity, that of a peptidoglycan transglycosylase (Suzuki et al. 1980).

Spratt and Pardee (1975) developed a powerful technique for the detection of ß-lactam sensitive enzymes, by using the ability of these enzymes to bind radiolabelled penicillin analogues. In this way seven penicillin binding proteins (PBPs) were identified and shown to have particular enzyme activities. By inhibiting the activity of these enzymes (using specific ß-lactam antibiotics or by studying mutants defective in the particular structural gene), information about the role that each enzyme plays in cell growth and division was obtained. This information is summarised below.

TABLE 1.2 The Penicillin Binding Proteins

PBP	Gene	Activity	Function
1A	ponA	TPase TGase	Elongation
1B	ponB	TPase TGase	Elongation
2	pbpA	TPase	Cell Shape
3	ftsI (pbpB)	TPase TGase	Septation
4	dacB	CPase	Unknown
5	dacA	CPase	Switch ?
6	dacC	Unknown	Unknown

TPase: Transpeptidase CPase: Carboxypeptidase

TGase: Transglycosylase

Mutants carrying mutations in either PBP1A or 1B are viable and appear to grow and divide normally, although there is one report which claims that a particular PBP1B conditional mutant is lethal at the non-permissive temperature (Tamaki et al. 1977). A double mutant which lacks PBP1A and has a temperature sensitive PBP1B mutation, also causes lysis at the non-permissive temperature (Spratt 1983, Suzuki et al. 1978). Both PBP1A and 1B have transglycosylase and transpeptidase activities and are involved in cell elongation. Since PBP1B has the greater affinity for the β-lactam antibiotics, and mutants of PBP1B have PBP1B significantly reduced peptidoglycan synthetic ability, is believed to be the major enzyme of the two in terms of activity (PBP1A and PBP1B) (Tamaki et al. 1977).

PBP2 is specifically inhibited by the β-lactam antibiotic mecillinam (Spratt 1975). In the presence of this antibiotic large osmotically-stable, spherical cells grow which lyse after several hours growth. Conditional mutations in pbpA produce an identical phenotype at the non-permissive temperature. PBP2 is proposed to be involved in laying down the cell wall at new sites of elongation, and to ensure that the peptidoglycan is introduced with the correct orientation (Canepari et al. 1984). The restricted distribution of PBP2 in the cell membrane agrees with this postulated role (Bucanan 1981).

Unlike PBP1A, 1B and 2, PBP3 seems to be required for peptidoglycan cross-linking at the septum. It possesses tranglycosylase and transpeptidase activities and is inhibited by the antibiotics cephalexin, cefuroxine, furazlocillin, piperacillin and azthreonam. These antibiotics (and mutations in the ftsI gene) cause the cell to grow into long aseptate filaments (Pisabarro et al. 1986).

PBPs 4,5 and 6 all show D-alanine carboxypeptidase activity but appear not to be essential for cell growth or maintaining morphology since mutants lacking these proteins have a completely normal phenotype (Broome-Smith and Spratt 1982). However over-expressing PBP5 results in the formation of ovoid cells which eventually die. Markiewickz (1982) has suggested that the role of PBP5 is to switch PG synthesis from the elongation site(s) to the septal site.

1.3.5 A Model for Cell Growth and Division

Based on the information discussed above it is possible to form a basic, speculative model for cell growth and division.

A cell elongates from a particular growth site in a particular direction, PBP2 has a role in initiating PG insertion at this site. Along with RodA, PBP2 is required to maintain the cylindrical shape of the cell. PBPs 1A and 1B are required for

cross-linking the PG chains during cell wall elongation.

At a particular stage of the cell cycle elongation ceases and septum formation is initiated. PBP5 is implicated in this switching from PG incorporation in the lateral wall to incorporation into the septum. Its carboxypeptidase activity results in the cleavage of the terminal D-alanine residue from the PG monomer. The resulting tetrapeptide derivative may be a prefered substrate for the septal construction enzymes. PBP3 seems to be the major transpeptidase/transglycosylase enzyme at the septum.

An initial step in septum formation is modification of the PG wall, possibly by the hydrolytic transglycosylase and endopeptidase activities involved in autolysis (Kitano et al. 1986).

Of the other components thought to be involved in septum formation (the fts gene products) few have a defined function. Based on morphological evidence FtsZ acts at an early stage, FtsQ and PBP3 slightly later and FtsA later still. This information was obtained in a series of experiments by Begg and Donachie (1985). They constructed double mutants impaired in both elongation (rodA/pbpA) and division (ftsA, ftsQ, ftsZ, ftsI). All the double mutants grew as stable spherical cells suggesting that there is a general system for PG synthesis in the absence of specific elongation or crosswall determinants. The temporal order

was determined by the amount of septum that could be observed in these double mutants.

FtsA is believed to have a more complex role in cell division than as a basic component of the septum. It has been found to interact (either directly or indirectly) with PBP3 (Tormo et al. 1986). Furthermore FtsA is also believed to have a regulatory role. Similarly FtsZ has a role in the control of cell division, both of these regulatory actions will be discussed in following sections (1.4.1 and 1.5.8).

1.3.6 The Role of cAMP in Cell Growth and Division

Various workers have studied the effect that cAMP has on the cell cycle of E.coli. Cyclic AMP has been implemented in many regulatory processes in both eukaryotic and prokaryotic cells. In association with its receptor protein (CRP), the role of cAMP as a positive effector for transcription has been well studied in E.coli (Ullman and Danchin 1983).

An inverse relationship between growth rate and cellular cAMP levels have been reported for both bacterial and mammalian cells. Holtje and Nanninga (1984) found that both intracellular and extracellular concentrations of cAMP increased exponentially in synchronously growing cultures, and concluded that cAMP could not regulate cell growth and division. Utsumi et al. (1986)

argued that it was technically too difficult to measure small changes in cAMP throughout the cell cycle. Instead they used a *cya::lacZ* fusion to measure transcription from the adenylate cyclase gene (*cya*) promoter. Their results showed that *cya* is expressed during cell elongation but repressed during division. By constructing a fusion between *cya* and the *lac* promoter they were able to induce transcription of *cya* with IPTG. Such induction resulted in the inhibition of cell division and the formation of filaments. They further suggested that a possible target for this cAMP-mediated control over cell growth might be the *fic* gene product. The *fic* allele was identified as causing a temperature sensitive inhibition of cell division upon the addition of cAMP. Utsumi *et al.* cloned the *fic* gene and showed that it was distinct from *crp*.

1.4 The Co-ordination of Cell Division with DNA Replication

There are two main discontinuous events in the cell cycle of E.coli: the first is the initiation of a new round of chromosome replication and the second, the initiation of cell division. The virtual absence of DNA-less cells indicates that the two processes are tightly coordinated. This could be the result of some aspect of the DNA replication cycle triggering cell division or vice versa. Alternatively, the two processes could be independently triggered by some common signal.

1.4.1 Termination of Replication as a Division Signal

Most attention has been focussed on the possibility that the DNA replication cycle controls the onset of division. Helmstetter and Pierucci (1963) showed that blocking DNA replication prevented cell division and thus concluded that replication was a necessary and sufficient requirement for cell division. Cell division is known to occur at a constant time after termination of replication (D period, section 1.1) which suggests that termination might provide the signal for division. However since the time taken to replicate the chromosome (C period) is also constant it is equally possible that initiation of replication, or indeed any part of the replication cycle, could provide such a signal. Grossman and Ron (1980) managed to prolong the replication time by starving the cell of an essential amino acid

in the presence of excess methionine. This served to increase the C period but did not increase the D period. This result made termination of chromosome replication a more likely signal for division than initiation.

Jones and Donachie (1973) found that a short period of RNA and protein synthesis was required immediately after termination of replication, in order that division could occur. They suggested that during this period of RNA and protein synthesis a termination protein is synthesized. This termination protein can only be synthesized after completion of chromosome replication and its role is to initiate the formation of a septum.

FtsA has been proposed as a candidate for such a termination protein. Conditional mutants containing mutations in the ftsA gene show a typical cell division mutant phenotype of filamenting at the non-permissive temperature. Tormo and Vicente (1984) have indeed shown that the ftsA gene product has a structural role in the construction of the E.coli septum, but there is also evidence that FtsA has a regulatory role.

Donachie et al. (1979) and Tormo et al. (1980) showed that an active ftsA gene product was only required at the latter stages of the cell cycle and that failure to synthesize the protein during these latter stages prevented division, even if the protein had been synthesized earlier in the cycle. Tormo et al. (1985) later showed that a period of DNA synthesis was also

required for the synthesis (but not activity) of FtsA. They also showed that the division block produced by an inactivated FtsA protein was independent of the SOS response.

A termination protein needs to be synthesized at the end of the chromosome replication cycle, and then initiate the septation process. The evidence discussed above suggests that FtsA could satisfy the first criterion. There is little evidence, though, that FtsA is capable of initiating septation. Although it is thought to interact with FtsQ, FtsZ (Descoteaux and Drapeau 1987) and PBP3 (Tormo et al. 1986), mutants containing a variety of mutations in ftsA seem perfectly capable of initiating septum formation (Begg and Donachie 1985). Indeed it is generally believed that FtsA acts at a late stage of the septation process.

A replication dependent expression of genes located at the terminus region of the chromosome, could also provide a mechanism for a termination control of cell division. Indeed several cell division genes have been identified that map to the terminus region, these include

ftsS (Dwek et al. 1984)

ftsT (Dwek et al. 1984)

ts52 (Zusman et al. 1972)

dif (Kuempel 1987)

dicABC (Bejar et al. 1986).

Of these only the dicABC cluster has been extensively studied. DicB was found to be an inhibitor of cell division and was repressed by both DicA and DicC. However the region containing these genes could be completely deleted, suggesting that they probably do not have an essential physiological role. This will be discussed further in section 1.5. The potential for a termination control for cell division still exists with the presence of these terminus linked genes but as yet no mechanism has been established.

1.4.2 Uncoupling of DNA Replication and Cell Division

Koch (1977) argued that if the process of chromosome replication were to trigger cell division, then the latter process would be less precisely timed, because unrelated sources of variance would be additive. In fact the two processes show a similar precision of timing (Koppes 1987). This could be explained if both chromosome replication and cell division were independently triggered by some common signal, which would be a critical controller of the cell cycle.

If there is no direct link between chromosome replication and cell division then it may be possible to uncouple the two processes, that is it should be possible to observe cell division in the absence of chromosome replication. As mentioned earlier Helmstetter and Pierucci (1963) found that preventing replication

with inhibitors such as UV light, nalidixic acid and Mitomycin C prevented cell division. However these inhibitors are now known to induce the SOS response (section 1:5) a facet of which is to inhibit cell division. This problem has been overcome by the use of conditions which will prevent DNA replication but will not induce the SOS response. This is based on work with various mutations that affect DNA replication (Tang and Helmstetter 1980). Mutants containing mutations in the dnaA gene are unable to initiate new rounds of replication at the restrictive temperature but are able to complete any existing rounds. This type of block does not elicit the SOS response. Upon a shift to the restrictive temperature there is a temporary block in division after which growth and division continue producing normal sized but DNA-less cells.

More recent work has also shown the formation of DNA-less cells after a temporary division block. Jaffe et al. (1986) studied the effects of blocking DNA synthesis in a strain incapable of eliciting the SOS response due to mutations in sfiA sfiC or ftsZ. DNA sythesis was blocked either at initiation (dnaA) or elongation (dnaB or thymine starvation). In all cases both filamentation and the production of anucleate cells were observed. In the case of thymine starvation the whole population forms filaments before a new population of normal sized anucleate cells appear. Since both filamentation and anucleate cell production were observed, these results could not completely prove the absence of a causal relationship between DNA

replication and cell division.

1.4.3 The Search for a Common Signal

Much work has concentrated on establishing the signal which results in the initiation of chromosome replication which may also be the signal for cell division. Donachie (1968) observed that cells initiated chromosome replication at a certain mass (the initiation mass), which was independent of the size at birth. Koppes et al. (1980) measured this correlation between size and time of initiation and found it to be very strong. This was in contrast to the correlation between cell age and time of initiation which was very poor, suggesting a size rather than age control over initiation of chromosome replication. koppes data was inconsistent with a general model of cell division proposed by Smith and Martin (1973). In this model, which is essentially time controlled, there is a probabilistic stage between birth and initiation; such that the cell will enter the deterministic stage of chromosomal replication at a given probability per unit time. Modifications of this model though, reviewed by Tyson (1985), made it more amenable to experimental analysis with a series of cell types.

Churchward et al. (1981) found that the size of the cell at initiation was not totally invariant but was dependent on the growth rate, at least at very slow growth rates. They state, however, that this does not diminish the significance of an

initiation size, but introduces a growth rate dependent factor.

Other workers have attempted to uncouple initiation of chromosome replication and cell size by various methods. Grossman and Ron (1980) found that upon amino acid starvation initiation occurred at a smaller size than normal and concluded that the coupling was dependent on the cell's ability to synthesize proteins. In these experiments the cells also divided at a smaller size. Chromosome initiation thus seems to be triggered by some aspect of cell growth. Two types of model have been proposed to explain this basic observation.

a: Dilution of an inhibitor due to increase in mass.

b: Growth dependent accumulation of a positive effector.

The study of chromosome initiation control has been extensive and is beyond the scope of this review, especially since no consistent mechanism has been derived. Messer (1987) has recently reviewed the problems encountered in forming any unifying theory for the initiation of replication and provides a good starting reference for further information. Whilst the mechanism for the control of cell division for E.coli undergoing balanced growth remains unclear alternative systems, operating under certain conditions, are better understood. The following section briefly describes the responses of a cell to stressful conditions, under which the cell often has to adjust its normal cell cycle.

1.5 The Response of E.coli to Stress, and the Role of FtsZ in Cell Division

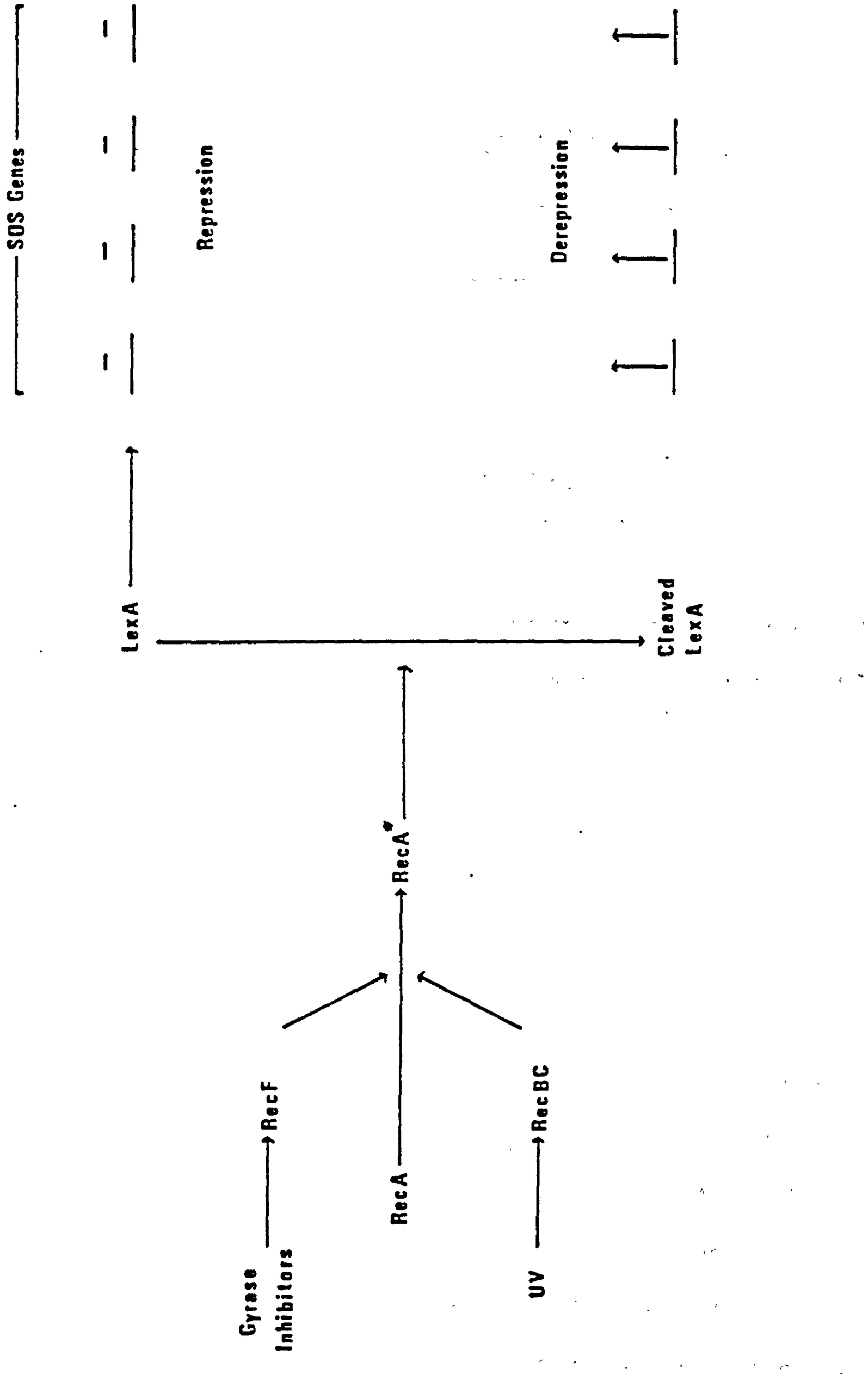
Drastic changes in the environment can disrupt the physiology of the E.coli growth cycle. As a result it has developed responses to potentially damaging environmental factors, such as UV light or heat. Some of these responses are discussed below, both individually and as part of a global regulatory system.

1.5.1 The SOS Response

This response is an inducible repair system evolved to combat damage to DNA. Upon such damage (by UV light or other mutagens) the cell responds by inducing a DNA repair system and also inhibits further cell division to allow time for the repairs to be completed. Upon completion nucleoid segregation and cell division are re-established as normal (reviewed by Walker 1984).

The basis of the response is shown in figure 1.7. Damage to DNA results in the activation of the RecA protease by one of two (RecBC (Chaudhurge and Smith 1985) or RecF (Thoms and Wackernagel 1987)) pathways. The activated protease (RecA*) can then cleave LexA which is a repressor of many genes and cleavage results in the derepression of all these SOS genes.

FIGURE 1.7 The SOS Response



Some of these genes and the proposed function of their gene products are given in table 1.3.

TABLE 1.3 The Main SOS Gene Products and Their Function

RecA, LexA	Modulation of Response
Uvr ABCD, Umu CD	DNA Repair
SfiA	Inhibition of Cell Division

Once the DNA is repaired, the inducing signal is removed and the RecA* protease activity is reduced, by proteolytic degradation, and so LexA repression is restored.

1.5.2 SOS Induced Division inhibition

A response of the SOS system is to prevent further cell division whilst repair of the DNA is attempted; this results in the temporary formation of filaments since cell growth continues as normal. A conditionally constitutive mutant of recA (tif) results in the induction of the SOS response at the restrictive temperature in the absence of DNA damage. The resulting filamentation can be suppressed by mutations in either sfiA or

sfiB (George et al. 1975).

sfiA was shown to be non-essential and induced as part of the SOS response, and therefore a good candidate for the cell division inhibitor proposed by Witkin (1967). sfiB was then found to be an allele of ftsZ, an essential gene involved in septation (Lutkenhaus 1983). It was thus proposed that SfiA and FtsZ interact directly resulting in the inhibition of cell division.

The mechanism of this inhibition and its subsequent reactivation was studied by Dopazo et al. (1987). They showed that only septa that had been initiated at the time of DNA damage by UV light were inactivated, whilst those initiated during the recovery were readily synthesised. This suggested that the SOS response resulted in the specific structural blocking of active septa rather than a general inhibition of all septal activity.

They also showed that the inhibition of septation resulted in resistance to benzyl penicillin induced lysis, which is observed when septation is structurally blocked in some fts mutants.

The structural blocks induced by the ftsA3 allele were not permanent, but could be reactivated with the action of Lon protease. The same seems to be true for the SOS-induced division block. After such inhibition no further protein synthesis is required for recovery of division, thus active FtsZ can be recovered from blocked septa by the degradation of SfiA.

1.5.3 The Heat Shock Response

The response of cells to heat has been known as a phenomenon for many years. After a shift-up in temperature, cells of many types induce a subset of polypeptides, some of which are highly conserved even between prokaryotes and eukaryotes (Bardwell and Craig 1984 and 1987).

In E.coli there are 17 known heat shock proteins some of which are listed in table 1.4. Some have no known function, whereas others (eg DnaK) seem to have an array of different activities (Niedhardt et al. 1984). Table 1.4 also shows known effects of the heat shock response, but for which no gene product has been implicated. Many different and varied stimuli are known to induce the heat shock response and these are listed below.

Hydrogen Peroxide	VanBogelen et al. 1987
Ethanol	Neidhardt et al. 1984
Nalidixic Acid	Kruegar and Walker 1984
Alkaline Shifts	Taglicht et al. 1987
Abnormal Proteins	Goff and Goldberg 1985
Viral Infection	Drahos and Hendrix 1982
Ultra Violet Light	Kruegar and Walker 1984
Topoisomerase Inhibition	Travers and Mace 1982

TABLE 1.4 The Main Heat Shock Gene Products and Their Possible Functions

Gene Product		Proposed Functions		
RpoD	σ^{70}			
DnaK, DnaJ	Charging of tRNAs	Involvement in cellular DNA and RNA Synthesis		Modulation of heat shock response
GroEL, GroES	Interaction with DnaA			
Lon		Proteolysis		
?				
?		Cell Division		
?		F Plasmid Replication		
?		Cell envelope composition		

It is now known that the heat shock response in E.coli is positively regulated by rpoH (htpR), the alternative sigma factor σ^{32} (Grossman et al. 1984). In vitro work has confirmed that this sigma factor recognises a unique promoter sequence which is different to that recognised by the normal sigma factor σ^{70} (Cowing et al. 1985). Thus expression of rpoH by heat shock induction, results in the preferential expression of genes under the transcriptional control of the heat shock promoter.

Exactly how a rise in temperature induces expression of rpoH is

unclear. After a rise in temperature the levels of rpoH mRNA increase (Tilly et al. 1986), suggesting control, either directly or indirectly, at the level of transcription.

The role of the heat shock response in adaptation to the altered enviroment is also unclear. It has been proposed that a major role of the response is to sequester and degrade proteins that have become denatured as a result of the temperature increase. In eukaryotes Hsp70 (which is highly homologous to DnaK), has been proposed to have a role in the sequestering of abnormal proteins (Pelham 1986). Furthermore the ATP dependent protease Lon is a heat shock protein and in strains defective in the heat shock response, due to a mutation in rpoH, there is also a defect in proteolysis (Goff et al. 1984, Baker et al. 1984).

1.5.4 The Stringent Response

This response is induced when the availability of an aminoacyl-tRNA species becomes limiting for protein synthesis (Gallant et al. 1979). Under these conditions the nucleotide phosphates ppGpp and pppGpp accumulate, which results in a variety of metabolic changes designed to reduce the stress caused by the limitation. These include a reduction in the rate of synthesis of stable RNA and ribosomal proteins and an increase in the rate of synthesis of other, unidentified, proteins.

1.5.5 The Adaptive Response

This is a longer term response to damage to DNA caused by methyl or ethyl alkylating agents (Jeggo et al. 1977), which tag alkyl groups onto the purine bases. This damage results in the induction of two enzymes, O^6-alkylguanine-DNA alkyl transferase and 3-methyladenine-DNA glycosylase 11, under the control of the positively acting regulator ada (Jeggo 1979). These enzymes remove the alkyl groups which, if left, would result in mutation due to mis-pairing during DNA replication.

1.5.6 A Global Regulatory Network

In his review of mutagenesis and inducible responses to DNA damage, Walker (1984) discussed interactions between the heat shock, SOS and adaptive responses. These interactions were based on the observations that certain methylating agents could induce both the SOS and adaptive responses and that UV light and nalidixic acid could induce both SOS and heat shock respones. Since then it has also been reported that the stringent response and the heat shock response can be induced by a common pathway (Grossman et al. 1985). Figure 1.8 shows these interactions and is based on Walker's initial diagram.

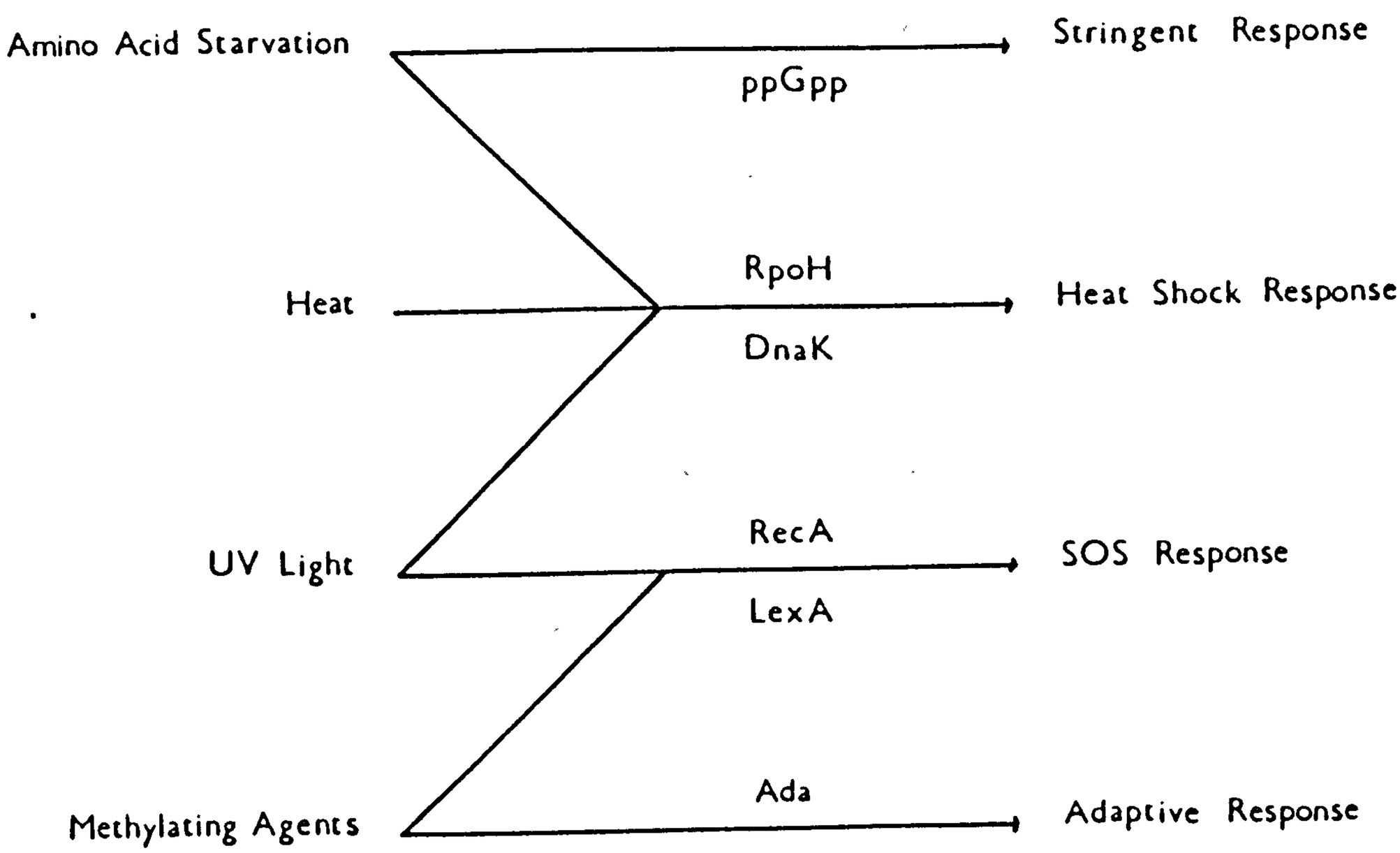
Of these four the heat shock and SOS responses have been studied in most detail, and it appears that the heat shock response may play a much broader role than just a response to a rise in temperature.

Unlike the regulatory genes of the SOS system (recA and lexA), those of the heat shock response (rpoH and dnaK) are essential for cell viability (C. Gross pers. comm., Paek and Walker 1987), although there is some controversy over rpoH (Yammamori and Yura 1982). The heat shock proteins are synthesised at all temperatures studied to date and are induced by any rise in temperature. This suggests that perhaps the heat shock response is a general stress response system in E.coli that is coupled to other more specific systems.

FIGURE 1.8 Interactive Stress Responses

This figure is based on one drawn by Walker (1984) but also includes the stringent response.

The inducers and modulators of each response are shown as well as the interactions between different responses.



1.5.7 Cellular Mechanisms for Inhibition of Cell Division

A well characterised response of the SOS response, and to a lesser extent for the other stress responses, is the ability to inhibit cell division. Several mechanisms have been established that result in such inhibition and are summarised in Table 1.5.

TABLE 1.5 Cell Division Inhibitors

SfiA	SOS induced division inhibiton
Sfi C	SOS induced division inhibition
FtsM	LexA dependent division inhibition
DicABC	TerC linked division inhibitor
CcdAB	F plasmid linked division inhibitor

sfiA (sulA) is found in many members of the Enterobacteriaceae and the gene contains a highly conserved domain (Freudl et al. 1987). The gene is induced during the SOS response and is

responsible for the resulting inhibition of cell division. The gene was identified as being a suppressor of lon induced filamentation resulting from DNA damage. In a lon strain filamentation persists even after the DNA damage has been repaired, presumably due to the cells inability to degrade a cell division inhibitor. Suppressors of this effect mapped to two genes sfiA and sfiB (Gottesman et al. 1981). sfiA was later shown to be SOS inducible and to be preceded by the characteristic lexA box (Huisman and D'Ari 1981, Cole 1983), discussed in section 1.5.1. The target for SfiA was then shown to be SfiB which was identified as being the gene product of the cell division gene ftsZ (Lutkenhaus 1983). Unlike sfiA, ftsZ was found to be an essential gene and was presumed to be a normal component of the cell division machinery (Gottesman et al. 1981).

A constitutive temperature sensitive mutation (tif), in recA, results in constitutive expression of the SOS response and mutants carrying the tif allele show SfiA induced filamentation at the non-permissive temperature. D'Ari and Huisman (1983) found that filamentation could also be observed in tif sfiA double mutants. This led to the discovery of SfiC, an alternative cell division inhibitor. Both SfiA and SfiC are induced as part of the SOS response and both utilize FtsZ as their target for division inhibition. There were, though, certain differences ; SfiC was not regulated by LexA (although it was regulated by RecA) and also resulted in an irreversible division inhibition. Furthermore SfiC was only found in certain E.coli K12 strains, and it is now

believed to be part of the e14 cryptic prophage.

ftsM was identified by Drapeau et al. (1983) as a temperature sensitive mutation that caused abnormal sensitivity to UV light. The mutation was found to be dominant over the wild type gene resulting in an enhanced division inhibition after DNA damage. Drapeau et al. (1984) showed that ftsM was under LexA control and did not require SfiA for its action, but was believed to act on FtsZ (Belhumeur and Drapeau 1984). The recent observation that ftsM is a suppressor of the closely linked leu gene (D.Gill pers.comm. from G.Drapeau) casts some doubt on any physiological role the wild type gene may play in the control of cell division.

dicB is another E.coli gene believed to encode a cell division inhibitor. The dicA, dicB and dicC genes were identified by Bouche and co-workers (Bejar and Bouche 1985, Bejar et al. 1986, Bejar et al. 1987) by chemical mutagenesis of the terminus region of the chromosome. Division inhibition was found associated with DicA, which was presumed to be an inhibitor of DicB. In strains carrying mutations in dicA the inhibition phenotype could be suppressed by a multicopy clone of the third gene in the cluster dicC. Since the whole region could be deleted there seemed to be no normal role for these genes in the control of cell division. Furthermore sequence analysis revealed that dicA and dicC were homologous to the C2 and cro genes of P22 immC suggesting that, like sfiC, these genes may also be part of a cryptic prophage.

A similar system to dicABC is found associated with the F plasmids of E.coli. Division inhibition was observed when replication of these plasmids was impaired. Two genes identified as being involved were ccdA and ccdB. It was proposed that CcdB acted as the division inhibitor and was repressed by CcdA (Jaffe et al. 1985).

1.5.8 The Role of FtsZ in Cell Division

Jones and Holland (1985) investigated the role of FtsZ in SfiA mediated division inhibition. They showed that FtsZ could stabilize SfiA in a maxicell system, increasing its half life from 3 minutes to 12 minutes. This confirmed the hypothesis that SfiA interacted physically with FtsZ to bring about division inhibition. Further confirmation was found with the observation that a sfiB mutant allele of ftsZ (in which SfiA mediated inhibition is repressed) did not affect the stability of SfiA.

Ward and Lutkenhaus (1984) created a LacZ-FtsZ protein fusion, ZZ, which fortuitouly acted as an analogue of SfiA. Upon induction of ZZ with IPTG a reversible inhibition of division was observed resulting in filamentation. This inhibition could be repressed by either the introduction of a sfiB allele or the introduction of a multicopy clone of ftsZ. Ward and Lutkenhaus

proposed two models that would explain the ZZ induced division block and its suppression.

a: Wild type FtsZ normally functions as a multimer, a mixed multimer containing ZZ was presumed to be inactive.

b: The ZZ hybrid protein would compete with wild type FtsZ for certain, limiting, target sites.

Lutkenhaus (1987) has recently extended the results obtained with the ZZ protein by constructing other truncates of FtsZ. It appears that the synthesis of peptides containing the central portion of FtsZ was sufficient to block division. Favouring the multimer model he suggests that this central region may represent a site for oligomerisation.

Holland and Jones (1985) also considered the mechanism of SfiA mediated division block and postulated two possible models for the role of FtsZ in the control of cell division.

1: Positive control; in this model it is the achievement of a critical concentration of FtsZ or its activation by a cell cycle dependent effector that triggers the initiation of cell division. Titration of (active) FtsZ by SfiA would then delay the onset of division.

2: Negative control; in this model FtsZ acts as an inhibitor of

cell division, preventing association of the septal components. This inhibition is removed at a critical stage of the cell cycle by, for example, a low molecular weight effector. SfiA binding to FtsZ would prevent removal of inhibition.

The authors noted that the two models could be distinguished by the response of a cell to an increased concentration of FtsZ. Model 1 would predict an increase in cell division resulting in the production of a smaller cell population. Model 2 would predict a greater inhibition of division resulting in filamentation. Ward and Lutkenhaus performed this experiment and found that overexpression of ftsZ from a lac promoter resulted in the formation of minicells. This suggested that FtsZ is a positive and rate limiting effector of cell division.

Holland and Jones (1985) postulated the role of FtsZ as organising the formation of a septalsome, this being a collection of cell division gene products at the septal site which, upon formation, initiates septation.

Most mutations in ftsZ were isolated as having the SfiB phenotype and for many years only one mutant (PAT84, Lutkenhaus et al. 1980) existed which showed a typical cell division phenotype. Belhumeur and Drapeau (1984) used a novel selection procedure in which they isolated new ftsZ mutants under conditions which brought no selective pressure. Among the phenotypes found associated with the new mutants were UV sensitivity, a defect in

bacteriophage lysogeny and filamentation following a nutritional shift-up. The role of FtsZ in the control of cell division after a nutritional shift-up has recently been studied by Kepes and D'Ari (1987). After a nutritional shift-up cells immediately increase their rate of growth to the new level, but their rate of cell division remains at the preshift level for some time after (section 1.1). Kepes and D'Ari showed that a brief exposure to the richer medium also resulted in a postponement of division. Thus whilst not responding immediately to the new conditions the cell could obviously detect the change and subsequently respond to it. This detection and "memory" response did not require de novo protein synthesis since it could be observed in the presence of chloramphenicol. In sfiB mutants the delay in cell division was found to have more than doubled after the shift up, implicating FtsZ in sensing the state of growth of the cell. The state of growth might be detected by changes in metabolic pools or tRNA acylation, for example.

1.6 Organisation of the Cell Division Genes

Donachie et al.(1984) termed any gene affecting the cell growth and division cycle of E.coli a morphogene. Figure 1.9 shows the distribution of morphogenes around the E.coli chromosome map. A striking aspect of this distribution is the presence of many genes clustered into several large groups. Such clustering could provide a mechanism for co-ordinated expression of these genes. For this reason special interest has been shown in these clusters which are briefly described below.

1.6.1 The 2 Minute Cluster

This region provides the most remarkable clustering of genes involved in cell growth and division. Moving clockwise around the E. coli chromosome map are found first a group of genes required for murein biosynthesis: mraA, mraB, ftsI, murE, murF, murG, murC and ddl (Bachmann 1983). Following these are the cell division genes ftsQ, ftsA and ftsZ (Robinson et al. 1986) and finally envA (Normack 1970) and secA (Oliver and Beckwith 1981). The region from ddl to envA has been extensively studied and its transcriptional organisation determined (figure 1.9).

FIGURE 1.9 Distribution of Morphogenes around the E.coli Chromosome

The circle represents the genetic map of E.coli K-12. The map is divided into 100 units and starts at the top of the circle , moving clockwise around the circle. The OriC and TerC regions are shown for reference.

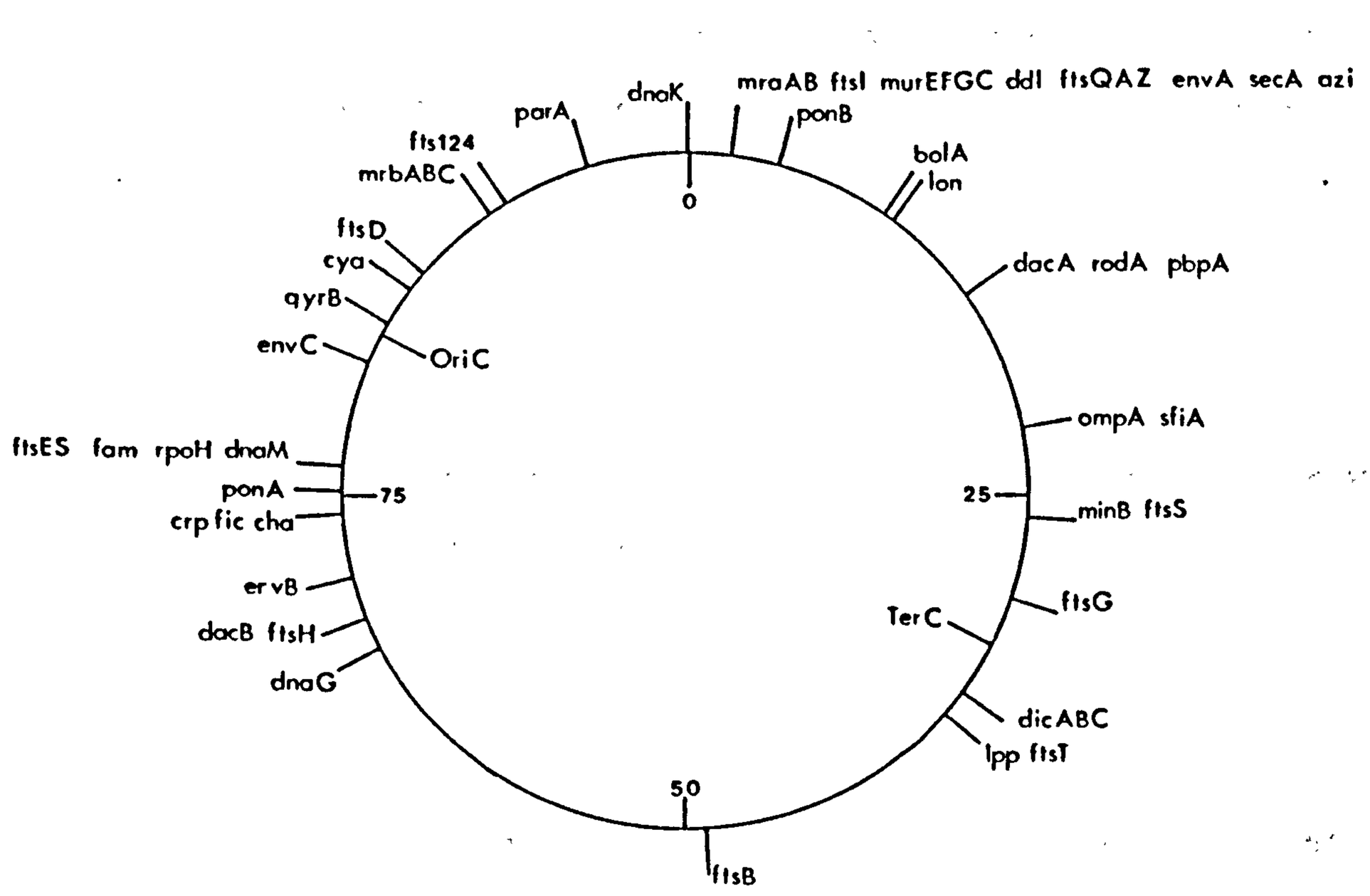
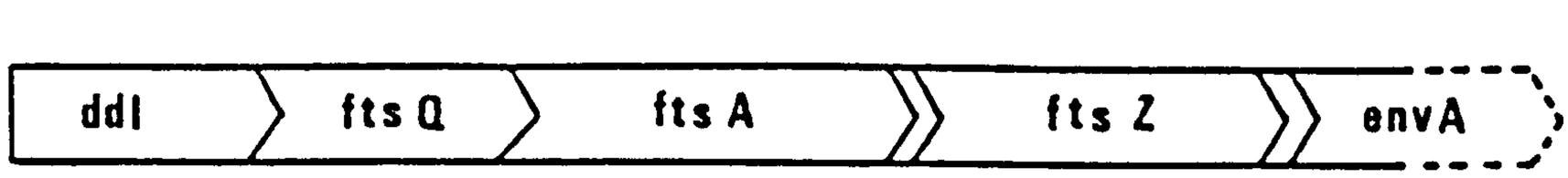


FIGURE 1.10 The 2 minute Gene Cluster



All the genes were found to be transcribed in the same direction, yet no evidence of any operons could be established. All the genes could be expressed independently of the others and individual promoters were identified for all the genes. Transcriptional control of this region has proved complex, at least *in vitro*, and will be considered further in chapters six and eight.

1.6.2 The 15 Minute Cluster

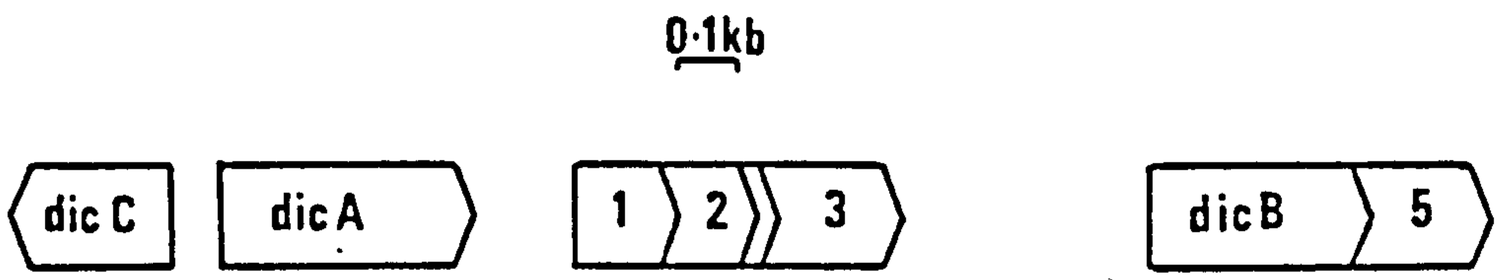
This smaller cluster contains three genes involved in cell growth and division *pbpA*, *rodA* and *dacA* (Spratt *et al.* 1980)

1.6.3 The TerC Cluster

The possible reason for the presence of cell division genes at the terminus of replication was discussed in section 1.4.1. Several genes have been mapped to this region including *ftsS*, *ftsT*, *ts52*, *dif* and *dicABC*, though some of these may prove to be

allelic. The organisation of the dic genes has been determined and is shown below.

FIGURE 1.11 The TerC Gene Cluster



This region has proved dispensible since deletions can be constructed without affecting cell viability.

1.6.4 The 76 Minute Cluster

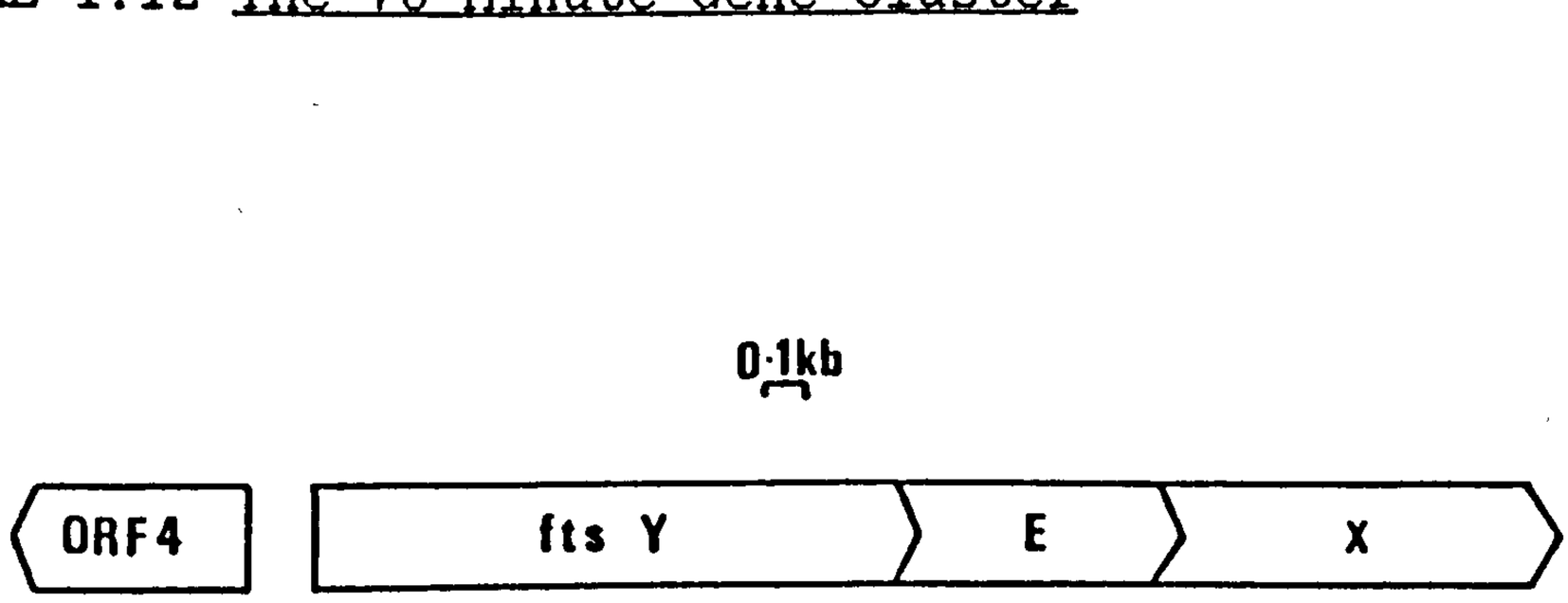
The cell division gene ftsE, as characterised by the mutation in MFT1181 (Ricard and Hirota 1973), was originally mapped to 73 minutes (Bachman and Low 1980) but was later placed at 76 minutes (Bachman 1983). Also located at 76 minutes was the rpoH (htpR) gene characterised by the mutation in TSN K165. RpoH has been shown to be the regulator of the heat shock response (section 1.5.3) and is also believed to be essential at all temperatures (C. Gross pers. comm.). Three other essential genes have also been mapped to the 76 minute region : fam, dnaM and ftsS22. fam

was characterised by a mutation which resulted in a defect in lipoprotein synthesis (Torti and Park 1980), but showed other pleiotrophic effects, including filamentation. dnaM is not actually involved in DNA replication, as its name suggests, but was isolated by accident (Glassberg et al. 1979) and has no major phenotype other than the temperature sensitive nature of the dnaM710 mutant allele.

Salmond and Plakidou (1984) cloned ftsE on a lambda phage containg a 6.5kb partial HindIII fragment, consisting of 4.5kb, 1.1kb and 0.9kb sub-fragments. By recombining the ftsE mutation from MFT1181 onto this phage they showed that the mutated gene in OV22, which also resulted in filamentation and mapped to 76 minutes, was distinct from ftsE and was thus named ftsS.

Gill et al. 1986 then subcloned and sequenced the 4.5kb HindIII fragment (see appendix A) and showed that it encoded four genes. These included ftsE but not ftsS, rpoH, fam or dnaM and are shown below.

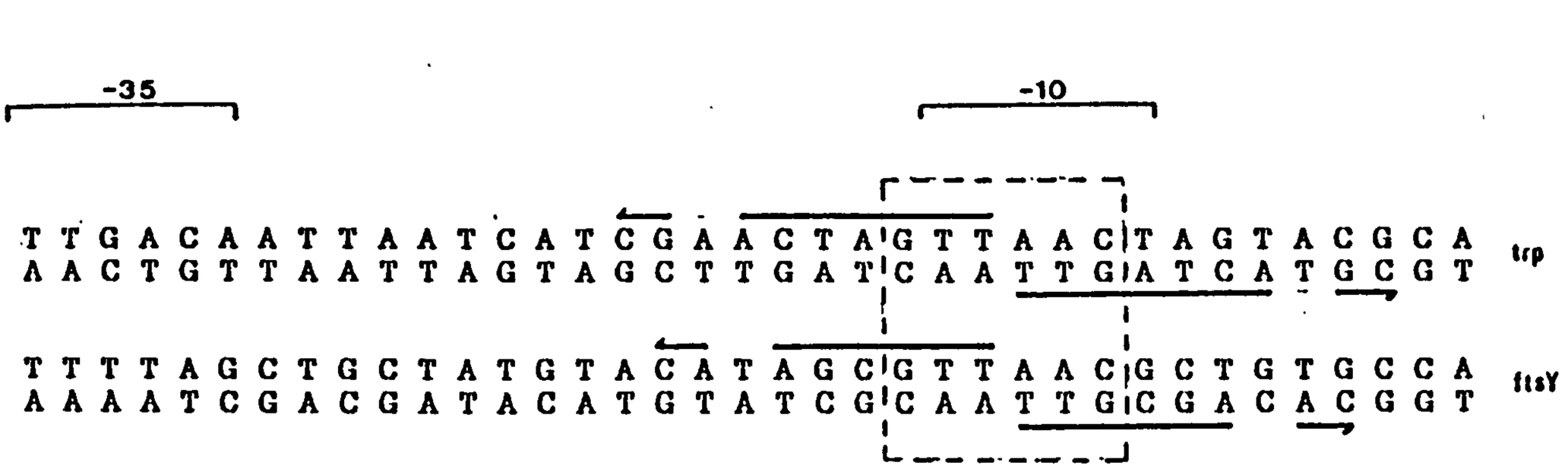
FIGURE 1.12 The 76 Minute Gene Cluster



The use of transposon insertions provided some evidence for an ftsYEX operon, a hypothesis which was strengthened by the finding of a region 5' of ftsY with an homology to the operator region of the tryptophan operator, as shown in figure 1.13.

Figure 1.13 Comparison of ftsY Leader Sequence with the trp Operator

The hexanucleotide homology, the trp -10 and -35 regions and the repeats are all shown on this diagram



The location of the transposon insertions, however, could not totally disprove the existence of internal promoters as found in the 2 minute gene cluster. Thus no clear picture has been established to describe the transcriptional organisation of the 76 minute cluster.

1.7 Aims of this Work

The mapping of seven essential genes (ftsYEXS, rpoH, fam and dnaM) to the 76 minute region of the E. coli chromosome suggests the existence of another morphogene cluster. It seems likely that the clustering of morphogenes is not fortuitous, yet the reason for it remains unknown. The aim of this work was to study the clustering of the 76 minute genes as follows:

1. To clone the fam, dnaM and ftsS genes and establish their proximity to the ftsYEX operon.

2. To determine the transcriptional organisation of the cluster and to identify promoters.

3. To study the control of cell division by characterising mRNA transcripts from the operon.

CHAPTER TWO

Materials and Methods

2.1 Bacterial Strains

All bacterial strains used in this study were derivatives of E.coli K12 and are listed in table 2.1.

2.2 Plasmids

All plasmids used in this study are listed in table 2.2, their construction, where applicable, is described in the relevant chapter.

2.3 Bacteriophage

All bacteriophage used in this study are listed in table 2.3.

TABLE 2.1 Bacterial Strains

Strain	Genotype	Source	Reference
OV2	F- *trp*(am) *leu ilv his thyA deo ara*(am) *lac*(am) *galU*(am) *galE tsx*(am) *supF*(ts)	G. Salmond	Donachie *et al.* 1976
OV22	As OV2 but *ftsS*(am)	G. Salmond	Salmond & Plakidou 1984
OV22 *recA*	As OV22 but *recA56* *srl*::Tn10	D. Gill	Gill *et al.* 1986
OV32	As OV2 but *ftsX*	D. Gill	ibid
OV32 *recA*	As OV32 but *recA56* *srl*::Tn10	D. Gill	ibid
GSY	As OV2 but *htpR165* *zhf*::Tn10	G. Salmond	

MFT1181	F- leu thr argH his trp ftsE thyA lacY mtl xyl tonA supE44 Str^R	D. Gill	Ricard & Hirota 1973
MFT1181 recA	As MFT1181 but recA56 srl::Tn10	D. Gill	Gill et al. 1984
JGC127	dnaM-710 zhg::Tn10 thy leu his met arg	M.C.Jones-Mortimer	Glassberg et al. 1979
OJ127	As OV2 but dnaM-710 zhg::Tn10		This Study
ST715	fam-715 (am) pro his thi	G. Salmond	Torti and Park 1980
ST715 recA	As ST715 but recA56 srl::Tn10		This Study
DH1	F- recA1 endA1 gyrA96 thi hsd R17 (rk- mk-) supE44 relA1	Lab Stock	Hanahan (1983)
CSHDF6	F- ara Δ(lac-pro) rpsL thi Δ(recA-srl)F6 sup^0	D. Gill	Jones & Holland 1984

JC10240	Hfr PO45 srl300::Tn10 recA56 thr ilv rpsE	G. Salmond	Csonka et al. 1980
GS003	Δ(gal attλ bio uvrB) nadA::Tn10	G. Salmond	Salmond & Plakidou 1984
N100	galK2 recA13 pro	K. Begg	McKenney et al. 1981

TABLE 2.2 Plasmids

Plasmid	Construction/Comments	Reference/Source
pBR322	Multicopy Ap^R Tc^R	Bolivar *et al.* 1977
p0.9	0.9kb HindIII fragment from λS3d into pBR322	This study Chapter 3
p1.1	1.1kb HindIII fragment from λS3d into pBR322	This study Chapter 3
pH3E	10kb HindIII fragment from λS3d into pBR322	This study Chapter 3
pH3C	4.5kb HindIII fragment from λS3d into pBR322	This study Chapter 3
pH3C'	As pH3C but opposite orientation	This study Chapter 3
pCΔr	0.4kb EcoRV deletion from pH3C	This study Chapter 4

pCΔs	1.7kb Sph1 deletion from pH3C	This study Chapter 6
pCΔb	65bp BstE2 deletion from pH3C	This study Chapter 6
pCΔsr	3.3kb Stu1-EcoRV deletion from pH3C	This study Chapter 6
pCΔa	2.4kb AatII deletion from pH3C	This study Chapter 6
pCΔaΔp	2.6kb PvuII deletion from pCΔa	This study Chapter 6
pC'Δs	3.8kb Sph1 deletion from pH3C'	This study Chapter 6
pSPHa	11kb Sph1 fragment from λS3d	This study Chapter 3
pSPHb	4.6kb Sph1 fragment from λS3d	This study Chapter 3
pSPHd	12.5kb Sph1 fragment from λS3d	This study Chapter 3

pSPHd'	As pSPHd but opposite orientation	This study Chapter 3
pdΔH	1.5kb HindIII deletion from pSPHd	This study Chapter 3
pdΔSS	8.5kb Stu1 deletion from pSPHd	This study Chapter 3
pdΔSSΔH	1.5kb HindIII deletion from pdΔSS	This study Chapter 3
pdΔSSΔCla	1.7kb Cla1 deletion from pdΔSS	This study Chapter 3
pdΔSSΔRV	2.7kb EcoRV deletion from pdΔSS	This study Chapter 3
pdΔSS4.5	4.5kb HindIII fragment from pH3C into pdΔSSΔH	This study Chapter 3
pR2	1.3kb EcoRV fragment from pSPHd into pBR322	This study Chapter 3
pKO2	galk expression vector	McKenney et al. 1981

P18	1.9kb PvuII fragment from pH3C into pKO2	This study Chapter 6
P7	As P18 but opposite orientation	This study Chapter 6
P3	1.8kb PvuII fragment from pH3C into pKO2	This study Chapter 6
P9	As P3 but opposite orientation	This study Chapter 6
HP14	0.4kb Pst1 fragment from pH3C into pKO2	This study Chapter 6
PST4	As HP14 but opposite orientation	This study Chapter 6
R4	0.3kb EcoRV fragment from pSPHd into pKO2	This study Chapter 6
R6	As R4 but opposite orientation	This study Chapter 6

TABLE 2.3 Bacteriophage

Phage	Characteristics	Source	Reference
λpGS22	ftsYEXS$^+$	G. Salmond	Salmond and Plakidou 1984
λS3d	ftsYEX$^+$ rpoH$^+$	This Study	
λ4.5	ftsYEX$^+$	This Study	
λ540	HindIII Cloning Vector	G. Salmond	Borck et al. 1976
λpsupF	λ540, supF	K. Hussain	ibid
λb2red	recombination deficient	G. Salmond	Lab stock
λvir	virulent (λ$^+$ immunity)	G. Salmond	Lab stock
λimm21^c	virulent (immunity 21)	G. Salmond	Lab stock
P1 vir	virulent form of P1	K. Hussain	Lab stock
T4GT7	generalised transducing derivative of T4	J. Hinton	Wilson et al. 1979

2.4 Media

The various media and other solutions routinely used in this study are described in table 2.4. They were prepared in double distilled water and sterilized by filtration or by autoclaving at 120°C for 20 minutes.

Agar plates contained 1.5% (w/v) Difco Bacto agar; top agar contained 0.5% (w/v) Difco Bacto agar.

Ampicillin (Ap) was added to a final concentration of 50ug/ml.

Tetracycline (Tc) was added to a final concentration of 10ug/ml.

Amino acids and sugars were added to media at final concentrations of 20ug/ml and 0.2% (w/v) respectively.

TABLE 2.4 Media and Solutions

Media	Constituents per litre
Nutrient Broth (NB)	13g Oxoid Nutrient Broth
Luria Broth (LB)	10g Bacto Tryptone 5g Bacto Yeast Extract 5g Sodium Chloride
Double Difco (DD)	20g Bacto Tryptone 8g Sodium Chloride 2ml 1M Magnesium Sulphate
M9 Salts Solution pH 7.4	6g Disodium Hydrogen Phosphate 3g Potassium Dihydrogen Phosphate 10g Ammonium Chloride 5g Sodium Chloride
K Medium	M9 Salts solution (1 litre) 50ml 20% (w/v) Bacto Casamino acids 0.1ml 0.1% (w/v) thiamine 10ml 20% (w/v) Glucose 2ml 1M Magnesium Sulphate 0.1ml 1M Calcium Chloride

Hershey Salts Solution pH7.4	3.0g Potassium Chloride 5.4g Sodium Chloride 1.1g Ammonium Chloride 15mg Calcium Chloride 0.2g Magnesium Chloride 0.2mg Iron (III) Chloride 87mg Potassium Dihydrogen Phosphate 121g Tris HCl
Hershey Medium	Hershey Salts 20ml 20% (w/v) Glucose 10ml 2% (w/v) Proline 1ml 0.1% (w/v) Thiamine
20X SSC	175g Sodium Chloride 88g Sodium Citrate
TE Buffer pH8	60.6g Tris HCl 14.6g EDTA
50X TAE Buffer pH8	242g Tris HCl 57.1ml Glacial Acetic Acid 100ml 0.5M EDTA pH8

5X TBE Buffer	54g Tris HCl 27.5g Boric acid 20ml 0.5M EDTA pH8
SM	5.8g Sodium Chloride 2g Magnesium Sulphate 50ml 1M Tris HCl pH7.5 5ml 2% Gelatin
Denhardts Solution	10g Ficoll 400 10g Polyvinyl pyrrolidone 10g BSA

2.5 Growth of Bacterial Cultures

The E.coli strains used in this study were grown at 30°C, 37°C or 42°C depending on the particular strain or experiment.

Liquid cultures, unless otherwise stated, were grown in 25ml Universal bottles (1-15ml) or conical flasks (25-500ml) in a Gallenkamp orbital shaker (150rpm). Culture density was measured at 600nm using a Unicam sp500 spectrophotometer.

Cells were harvested from liquid culture by centrifugation in an MSE Micro Centaur (high speed, 3 minutes, 1-1.5ml) or an MSE Chilspin (5krpm, 10 minutes, 2-15ml) or an MSE Hi-Spin (7krpm, 10 minutes, 25-500ml).

2.6 Testing of Temperature Sensitivity

The testing of temperature sensitivity was performed with both solid and liquid media. Using solid media a single colony, isolated at the permissive temperature, was streaked onto two identical plates. One plate was incubated at the permissive temperature, the other at the non-permissive temperature. Growth at the two temperatures was compared by light microscopy. In

liquid culture a single colony was inoculated into broth and grown up to early log phase at the permissive temperature; the culture was then divided into two. Incubation at the permissive temperature was continued for one half whilst the other half was incubated at the non-permissive temperature. Growth was followed by a combination of cell density measurement and light microscopy.

2.7 Use of Bacteriophage P1

2.7.1 Preparation of Lysates

A 0.1ml sample of an overnight nutrient broth culture was mixed with 3ml top nutrient agar and $CaCl_2$ (5mM) in a test tube prewarmed to 45°C. Several such tubes were set up to which P1 was added to give a range of moi. The top agar was poured onto a double difco agar (DDA) plate. Once set the plates were inverted and incubated at the appropriate temperature until confluent lysis occured. The top agar in which lysis was observed was scraped off the plate with a sterile spatula into a Universal bottle. 5ml of NB were then swilled round the agar plate before being poured into the universal. This was then vortexed (Fisons Whirlimixer) at room temperature for 5-10 minutes and centrifuged (5k, 10min). The supernatant was removed from the agar and stored

at 4°C over a few drops of chloroform.

The lysate was titred by setting up a series of top agar lawns as above, using serial dilutions of phage , to give well isolated, scorable plaques.

2.7.2 Transduction

A 1ml sample of an overnight culture grown in nutrient broth was mixed with P1 lysate (moi about 1) and $CaCl_2$ (5mM) in a 1.5ml Eppendorf tube. The tube was incubated without shaking at the appropriate temperature for 30 minutes. The cells were then pelleted and washed in 5mM sodium citrate before being resuspended in nutrient broth containing 5mM sodium citrate. Expression time was given if required before plating out on the appropriate media.

2.8 Use of Bacteriophage T4

Preparation of lysates and transduction with T4 was performed as with P1 but with the following differences. For adsorption of the phage, tryptophan (50ug/ml) was used instead of $CaCl_2$. No citrate was required in the washing or resuspension steps.

2.9 Use of Bacteriophage Lambda

2.9.1 Plaque Purification

A 0.1ml sample of a lysate was mixed with 0.1ml of an overnight culture of DH1 (grown in LB + 0.2% maltose) and 3mls of top agar containing 10mM $MgSO_4$. The top agar was then poured onto a DDA plate and incubated at 37°C for about 6 hours. Various dilutions of the lysate were used in order to give single isolated plaques. One plaque was picked, using a Pasteur pipette, and resuspended in 1ml of SM. This was stored over a few drops of chloroform at 4°C.

2.9.2 Preparation of Lysates

Phage lambda lysates were prepared as for plaque purification except that a dilution of plaque-purified stock was used that gave confluent lysis. The top agar was recovered with a spatula and added to a universal. The plate was then washed with 3ml of SM which was added to the universal followed by vortexing and centrifugation to give the lysate; which was stored over chloroform at 4°C.

2.9.3 Isolation of Lysogens

A seeded top nutrient agar lawn, containing the lambda sensitive strain grown overnight in LB + 0.2% maltose, was poured onto a DDA plate. 10ul of a plaque-purified lysate was spotted onto this lawn and incubated overnight at the appropriate temperature. Cells were removed from the turbid spot and streaked to single colonies. These colonies were tested for lysogeny by their ability to release phage when stabbed into a lawn of DH1. These putative lysogens were then tested for immunity to a homoimmune phage whilst remaining sensitive to λvir.

2.9.4 Superinfection Curing of Lysogens

λb2red (10ul) was spotted onto a seeded top agar lawn of the lysogen and incubated overnight at 30°C. Cells were picked from the turbid spot, streaked to single colonies and tested for lysogeny as described in the previous section.

2.9.5 UV Induction of Lysogens

An overnight culture of the lysogen (1ml) was diluted into 50ml of LB and grown to mid-log phase. The cells were then spun down and resuspended in SM to give an OD_{600} of 1. This suspension

(10ml) was added to a petri dish and irradiated (300 ergs/mm^2) with short wave UV light. A 200ul sample was removed and added to 2mls of LB + 10mM $MgSO_4$ and incubated with shaking at 30°C until lysis occured. The cell debris was removed by centrifugation and the lysate was stored over chloroform.

2.9.6 Isolation of Phage DNA

A 10ml sample of lysate prepared as above was extracted with an equal volume of phenol mix (section 2:10) followed by a chloroform extraction. The aqueous layer (5ml) was then centrifuged in a CsCl gradient in a VTi65 rotor (section 2:12) in order to purify the DNA.

2.9.7 Transfection of Phage DNA

About 100ng of DNA were added to 0.1ml of competent DH1 cells (section 2:21) and left on ice for one hour. This was then added to 3ml of prewarmed top agar containing 10mM $MgSO_4$ and poured onto a DDA plate. This was incubated overnight to allow the plaques to form.

2.10 Extraction of DNA with Phenol/Chloroform

Phenol mix was prepared as described in Maniatis et al. (1982) and stored in the dark. In the extraction procedure an equal volume of phenol mix was added to the DNA solution. The total mix was vortexed to form an emulsion which was separated by centrifugation (high speed, 5 minutes). The upper aqueous phase was then re-extracted with chloroform/IAA (24/1 v/v) to remove traces of phenol.

2.11 Precipitation of DNA with Ethanol

Ammonium acetate (0.5 volumes, 7.5M, pH7.5) and 2 volumes of ethanol were added to the DNA solution, which was then left at -20°C for 30 min to precipitate. The DNA pellet was recovered by centrifugation (Micro-Centaur, high speed, 10min), dried in a vacuum desiccator, and resuspended in TE buffer

2.12 Caesium Chloride Gradient Purification of DNA

Caesium chloride gradients were run in either a VTi70 (14ml) or VTi65.2 (5ml) rotor for 48h at 36krpm or 4h at 65krpm respectively. The crude DNA solution was mixed with caesium chloride (0.9g/ml) and

ethidium bromide (0.4 mg/ml) before being added to the centrifuge tube and spun in the appropriate rotor. After centrifugation the DNA band was visualised by long wave UV light and removed in 0.5-1ml using a 1ml syringe and needle. Ethidium bromide was removed by several extractions with propan-2-ol equilibrated with caesium chloride solution (0.9g/ml). The caesium chloride was removed by dialysis against TE Buffer (1 litre) with two buffer changes spread over at least 3 hours.

2.13 Small Scale Preparation of Plasmid DNA

The method used was that of Holmes and Quigley (1981). An overnight culture (5ml), grown in the presence of the appropriate antibiotic, was pelleted and resuspended in 350ul of lysis mix, the composition of which is given below,

8% w/v sucrose

0.5% Triton X100

50mM EDTA

10mM Tris pH8 (HCl)

in an Eppendorf tube. Lysozyme (25ul of a 10mg/ml solution in 10mM Tris pH8) was added and the tube boiled for 40 seconds. After centrifugation (high speed, 15mins) the pellet was removed and the lysate extracted wih phenol mix (section 2:10). After precipitation by ethanol the DNA pellet was resuspended in TE.

2.14 Large Scale Preparation of Plasmid DNA

The procedure used was essentially that of Clewell and Helinski (1970). An overnight culture (5ml) of the plasmid containing strain was diluted into 500ml of LB containing the appropriate antibiotics. The culture was grown for several hours until it reached mid-exponential phase. Chloramphenicol was then added to a final concentration of 250ug/ml. The incubation was then continued overnight. The cells were harvested by centrifugation and resuspended in 16ml of the following buffer:

25% sucrose (w/v)

0.05M Tris pH8.
HCl

Lysozyme (5ml of a 5mg/ml solution in 0.25M Tris pH8) were added and the mixture left on ice for 5 minutes. EDTA (4.5 ml of a 0.25M, pH8 solution) were added and again the mixture was left on ice for 5 minutes. Finally 18ml of lysis mix, with the following composition,

1%	w/v Brij 35
0.4%	w/v sodium deoxycholate
0.06M	EDTA
50mM	Tris pH 8 HCl

were added and the cells lysed by gentle mixing and incubation in a 42°C water bath. When lysed the cell debris was removed by centrifugation (hi spin, 18krpm, 15') and the cleared lysate stored at 4°C.

Caesium chloride gradient purification of the DNA was then performed in either a VTi70 or VTi65.2 rotor, followed by a propan-2-ol extraction and dialysis as in section 2:12

2.15 Preparation of Chromosomal DNA

An overnight culture of the strain (5ml) was diluted into 500ml of LB and incubated until the culture had reached mid-log phase. The cells were harvested and lysed by the method outlined in section 2:14. The lysed cell extract was treated twice with phenol mix and once with chloroform (section 2:10). The partially cleared lysate was then loaded onto a caesium chloride gradient (section 2:12) to purify the chromosomal DNA.

2.16 Agarose Gel Electrophoresis of DNA

Horizontal gel slabs were prepared by boiling agarose in TAE buffer and then adding ethidium bromide (0.5ug/ml final concentration) before pouring. The gels were run submerged in TAE (+ 0.5ug/ml ethidium bromide) at a constant voltage, which depended on the apparatus used. Agarose concentrations between 0.4% and 2% (w/v) were used depending on the resolution required, but 0.6% was used routinely.

The DNA bands were visualised using a transilluminator and photographed using a Polaroid instamatic camera system. The fragment sizes, relative to known molecular weight markers, were determined using the program DNAGEL written in BBC basic for a BBC microcomputer based on the algorithm of Schaffer and Sederoff (1981).

2.17 Recovery of DNA Fragments from Agarose Gels

2.17.1 Hot Phenol Method

The DNA band was visualised under long wave UV light and cut out of the gel with as little excess agarose as possible. The agarose block was added to 300ul of TE buffer in an Eppendorf tube. Phenol mix (300ul, section 2:10) was then added and the tube

incubated at 65°C for 30 minutes with occasional vortexing. After centrifugation (high speed, 10') the aqueous layer was re-extracted with phenol and precipitated with ethanol.

2.17.2 DE81 Method

This method is derived from that of Dretzen et al. (1981). After electrophoretic separation of the DNA fragments a piece of DE81 paper, pre-soaked in TAE, was slipped into a slit cut beneath the fragment to be removed. This DE81 paper was previously prepared by soaking in 2.5M NaCl and storage in 1mM EDTA. Electrophoresis was continued with the fragment running into and adsorbing onto the DE81 paper. The movement of the fragment through the gel was followed using long wave UV light. After the fragment had run onto the paper the paper was removed, washed briefly in water and then vortexed with 0.5ml of elution buffer (shown below) in an Eppendorf tube.

1.5M NaCl

1mM EDTA

20mM Tris pH8
HCl

This was incubated for at least two hours at 37°C. A small hole was pierced in the base of the tube and the fluid contents centrifuged through the hole into another tube. The DE81 paper remained in the original tube. The ethidium bromide was removed by extraction with water-saturated butanol and the DNA

precipitated by the addition of two volumes of ethanol.

2.18 Endonuclease Digestion of DNA

Endonuclease digestion of DNA was carried out in either high salt, medium salt, low salt or Sma1 buffer, as described by Maniatis et al. (1982), depending on the enzyme used. These buffers were made up as 10X stocks and stored at -20'C. digestions were carried out at the recommended temperature for at least 60 minutes. Heat treated RNAseA (100ug/ml) was added where necessary.

2.19 End filling of DNA

The DNA fragment to be end filled was added to the following reaction mix:

16ul DNA fragment
2ul 10x T4 buffer
1ul 2mM dNTPs
1ul T4 DNA polymerase (2 units)

The incubation was carried out at 37°C for one hour. 10x T4 buffer has the following composition:

0.33M Tris Acetate pH 7.9

0.66M Potassium Acetate

0.10M Magnesium Sulphate

5mM DTT

1mg/ml BSA

2.20 Ligation of DNA

The DNA samples to be ligated were diluted and mixed to the appropriate concentration in TE. When ligating "sticky" ended DNA, the mixture was heated at 65°C for 5 minutes then left on ice for 60 minutes to allow melting and re-annealing of the ends. 10x ligation salts were added as a ten fold dilution.

10x ligation salts:

4mM ATP

66mM $MgCl_2$

100mM DTT

660mM Tris pH 7.6
HCl

After the addition of T4 DNA ligase the mixture was incubated for at least 4 hours at 15°C.

2.21 Transformation

Recipient cells were grown in 15mls of NB to an OD_{600} of about 0.4, at which point they were harvested by centrifugation (5k 5 minutes), and resuspended in 5ml of ice cold 100mM $CaCl_2$ (Sigma grade 1). After 30-60 minutes incubation on ice the cells were again pelleted and resuspended in 1ml of ice cold 100mM $CaCl_2$. After 5 minutes on ice these 'competent' cells were ready for transformation, although their 'competence' increased after overnight storage at 4°C.

In the tranformation procedure 0.1ml of competent cells were mixed with the DNA to be transformed and left for 30 minutes on ice. The cells were then heat shocked (42°C 2 minutes) and diluted with 0.1ml of NB. If expression time was required they were then incubated at the appropriate temperature for one hour before plating out.

2.22 Polyacrylamide Gel Electrophoresis (PAGE)

2.22.1 DNA

A 3.5% gel was routinely used for sizing DNA fragments between 100 and 1000 base pairs in length. The gel was prepared by mixing the following stock solutions:

10.0ml 10X TBE

11.6ml 30% acrylamide/bis (30%:1% w/v)

2.1ml 3% (w/v) ammonium persulphate

76.3ml water

After degassing, 30ul of TEMED was added , the gel poured and left for 1 hour to set. DNA samples were mixed with loading buffer (section 2:16) before loading. The gel was then run in 1X TBE at 80V for about 6h. After staining in 1X TBE + 2ug/ml ethidium bromide for 45', the DNA fragments were visualised and photographed as in section 2:16.

2.22.2 Proteins

Proteins were analysed on 10% linear SDS-PAGE gels, consisting of a stacking and a resolving gel, which were prepared by mixing the following stock solutions:

	Stacking	Resolving	
Acrylamide/bis	2.5ml	10.0ml	(30%:1% w/v)
Stacking buffer	5.0ml		(0.5M Tris Hcl pH 6.8)
Resolving buffer		3.8ml	(3.0M Tris Hcl pH 8.8)
10% SDS w/v	0.2ml	0.3ml	
3% APS w/v	0.5ml	0.8ml	
Water	11.8ml	15.2ml	
TEMED	15.0ul	15.0ul	

Gels were run in the following buffer:

1% SDS

1.92M glycine

0.25M Tris pH 8.3
Hcl

at 25mA for about four hours.

2.23 Treatment of SDS-PAGE Gels

2.23.1 Autoradiography

After electrophoresis the gels were fixed in 50% (v/v) methanol

for 30 minutes and dried at 80°C using a Bio-Rad model 1125B gel drier. The gels were then placed in a film cassette with a sheet of Kodak X-omat s X-ray film. The film was exposed to the gel at room temperature. The film was developed using Kodak LX24 developer (4 minutes) and fixed in Kodak FX-40 (2 minutes).

2.23.2 Fluorography

The method of Skinner and Griswold (1983) was used. Gels were fixed for 5 min in 200ml of glacial acetic acid (GAA) and then soaked in 200ml of 20% (w/v) 2,5-diphenyloxazole (PPO) in GAA for 90 min. Finally, the gels were soaked in distilled water for 30 min before drying at 60°C and exposure to X-ray film at -70°C.

2.24 The Maxicell System

Plasmid encoded gene products were identified using the maxicell system of Sancar et al. (1979). The recA strain CSH26 was transformed with the plasmid of interest. The strain was then grown up in K-medium to an OD_{600} of 0.5. A 10ml sample was irradiated with short wave UV light at a dosage of 50 J/m^2 in a petri dish. After transferring the irradiated cells to a universal bottle, Cycloserine (200ug/ml) was added and the culture incubated for 14-16 hours. The cells were then harvested by centrifugation and washed twice in Hershey salts before being resuspended in 5ml of Hershey medium. Following incubation for 1

hour, 30uCi ^{35}S-methionine were added and the incubation continued for a further hour. The cells were then harvested and washed in 10mM Tris$_{HCl}$ pH8 and finally resuspended in 50ul of 10mM Tris pH8. The cell proteins were solubilised by adding 50ul of SDS sample buffer:

10% v/v glycerol
2% w/v SDS
5% v/v 2-mercaptoethanol
50mM Tris pH 6.8

and boiling for 5 min. The samples were stored at -20°C and reboiled for 3 min before electrophoresis.

2.25 ^{32}P Labelling of DNA by Nick Translation

DNA Fragments for nick translation were routinely prepared by either the hot phenol (section 2:17:1) or the DE81 (section 2:17:2) method. The reaction mixture shown below was incubated at 15°C for 1 hour.

DNA solution	28ul
10X Nick Translation Buffer	4ul
1mM dATP	1ul
1mM dGTP	1ul
1mM dTTP	1ul
25uCi ^{32}P dCTP	2.5ul
DNAse1 (100ng/ml)	1ul
E.coli DNA polymerase	1.5ul (5 units)

The reaction mixture was then diluted into 200ul of TE and the unincorporated nucleotides removed by passage through a column of Sephadex G50 equilabrated with TE.

10X Nick Tranlation Buffer:

0.5M	Tris HCl pH7.2
0.1M	Magnesium Sulphate
1mM	DTT
500ug/ml	BSA

2.26 Southern Blotting

2.26.1 Transfer of DNA to the Nitrocellulose Filter

The DNA samples to be probed were electrophoresed on an agarose gel and photographed as in section 2:16. The DNA was denatured by soaking the gel in the following solution:

1.5M NaCl

0.5M NaOH

for 1 hour at room temperature with constant shaking. The gel was then neutralised by soaking in the following solution:

1.5M NaCl

1M Tris pH 8
HCl

with constant shaking for 1 hour.

The denatured gel was placed on a sheet of Whatman 3MM paper soaked in 10X SSC. On top of the gel was placed the nitrocellulose filter and on top of that two sheets of 3MM paper and a stack of paper tissues. Transfer was allowed to continue overnight. The filter was then soaked in 6X SSC before being left to dry on a sheet of 3MM paper. When dry the filter was baked at

80°C in a gel drier between two sheets of 3MM paper. After baking for 2 hours the filter could be stored dry at room temperature until required for hybridisation.

2.26.2 The Hybridisation of Southern Filters

The baked filter was wetted with 6X SSC and placed inside a heat sealable plastic bag with 0.2ml of prehybridisation fluid, which had the following composition, for each square centimetre of filter.

6X SSC

0.5% SDS w/v

5X Denhardts Solution

100ug/ml denatured salmon sperm DNA

The bag was then sealed and incubated for 2-4 hours submerged in a 68°C water bath. After this time the bag was opened and the prehybridisation fluid squeezed out. 50ul of hybridisation fluid ,with the following composition,

6X SSC

10mM EDTA

5X Denhardts Solution

0.5% SDS w/v

100ug/ml denatured salmon sperm DNA

denatured ^{32}P DNA prbe

were added for each square centimetre, and incubation at 68°C was continued overnight. The filter was removed from the hybridisation fluid and initially washed in

2X SSC

0.5% SDS w/v

followed by a 15 min soaking in

0.2X SSC

0.1% SDS w/v

Finally the filter was washed in

0.1X SSC

0.5% SDS w/v

at 68°C for 2 hours. After washing the filter was dried at room temperature before autoradiography at -70°C.

2.27 Preparation of RNA

All glassware used in the preparation of RNA was baked overnight

at 150°C. All tubes, tips and reagents (except phenol and ethanol) were autoclaved before use. After baking, and/or autoclaving, all equipment and reagents were handled with gloves.

The relevant strain was grown up in NB to an OD_{600} of about 0.6. The cells were harvested by centrifugation and resuspended in 450ul of SAE, whose composition is shown below.

0.5% SDS, w/v

1mM EDTA

20mM NaAc pH 5.5.

BRL ultra pure phenol (450ul, equilibrated with 20mM NaAc pH5.5) were added and an emulsion formed by vortexing. The mixture was then incubated at 65°C for 5 min with regular mixing. After centrifugation (high speed, 10 min) the phenol extraction was repeated on the aqueous phase. After the second extraction, the RNA in the aqueous phase was precipitated at -70°C for 30 min by the addition of 1ml of ethanol. The RNA was then pelleted (high speed, 10 min), resuspended in 333ul of SAE and precipitated as before. After pelleting for the second time the RNA was dried and resuspended in 200ul of SAE, before being stored at -70°C.

2.28 Northern Blotting

2.28.1 Electrophoresis and Transfer of RNA

The RNA to be probed was electrophoresed on a formaldehyde denaturing agarose gel. The gel was prepared by boiling 1.5g of agarose with 64ml of distilled water. When the agarose had dissolved 10ml of 10X MOPS buffer ,whose composition is shown below,

10mM EDTA pH 8

100mM NaAc

400mM MOPS pH7

were added followed by 16ml of formaldehyde; the gel was then immediately poured. The RNA samples were denatured by heating 5ul with 15ul of sample buffer

50% formamide v/v

15% formaldehyde v/v

1x MOPS buffer,

10% glycerol v/v

100ug/ml ethidium bromide

250ug/ml xylene cyanol

250ug/ml bromophenol blue

at 68°C for 3 min before loading. The gel was run at 80V in 1X MOPS buffer. After electrophoresis the gel was washed in water, photographed, then soaked twice in 10X SSC for 20 min. The RNA was then transferred to nitrocellulose and probed exactly as described for Southern blotting (section 2:26).

2.28.2 Staining the Nitrocellulose Filter

After hybridisation and autoradiography the filter was soaked in 5% (v/v) glacial acetic acid for 15 minutes. It was then soaked in

0.04% (w/v) Methylene blue

0.5M Sodium Acetate pH 5.1

for 10 minutes before washing in water. The RNA bands appeared dark blue on a light blue background.

2.29 S1 Mapping

2.29.1 End Labelling

The DNA fragment to be end labelled was recovered from an agarose

gel using the DE81 method (2:17:2) and added to the following reaction mix.

DNA fragment	38ul
10x PNK buffer	5ul
^{32}P γ-ATP	5ul (50uCi)
PNK	2ul (20 units)

10x PNK buffer had the following composition

500mM	Tris HCl (pH 7.6)
100mM	$MgCl_2$
50mM	DTT
1mM	Spermidine
1mM	EDTA

The reaction was incubated at 37°C for one hour.

2.29.2 Hybridisation

5ul of the end-labelling reaction was added to 100ug (in 100ul) of RNA in SAE. The nucleic acids were precipitated by the addition of three volumes of ethanol (-70°C, 15 minutes). The RNA and probe were recovered by centrifugation and resuspended in 30ul of hybridisation buffer, which had the following composition,

80% formamide v/v

20mM PIPES (pH 6.5)

400mM NaCl

in a 1.5ml Eppendorf tube and heated to 75°C for 10 minutes to denature any duplexes. The tube was then transfered to a water bath at 52°C where it was left for 3 hours.

2.29.3 S1 Nuclease Digestion

After 3 hours hybridisation 300ul of ice cold 10x S1 buffer were added followed by 200 units of S1 nuclease. The reaction tube was incubated at 37°C for one hour. The resulting RNA:DNA hybrid was recovered by precipitation with ammonium acetate/ethanol (-70°C, 15 minutes) and resuspended in 20ul of TE. This sample was then electrophoresed on an agarose gel. The gel was dried (80°C, 90 minutes) and autoradiographed.

10x S1 buffer:

300mM NaAc (pH 4.6)

500mM NaCl

10mM ZnSO4

50% Glycerol v/v

2.30 Primer Extension Mapping

2.30.1 Preparation of Oligonucleotide Primer

Oligonucleotide primers (20mers) were synthesized using an Applied Biosystems 380B DNA synthesizer. The primer was end labelled in the following reaction mix.

Primer	5ul	
^{32}P δ ATP	3ul	(30uCi)
10x PNK buffer	1.5ul	(section 2:29:1)
water	3.5ul	
PNK	2ul	

The reaction was incubated at 37°C for one hour.

2.30.2 Hybridisation

5ul of the end labelling reaction was added to 50ug (in 50ul) of RNA in SAE and precipitated by the addition of three volumes of ethanol (-70°C, 15 minutes). The recovered nucleic acids were resuspended in 10ul of the following hybridisation buffer

200mM NaCl
5mM PIPES (pH 6.5)

and sealed in a glass capillary tube. The capillary tube was heated to 85°C for 10 minutes to denature any duplexes before being transfered to a 52°C waterbath for three hours.

2.30.3 Primer Extension Reaction

After hybridisation, the mixture was recovered from the capillary tube and added to 80ul of the following reaction mix:

100mM	Tris HCl (pH 8)
0.5mM	dNTPs
10mM	DTT
12mM	$MgCl_2$
25ug/ml	Actinomycin D

Reverse transcriptase (10 units) was added and the reaction incubated at 42°C for one hour. The addition of 10ul of 1M NaOH followed by boiling for 5 minutes degraded the RNA. After neutralisation with 10ul of 1M HCl the mixture was precipitated with ammonium acetate/ethanol (-70°C, 15 minutes). The extended primer was recovered by centrifugation and resuspended in formamide dyes (2:31) ready for loading on a sequencing gel.

2.31 DNA Sequencing

The dideoxy chain termination method was used as described by Bankier and Barrell (1984) using the tg130 and tg131 M13 vectors.

2.32 Galactokinase Assay

An overnight culture of the strain to be assayed was diluted 1 in 10 into 10ml of K medium containing 50ug/ml ampicillin. When the culture had reached an OD_{600} of about 0.5 a 1ml aliquot was dispensed into a test tube. To this 40ul of the following lysis mix

100mM EDTA pH8

100mM DTT

50mM Tris HCl

and 20ul of toluene were added and the tube vortexed for one minute. After 5-10 minutes incubation at 37°C (to evaporate the toluene) 20ul of the cell extract was added to the following reaction mix

20ul Mix 1	5mM	DTT
	16mM	Sodium Fluoride

50ul Mix 2	8mM	Magnesium Chloride
	200mM	Tris HCl pH 7.6
	3.2mM	ATP
10ul Mix 3	9mM	^{14}C Galactose ($4x10^6$ dpm/umole)

and the reaction incubated at 32°C for 20 minutes. Two 45ul samples were then removed and spotted onto a 3cm diameter DE81 filter. One filter was clamped in a vacuum filter unit and washed with 50ml of distilled water. Both filters were then placed in 4ml of an aqueous scintillation fluid and counted in a β counter.

The galactose activity was calculated using the following formula

$$\frac{\text{Counts on unwashed filter}}{\text{Counts on washed filter}} \times \frac{232}{OD_{600}}$$

nmoles galactose phosphorylated per minute per OD_{600}.

CHAPTER THREE

The Cloning of *fam* and *dnaM*

3.1 Introduction

Bacteriophage P1 mapping studies have shown that the genes fam, dnaM, rpoH and ftsS are tightly linked to the operon (Salmond and Plakidou 1984). These studies, however, could not determine the relative positions of these genes (with respect to the operon). In order to determine their relative locations precisely it was necessary to clone the genes. With no probes available clones could only be identified by complementation or by hybridisation to operon sequences, which would require cloning the genes on a fragment containing at least some of the 6.5kb sequence. In order to identify suitable sites for cloning into a plasmid vector, Southern blot analysis would have to be performed on the region surrounding the operon. Alternatively larger chromosomal fragments could be cloned into lambda or cosmid vectors. A third approach can also be used since the genes are known to be very tightly linked to the operon. This is to extend the sequence found in λpGS22 in vivo and select the clones by complementation. It is this approach that was finally used.

Strain GS003 is deleted for the primary lambda attachment site attλ. Lambda DNA injected into this strain can integrate ,by homology, at sites other than attλ. λpGS22 therefore should be able to integrate by homology within the 6.5kb partial HindIII region. Such a "prophage" can be forced to excise by irradiation

with short wave ultra-violet light. Upon excision the "prophage" may acquire sequences that were adjacent to those initially contained in the phage, this is the basis of the method used.

3.2 The Isolation of λS3d and λG3b

Strain GS003 lysogenised with λpGS22 was grown in LB to an OD_{600} of 0.6. The culture (20ml) was pelleted and resuspended to an OD_{600} of 1 in 10mM magnesium sulphate. Resuspended cells (10ml) were transferred to a Petri dish and irradiated with short wave ultra-violet light. A sample (200ul) was removed and added to 2ml of LB (with 10mM magnesium sulphate) and incubated at 30°C, with vigorous shaking, for 3-4 hours until lysis occured. Lysis was completed by vortexing with a few drops of chloroform and the cell debris removed by centrifugation. A range of ultra-violet light exposure times were used to optimise the phage recovery. The various lysates were titred and the results are shown in table 3.1. The 1.2×10^9 pfu/ml lysate was chosen for use in the transduction experiment.

TABLE 3.1 Phage Titres from UV Irradiation of GS003 (λpGS22)

Time	Dose	Phage Titre
1 second	150ergs/mm^2	4 x 10^8 pfu/ml
2 seconds	300ergs/mm^2	12 x 10^8 pfu/ml
4 seconds	600ergs/mm^2	11 x 10^8 pfu/ml
6 seconds	900ergs/mm^2	6 x 10^8 pfu/ml

10^8 pfu of this lysate was added to 2 x 10^8 cells of an exponential culture of each of the mutants growing in LB with 10mM magnesium sulphate and 0.2% maltose. The four mutants used were ST715 *recA* (*fam*), OV22 *recA* (*ftsS*), GSY (*rpoH*) and JGC127 (*dnaM*). After 30 minutes absorption time at 30°C, 10^9 pfu of a λ*imm*21^c phage were added and incubation continued for a further 60 minutes to allow killing of all non-lysogens. The cells were then plated out on nutrient agar plates at the non-permissive temperature (42°C).

After overnight incubation small colonies were seen on each plate and larger colonies were seen on the ST715 recA and the GSY plates. The small colonies did not grow when patched to another plate at 42°C and so were not studied further. The larger colonies remained viable at 42°C, and were tested for the presence of a lambda prophage by the methods discussed in chapter two. Twelve lysogens were chosen from each plate (G1-12, S1-12), inoculated into LB (and 10mM magnesium sulphate) and incubated at 30°C overnight to allow release of phage into the supernatant. Phage isolated in this way (λG1-12, λS1-12) were spotted onto a top agar lawn seeded with either GSY or ST715 recA and the plates incubated at 42°C overnight. Phages λG3 and λS3 both produced large areas of growth where they had been spotted onto GSY and ST715 recA lawns respectively and were chosen for further study.

The two phages were plaque purified on DH1 and the spot test repeated with individual plaques. Two plaques which gave good growth (λG3b and λS3d) were chosen. Lysogens of GSY and ST715 recA with λG3b and λS3d respectively were isolated and shown to be temperature resistant, suggesting that the rpoH and fam genes were cloned on the respective phages.

3.3 Complementation Analysis of λG3b and λS3d

In order to establish which genes were carried on the above phages, lysogens were constructed in the following strains; MFT1181 recA (ftsE), OV32 recA (ftsX), OV22 recA (ftsS), GSY (rpoH), ST715 recA (fam) and JGC127 (dnaM). Problems were encountered in constructing stable lysogens in JGC127, but this was circumvented by transducing the temperature sensitive mutation ,linked to a Tn10 marker, into OV2 to form the strain OJ127, and then lysogenising this strain. All lysogens were tested for temperature sensitivity and the results are shown in table 3:2 which also includes lysogens of λpGS22 and λpsupF.

These results show that both phages are phenotypically ftsE+, ftsX+, rpoH+, fam+, dnaM+ and apparently ftsS-.

Furthermore it shows that the mutations in OV22, OV32, ST715 and GSY are ambers (since they can be supressed by λpsupF), whereas those in MFT1181 and JGC127 are temperature sensitive missense.

TABLE 3.2 Complementation Data for λpGS22, λG3b and λS3d.

	MFT1181 *ftsE*	OV32 *ftsX*	OV22 *ftsS*	ST715 *fam*	GSY *rpoH*	OJ127 *dnaM*
λpGS22	+	+	-	-	-	-
λG3b	+	+	-	+	+	+
λS3d	+	+	-	+	+	+
λp*supF*	-	+	+	+	+	-

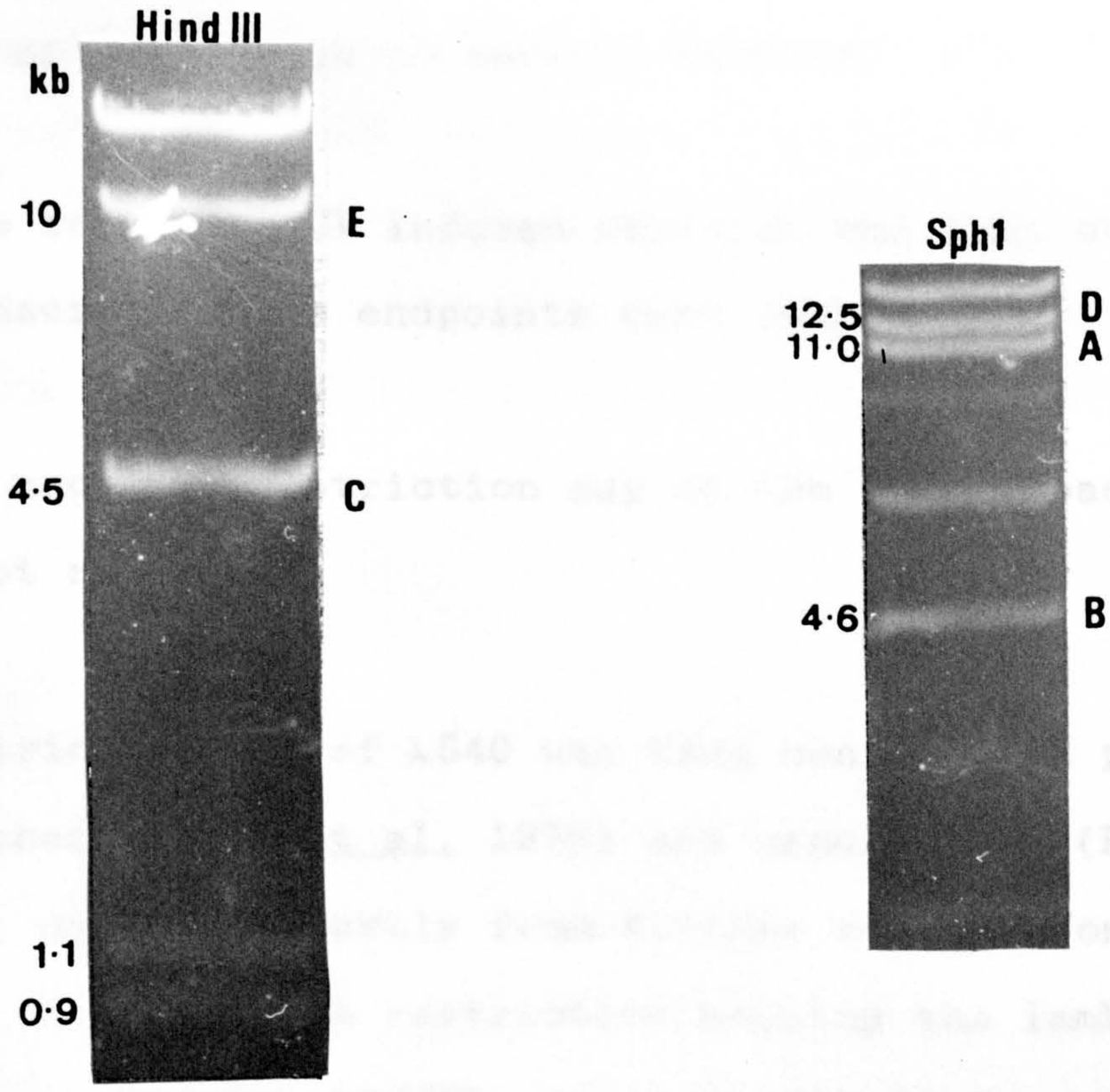
\+ Complementation (Supression)

\- No Complementation (Supression)

The MFT1181, OV32, OV22 and ST715 strains were all *recA* derivatives constructed by T4 transduction using JC10240 as donor.

FIGURE 3.1 HindIII and Sph1 Digestion of λS3d

The digests were electrophoresed on a 0.8% agarose gel. The standard fragment sizes were obtained from a HindIII digestion of λcI_{857}. The letters are referred to in section 3.5 and represent the fragments subcloned into pBR322.



3.4 Restriction Analysis of λS3d

Since λS3d and λG3b were phenotypically identical just one (λS3d) was chosen for further analysis. Phage DNA was prepared and restricted to determine the extent of alteration of its DNA due to the UV induced excision from GS003. Restriction by HindIII shows that the three fragments of λpGS22 (0.9kb, 1.1kb and 4.5kb) are still present (figure 3.1). A restriction map of λS3d was constructed and is shown in figure 3.2. Restriction mapping λS3d was complicated due to several reasons.

1. The result of UV induced excision was that both the length of the insert and its endpoints were unknown.

2. An accurate restriction map of the parent bacteriophage (λ540) was not available.

A restriction map of λ540 was thus constructed partly from published (Borck et al. 1976) and unpublished (R. Walters pers. comm.) data, and partly from further restriction analysis. In order to facilitate restriction mapping the lambda phages a computer program (DNAGEL, see appendix B) was written in BBC Basic for a BBC model B microcomputer. The program was based on the algorithm of Schaefer and Sederoff (1981) and also on a previous program by G. Russell (1984). The program calculated the

size of DNA bands, relative to standard bands, based on their mobility through an agarose or an acrylamide gel. The program presented the results both numerically and graphically, and calculated the errors in sizing each band. A sample readout of the program is shown in appendix C.

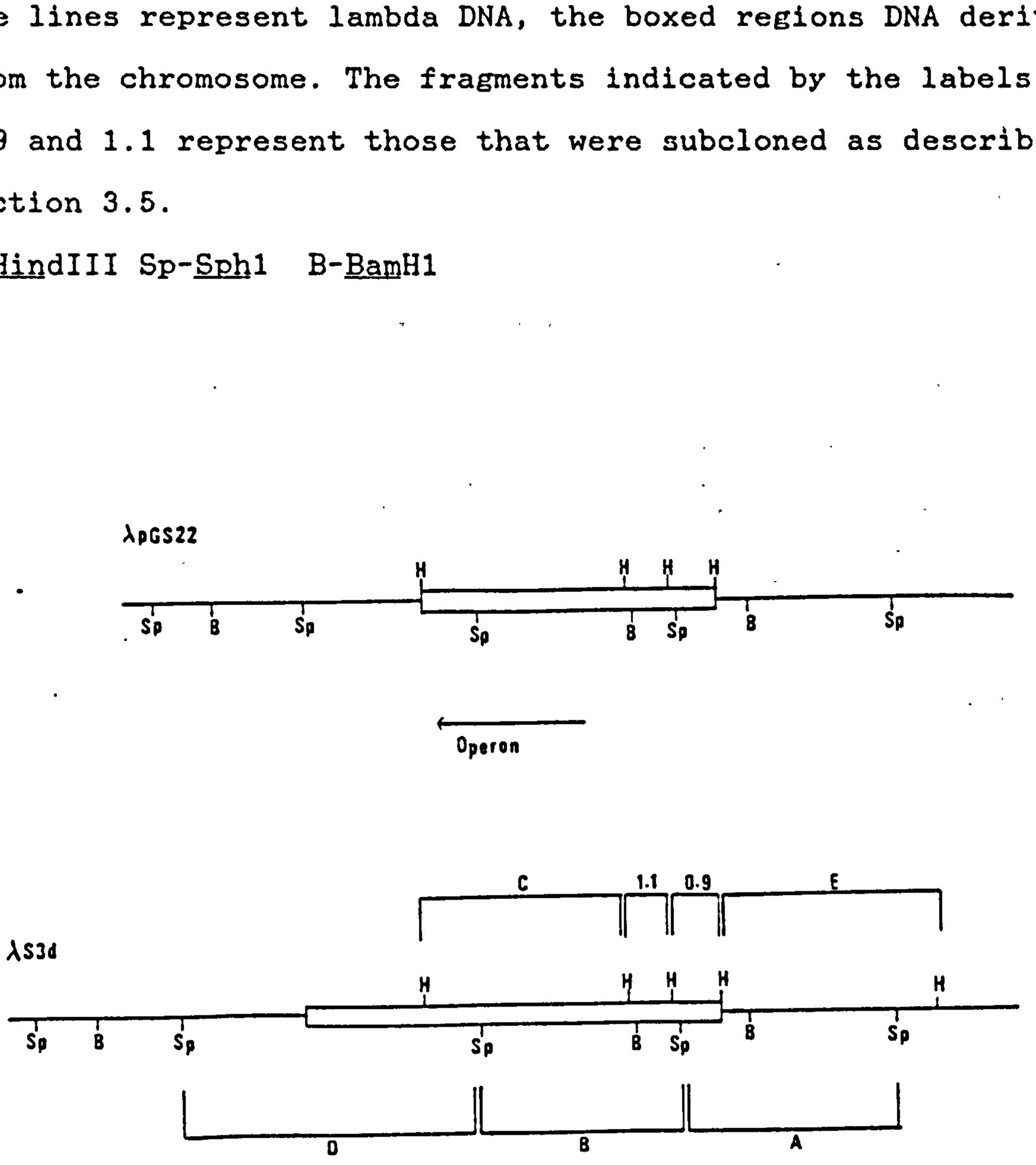
From the restriction map of λS3d it can be seen that the phage contains extra chromosomal DNA 5' to the 4.5kb HindIII fragment that was not present in λpGS22. The λS3d phage has also picked up another HindIII site near its 3' end, probably by recombination with the λimm21^{c} phage used in the selection of lysogens.This map also shows the relative locations of the 0.9kb, 1.1kb and 4.5kb HindIII fragments which was previously unknown.

Since these were the only obvious differences between λS3d and λGS22 it seemed likely that the extra chromosomal DNA 3' of the operon coded for rpoH, fam and dnaM.

FIGURE 3.2 Comparison Between the Restriction Maps of λpGS22 and λS3d.

The lines represent lambda DNA, the boxed regions DNA derived from the chromosome. The fragments indicated by the labels A-E, 0.9 and 1.1 represent those that were subcloned as described in section 3.5.

H-HindIII Sp-Sph1 B-BamH1



3.5 Subcloning from λS3d

To test whether this extra chromosomal DNA ,about 2-3kb in length, did actually code for fam, rpoH and dnaM it was necessary to subclone this region. From the restriction map shown in figure 3.2 it was decided to subclone the fragments generated by Sph1 and HindIII. In two independent experiments λS3d was restricted to completion with either HindIII or Sph1, as shown in figure 3.1. The resulting fragments were then ligated into pBR322, previously restricted with HindIII or Sph1 and (treated with phosphatase with calf alkaline phosphatase. After DH1 was transformed with the two ligations , subclones were selected on ampicillin, and screened for insertions on tetracycline, agar plates. All plasmids of Ap^R Tc^S colonies were "mini-prepped" and restricted to determine the size of the insert. Basic restriction mapping was then used to characterise three Sph1 inserts (SPHa, SPHb and SPHd), which together spanned the chromosomal insert in λS3d. The 0.9kb, 1.1kb and 4.5 kb HindIII fragments were also subcloned in this experiment. The position of these six subcloned fragments in λS3d is shown in figure 3.2.

Plasmid pSPHd contains all the extra DNA acquired by λS3d flanked by some lambda DNA and 1.3kb of the 4.5kb HindIII fragment.It was believed that the three unmapped genes (fam, rpoH and dnaM) all lay on this fragment. However this plasmid failed to complement

any of the mutations in these genes. Although this could mean that the clone did not contain any of the genes it could also represent the absence of regulatory regions or some artefact of construction.

Since one of the unmapped genes (rpoH) had previously been cloned and its restriction map had been determined (Neidhardt et al. 1983), pSPHd was restridion mapped more fully to determine whether or not it contained rpoH. Figure 3.4 shows one of the agarose gels used for the construction of the pSPHd restriction map. The comparison between the two maps is shown in figure 3.3 and clearly shows that pSPHd does indeed contain the rpoH gene.

FIGURE 3.3 Comparison Between pSPHd and a Restriction Map of rpoH

The lines represent DNA derived from either lambda or pBR322 vectors, the boxed regions depict DNA from the chromosome.
P-PvuII Sp-Sph1 St-Stu1 H-HindIII C-Cla1 R-EcoRV

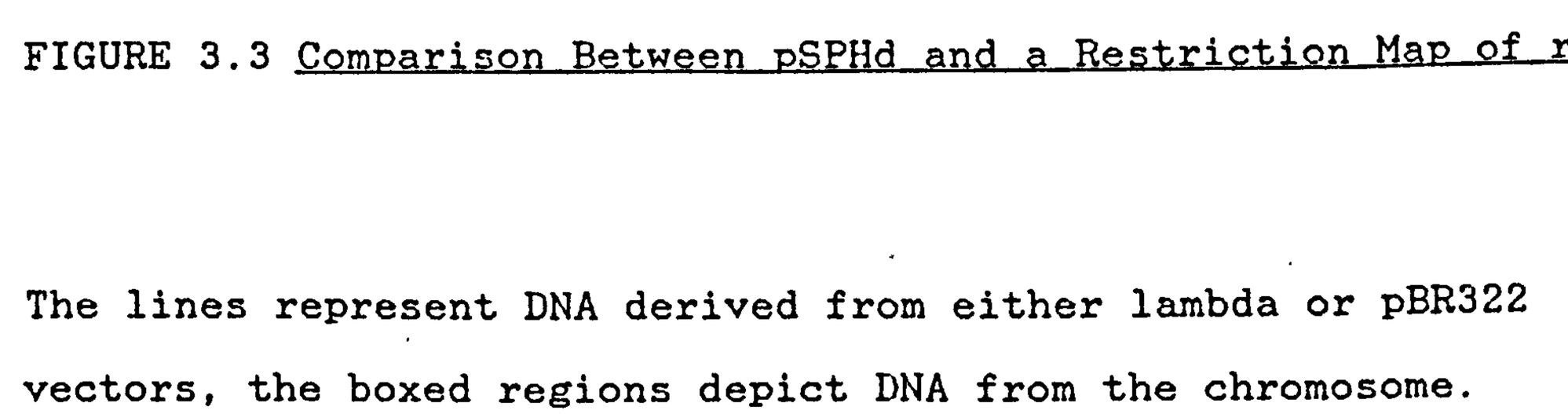


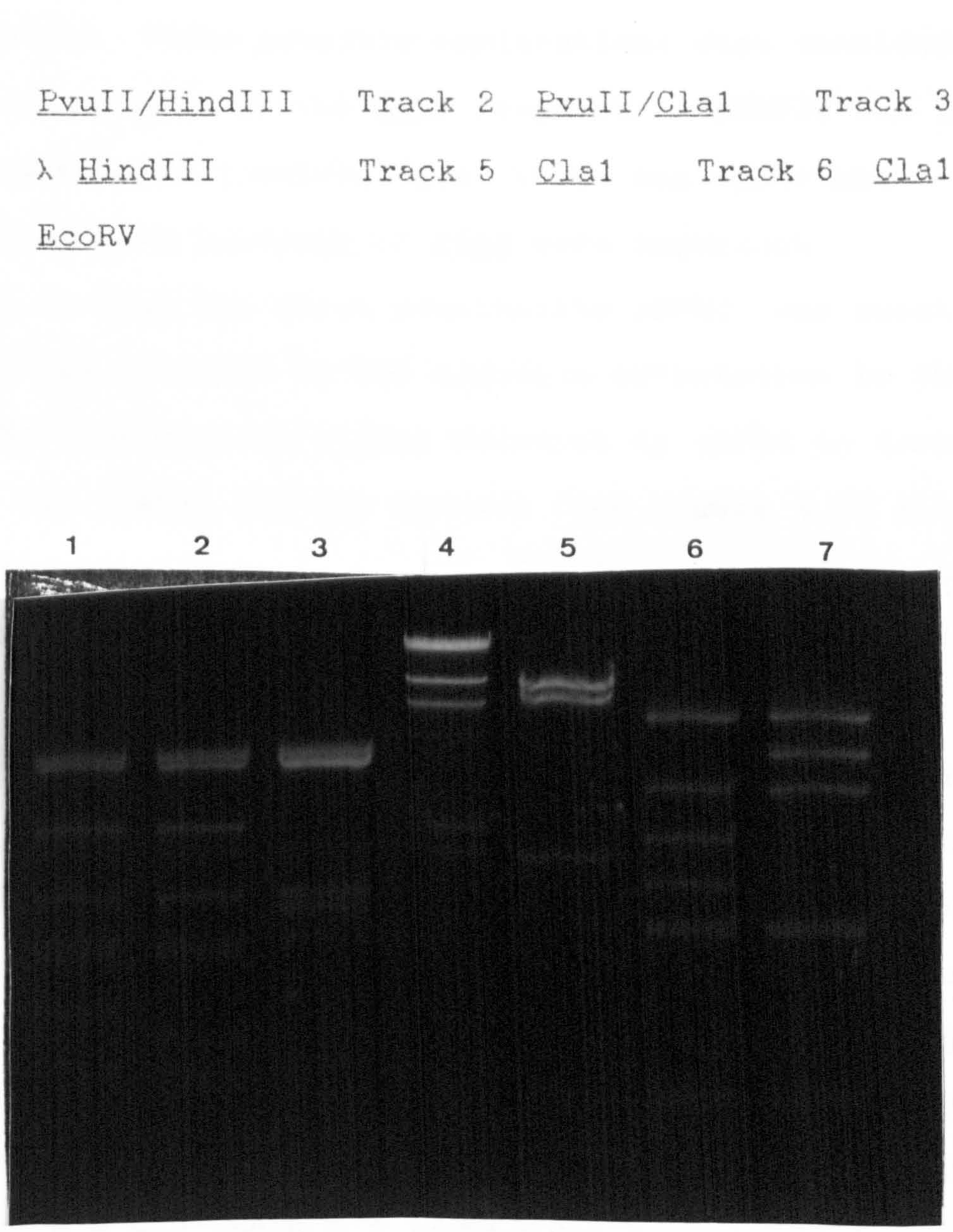
FIGURE 3.4 Endonuclease Digestion Analysis of pSPHd

The plasmid DNA was digested and electrophoresed on a 1% agarose gel as described in chapter 2. In order to calculate the size of the bands the computer program DNAGEL was used. The results obtained from this program for this gel is shown in appendix C. The various enzymes used were as follows:

Track 1 PvuII/HindIII Track 2 PvuII/Cla1 Track 3 PvuII

Track 4 λ HindIII Track 5 Cla1 Track 6 Cla1 /EcoRV

Track 7 EcoRV



3.6 Further Constructs Based on pSPHd

From the comparison with published data pSPHd appeared to contain both the rpoH coding and promoter regions, yet it was found not to complement the classical rpoH mutation rpoH165 (in GSY). Since the parent phage λS3d did complement the rpoH165 mutation the reason seemed to be due to some aspect of the plasmid construction. Three possible explanations were considered :

a) The orientation of the Sph1 fragment in pBR322 was important.

b) The lambda DNA (derived from λS3d) was important.

c) The sequences upstream of rpoH were important.

In order to test the first possibility pSPHd' was constructed with the Sph fragment in the opposite orientation to that in pSPHd. By constructing a Stu1 deletion in pSPHd to form pdΔSS most of the lambda DNA was removed (see figure 3.3) allowing the second possibility to be tested. Finally, in order to test the third possibility a series of plasmids were constructed with various amounts of chromosomal DNA 5' to the rpoH gene. In pdΔSSΔH the deletion of a HindIII fragment left a plasmid very similar to the rpoH complementing plasmid pFN97 (Niedhardt et al. 1983). A Cla1 deletion in pdΔSSΔCla removed even more of the 5' chromosomal DNA. pdΔSS4.5 was constructed by cloning the 4.5kb operon-containing fragment into the HindIII site of pdΔSSΔH. The construction of these plasmids is outlined in figure 3.5. Their structure and ability to complement the mutations are then shown in figure 3.6 and table 3.3 respectively.

FIGURE 3.5 Construction of Various Plasmids Derived from pSPHd

The solid lines represent insert DNA: the thin lines represent pBR322 DNA

1/2 The 12.5kb Sph1 fragment from λS3d was cloned into the Sph1 site of pBR322 in both orientations to give pSPHd and pSPHd'.

3 An internal deletion of the 1.5kb HindIII fragment from pSPHd giving pdΔH.

4 An internal deletion of the 8.5kb Stu1 fragment from pSPHd giving pdΔSS.

5/6 The 1.3kb EcoRV fragment from pdΔSS was cloned into the EcoRV site of pBR322 to give pR2.

7 An internal deletion of the 2.7kb EcoRV fragment from pdΔSS giving pdΔSSΔRV.

8 An internal deletion of the 1.7kb Cla fragment from pdΔSS giving pdΔSSΔCla.

9 An internal deletion of the 1.5kb HindIII fragment from pdΔSS giving pdΔSSΔH.

10/11 The 4.5kb HindIII fragment from pH3C was cloned into the HindIII site of pdΔSSΔH.

FIGURE 3.5 Continued

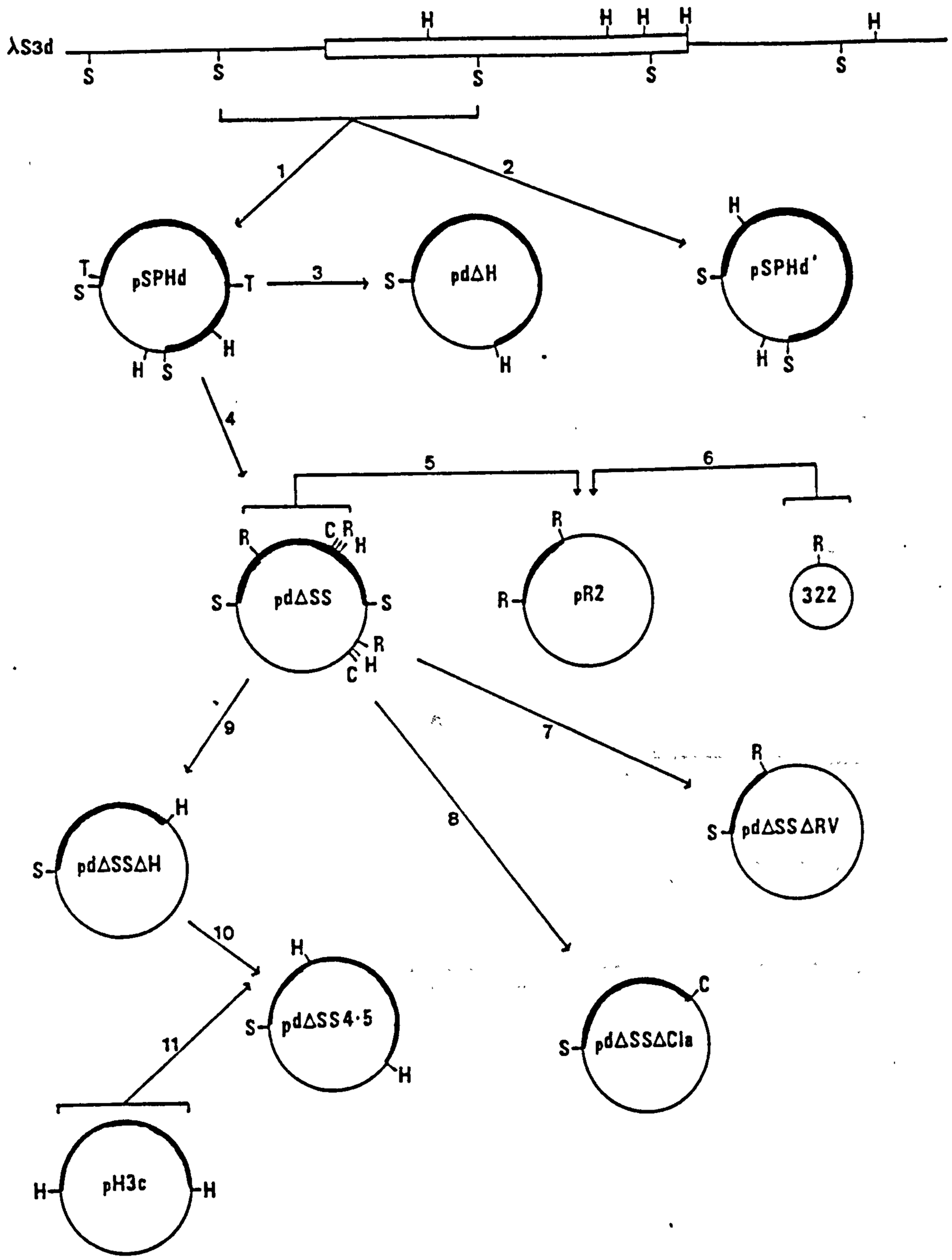


FIGURE 3.6 Structure of the pSPHd Derived Plasmids

The boxed regions represent chromosomal derived DNA, the lines, lambda DNA.

H- HindIII Sp- Sph1 C- Cla1 R- EcoR1 St- Stu1

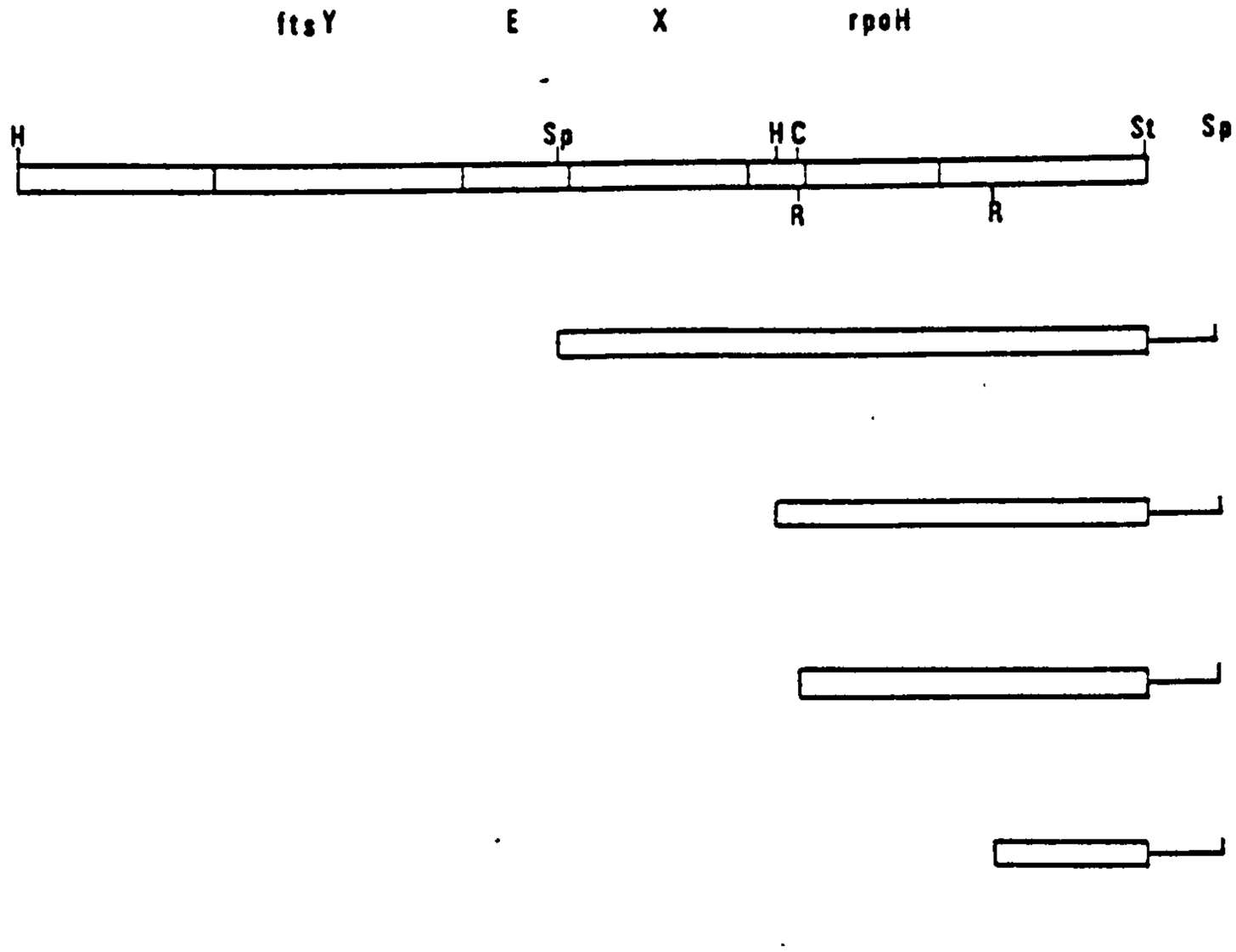


TABLE 3.3 Complementation Data for the pSPHd Based Constructs

	MFT1181 *ftsE*	OV22 *ftsS*	ST715 *fam*	GSY *rpoH*	OJ127 *dnaM*
pSPHd	-	-	-	-	-
pSPHd'	-	-	-	-	-
pdΔSS	-	-	-	-	-
pdΔSSΔH	-	-	+	+	+
pdΔSSΔCla	-	-	+	+	+
pdΔSSΔRV	-	-	-	-	-
pdΔSSΔ4.5	+	-	+	+	+
pR2	-	-	+	+	+

+ Complementation - No Complementation

recA derivatives of MFT1181, OV22 and ST715 were used.

The data shows that removal of the Sph1-HindIII fragment 5' of rpoH in pSPHd or its replacement by the 4.5kb HindIII fragment produces a plasmid that can now complement the rpoH mutation in GSY. Furthermore these plasmids can also complement mutations in fam and dnaM, suggesting that the three mutations may be allelic.

Since a 1.3kb EcoRV fragment is known to have rpoH as its only open reading frame, complementation of the fam and dnaM mutations by this fragment would provide stronger evidence that the mutations are allelic. This fragment was subcloned from pdΔ55 and placed under the bla promoter of pBR322, since the fragment lacks the normal rpoH promotor region. The resulting plasmid pR2 complemented the mutations in both ST715 recA and OJ127.

Thus fam and dnaM are allelic with rpoH and the mutant alleles can be renamed rpoH715 and rpoH710 respectively. The subcloning data also shows that rpoH lies downstream of the cell division operon. Comparison of the published sequence of rpoH (Landick et al. 1984) and that of the operon shows a small but significant overlap confirming their relative positions.

The complementation data with respect to the plasmids pSPHd, pSPHd' and pdΔ55 remained confusing. Some growth was observed and more with pSPHd' than with pSPHd. The possible reasons for this will be discussed in chapter 5.

CHAPTER FOUR

Physiology of the *rpoH* Alleles

4.1 Introduction

The genetic analysis discussed in chapter 3, clearly suggested that dnaM and fam are allelic with rpoH. The latter gene was first identified as htpR/hin the regulatory gene for the heat shock response. It was later shown to encode an alternative sigma factor and was renamed rpoH. The fam and dnaM alleles were both isolated as temperature sensitive conditional lethal mutations but with differing phenotypes to rpoH.

The fam allele was identified by Torti and Park (1976) during a search for mutants defective in murein lipoprotein synthesis. The fam715 mutation resulted in reduced levels of lipoprotein at its restrictive temperature, but differed in phenotype from mutations in the lipoprotein structural gene lpp. Torti and Park (1980) later showed conclusively that the mutation did not lie in the lipoprotein structural gene and concluded that fam must have a processing or regulatory function.

The dnaM allele was isolated by accident in a search for mutations affecting DNA synthesis (Glassberg et al. 1979). The workers isolated a double mutant defective in its single stranded binding protein, but also carrying an unknown mutation. When this mutation was transduced out it gave rise to a temperature sensitive growth phenotype and was named dnaM710. The mutation

was mapped by Hfr crossing to near 75 minutes.

If fam and dnaM are allelic to rpoH then it is possible that the mutations in fam and dnaM, like rpoH, will be defective in the heat shock response. Upon a shift in temperature from 30°C to 42°C E.coli synthesizes a set of about 20 polypeptides which represents the "heat shock response" as discussed in section 1.5.3. Upon shifting the temperature from 30°C to 50°C the only polypeptides synthesized to any extent are these 20 heat shock proteins (HSP); mutations in rpoH prevent this response. Whether the fam and dnaM mutations also result in this defect will be considered in this chapter.

Since rpoH lies immediately downstream of the ftsYEX operon the possibility exists that its expression may be regulated in some way by the operon. The control of rpoH expression during the heat shock response is unclear although it has been reported that there may be a transcriptional control of rpoH (Tilly et al. , 1986) and that sequences some distance upstream may be important (C. Gross pers. comm.). The possible involvement of the operon in transcriptional control of rpoH expression will also be discussed.

4.2 Strains ST715 (fam), OJ127 (dnaM) and GSY (rpoH) are Morphologically Similar.

A characteristic of rpoH-containing mutants is that at the non-permissive temperature they form short filaments. The strains ST715 and OJ127 were studied to see whether they showed a similar phenotype. Figure 4.1 shows the result of shifting the strains OV2, OV32, MFT1181, GSY, ST715 and OJ127 from 30°C to 42°C and observing the changes in cell morphology. The photographs clearly show that the three mutations do indeed result in a similar morphological phenotype at the non-permissive temperature. The resulting filaments, however, are in contrast to those produced by the cell division mutants OV32 (ftsX) and MFT1181 (ftsE). The latter filaments are much longer than those associated with the rpoH mutations.

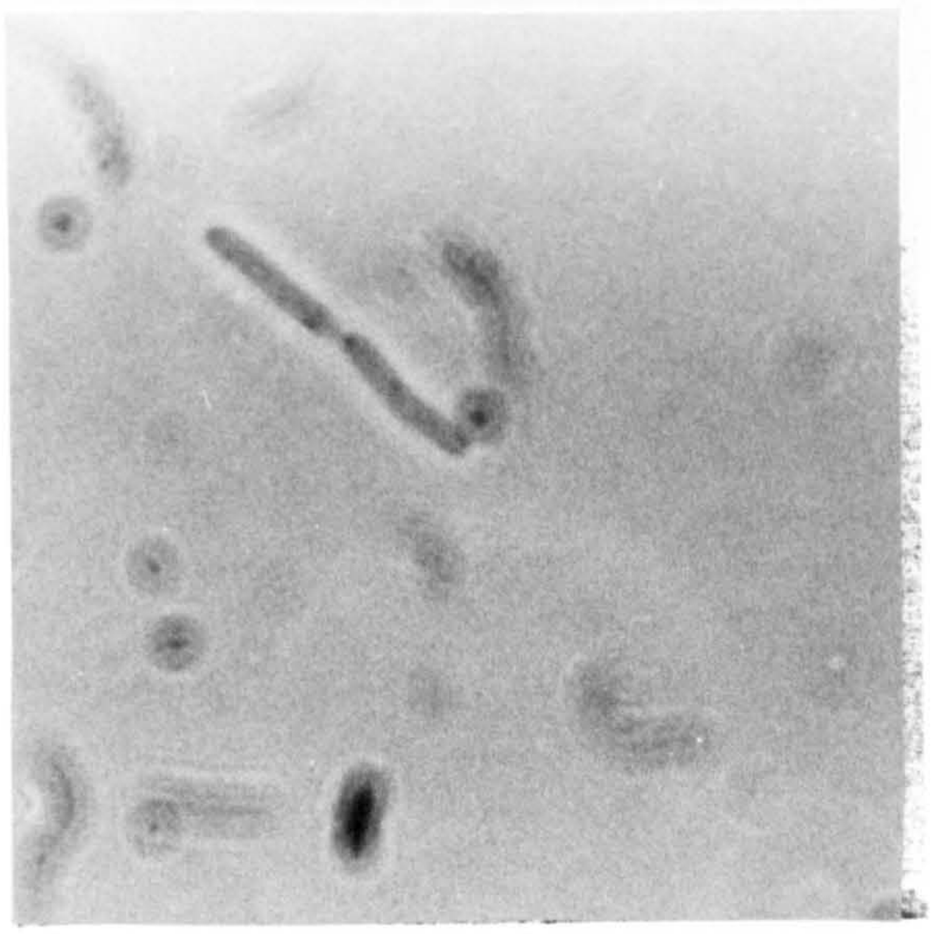
4.3 Strains ST715 (fam) and OJ127 (dnaM) are Defective in the Heat Shock Response

The ability of the mutations in fam and dnaM to induce the heat shock response upon a temperature shift from 30°C to 42°C or to 50°C was studied. The mutants were grown to mid-log phase in nutrient broth, a 1ml aliquot was removed and incubated at either 42°C or 50°C . After 30 minutes at the higher temperature 10uCi of ^{35}S-methionine was added and labelling of total cell protein was continued for 5 minutes.

FIGURE 4.1 Photomicrographs Comparing the Morphologies of ST715, OJ127 and GSY

The photomicrographs were taken using a Leitz Dialux 22 camera system. All the photographs are shown at the same magnification to allow direct comparison between the strains.

ST715



OJ127

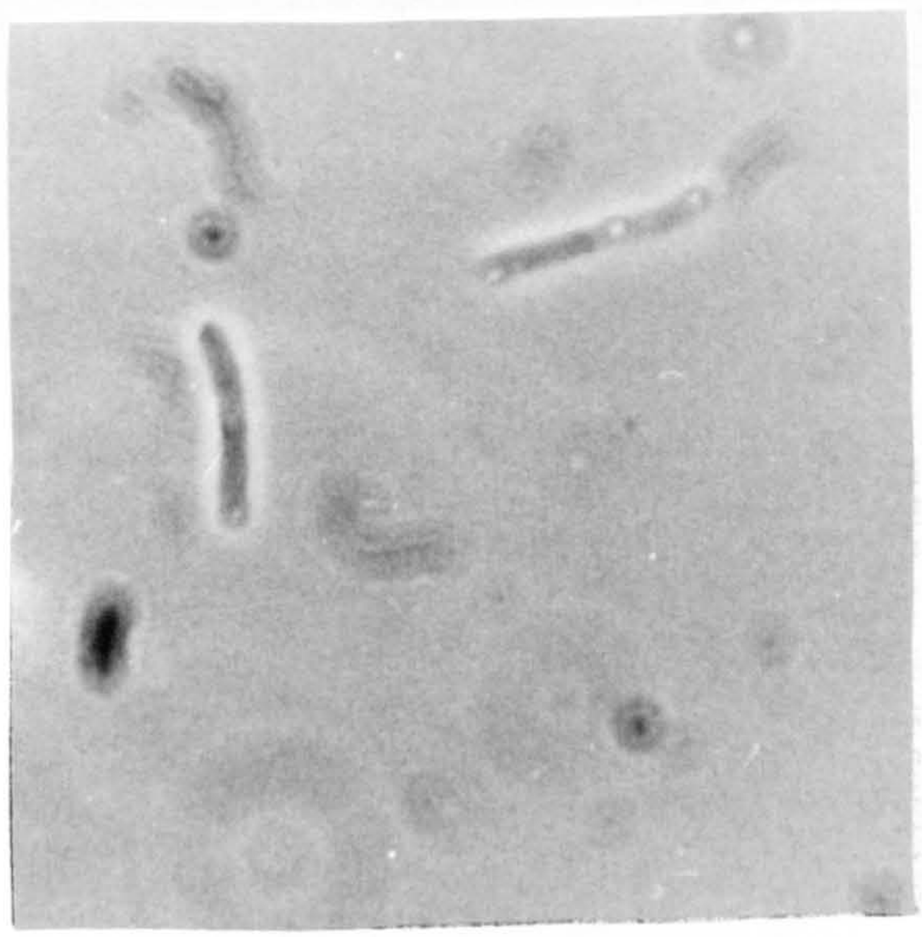
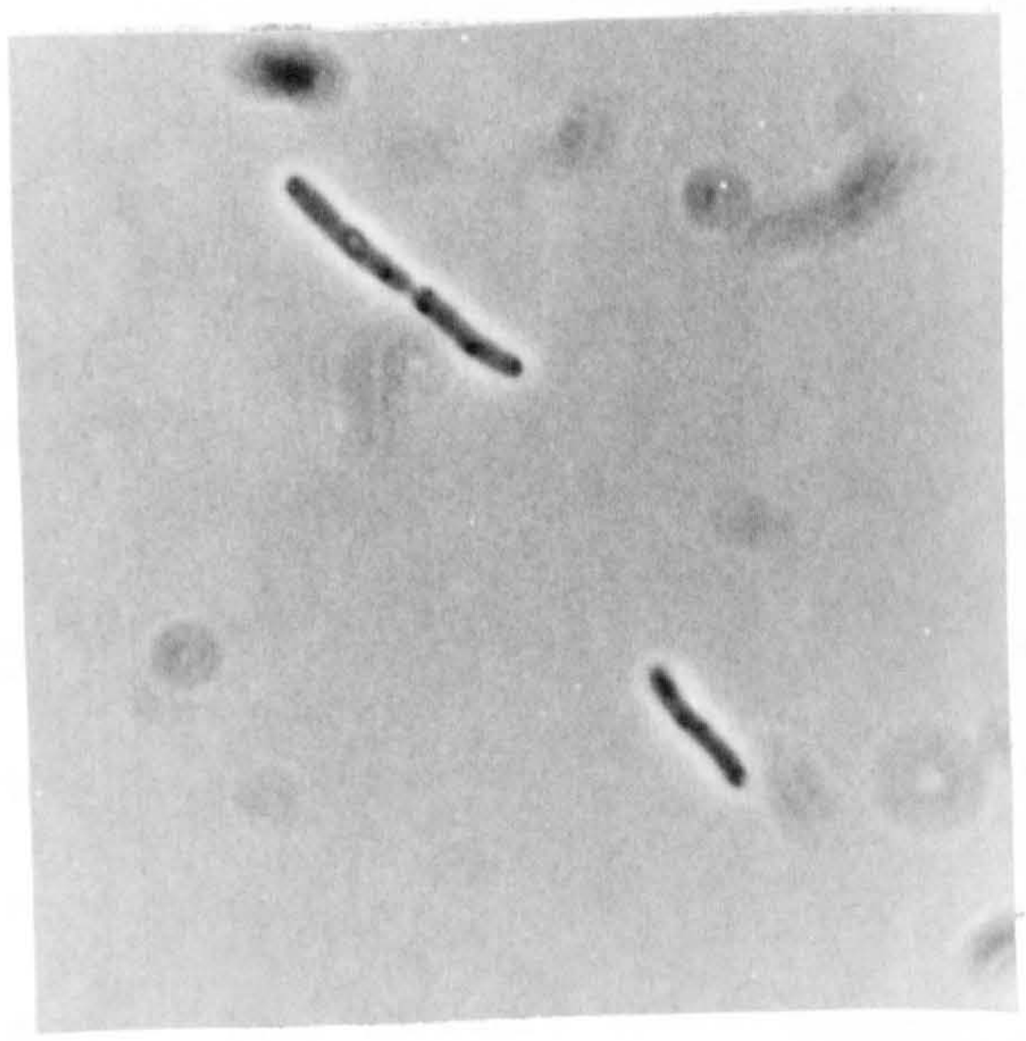
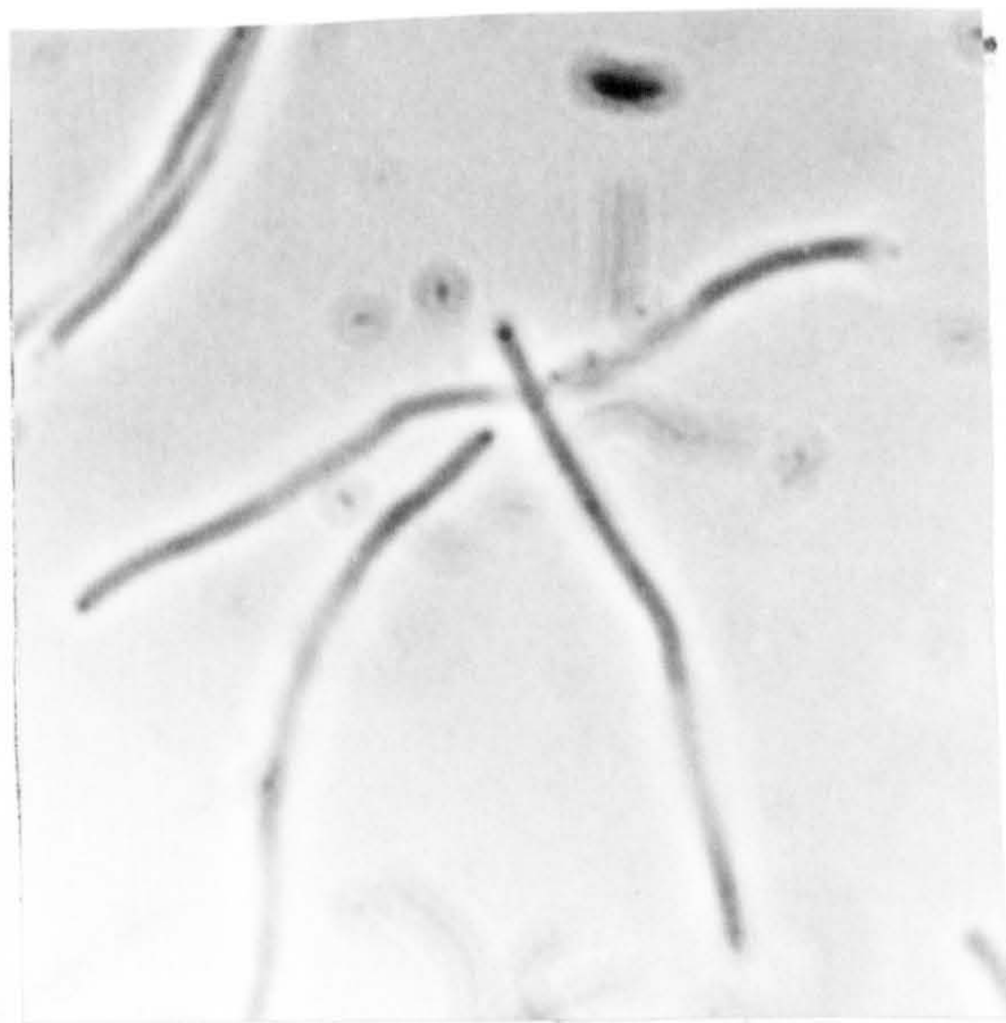


FIGURE 4.1 Continued

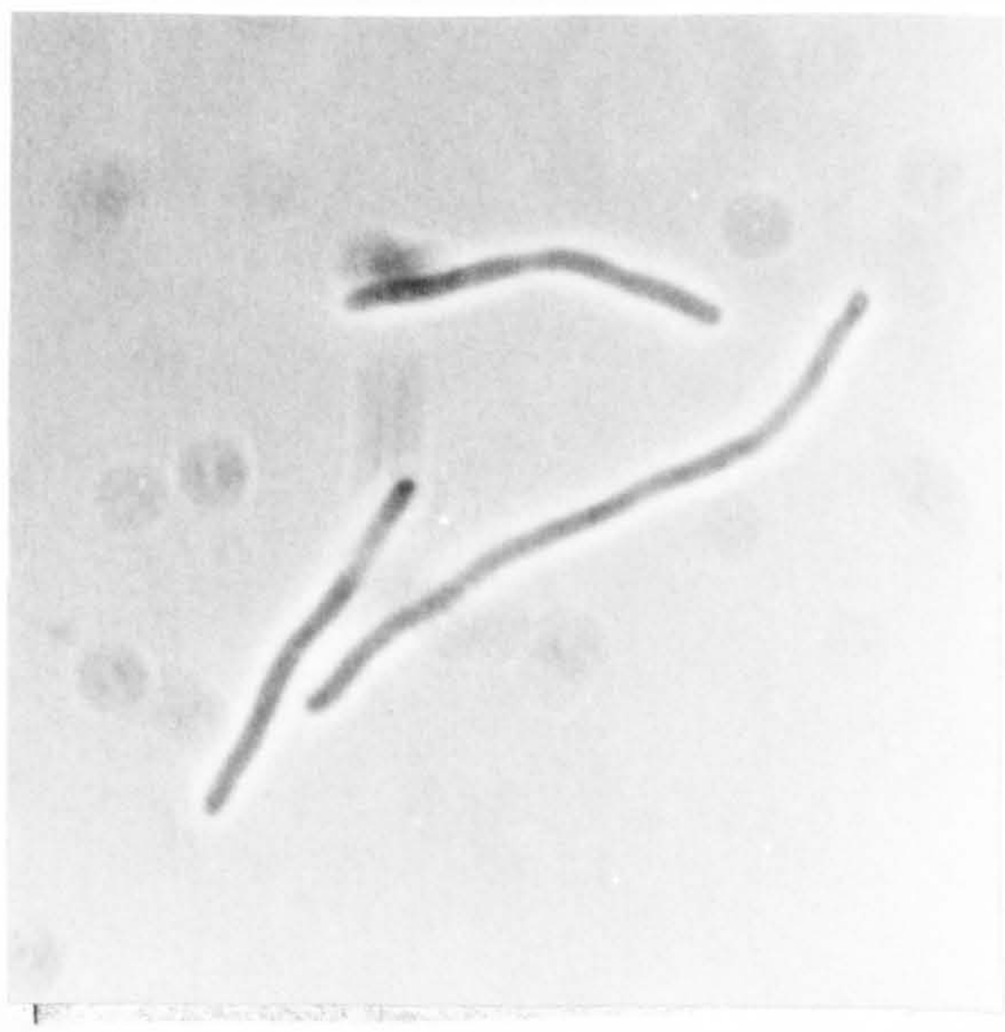
GSY



MFT1181



OV32



The cells were then harvested, washed, resuspended in SDS sample buffer, denatured by boiling and electrophoresed on a 10% SDS polyacrylamide gel.

Figure 4.2 shows the protein profiles obtained for GSY, ST715, OJ127 and OV2 when shifted from 30°C to 42°C or 50°C. Track 11 shows the effect of shifting OV2 to 50°C, the only bands present are those of the heat shock proteins . None of the rpoH mutants synthesized any proteins at this temperature. At 42'C the picture is less clear. Track 7 shows the normal heat shock response associated with OV2. Comparing this with tracks 1-3 (GSY, ST715 and OJ127 respectively) one of the heat shock proteins (arrow) appears to be absent in the rpoH mutants. however when the same mutants are transformed with the rpoH-expressing plasmid pR2 (tracks 4-6) their ability to synthesise this heat shock protein is restored.

Tracks 12-15 show that pR2 can also correct the heat shock defect in OJ127 at 50°C. Track 13 shows the normal heat shock response of OV2 at 50°C. Track 14 shows the absence of a heat shock response from OJ127 at this temperature, which was restored upon transformation with pR2 (track 15)

These results show that although the fam and dnaM mutations were not specifically isolated as being defective in the heat shock response, they nevertheless show such a defect.

FIGURE 4.2 Synthesis of Heat Shock Proteins by OV2, ST715, OJ127 and GSY

Track 1	GSY		42°C	Track 2	ST715		42°C
Track 3	OJ127		42°C	Track 4	GSY	(pR2)	42°C
Track 5	ST715	(pR2)	42°C	Track 6	OJ127	(pR2)	42°C
Track 7	OV2		42°C	Track 8	GSY		50°C
Track 9	ST715		50°C	Track 10	OJ127		50°C
Track 11	OV2		50°C				

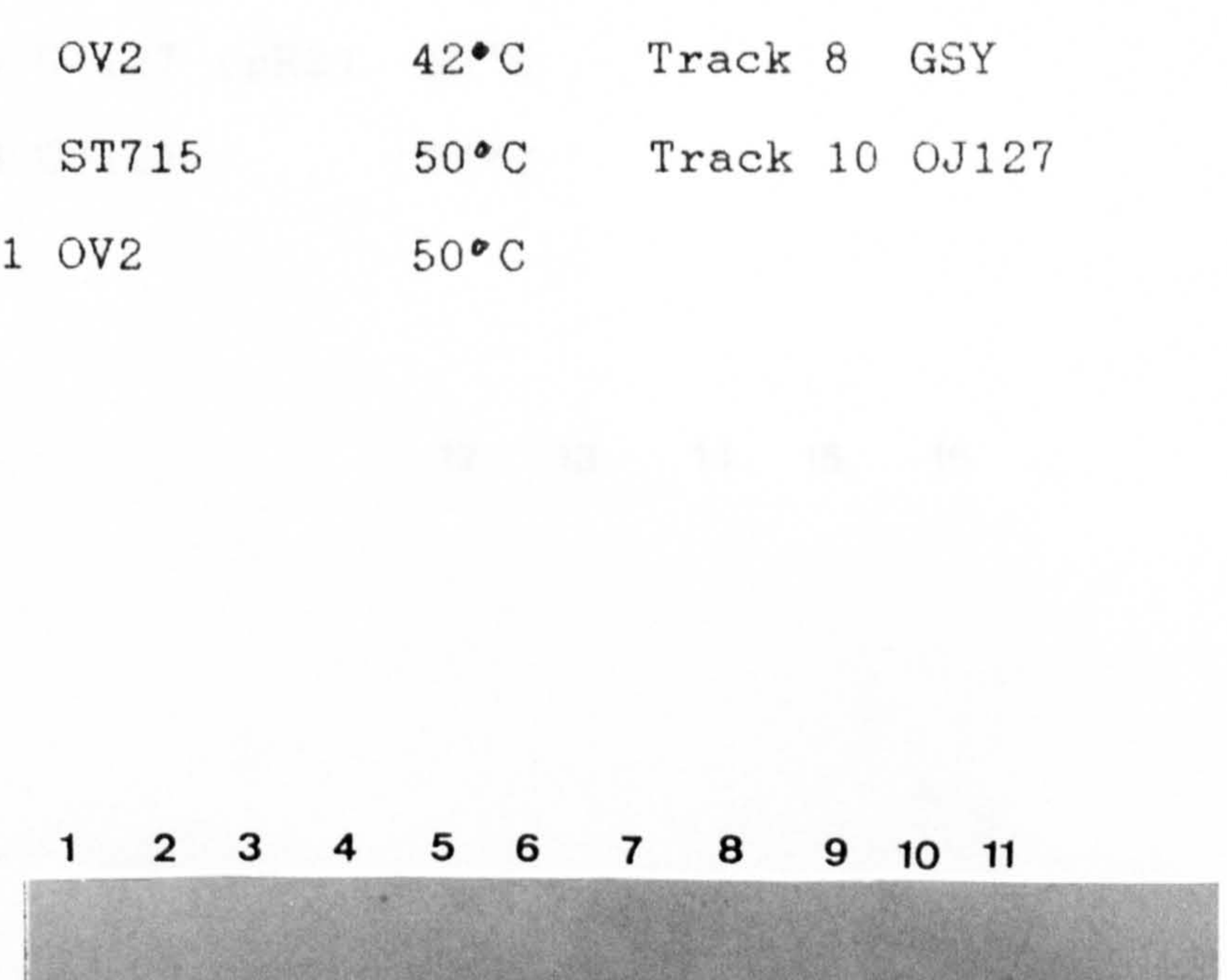


FIGURE 4.2 Continued

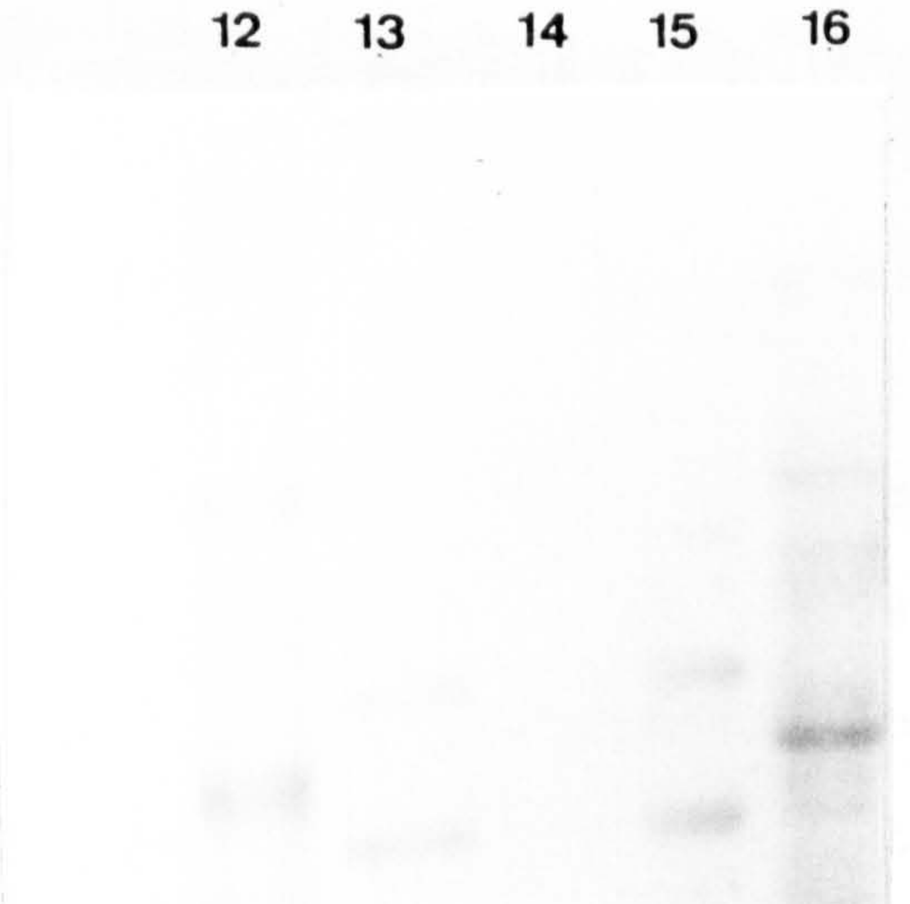
Track 12 ^{35}S Molecular Weight Standards

Track 13 OV2 50°C

Track 14 OJ127 50°C

Track 15 OJ127 (pR2) 50°C

Track 16 OJ127 30°C



4.4 Discussion

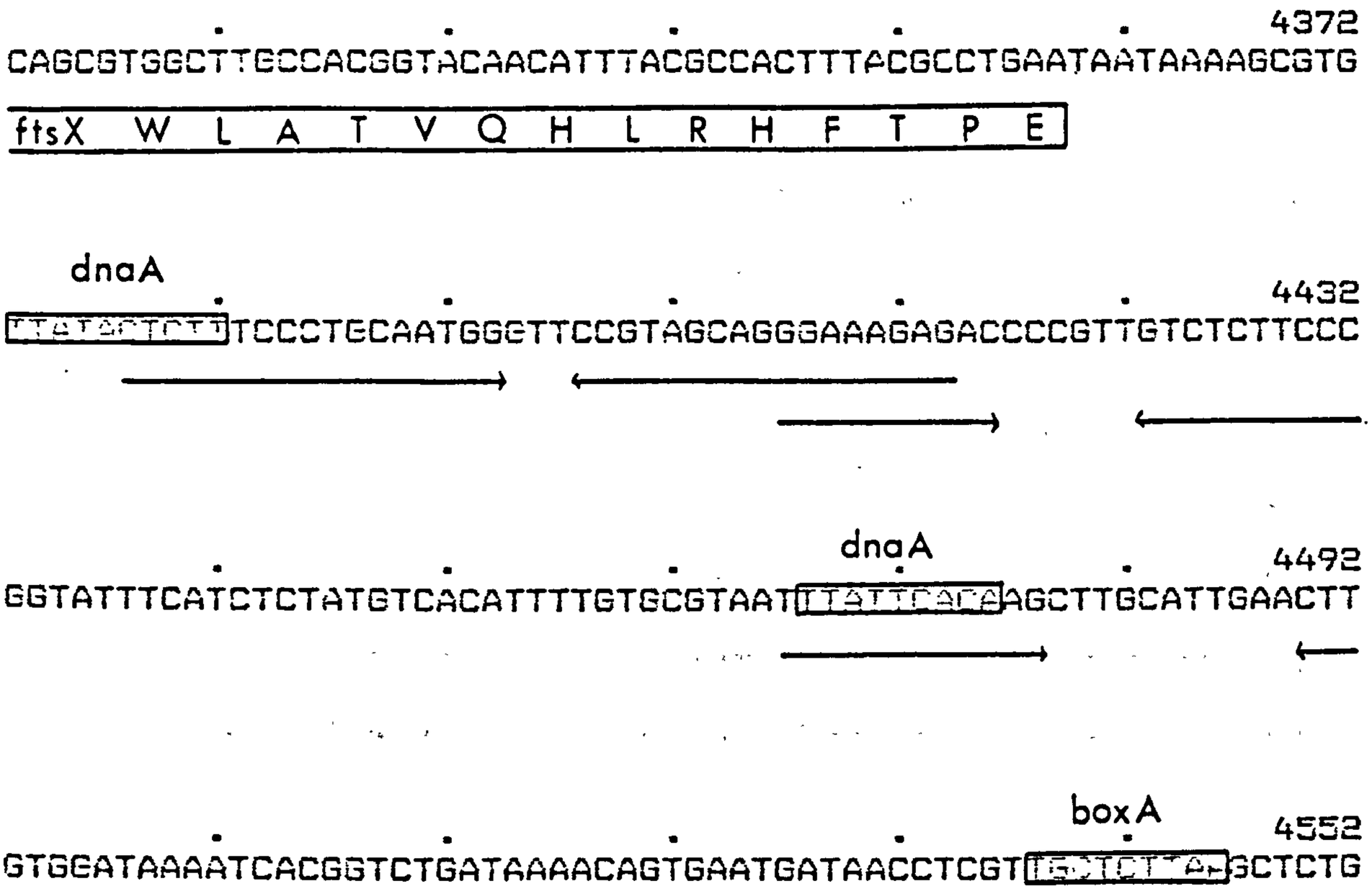
Data presented in this chapter and in the last, clearly indicate that the fam and dnaM mutations are allelic with the heat shock regulatory gene rpoH , and that rpoH lies downstream from the cell division operon. The mechanism by which the initial stages in the heat shock response are evoked remain unclear; in particular there is evidence for and against a transcriptional activation of rpoH. If there is indeed a transcriptional activation step then the DNA sequences upstream of rpoH should prove essential in the elucidation of any such mechanism. Figure 4.3 shows the intergenic sequence between ftsX and rpoH, possible regulatory sequences have also been included in this figure. Notably there are several inverted repeats within this region, which are potentially capable of forming stable stem loop structures in an RNA transcript. The presence of such stem loop structures at the end of a gene are characteristic signals for transcription termination, suggesting that

any readthrough of an ftsX transcript into rpoH is unlikely. On the other hand the presence of potential stem loop structures are also known to have regulatory functions at the start of a gene. The classical example is that of attenuation in the tryptophan operon in E.coli. Here the 5' end of the trp operon mRNA is capable of forming two different stem loop structures; since one stretch of sequence (b in figure 4.4) participates in both structures only one can form at any time.

FIGURE 4.3 Sequence of the Region Between ftsX and rpoH

The numbering of the sequence is the same as that in appendix A.
The arrows represent stretches of inverted repeat.

SD (Shine Dalgarno) Ribosome Binding Site



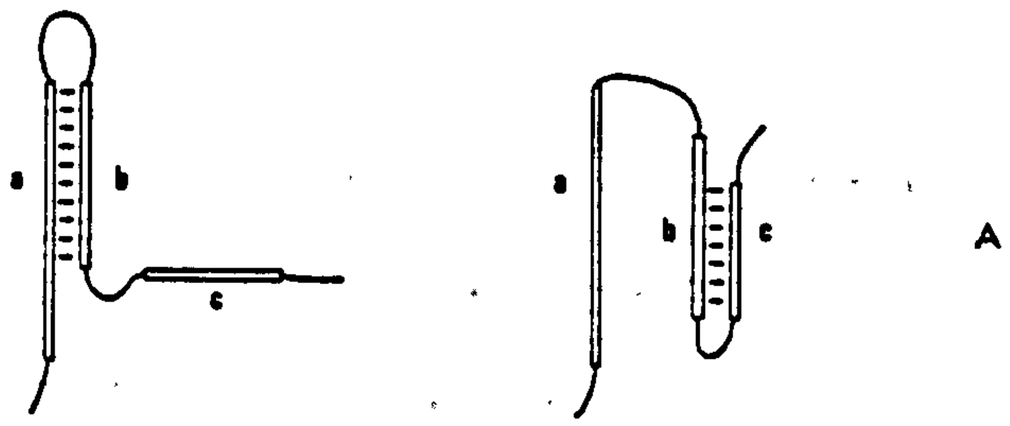
Which one forms depends on the rate of translation of the RNA by the ribosome, which in turn is affected by the concentration of tryptophan-tRNA within the cell. The heart of the control mechanism is that one stem loop structure (b+c) allows efficient translation of the message whereas the other (a+b) induces termination of translation. This mechanism is depicted graphically in figure 4.4. The potential for forming two mutually exclusive stem loop structures also exists in the region 5' of rpoH. The two possible structures are also shown in figure 4.4 and it is purely speculative that some physiological parameter, perhaps heat itself, might induce the formation of one particular structure and thus affect translation of the rpoH gene. Screening the intergenic region for recognition sequences of known regulatory proteins revealed two possible DnaA binding sites at the positions shown. Landick et al.(1984) had previously identified a putative BoxA sequence (NusA binding site) 5' to rpoH, which is also shown in figure 4.3. The physiological significance, if any, of either of these potential binding sites is unknown.

Tilly et al.(1986) measured rpoH transcript levels before and after heat shock and found a five-fold increase within 5 minutes of shifting from 30°C to 43.5°C. They also quote work involving the induction of a tac promoter::rpoH coding region fusion, which was found to increase the concentration of rpoH mRNA and proved sufficient to induce the heat shock response at 30'C.

Figure 4.4 Comparison of the pre-rpoH Sequence with the trp Attenuator

A shows diagramatically the secondary structures that can occur within the leader sequence of the trp operon mRNA. The consequence of having two mutually exclusive structures are discussed in the text.

B shows a region of pre-rpoH sequence (4373-4438) which also has the potential for forming two mutually exclusive stem loop structures.



It was concluded that an increase in the concentration of rpoH mRNA was an essential step in the induction of the heat shock response and could be the result of either increased transcription of rpoH and/or stabilisation of the message.

Stabilisation of mRNA as a regulatory response has been reported before. In Klebsiella pneumoniae a specific gene product (NifL) participates in the stabilisation of nif mRNA in response to various enviromental changes (Collins et al. 1986). Increased translation of mRNA can also stabilise the message as has been observed for the ribosomal protein RNAs in E.coli (Cole and Nomura 1986).

An increase in rpoH mRNA would presumably result in an increase in the concentration of σ^{32} protein, and this was observed by Lesley et al. (1987) who found a four-fold increase in concentration at 5 minutes after a temperature upshift.

The increase in transcription of heat shock genes after a temperature upshift is very rapid, apparently less than one minute (Neidhardt et al. 1984). It seems unlikely that such a rapid response can be caused solely by an increase in transcription of rpoH. The ability of plasmid pR2 to complement the heat shock defect in the various rpoH mutants also argues against a transcriptional control. In this plasmid the rpoH gene is under the control of the pBR322 tet promoter and therefore

lacking any putative heat dependent promoters that may be found upstream of rpoH.

An increase in activity of σ^{32} is obviously the essential step in the heat shock response. It is possible, though, that this increase in activity could result not only from de novo synthesis but also by modification of existing σ^{32}. Modification of σ^{32} could increase its affinity for the heat shock promoter or for the core polymerase, for which it has to compete with σ^{70}.

Similarly, modification of σ^{32} could also be a mechanism for reducing its activity during the shut-off phase of the response. DnaK is known to be involved in this shut-off phase (Tilly et al. 1983); DnaK is also known to be a protein kinase leading to the possibility that σ^{32} might be a target for DnaK mediated phosphorylation. DnaK was initially shown to possess a weak DNA dependent ATPase activity and it was found that it could be phosphorylated either in vitro or in vivo, probably by an auto-phosphorylating activity (Zylicz et al. 1983). It was later shown to be capable of phosphorylating both glutamyl-tRNA and threonyl-tRNA synthetases (Wada et al. 1986). Interestingly Grossman et al. (1984) observed that overproduced σ^{32} migrates as two spots on 2D gels, one spot perhaps being a phosphorylated form. The same workers noted that when σ^{32} was purified on the basis of activity only one spot was observed, possibly just the non-phosphorylated form. Phosphorylation of proteins is a well

known regulatory process in eukaryotes. Recently though evidence is appearing that it may also be widespread in prokaryotes.

In section 1.5.3 the various external stimuli that can induce the heat shock response were discussed. The question arises as to how such a varied range of stimuli can induce a single response. Consider first the induction of the heat shock response under conditions that induce the stringent response, the stringent response is induced when the availability of an aminoacyl-tRNA becomes limiting for protein synthesis. An initial response is that the nucleotide phosphates ppGpp and pppGpp accumulate with the requirement of the rel gene. ppGpp has recently been shown to bind to RNA polymerase affecting its conformation (Woody et al. 1987) and also its ability to transcribe certain promoters. This could provide a mechanism for differential expression of heat shock (and stringent response) genes (Kajitani and Ishihama 1984). Since, however, the heat shock response is apparently normal in relA mutants the accumulation of ppGpp or pppGpp is not necessary for the response (Grossman et al. 1985).

When E.coli is exposed to oxidation stress, by hydrogen peroxide for example, various dinucleotide tetraphosphates accumulate, the most common of these being AppppA (Bochner et al 1984). This dinucleotide tetraphosphate is also accumulated during the heat shock response. Bochner et al. (1986) found that AppppA inhibits a 5' nucleotidase activity associated with DnaK. The function of this activity is unknown although the workers speculated that the

binding of AppppA to DnaK might help modulate the heat shock response by altering either DnaK's 5' nucleotidase, or even its protein kinase activity. Plateau et al. (1987) recently showed that the overproduction of dinucleotide tetraphosphate hydrolase (which degrades AppppA) had no effect on either the heat shock or hydrogen peroxide responses, casting doubt on any role played by AppppA in these responses.

The role of potential mediators such as ppGpp and AppppA remains confusing and there is good reason for seeking a simple unifying theory to explain the common effect of many varied stimuli. It has been postulated that the heat shock response is a general stress reponse, one function of which is to remove abnormal polypeptides from the cell (Pelham 1986). Indeed some of the heat shock proteins are themselves proteinases (Baker et al. 1984). The presence of abnormal polypeptides could be a consequence of various stress conditions and could also be the primary inducer of the heat shock response. A possible mechanism for this would involve the saturation of a cells proteolytic capability by an accumulation of these abnormal polypeptides. If σ^{32} is also a substrate for the proteolytic system then this saturation could result in stabilisation of σ^{32} and an increased heat shock response.

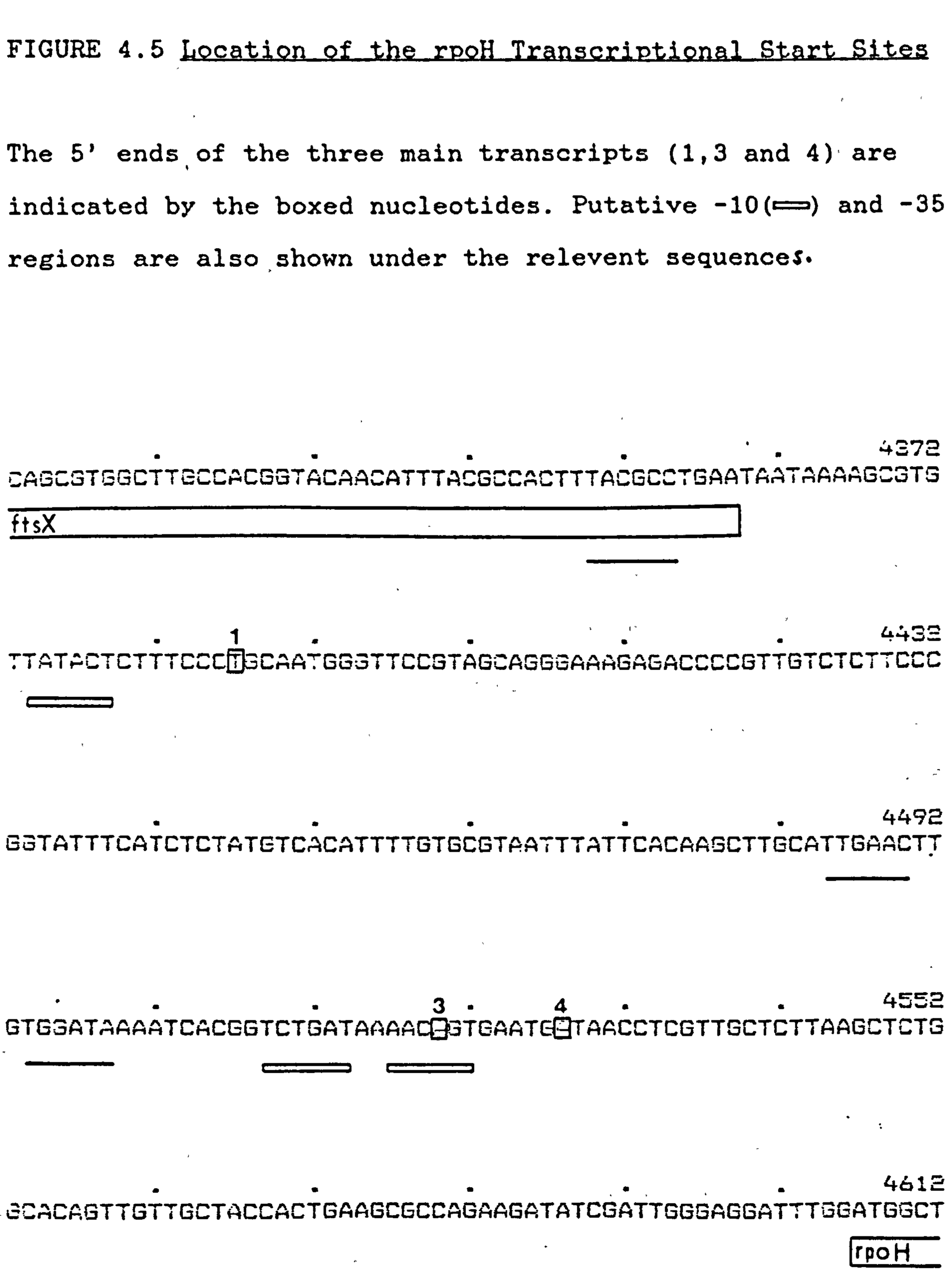
A recent series of papers, in particular three from the laboratory of Carol Gross, have concerned themselves solely with regulatory aspects of the heat shock response. Erickson et al.

(1987) studied the rpoH promoters and transcripts. S1 mapping showed four distinct transcripts with 5' end points 221bp (1), 132bp (2), 88bp (3) and 82bp (4) upsteam from the rpoH translational start codon. Another report (Tobe et al. 1987) identified three main transcripts with 5' ends at around 235bp, 90bp and 85bp from the start of rpoH. With the exception of the -132bp transcript the results are consistent given the inaccuracy in sizing the transcripts from Tobes publication. The -132bp transcript was only identified in one particular strain and may be due to a processing event. The location of the 5' ends of the three main transcripts are shown in figure 4.5. From this it can be seen that the promoter (P1) for the longest transcript (mRNA1) lies within the putative transcription terminator of ftsX.

The use of in vitro studies and rpoD mutants by Erickson et al. revealed that P1 and P4 were read by σ^{70} but not σ^{32}. P3 however was read by neither σ^{70} nor σ^{32}. Since this transcript could be detected both in vivo and in vitro this suggested the existence of yet another sigma factor. Upon a shift from 30'C to 43.5'C all three transcripts were found to increase in concentration, mRNA3 increasing most, but at a slower rate. Upon a shift to 50'C however, mRNA3 was the predominant transcript. The reduction in levels of mRNA1 and mRNA4 could represent inactivation of σ^{70} since very few other polypeptides are known to be synthesised at 50°C (see section 4.2). A novel, heat insensitive, sigma factor recognising P3 would then ensure a prolonged heat shock response ,essential for survival, under such extreme conditions.

FIGURE 4.5 Location of the rpoH Transcriptional Start Sites

The 5' ends of the three main transcripts (1,3 and 4) are indicated by the boxed nucleotides. Putative -10(═) and -35(——) regions are also shown under the relevent sequences.



In agreement with the work of Tilly et al (1986), Erickson et al. found a five fold increase in rpoH mRNA after heat shock. The use of promoter fusion vectors though, showed that this increase was not due to an increase in transcription, instead it probably resulted from increased stability of the message as discussed earlier in this section.

Grossman et al. (1987) studied the kinetics of σ^{32} synthesis and degradation. They found that overexpression of rpoH from a P_L promoter did not result in an appreciable accumulation of σ^{32}. This was found to be due to a rapid turnover of σ^{32}, its half life being calculated at 4 minutes. Straus et al.(1987) later showed that chromosomally synthesised σ^{32} had a half life of just one minute. This inherent instability is consistent with a model where a factor affecting the stability of σ^{32} can readily affect transcription of the heat shock genes. Increasing the rate of synthesis of σ^{32} using a P_{lac} or P_{tac} promoter proved sufficient to induce the heat shock reponse at 30°C.

Grossman et al. also measured the transcriptional activity of rpoH during the shut off phase of the heat shock response. They found that it did not decrease, suggesting that σ^{32} is repressed post-transcriptionally. The role of DnaK in this process was also considered but no evidence of phosphorylation of σ^{32} could be found. It was proposed though that DnaK could act as a repressor of rpoH translation.

Straus et al. (1987) measured the cellular levels of σ^{32} using antibodies. At 30°C they estimated that there were 50 molecules per cell (compared with 3000 for σ^{70}), within 5 minutes of a temperature shift to 42°C the level had increased 17 fold. After 15 minutes it had dropped to five times its initial level. At 42°C the stability of σ^{32} had also increased giving a half life of 8 minutes, compared with 1 minute at 30°C. The magnitude and kinetics of the change in σ^{32} levels were sufficient to account for the observed increase of dnaKdnaJ mRNA during heat shock. The comparison between a lacZ operon fusion and a lacZ protein fusion to rpoH confirmed the observation of Grossman et al. that the increase in σ^{32} was not due to an increase in rpoH transcription.

The main conclusion drawn from these recent reports is that the changes in concentration of σ^{32} throughout the heat shock response seem sufficient to account for the response. Changes in σ^{32} concentration are not mediated at the level of transcription but probably as a result of the balance between translation of the mRNA and proteolytic degradation of σ^{32}. A final paper (Bahl et al. 1987) reported that σ^{32} was stabilised by the lambda cIII protein which supports the proposal that stabilisation of σ^{32} may be physiologically important.

CHAPTER FIVE

Restriction Mapping the 76 Minute Region and the Localisation of *ftsS*

5.1 Introduction

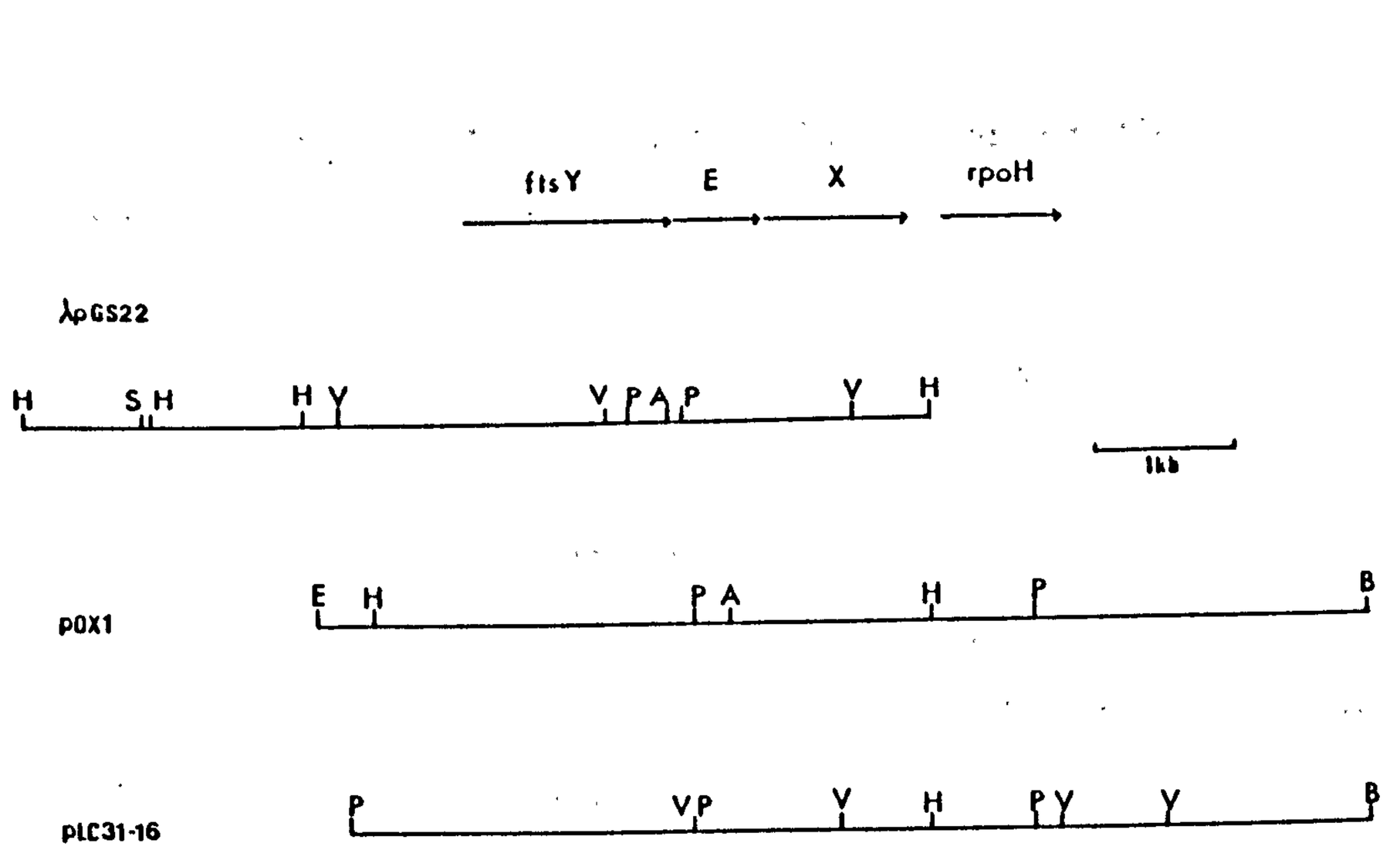
Other than λpGS22 there have been two other published reports of DNA being cloned from the 76 minute region of the chromosome. Neidhardt et al. (1983) cloned the htpR gene from pLC31-16, part of the library constructed by Clarke and Carbon (1976), and Oxender et al. (1980) cloned a 13kb EcoR1 fragment via a lambda vector into pACYC184 to give pOX1. The restriction maps of these three sources of DNA are shown in figure 5.1.

Their comparison shows a good agreement to the right of the common HindIII site, this being the region containing the rpoH (htpR) gene. The insert/vector junction in the Clarke and Carbon plasmid is unknown though it seems likely that the 5' Pst1 site is not within the insert which explains its absence in the other two clones. Within the errors of restriction mapping it also seems probable that the 4.0kb HindIII fragment in pOX1 is identical to the 4.5kb, operon containing fragment, in λpGS22. The approximate locations of the Pst1 and PvuII sites are consistent with this assumption. There is, however, a discrepancy between the restriction maps of λpGS22 and pOX1 in the fact that pOX1 (but not λpGS22) contains an EcoR1 site 400bp 5' of the leftmost HindIII site. Several possibilities exist to explain this discrepancy

FIGURE 5.1 Comparison Between the Restriction Maps of λpGS22, pOX1 and pLC31-16

H-HindIII S-Sph1 V-PvuII A-Ava1 P-Pst1 E-EcoR1

B-BamH1



1) A divergence in sequence between the source DNA of λpGS22 and pOX1

2) An error in the restriction mapping of either of them

3) That the 1.1kb HindIII fragment of λpGS22 is not adjacent to the 4.5kb fragment in the chromosome.

Of the three the third possibility seemed the most likely and could easily be tested.

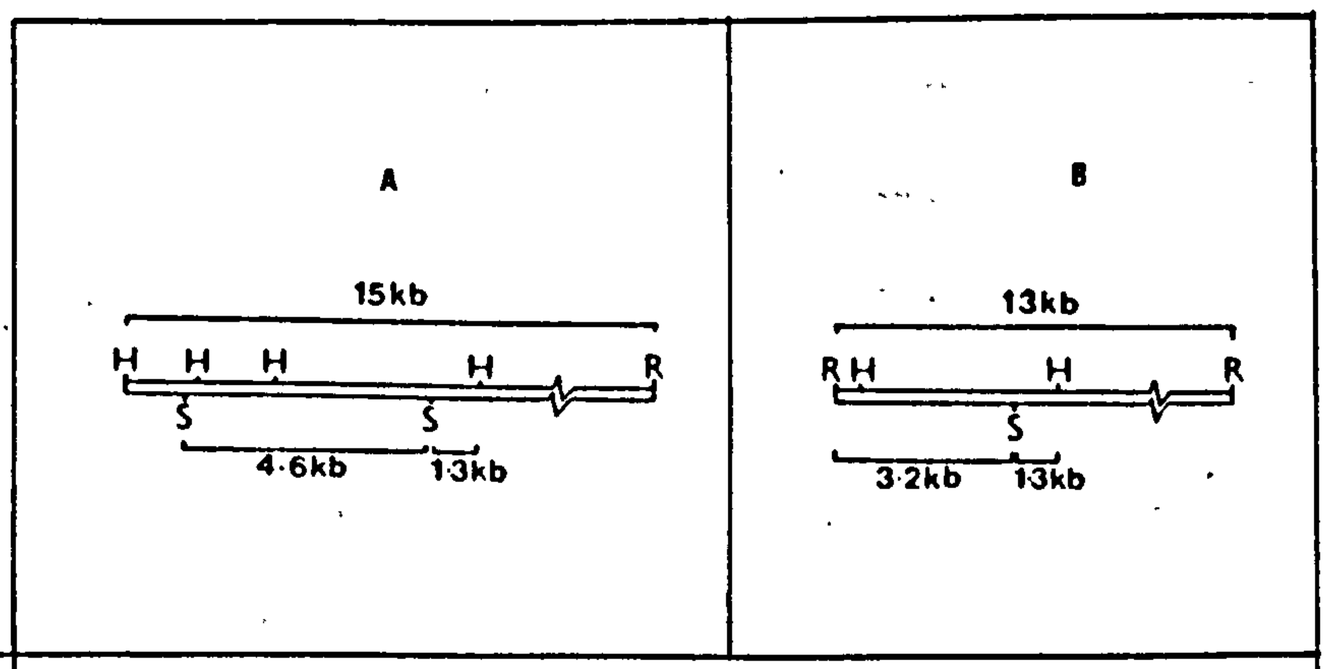
5.2 Determination of the Relative Positions of the 1.1kb and 4.5kb HindIII Fragments

The relative positions of the 4.5kb and 1.1kb HindIII fragments was determined by a Southern blotting experiment. In pOX1 the 4.5kb fragment lies within a 13kb EcoR1 fragment with an EcoR1 site 400bp from the 5' end of the 4.5kb fragment. No such EcoR1 site is found in λpGS22 which instead contains an extra 2kb of DNA 5' to the 4.5kb fragment. Within this 2kb lies a Sph1 site which gives a 4.6kb fragment when λpGS22 is cut with this enzyme. The presence of either the 13kb EcoR1 fragment or the 4.6kb Sph1 fragment in a chromosomal digest should discriminate between the two possible chromosomal situations. The scheme used for the actual experiment is shown in figure 5.2.

FIGURE 5.2 Experimental Protocol for the Determination of the Relative Locations of the 1.1kb and the 4.5kb Fragments

A represents the chromosomal situation predicted from the λpGS22 restriction map

B represents the chromosomal situation predicted from the pOX1 restriction map



Probe	Digest	Length of Hybridising Fragments	
	Sph1	4.6kb	?
1.1kb	HindIII	1.1kb	1.1kb
	EcoR1	>15kb	?
	Sph1	4.6kb + >1.3kb	>3.6kb + >1.3kb
4.5kb	HindIII	4.5kb	4.5kb
	EcoR1	>15kb	13kb

Chromosomal DNA was restricted in three independent experiments with HindIII, Sph1 and EcoR1. The restricted DNA was then split into two and electrophoresed on two identical agarose gels. The gels were then blotted onto two nitrocellulose filters, one filter was hybridized to a ^{32}P labelled 1.1kb fragment probe and the other with a ^{32}P labelled 4.5kb fragment probe. The results of the hybridisation are shown in figure 5.3.

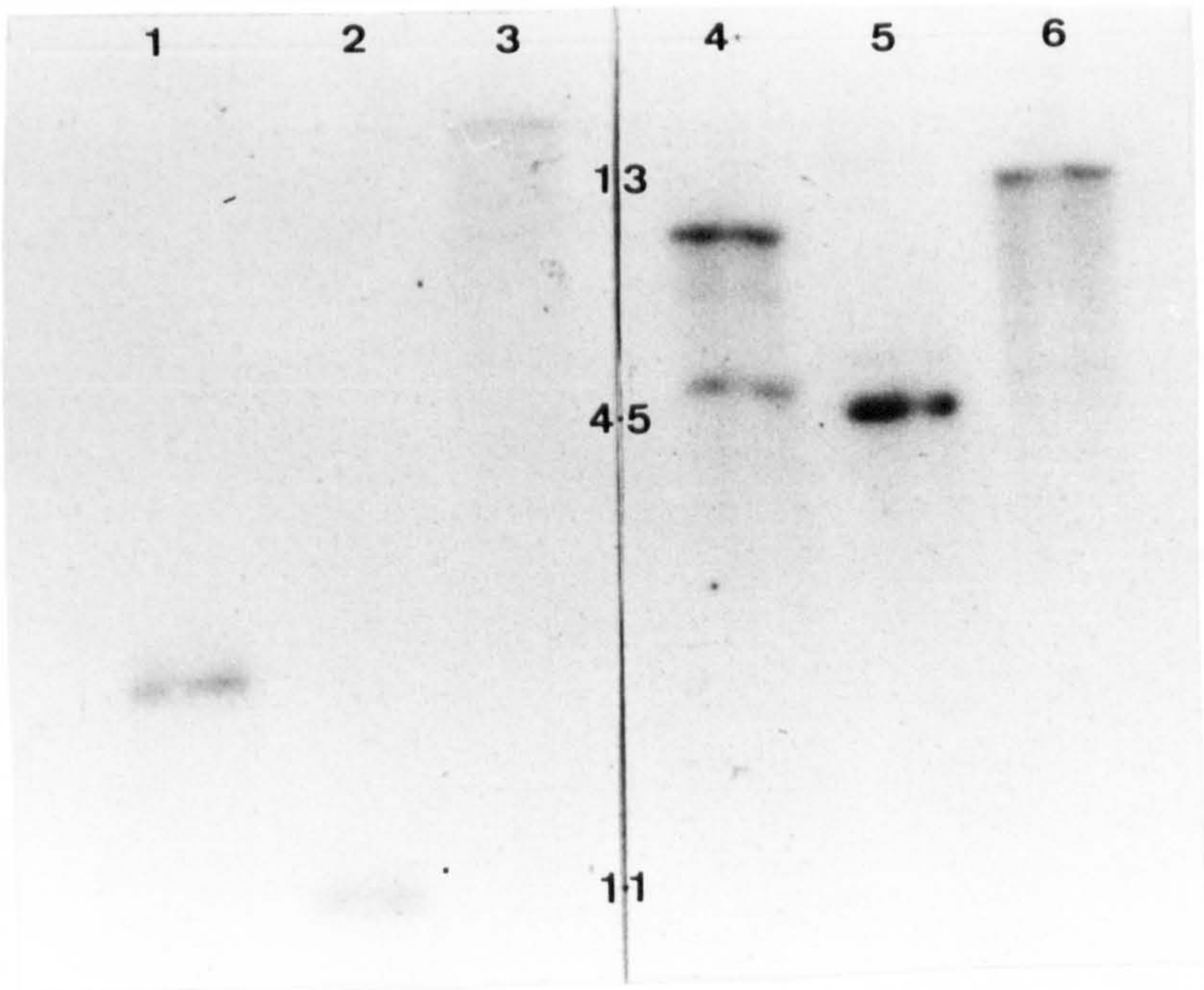
The results are consistent with the 4.5kb HindIII fragment lying witin a 13 kb EcoR1 fragment. The absence of a common 4.6kb Sph1 band suggests that the 1.1kb and 0.9kb HindIII fragments are not adjacent to the 4.5kb fragment on the chromosome. Since λpGS22 was constructed as a library of partial HindIII fragments it seems likely that the three fragments came together randomly during the formation of the library. The 0.9kb and 1.1kb fragments may themselves be adjacent within the chromosome, but this was not investigated.

The probability of such an event occurring (that is the coming together of unassociated fragments) was increased by certain selection procedures. λpGS22 was selected by its ability to complement the cell division mutant OV22 (ftsS). Since a lambda cloning vector (λNM540), was used this would result in the enrichment of phage containing a certain size of insert. It is possible that a phage containing just the 4.5kb HindIII fragment would be unstable relative to other inserts.

FIGURE 5.3 Determination of the Relative Positions of the 1.1kb and 4.5kb HindIII Fragments

The digests were electrophoresed on a 0.8% agarose gel prior to blotting. The numbers represent the size (in kb) of known fragments.

1.1kb Probe		4.5kb Probe	
Track 1	Sph1 Digest	Track 4	Sph1 Digest
Track 2	HindIII Digest	Track 5	HindIII Digest
Track 3	EcoR1 Digest	Track 6	EcoR1 Digest



A mechanism for increasing the chances of cloning the 4.5kb fragment, would be to fortuitously clone it, along with other more conveniently sized, but unlinked, fragments. This would appear to be the situation with λpGS22 and serves as a warning when constructing partial genomic libraries in lambda vectors.

5.3 Restriction Mapping the Region Clockwise of the Operon

Having established that the 0.9kb and 1.1kb HindIII fragments are not located next to the 4.5kb fragment little was known about the region upstream of ftsY and orf4. Since the 5.6kb, including and downstream from the operon was known to contain at least four important genes (ftsY, ftsX, ftsE and rpoH), the possibility existed that other equally important genes might be located upstream (clockwise on the E.coli map) of this cluster. The nearest reported genes in this direction were pit and gor though the distance between them and the operon was not known precisely (Elvin et al. 1986). In order to attempt to determine this distance and to facilitate any further study of this region a restriction map was constructed by Southern blotting.

Chromosomal DNA was restricted with a variety of restriction enzymes that were known to cut within the 4.5kb HindIII fragment. The digestions were electrophoresed on an agarose gel and blotted onto a nitocellulose filter. Also electrophoresed and blotted were some DNA size markers (HindIII digest of λcI_{857}). After

blotting, the filter was cut into two, separating the standards from the chromosomal digests. The filter containing the standards was hybridised to ^{32}P labelled lambda HindIII standards, whilst the other filter was hybridised to a ^{32}P labelled 3.2kb HindIII-Sph1 fragment derived from the 5' (ftsY) end of the 4.5kb HindIII fragment. After hybridisation and washing, the two filters were joined together such that they reformed the uncut filter onto which the gel was initially blotted. This filter was then autoradiographed and the result is shown in figure 5.4.

From this, a restriction map of the region clockwise of the operon was constructed and this is shown in figure 5.5. Also shown in this figure is the published restriction map for the region surrounding the pit gene. There is no obvious overlap between the two maps. However it remains a formal possibility that the most 3' Pst1 site on the pit map is identical to the most 5' Pst1 site as determined by the above experiment. Due to the large size of the BamH1 fragment on the Southern blot it was not possible to determine its position precisely. The possibility that the two PstI sites were identical was resolved by the recent publication of a limited restriction map of the entire E.coli chromosome (Kohara et al. 1987). These workers constructed a total genomic library in a lambda vector, and then restriction mapped each insert with eight enzymes. A computer program was finally used to link up overlapping clones.

FIGURE 5.4 Southern Blot Analysis of Restriction Sites Clockwise of the Operon

The right hand track contains lambda HindIII standards from which the other bands were sized.

Track 1	BamH1	Digest	Track 6	Pst1	Digest
Track 2	Cla1	Digest	Track 7	PvuII	Digest
Track 3	EcoR1	Digest	Track 8	Sal1	Digest
Track 4	EcoRV	Digest	Track 9	Sph1	Digest
Track 5	HindIII	Digest	Track 10	Stu1	Digest

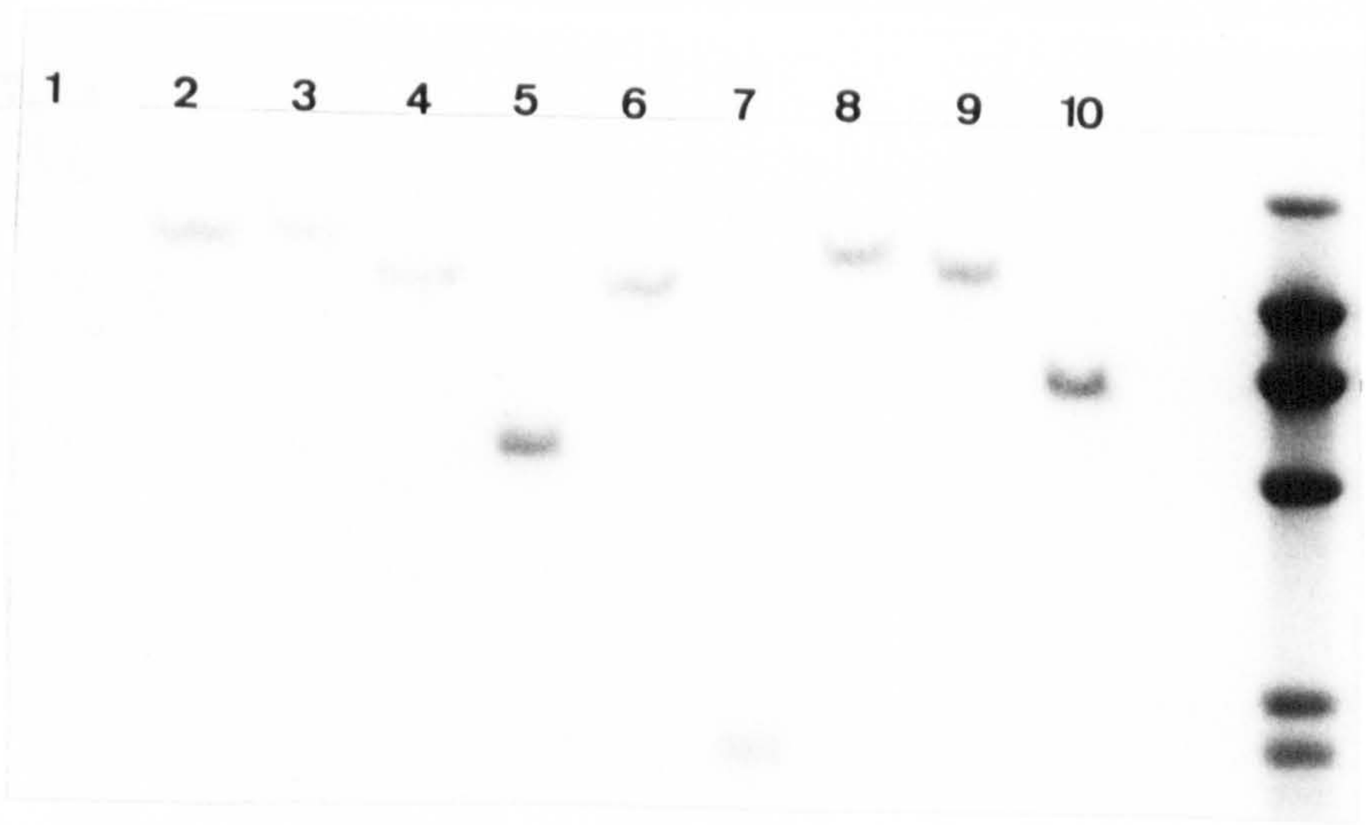


FIGURE 5.5 Restriction Maps of the 76 Minute and 77 Minute Regions of the E.coli Chromosome.

B-BamH1 P-Pst1 H-HindIII R-EcoR1 C-Cla1 Sp-Sph1 RV-EcoRV St-Stu1 S-Sal1

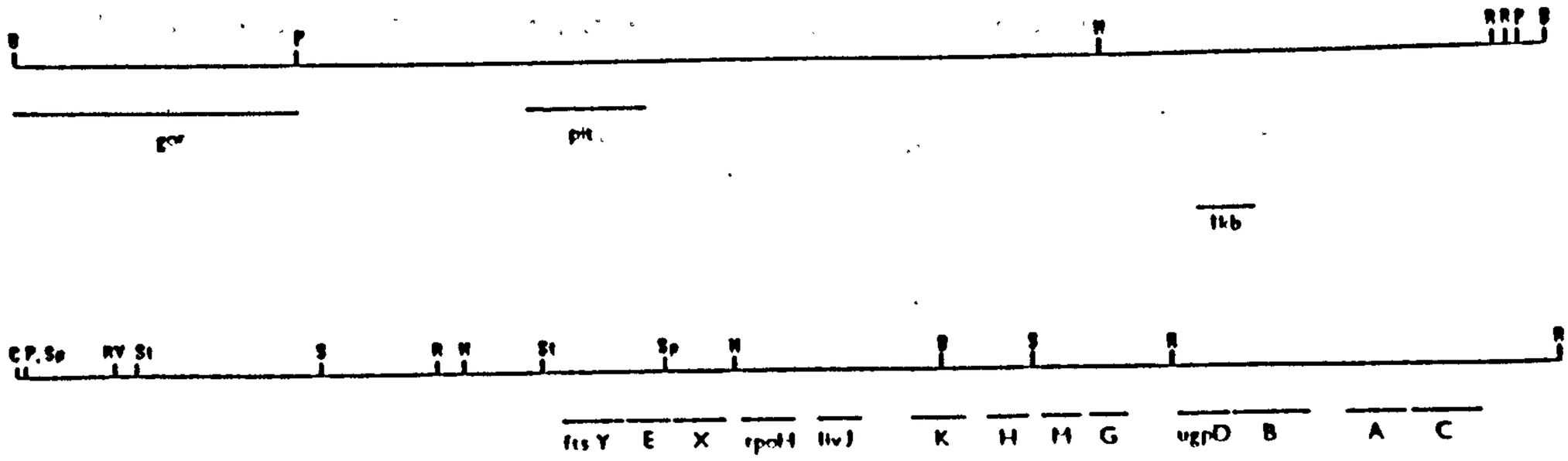


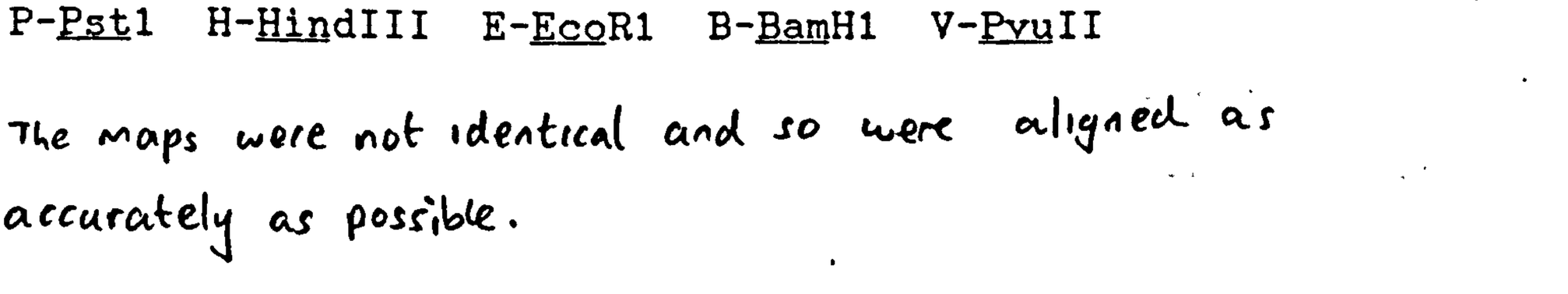
Figure 5.6 shows the restriction map they obtained for the 76-77 minute region. Under this map are aligned the maps for pit and ftsYEX shown in figure 5.5. From this figure it can be seen that the two PstI sites are not identical but are in fact separated by approximately 11kb. This means that there is a 30kb region between pit and ftsY within which no genes have been identified to date.

FIGURE 5.6 Complete Restriction Map of the 76-77 Minute Region

The top map was constructed by Kohara et al. (1987). The lower two maps are discussed in the text and also in figure 5.5.

P-Pst1 H-HindIII E-EcoR1 B-BamH1 V-PvuII

The maps were not identical and so were aligned as accurately as possible.



5.4 Localisation of ftsS

The location of ftsS within the 76 minute cluster has proved difficult to establish. Gill et al. (1986) found that a recA derivative of OV22 (ftsS) was not complemented by plasmid pDB1, which contained the 4.5kb HindIII fragment cloned into pBR322, but was complemented by λpGS22. The same workers found that on transforming a recA$^+$ strain of OV22 with pDB1 , temperature resistant colonies were readily isolated. This was attributed to "marker rescue" by a process of homologous recombination. Removal of the 3' end of the operon prevented such rescue. It was concluded that only part of the ftsS gene lay within the 4.5kb HindIII fragment and that the gene extended across the 3' boundary into sequences uniquely present in λpGS22, that is either the 0.9kb or 1.1kb fragments.

From the results discussed earlier in this chapter a dilemma obviously exists. Firstly the 0.9kb and 1.1kb fragments are known to lie at the 5' and not the 3' boundary and that secondly the two fragments are not truly associated with the operon anyway. The ability of λpGS22 to complement the OV22recA strain was tested and found to give mixed results. Some temperature resistant lysogens were isolated but many lysogens remained temperature sensitive, a fact also observed by Gill (1986).

In order to determine whether the subcloned 4.5kb fragment could truly complement OV22 (recA$^+$) or only rescue it λ4.5 was constructed. This was achieved by cloning the 4.5kb HindIII fragment into the lambda vector λNM540. Lysogens of OV22 with λ4.5 were isolated and all were found to be temperature resistant. This suggested complementation since the degree of rescue expected with a single copy clone of the wild type gene would be expected to be much less than the 100% observed. To confirm this, a lysogen was cured of the λ4.5 prophage by use of λb2red. All resulting colonies found to be non-lysogenic after this treatment had also reverted to temperature sensitivity, confirming that the 4.5kb HindIII fragment could complement the ftsS mutation in OV22.

However, neither pH3C (the 4.5kb HindIII fragment cloned into pBR322) nor λ4.5 could complement the mutation in OV22 recA suggesting that perhaps something was amiss with this strain. A new isolate was therefore constructed by T4 transduction using JC10240 as a donor. The new isolate was complemented by both λ4.5 and plasmid pH3C confirming the above suspicion.

Plasmid pCΔR was constructed by deleting a 233bp EcoRV-HindIII fragment from the 3' end of the 4.5kb fragment in pH3C, thus removing the 3' end of ftsX. This plasmid failed to complement the mutation in OV22 recA (new isolate) thus confirming ftsS as an allele of ftsX.

CHAPTER SIX

Identification of Promoters Within the 76 Minute Region

6.1 Introduction

The DNA sequence of the 4.5kb HindIII fragment containing the cell division operon has been completely sequenced (Gill et al. 1986), and is listed in appendix A. The designation of the region as an operon was on the basis of the following data.

a) From the sequence there were three adjacent open reading frames (orfs), which could be transcribed in the same direction.

b) All three orfs could be transcribed and translated in a maxi-cell system to give identifiable gene products.

c) Transposon insertions into the two most proximal genes prevented expression of the downstream gene(s).

d) There existed a region 5' to the most proximal gene (ftsY) with homology to the operator region of the trp operon.

Known cell division mutants had their mutations mapped to either ftsE (MFT1181, TC35, TOE22) or ftsX (OV32, JS10). No mutants, however, carried mutations in ftsY and thus its designation as a cell division gene was purely speculative. Furthermore the possibility remained that there was an independent promoter for ftsE since the transposon insertions in ftsY, that prevented ftsE expression, mapped very close to the ftsY-ftsE junction.

6.2 The Mapping of Promoters by Deletion Analysis

A series of deletions were made within the operon to determine which regions were required for expression of the three gene products. The effect of these deletions was determined by complementation analysis. Figure 6.1 shows, diagrammatically, a scheme outlining the construction of these plasmids. The deletion series is then shown in figure 6.2, and the results of the complementation analysis in table 6.1.

Plasmid pH3C contains the entire 4.5kb Hind III fragment and is identical to pDB1 (Gill et al. 1986), although it was independently cloned from a different source (section 3.5). It complements all the known mutations in ftsE and ftsX.

Plasmids pCΔs and pCΔr are the result of deletions between the 3' HindIII site and the Sph1 site of ftsE and the EcoRV site of ftsX respectively. In either case where a gene has been partially or totally deleted the plasmid failed to complement mutations within that gene.

FIGURE 6.1 Construction of the pH3C Deletion Series

The solid lines represent insert DNA; the thin lines represent pBR322 DNA.

1/2 The 4.5kb HindIII fragment from λS3d was cloned into the HindIII site of pBR322 in both orientations giving pH3C and pH3C'

3 An internal deletion of the 3.8kb Sph1 fragment from pH3C' giving pC'Δs

4 An internal 3.3kb deletion between the Stu1 site in the insert and the EcoRV site in pH3C giving pCΔsr

5 An internal deletion of the 0.4kb EcoRV fragment from pH3C giving pCΔr

6 An internal deletion of the 1.7kb Sph1 fragment from pH3C giving pCΔs

7 An internal deletion of the 2.4kb AatII fragment from pH3C giving pCΔa

8 An internal deletion of the 2.6kb PvuII fragment from pCΔa giving pCΔaΔp

FIGURE 6.1 Continued

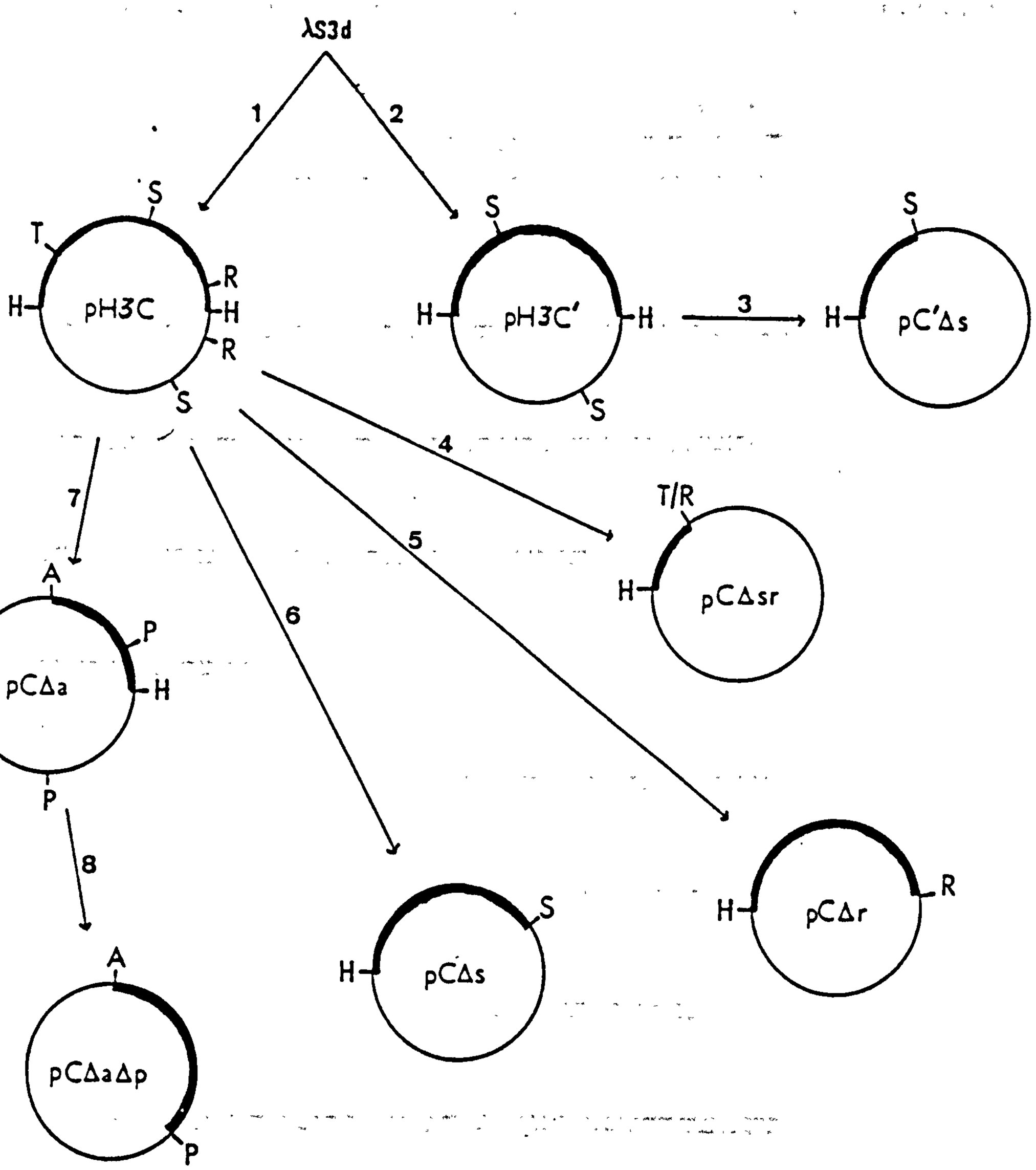


FIGURE 6.2 Structure of the pH3C Deletion Series

The boxed regions represent cloned DNA, no vector sequences are shown. The constructions are discussed in more detail in section 2.2 and in figure 6.1

H-HindIII St-Stu1 A-AatII B-BstE2 S-Sph1 P-PvuII R-EcoR1

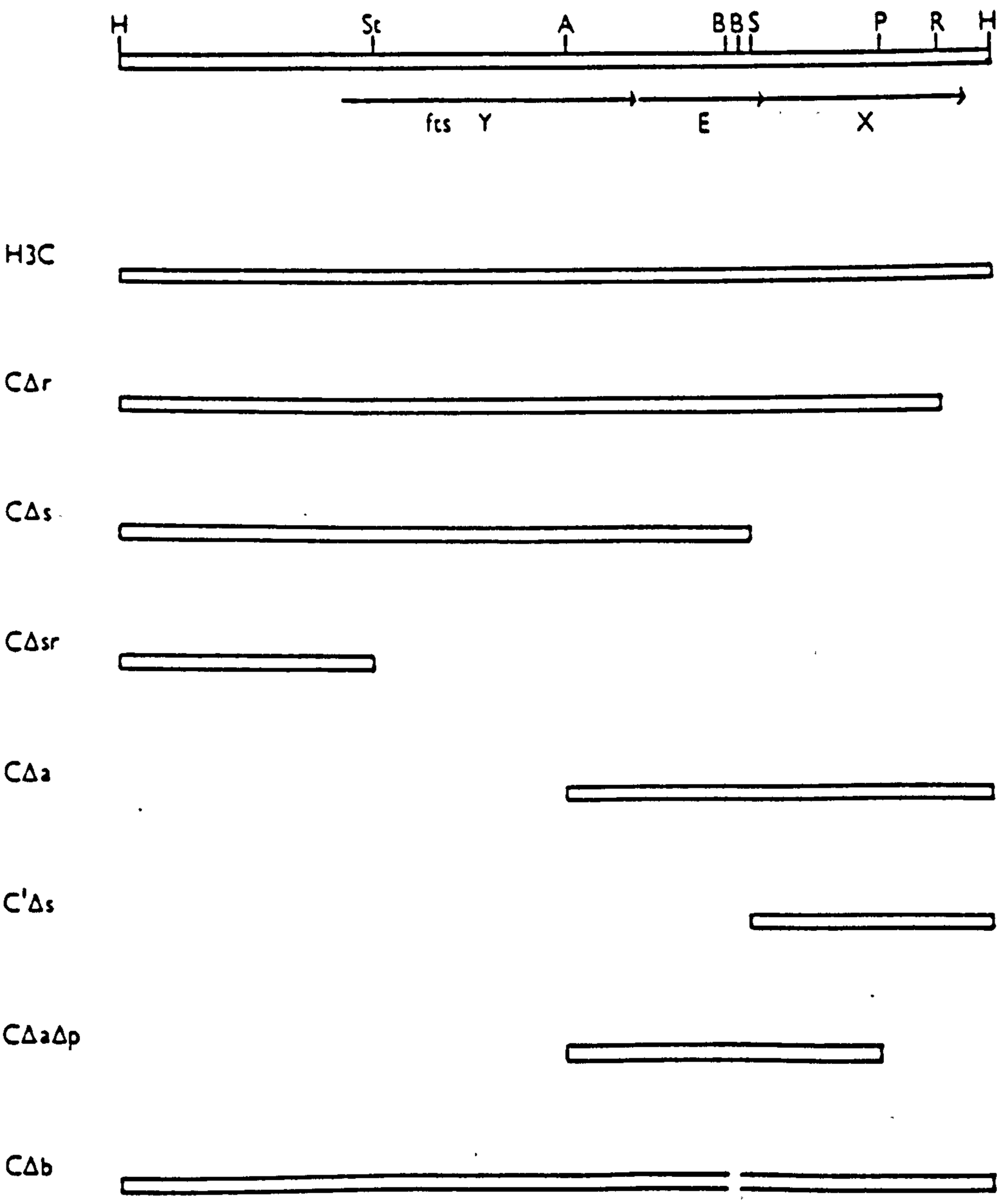


TABLE 6.1 Complementation Analysis of the pH3C Deletion Plasmids

	MFT1181 *recA* (*ftsE*)	OV32 *recA* (*ftsX*)
pH3C	+	+
pCΔr	+	-
pCΔs	-	-
pCΔsr	-	-
pCΔa	+	+
pC'Δs	-	-
pCΔaΔp	+	-
pCΔb	-	+

\+ Complementation - No Complementation

Plasmid pCΔsr lacks ftsE, ftsX and the distal portion of ftsY. As expected it failed to complement any of the mutations in ftsE or ftsX.

Plasmid pCΔa lacks the proximal portion of ftsY along with all its putative promoter and operator regions. The plasmid however was capable of complementing mutations in both ftsX and ftsE. This observation provided strong evidence for an internal promoter for ftsE lying downstream of the AatII site in ftsY.

Gill et al. (1986) showed that a transposon insertion in the middle of a plasmid-borne ftsE gene prevented expression of ftsX. This suggested that the two genes might be transcribed as a single unit, ftsX not having its own promoter. The inability of plasmid pC'Δs to complement mutations in ftsX confirms that there is no promoter for ftsX downstream of the Sph1 site in ftsE.

From the DNA sequence it can be seen that the coding regions of ftsE and ftsX overlap by 5 base pairs. Given that they also appear to be transcribed together the possibility exists that they may be translationally coupled. Two genes are said to be translationally coupled when the efficient expression of the more distal gene requires complete translation of the more proximal gene. An example can be found in the galactose operon where the expression of galK depends on the expression of the preceding

gene galE(Schumperli et al. 1982). This coupling can be altered in vitro by changing the position at which the more proximal gene terminates relative to the initiation position of the more distal gene. Schumperli et al. performed such an experiment with the galactose sytem and concluded that changing the relative sites for termination and initiation by some 40 base pairs drastically reduced the expression of the distal gene. A reduction in galK activity was also observed with changes as little as 2 base pairs.

Plasmid pCΔb contains an internal deletion in ftsE such as to induce a frameshift and thus alter the translational termination site. This change though did not seem to effect the expression of ftsX and thus could not provide any evidence for translational coupling. This lack of effect though was possibly due to the small displacement involved, just 4 base pairs (see section 6.3). The construction of ftsX gene fusions that allow a more accurate measurement of ftsX expression could be used to determine whether or not this small displacement does effect expression.

6.3 Mapping Promoters by use of galK Expression Vectors

The data discussed above clearly suggested that there are at least two promoters in the operon, one proximal to ftsY and another proximal to ftsE. The latter presumably lies within the ftsY coding region since only 5 base pairs separate the two genes. In order to localise these promoters more precisely it was decided to use the galK expression system developed by McKenney et al. (1981).

This system is based on the ability to accurately assay the galactokinase activity associated with the galK gene product. The pKO series of plasmids contain the galK gene lacking its normal promoter region, and so can not produce any galactokinase. Certain restriction sites have been incorporated in front of the promoterless galK gene to allow easy cloning of fragments into this position. If any cloned fragment contains a promoter, reading in the same direction as the galK gene, then it can direct the expression of galK. The level of expression can be determined by measuring the level of galactokinase in a strain containing a mutated chromosomal copy of galk. As well as indicating whether or not a particular fragment contains a promoter the system can also give an indication of the relative strengths of different promoters.

The experiment performed to identify the operon promoters is shown in figure 6.3. All the putative promoter-containing fragments are cloned into the galK expression vector pKO2 in both orientations to provide a control for non-specific promoter activity. All the values given for galactokinase activity are the result of several (2-3) experiments and are corrected for background levels.

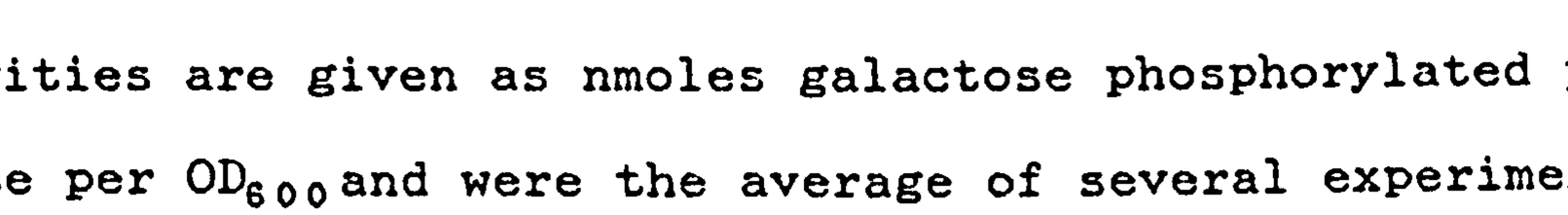
The aim of the galK promoter probe work was to confirm the existence of a promoter proximal to ftsE and to give more accurate information as to its location. The results obtained with plasmid pCΔa suggested that the promoter must lie 3' of the AatII site (in ftsY), which lies 350 base pairs upstream from the start of ftsE. The first set of plasmids constructed (pP3, pP7, pP9 and pP18) were based around the PvuII site 500bp upstream from ftsE.

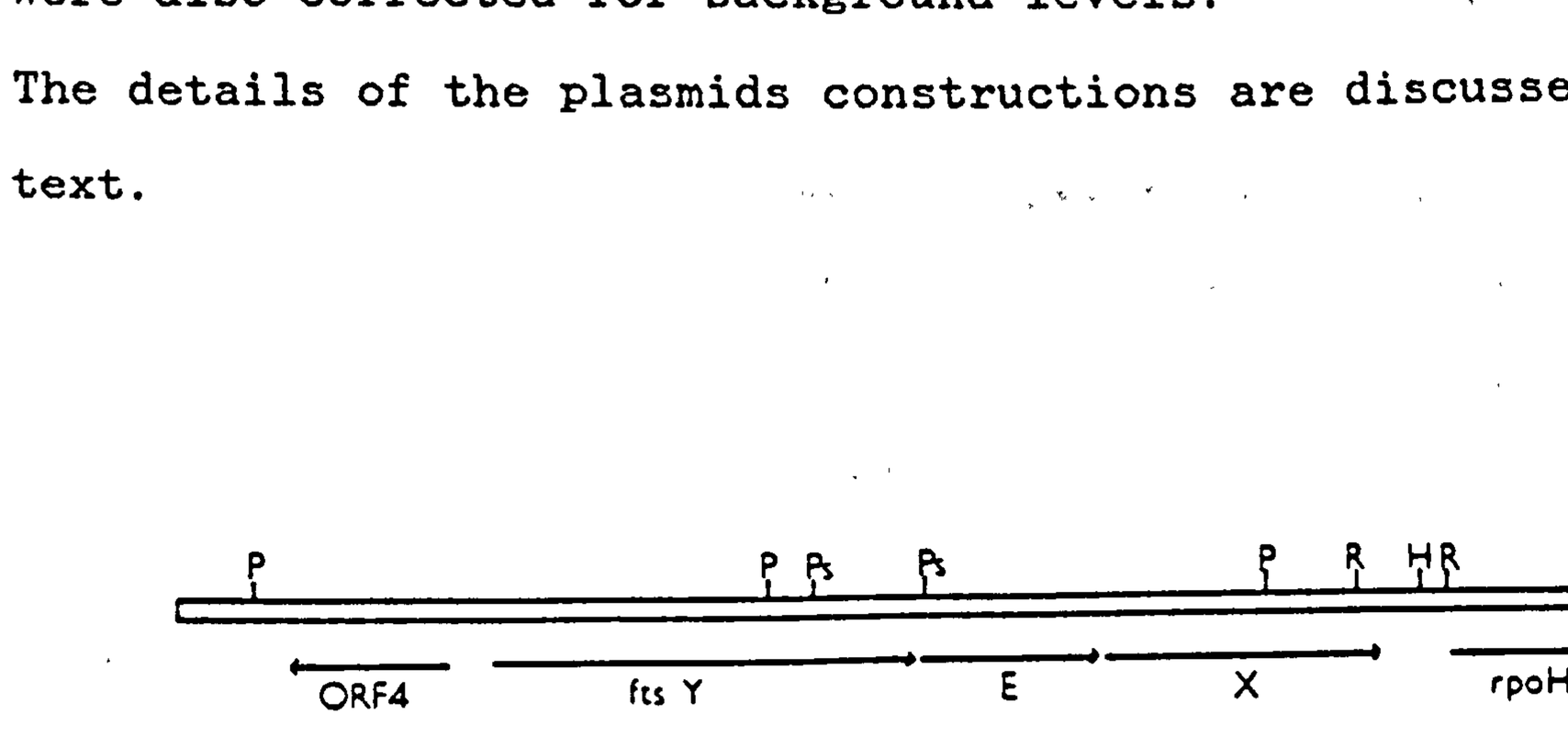
pP18 and pP7 were constructed by cloning the 1.9kb PvuII fragment (between positions 268 and 2147, see appendix A) into the SmaI site of pKO2. In pP18 the orientation of the fragment is such that the ftsY gene reads in the same direction as the galK gene, whereas in pP7 the fragment was cloned in the opposite orientation. Promoter activity was found to be associated with both of these constructs (4.2 and 9 nmoles respectively of galactose phosphorylated per minute per OD_{600}). The activity associated with pP18 was assumed to be due to an ftsY promoter.

FIGURE 6.3 Construction of galK Gene Fusions

Each cloned fragment, represented by boxes, was cloned into pKO2 in both orientations. The left to right arrows represent clones where the operon genes were cloned in the same orientation as galK. The right to left arrows represent clones where the operon genes were cloned in the opposite orientation. The galactose activities are given as nmoles galactose phosphorylated per minute per OD_{600} and were the average of several experiments and were also corrected for background levels.

The details of the plasmids constructions are discussed in the text.





The activity associated with pP7 could possibly be due to a promoter for orf4, this might not have been expected since the clone contains a potential termination region 3' of orf4. However it is possible that either no termination sequences exist here or that the construction of the plasmid disrupted any terminator structure.

The second pair of plasmids to be constructed were pP3 and pP9 which contain the 1.8kb PvuII fragment (2147 to 3927). In P3 the ftsE and ftsX genes are read in the same direction as galK, the opposite orientation exists in pP9. No promoter activity was found associated with pP9. Activity was found to be associated with plasmid pP3 however (11.5 units), thus providing confirmatory evidence for an internal promoter. Since the cloned fragment in pP3 contains not only ftsE sequences but also part of ftsX the location of this promoter can not be clearly defined. It could theoretically be due to an ftsX promoter. In any case there appears to be no strong termination sequences close to the 3' end of ftsE.

In order to try and localise this internal promoter to ftsE plasmids pPST4 and pHP14 were constructed. This was achieved by converting the 431bp PstI fragment (2305 to 2694) into a blunt-ended fragment by the use of T4 DNA polymerase before cloning into the Sma1 site of pKO2. In pHP14 the fragment was cloned such that the ftsE gene was read in the same direction as

galK, the opposite orientation was found in pPST4. Whereas no promoter activity was found associated with pPST4, an activity was found associated with pHP14 (2.9 units). This was attributed to an internal ftsE promoter located within a 335bp region upstream from ftsE.

A final pair of plasmids were constructed to confirm the presence of an independent promoter for rpoH. pR4 and pR6 were constructed by cloning the 342bp EcoRV fragment (4243 to 4585) into the SmaI site of pKO2. When cloned in the "correct" orientation promoter activity was indeed observed.

6.4 Complementation of an ftsX mutation by pSPHd

The construction of pSPHd was discussed in chapter three and the observation made that, although it encoded the rpoH gene, it complemented mutations in that gene very poorly, if at all. Whilst screening the cell division mutants for complementation by the deletion plasmids discussed earlier in this chapter, the mutants were also transformed with the plasmids pSPHd and pSPHd'. The results of temperature sensitivity tests with these plasmids are shown in table 6.2.

TABLE 6.2 Temperature Sensitivity Screening of pSPHd and pSPHd' Transformants

	OV2		MFT1181 (*ftsE*)		OV32 (*ftsX*)	
	30°C	42°C	30°C	42°C	30°C	42°C
pBR322	+++	+++	+++	-	+++	-
pSPHd	++	+	++	-	++	+
pSPHd'	++	+	++	-	++	-

+++ healthy growth ++ reasonable growth

\+ poor growth - no growth

The three strains used were all *recA* derivatives

A notable result was that the growth of any strain harbouring either pSPHd or pSPHd' seemed impaired, even at 30°C. The effect was greater at 42°C where even OV2 (pSPHd or pSPHd') grew poorly. The inability then of these plasmids to complement mutations in rpoH was probably due to a general growth inhibitory function.

It was observed though, that the growth of OV32 (pSPHd) at 42°C was significantly greater than that of OV32 (pSPHd'), OV32 (pBR322) or OV32 itself, suggesting that the plasmid might be complementing the ftsX mutation. This was confirmed by microscopic examination as shown in figure 6.4.

pSPHd contains only the 1.3kb 3' Sph1-HindIII portion of the 4.5kb HindIII fragment.Plasmids pSPHd' and pC'DS also contain this Sph1-HindIII fragment but neither complement the ftsX mutation in OV32. The difference between these two plasmids and pSPHd is that the fragment is cloned in different orientations in pBR322 (figure 6.5).

The inability of pC'As to complement ftsX mutations was considered to be due to the absence of a promoter within the cloned fragment (section 6.2). In pSPHd the ftsX gene lies downstream of, and in the same orientation as, the truncated tet gene of pBR322. The tet gene could thus provide a promoter for the transcription of the cloned ftsX gene.

FIGURE 6.4 Photomicrographs of OV32 (pSPHd)

FIGURE 6.5 Comparison between the Construction of pSPHd, pSPHd' and pC'As

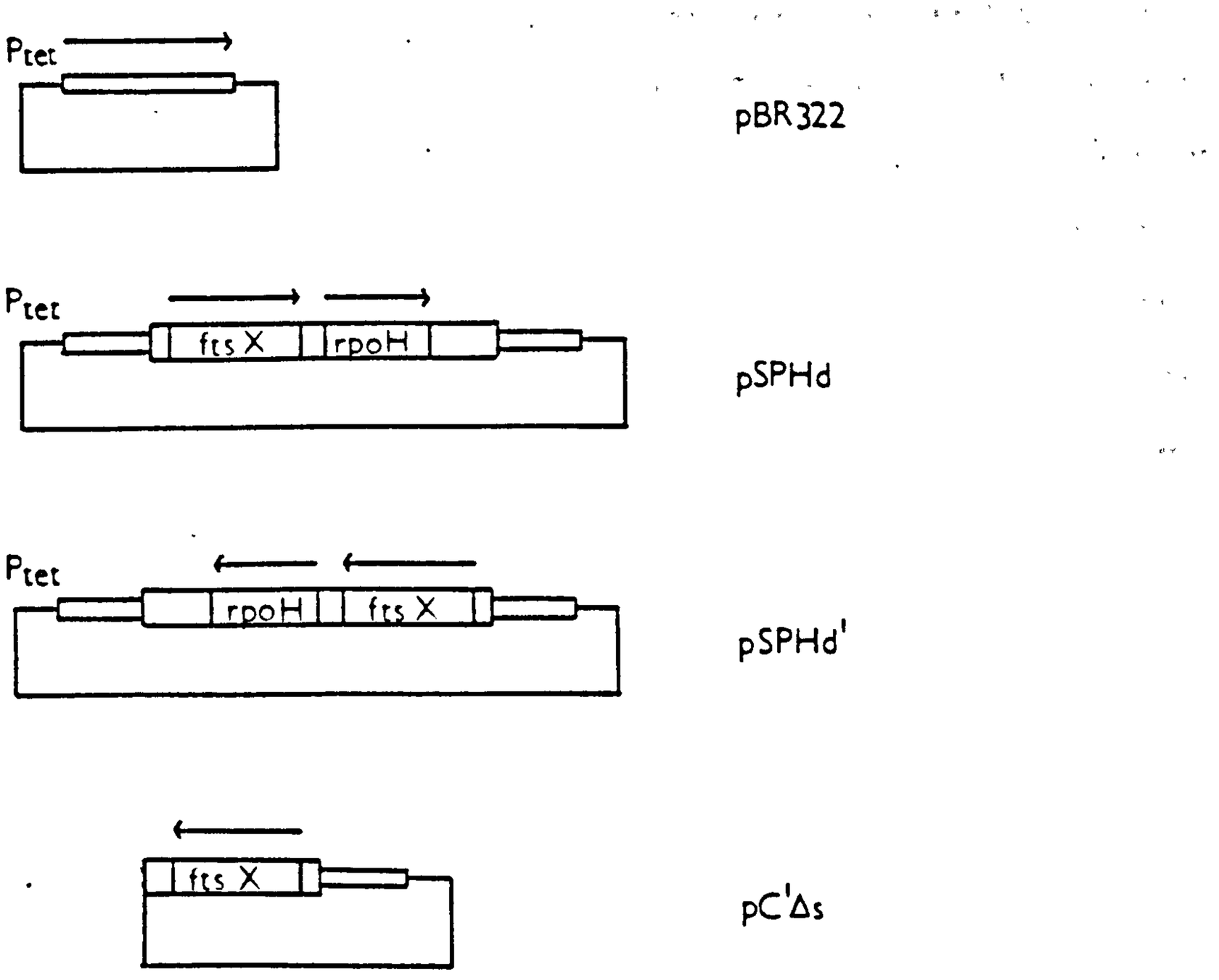
——— pBR322 vector sequence

═══ pBR322 *tet* gene

▭ cloned operon DNA

Ptet *tet* gene promoter

The arrows represent the direction of transcription. In all cases the *tet* gene is transcribed left to right.



To confirm that pSPHd can express ftsX , the gene products from this plasmid were examined in a maxicell system. The resulting gel is shown in figure 6.6, which also includes the plasmid pdΔH (pSPHd with the Sph1-HindIII fragment deleted). Three common bands can be seen on the gel: bla (30k), rpoH (38k) and an unidentified band (60k)-possibly a lambda protein (see figures 3.2 and 3.3 for the construction of pSPHd). There is also another band (36k) unique to pSPHd which is a good candidate for the ftsX gene product (Gill et al. 1986).

In the previous section the possibility of translational coupling between ftsE and ftsX was considered. If such coupling does exist then presumably it will be present between ftsX and the tet-ftsE gene fusion created in pSPHd. Figure 6.7 compares the sequence surrounding the translational start codon of ftsX in the three plasmids pH3C, pCΔb and pSPHd. In pH3C, which represents the normal sequence, there is an 5bp overlap between the two genes. In both pCΔb and pSPHd the frameshifted ftsE gene terminates at the TGA codon resulting in the 1bp overlap discussed in section 6.2.

FIGURE 6.6 Maxicell Analysis of Gene Products from pSPHd

The experiment was performed with CSH26 (pSPHd), track 1, and CSH26 (pdΔH), track 2, exactly as described in section 2.24 The bands were sized relative to ^{35}S labelled molecular weight standards (not shown).

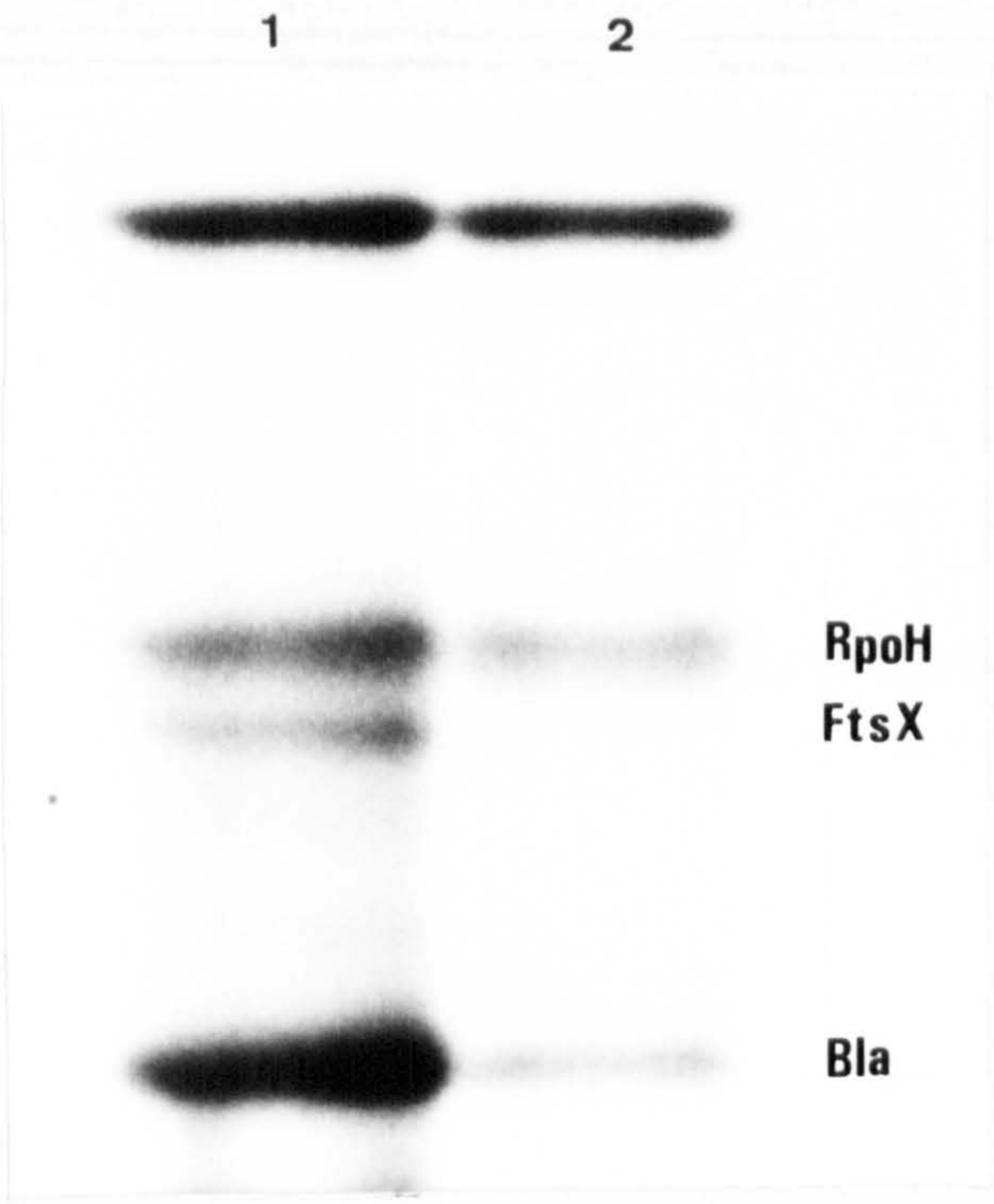
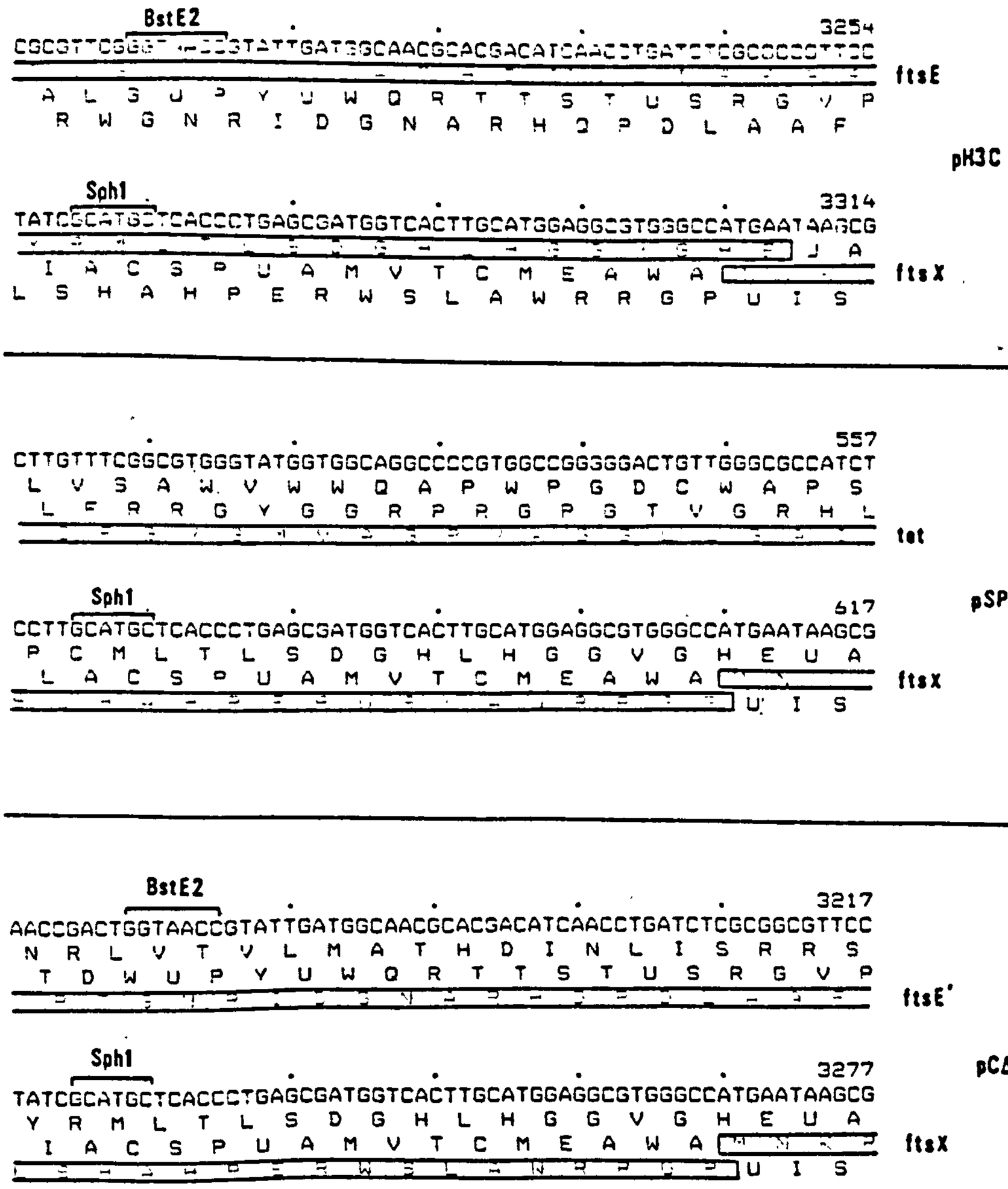


FIGURE 6.7 Comparison of the ftsE-ftsX Junction in pH3C, pSPHd and pCAb

The open reading frames of the appropriate genes are shown as boxed residues under the respective sequence. The BstE2 and Sph1 restriction sites are shown for reference.



6.5 Discussion

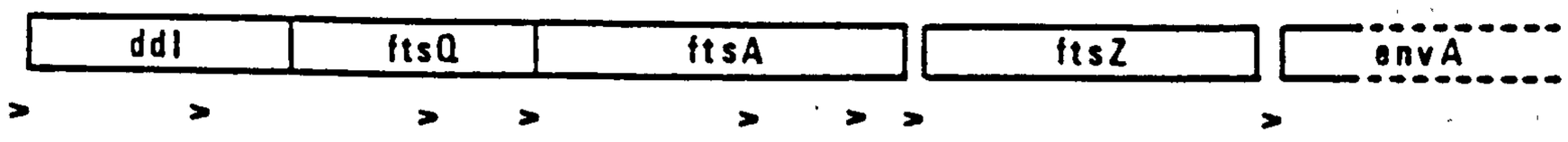
The use of deletion analysis and the pKO system has identified two promoters within the operon region. The first was found in a 900bp region upstream of the ftsY translational start codon. A second promoter was identified within a 350bp region upstream of ftsE, which placed it within the ftsY coding region.

A similar situation, where closely associated cell division genes are found with independent promoters, is found at 2 minutes on the E.coli chromosome map. This remarkable region contains at least 12 closely linked genes all involved in cell growth and division (section 1.6). Part of this cluster containing the genes ddl, ftsQ, ftsA, ftsZ and envA has been extensively studied and its transcriptional organisation determined (Yi et al. 1985, Robinson et al. 1986). All five genes were found to be transcribed in the same direction and all seemed to have their own promoters as shown in figure 6.8.

Donachie et al. (1984) suggested two possibilities to explain this clustering of genes with broadly related, but individually quite different, functions. The first is that they represent an evolutionary relic of some sort, perhaps due to duplication events. Secondly there may be a functional reason for the clustering, such as that found within an operon (Jacob and Monod 1981).

FIGURE 6.8 Organisation of the ddl-envA Region

The arrowheads represent promoters identified by a combination of *in vitro* techniques as described by Yi *et al.* (1985) and Robinson *et al.* (1986).



The presence, though, of many internal promoters suggests that it is unlikely that the 2 minute gene cluster represents a functional operon.

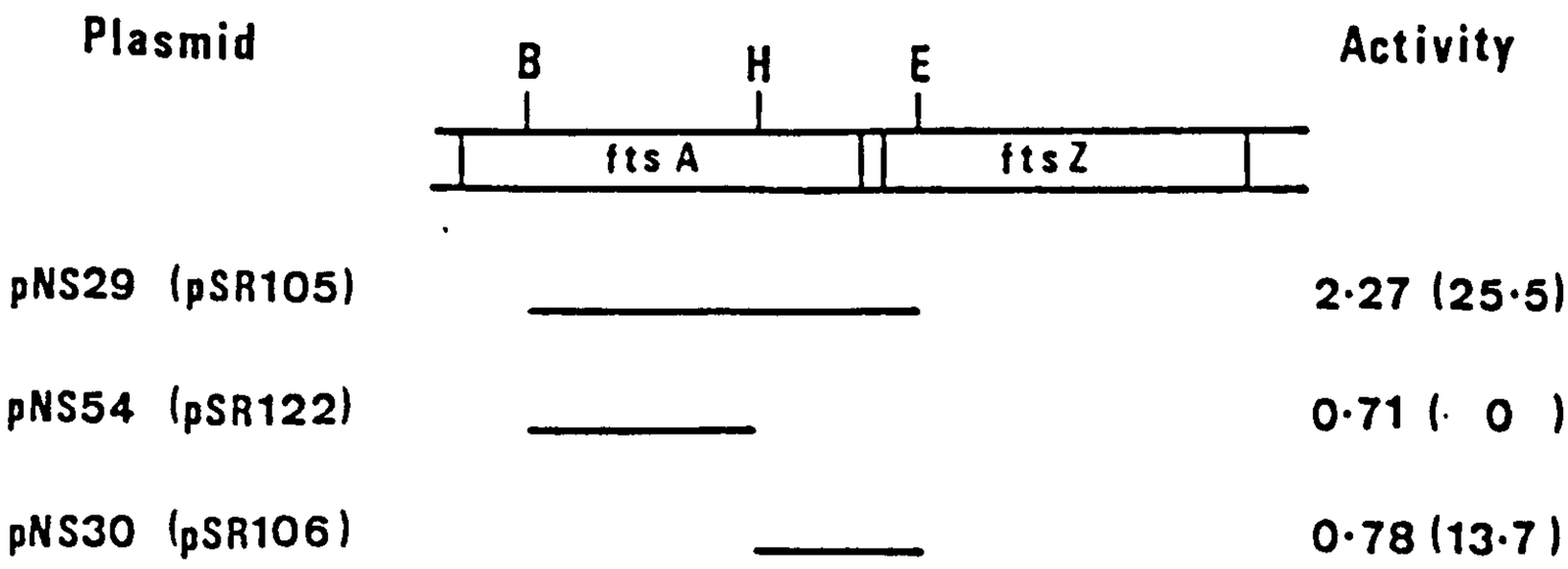
Sullivan and Donachie (1984) found an alternative form of regulation within the 2 minute cluster. They showed co-operative enhancement of transcription initiation of ftsZ between two promoters. The result was based on the measurement of promoter activity by the same galK expression system used in this chapter. Sullivan and Donachie found two promoters upstream from ftsZ which, when cloned together, gave a greater promoter activity than the sum of the activities when cloned individually. This result is shown in figure 6.9. Also shown in this figure is a result obtained by Yi et al. (1985) who constructed identical plasmids and again measured galactokinase activity. The results between the two groups are completely different in both magnitude and in their relative values. This does not diminish the use of galK expression vectors for measuring promoter activity, but highlights the problems of extrapolating too much information from them. In this thesis the galK expression results have been used to define the presence or absence of promoter activity on a cloned fragment, but no other conclusions have been drawn from the results obtained.

FIGURE 6.9 The Measurement of ftsZ Promoter Activity

The NS series of plasmids are those constructed by Sullivan and Donachie (1984). The galactokinase activity associated with these plasmids is shown by the unbracketed numbers. The SR series of plasmids are those constructed by Yi et al. (1985) and the galactokinase activities associated with these plasmids are shown within the brackets.

The lines represent the extent of the ftsA-ftsZ region cloned into the pKO vectors.

B-BglII H-HindIII E-EcoR1



CHAPTER SEVEN

Identification and Mapping of RNA Transcripts from the ftsYEX Operon

7.1 Introduction

The comparison between the ftsYEX-rpoH region and the 2 minute gene cluster discussed in chapter six, revealed some remarkable similarities.

a: The genes in both regions are very closely associated. In the ftsYEX-rpoH region over 90% of the DNA encodes genes, in the 2 minute region the value is near to 100% (Robinson et al. 1986). Neidhardt et al. (1983) have calculated that the average coding capacity of E.coli is approximately 70%, although this is probably an underestimate.

b: In both regions the genes are all transcribed in the same direction, yet many have their own promoters and there is little evidence for co-ordinated expression.

In both regions the use of the pKO system, deletion and insertion analysis, strongly suggested the presence of internal promoters. However they can not indicate whether these promoters were physiologically active and not just an artefact of the in vitro systems used. An alternative approach to study the expression of a chromosomally located gene is by the construction of in vivo gene fusions. In such constructs a gene such as galK, lacZ or phoA, whose gene product has an easily measureable enzymic

activity, is inserted downstream of a gene's promoter. The relative activity of this promoter can then be determined by measuring the enzyme activity of the gene product of the fused gene. Construction of the fusion can be achieved either *in vitro* and then recombined back into the chromosome, or by the use of various fusion vectors. These vectors can integrate randomly into the chromosome and in doing so form gene fusions, examples are the λp*lac*Mu series (Bremer *et al.* 1984) and λTn*phoA* (Manoil and Beckwith 1985). Fusions can be detected if the gene of interest has a readily selectable or scorable phenotype. This is where a problem is encountered when applying this method to cell division genes. Many cell division genes are essential and so the single chromosomal gene can not be disrupted, unless the cell also contains an extra cloned copy of the gene. This extra copy may well perturb the normal physiological situation within the cell.

The study of mRNAs from a wild type cell should provide a much better representation of the activity of a gene. The aim of the work in this chapter is to perform the initial characterisation of transcripts originating from the *ftsYEX* operon. Once characterised the expression of the cell division genes under normal physiological conditions can be more readily investigated.

7.2 Northern Blotting the *ftsYEX* Transcripts

In an initial attempt to identify the transcripts from the operon the technique of northern blotting was used (Alwine *et al.* 1977).

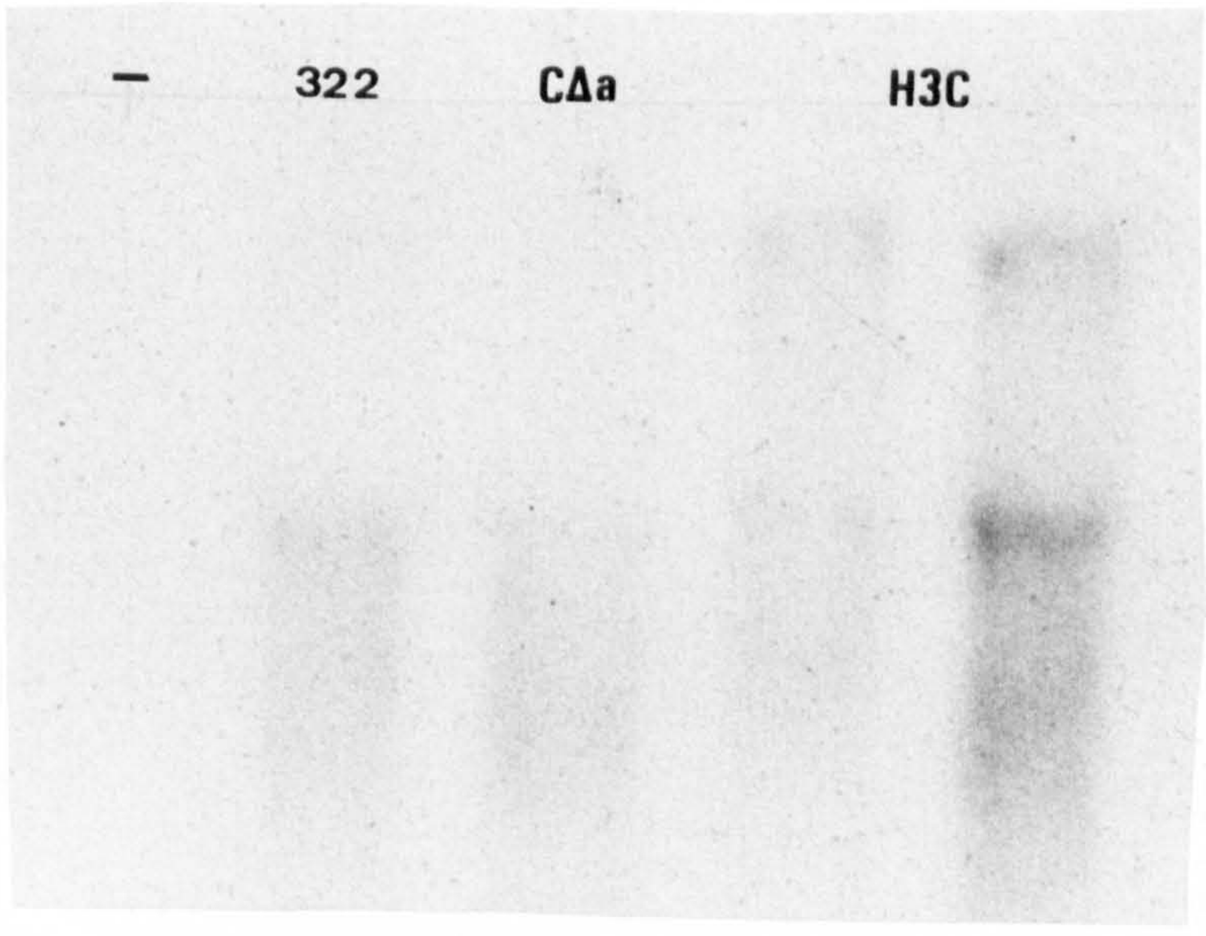
RNA was prepared from cells growing exponentially in LB and after several purification steps the total cell RNA was electrophoresed on a 1.5% agarose gel containing 16% formaldehyde (to maintain the RNA in a denatured state). After electrophoresis the RNA was transfered from the gel onto a nitrocellulose filter which was then hybridised to a ^{32}P labelled probe as described in section 2.28.

In this experiment RNA was prepared from four different strains; CSH26, CSH26 (pBR322), CSH26 (pCAA) and CSH26 (pH3C). Nick translated 4.5kb HindIII fragment DNA was used as the probe. The results of the hybridisation are shown in figure 7.1.

RNA (5ug) from each strain was electrophoresed on the agarose gel. In the case of CSH26 (pH3C) an extra lane containing 10ug was added. After hybridisation the nitrocellulose filter was stained with methylene blue to reveal the most abundant RNA species, the ribosomal RNAs; 23S (3.7kb), 16S (1.7kb) and 5S (0.1kb). These served as size markers. The autoradiograph shows bands in the four tracks containing RNA derived from plasmid-containing strains. The absence of any bands from the RNA derived from the plasmid-less strain could be due to one of the following explanations or a combination of all three.

FIGURE 7.1 Northern Blot of ftsYEX Transcripts

5ug of RNA prepared from the strains CSH26, CSH26(pBR322), CSH26(pCΔa) and CSH26(pH3C) were electrophoresed on a 1.5% agarose gel containing 16% formaldehyde. The fifth track contained 10ug of RNA prepared from CSH26(pH3C). After transfer to nitrocellulose paper the RNA was probed with ^{32}P labelled 4.5kb HindIII, operon containing, fragment. The sizes of the bands were estimated from the sizes of the 23S and 16S rRNA species visualised by staining the filter with methylene blue.



a) The hybridisation conditions may not have been efficient enough to detect the lower transcript levels.

b) The RNA preparation may have been partly degraded reducing its effective concentration.

c) The operon transcripts may exist normally at low levels.

Another problem was the presence of a band (or bands) common to all the other tracks, including one (CSH26 (pBR322)) not containing any of the cloned operon. This band was approximately the size of the bla transcripts from pBR322 (Kuriki 1987), it could then be due to either non-specific labelling of the abundant bla transcripts or to contamination of the probe with pBR322 sequences.

There is however another band unique to the CSH26 (pH3C) tracks. This could represent a transcript starting 5' of ftsY since it is not present in the RNA derived from the CΔa plasmid, which does not contain the putative ftsY promoter region. The length of this transcript is approximately 3-3.5kb. If one assumes that transcription terminates near the strong inverted repeat region, between the ftsX termination codon and the 3' HindIII site (section 4.4), then this would map the 5' end of the transcript near to the ftsY start codon. The 3-3.5kb transcript could thus represent a true polycistronic message encoding all three genes of the ftsYEX operon.

7.3 S1 Nuclease Mapping of ftsYEX Promoters

The northern blot experiment described in the previous section suggested the existence of a transcript encoding all three cell division genes. The nature of the technique was such that little information could be obtained about the actual promoter or terminator sites. The technique of S1 mapping is based on the sizing of a transcript from a fixed reference point and thus a more accurate localisation of a promoter or terminator can be established (Aiba et al. 1981). In this section S1 mapping was used to attempt to identify transcripts originating from either or both the ftsY or ftsE promoters and to map these promoters as accurately as possible.

The experimental protocol used is shown diagrammatically in figure 7.2. The BstE2 site towards the end of the ftsE gene was used as the reference point since this would allow transcripts originating from either an ftsY and an ftsE promoter to be visualised on the same gel. The probe used was the 3.1kb HindIII-BstE2 fragment shown and was 5' end labelled with ^{32}P ATP. Total cellular RNA was isolated from the same strains as used in section 7.2 (CSH26, CSH26(pBR322), CSH26(pCΔa) and CSH26(pH3C)).

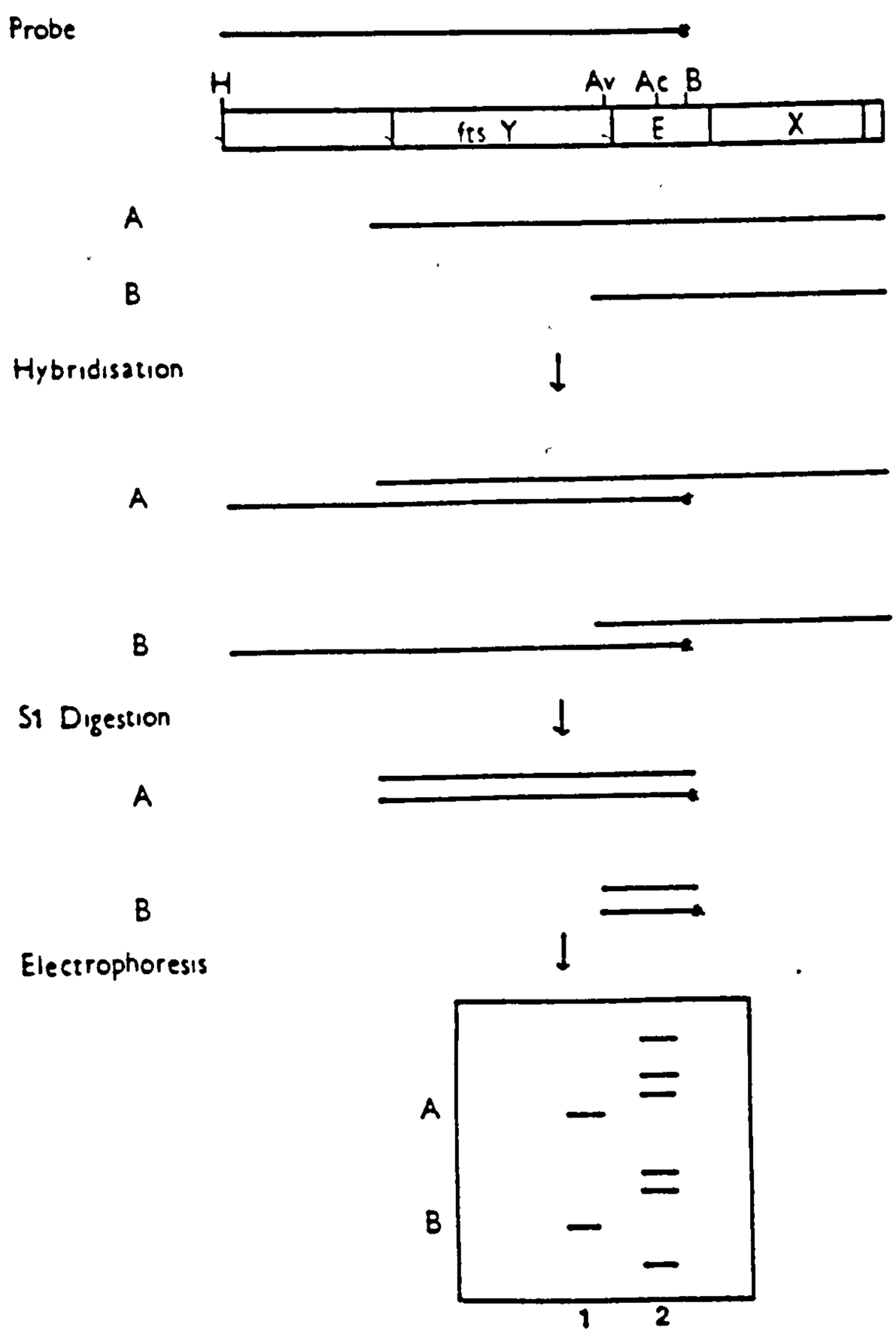
FIGURE 7.2 S1 Mapping ftsYEX Transcripts

A and B represent putative transcripts originating from ftsY and ftsE promoters respectively. * represents a 5' ^{32}P ATP residue

Track 1 RNA:DNA hybrids resulting from S1 digestion

Track 2 Standard size DNA markers

H-HindIII Av-AvaI Ac-AccI B-BstE2



The probe was hybridised to the RNA as described in section 2.29. After digestion with nuclease S1, the samples were electrophoresed on a 1% agarose gel along with DNA standard size markers. After electrophoresis the gel was dried and autoradiographed. Illumination of the dried gel with shortwave UV light allowed visualisation of the size standards and their position could be extrapolated onto the developed X-ray film, allowing the bands present to be sized.

The resulting autoradiograph from this experiment is shown in figure 7.3. The largest common band seen in each track is the full length probe (3.1kb). Beneath this band are a pair of bands which appear in all four tracks. The size of these two bands was calculated to be 2140bp and 1990bp, which would represent transcriptional start sites 150bp and 0bp upstream of the ftsY translational start codon. The larger transcript is thus a good candidate for a mRNA originating from an ftsY promoter. The smaller transcript that apparently maps to the start of the ftsY gene could represent an inaccuracy in sizing the band or could represent a processing event on the larger transcript. The intensity of the larger band is greater in the track containing RNA derived from the strain CSH26 (pH3C). This is the only strain used that contains the putative ftsY promoter region on a multicopy clone.

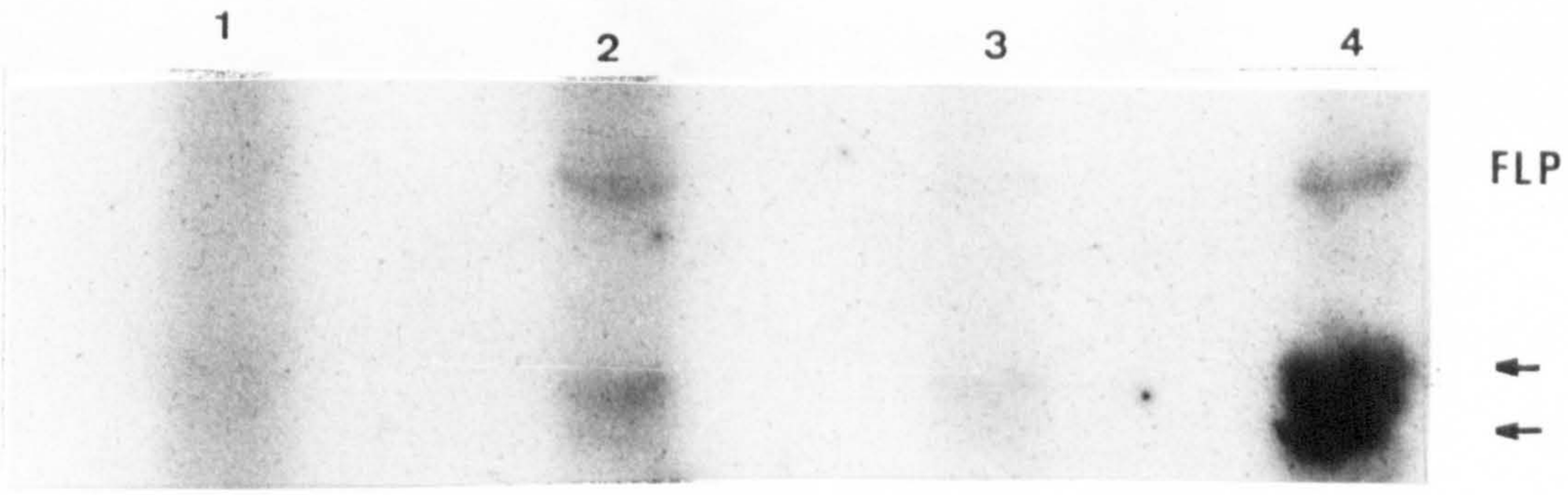
FIGURE 7.3 S1 Mapping ftsY and ftsE Promoters Using a HindIII-BstE2 Probe

Track 1 RNA from CSH26 Track 2 RNA from CSH26 (pBR322)

Track 3 RNA from CSH26 (pCΔa) Track 4 RNA from CSH26 (pH3C)

FLP- Full Length Probe

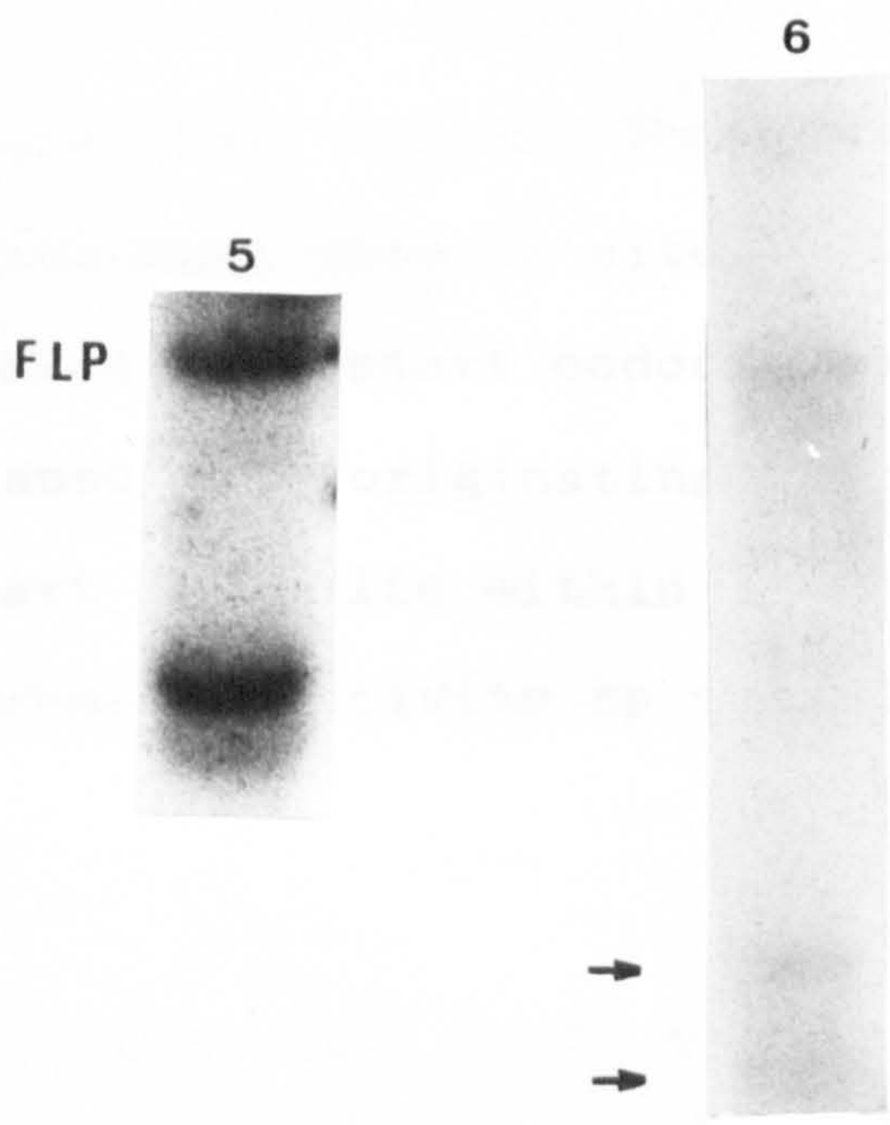
The major transcripts are labelled with arrows



Continued.....

Track 5 Shows a repeat experiment using RNA from CSH26 (pH3C) which has been exposed to X-ray film for less time than track 4 to show the different intensities of the two bands.

Track 6 Shows a repeat experiment using RNA from CSH26 (pCΔa) which shows the two smaller bands not visible in track 3 due to poor labelling.



The greater intensity of the larger band in this track could represent an increased synthesis of the transcript from a multicopy plasmid. The smaller of the two bands does not appear to be more intense in this strain. Both bands can be detected in RNA derived from CSH26, which does not contain any cloned DNA from the operon.

Two other bands are clearly visible in the track containing RNA derived from the strain CSH26 (pCAa), that are not obviously present in any of the other tracks. These two bands were sized at 830bp and 715bp which maps them to sites 335bp and 220bp upstream from the ftsE translational start codon. One or both of these could represent transcripts originating from an ftsE promoter, both calculated start sites lie within the 390bp Pst1 fragment shown to contain promoter activity by the pKO system (section 6.3).

In order to confirm that these bands discussed above do represent true transcripts from the operon, the experiment was repeated using probes with different end points. If the bands do represent true transcripts then the use of different probes should result in a similar banding pattern , the bands being of different sizes but all mapping to the same start points. The two new sites used were Ava1 and Acc1 whose locations within the operon are shown in figure 7.2

The results of this experiment are shown in figure 7.4. The location of Ava1 within the operon is such that no transcript from an ftsE promoter would be detected with the procedure used. It should have been possible to detect this transcript from the Acc1 site but the labelling was inefficient and smaller bands could not be detected. The full length probe and transcripts from the putative ftsY promoter could be seen and their size determined. The sizes of these transcripts and their extrapolated start sites are shown in table 7.1 which also contains the results from the previous experiment using the HindIII-BstE2 probe.

TABLE 7.1 S1 Mapped Transcripts

Probe Used length	Band 1 size	 start site	Band 2 size	 start site
HindIII-BstE2 (3.1kb)	2130bp	1010	1980bp	1160
HindIII-Ava1 (2.6kb)	1580bp	1050	1300bp	1330
HindIII-Acc1 (2.9kb)	1850bp	1080	1700BP	1230

FIGURE 7.4 S1 Mapping the ftsY Promoter Using HindIII-Ava1 and HindIII-Acc1 Probes

HindIII-Ava1 Probe

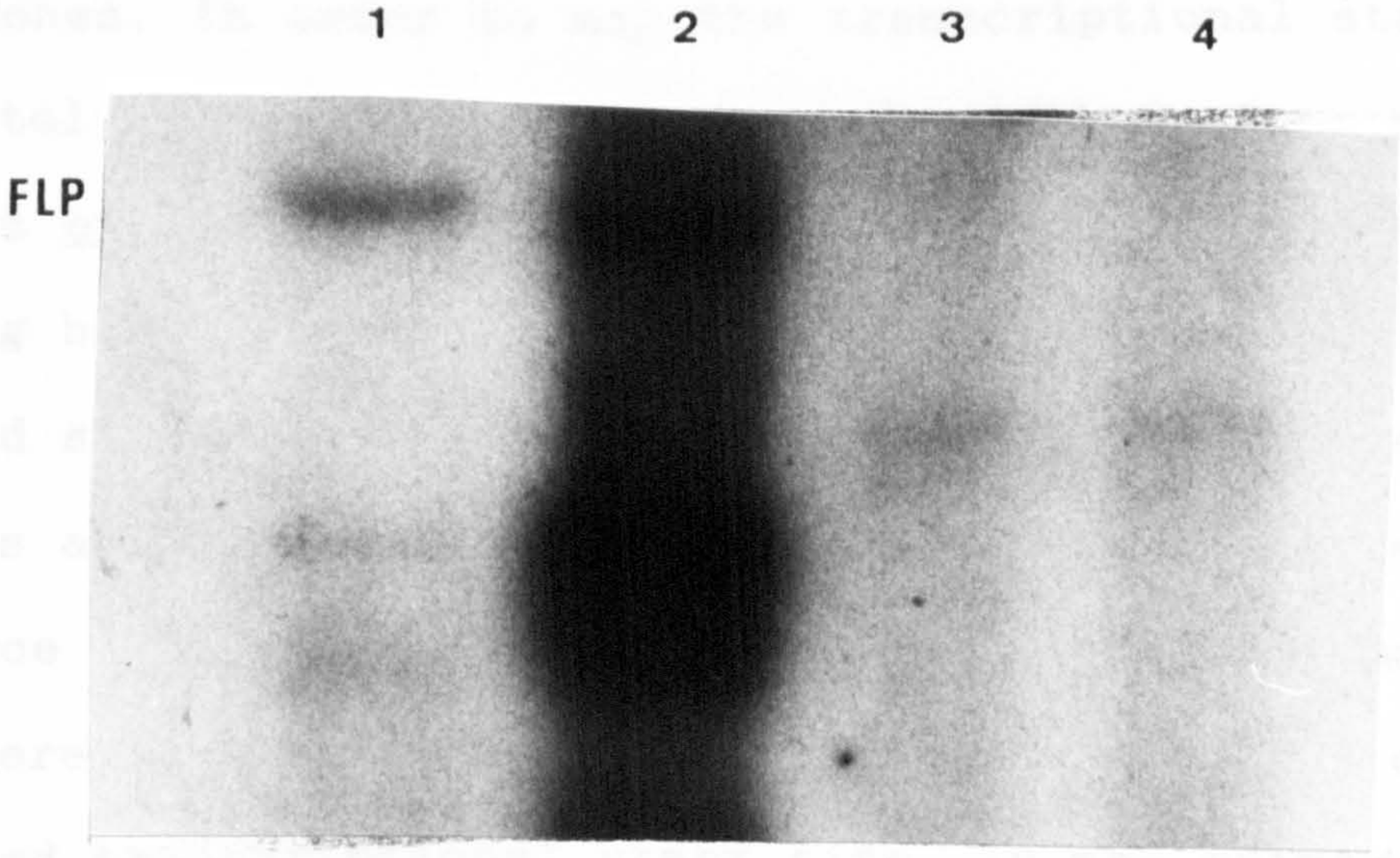
Track 1 RNA from CSH26 clearly showing chromosome derived transcripts

Track 2 RNA from CSH26 (pH3C)

HindIII-Acc1 Probe

Track 3 RNA from CSH26

Track 4 RNA from CSH26 (pH3C)



The data shows that in all three experiments the largest transcript seems to start at about position 1050, 100bp upstream of the ftsY translational start codon. The good agreement, considering the size of the probes, in all three cases strongly suggests that this is a polycistronic transcript encoding at least the ftsY and ftsE genes.

The agreement with respect to the lower band is not so good and gives no further clues as to the identity of the band.

7.4 Primer Extension Mapping of the ftsYEX Promoters

The data discussed to date suggests the presence of two promoters and has roughly mapped them to regions upstream of the ftsY and ftsE genes. In order to map the transcriptional start sites accurately it was decided to use the method of primer extension (Townes et al. 1985). In principle this method is similar to S1 mapping but instead of using a defined restriction site any defined stretch of sequence can be used as the reference point. This is achieved by synthesizing an oligonucleotide with a sequence complementary to the chosen stretch. S1 mapping was not used here due to the absence of appropriate sites close to the expected transcriptional start site. In the primer extension procedure the oligonucleotide is hybridised to the mRNA and used

as a substrate for reverse transcriptase. This enzyme will extend the primer until it runs out of template, the 5' end of the mRNA. The length of the extended primer can then be measured from a sequencing gel. This experimental scheme is shown diagramatically in figure 7.5

The choice of oligonucleotide was based on two criteria:

a: The extended primer should be between 30bp and 300bp in length, since this is the most accurate part of a sequencing gel.

b: The sequence of the primer should not be repeated elsewhere in the cloned DNA since this would result in multiple priming sites.

Two oligonucleotide primers were synthesized, one for the ftsY promoter and the other for the putative ftsE promoter. The positions of these two primers within the operon are shown in figure 7.7.

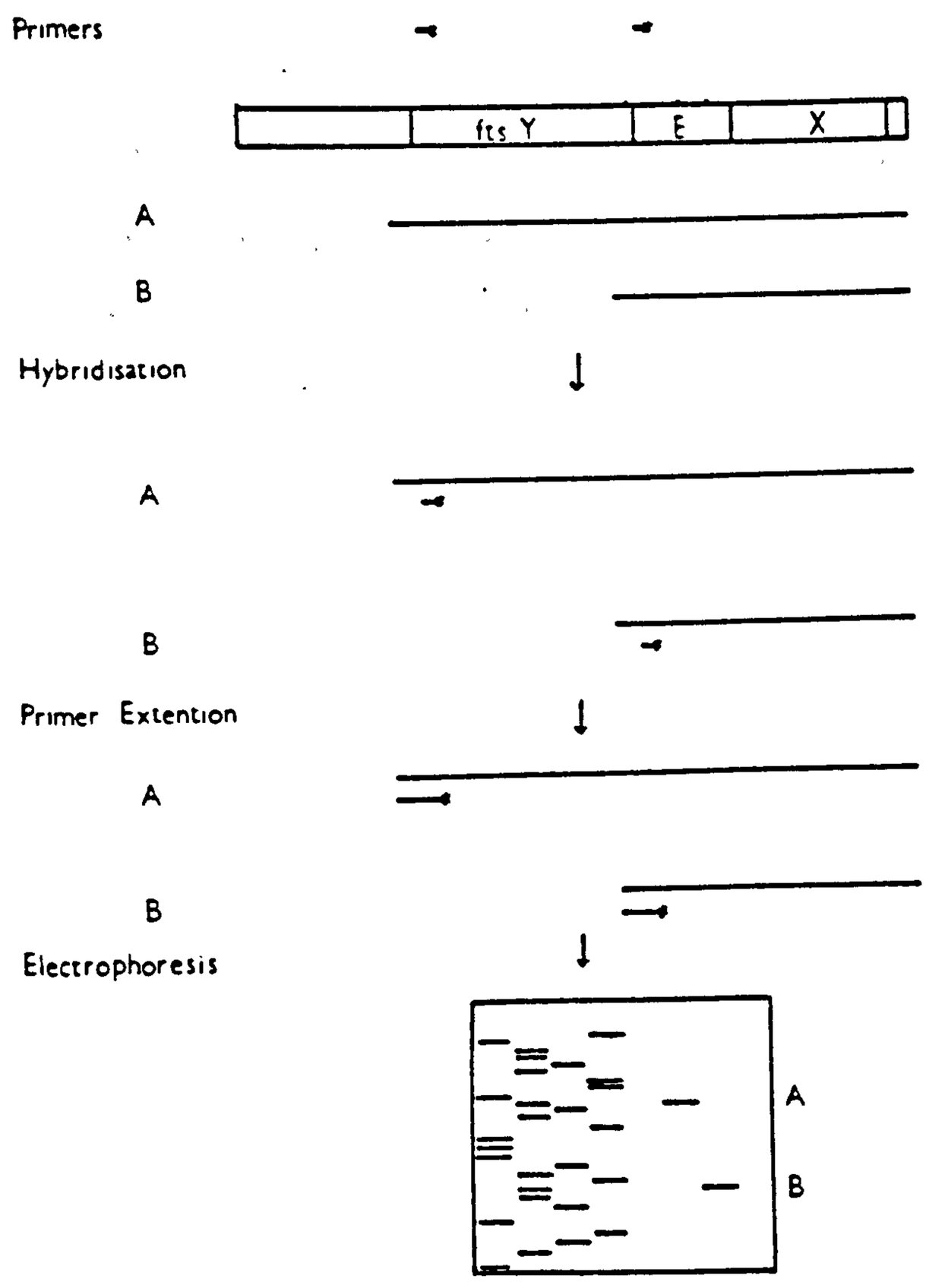
Total cellular RNA was prepared from the strain CSH26 (pH3C) and 50ug was hybridised to each primer which had been 5' end labelled with ^{32}P ATP. After the polymerisation reaction the RNA was removed by treatment with hot alkali and the single stranded extended primer electrophoresed on a sequencing gel. The resulting gel is shown in figure 7.6. Unfortunately sequencing clones containing the putative promoters could not be constructed in the time available. The sequence shown in figure 7.6 is an M13 clone of known sequence prepared by S. McGowan.

FIGURE 7.5 Primer Extension Mapping ftsYEX promoters

A and B represent the same transcripts as in figure 7.2

* represents a 5' ^{32}P ATP residue

The left hand four tracks on the gel represent a sequencing ladder



From the gel the extended ftsY primer was measured at 209bp and the ftsE extended primer at 132bp, because of possible differences in the structure of the extended primers and the sequenced DNA these lengths may be inaccurate by several base pairs. These values, however, mapped the 5' end of the ftsY transcript at position 1003 and the start of the ftsE transcript at position 2584. The location of these start sites are shown in figure 7.7 which also includes the location of putative promoters.

The choice of the putative promoters shown was based on the presence of regions with homology to the -10 and -35 regions of the promoter for E.coli σ^{70}. The spacing of the -10 and -35 regions also had to be around 16-18bp and the -10 region less than 10bp upstream of the transcriptional start site. Since there was no sequence present in the region with strong homology to the consensus sequence, the promoter sequences from other E.coli genes were used as a reference (Harley and Reynolds 1987).

FIGURE 7.6 Localisation of Transcriptional Start Sites by Primer Extension

The experimental protocol used is discussed fully in section 2.30

Track 1 Extended ftsY primer

Track 2 Extended ftsE primer

The sequenced DNA in the left hand tracks are from M13 clones of known sequence prepared by S. McGowan (pers. comm.). From this known sequence the sizes of the two extended primers (arrowed) were measured at 209bp (ftsY) and 132bp (ftsE).

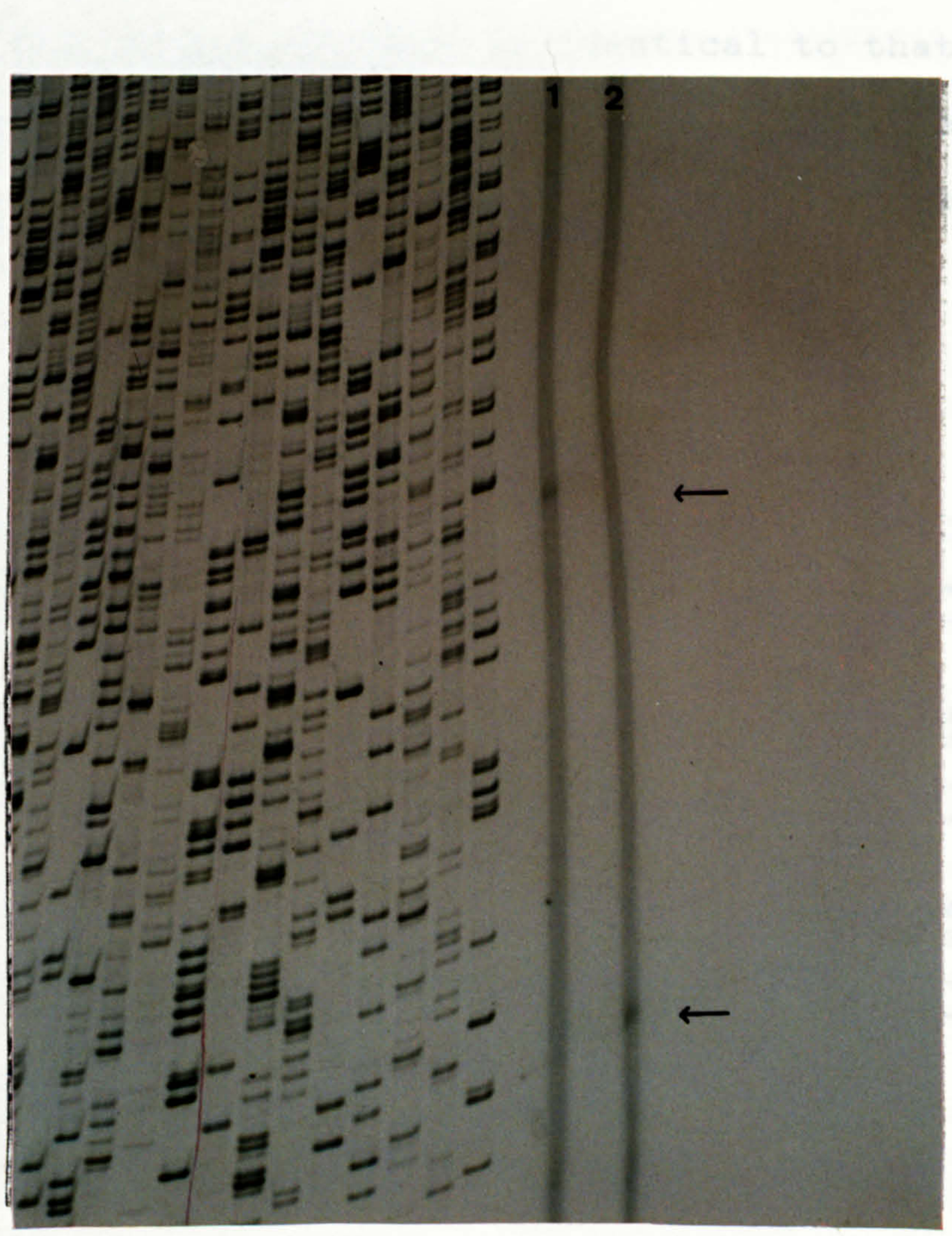


FIGURE 7.7 Location of the Putative ftsY and ftsE Promoters

The transcriptional start sites determined by primer extension are shown by the boxed and arrowed nucleotides. The putative promoters are shown by the boxed -10 and -35 regions.
Also shown are the locations of the oligonucleotide sequences used for the primers and the putative Shine Dalgarno (SD) sequences.
The boxed region upstream of ftsY labelled Op represents the potential promoter/operator region identified by Gill et al. (1986), as having homology with the trp operator region.
The numbering of the sequence is identical to that in appendix A.
The coding regions of the orf4, ftsY and ftsE genes are shown as boxes below (ftsY and ftsE) or above (orf4) the respective sequence.

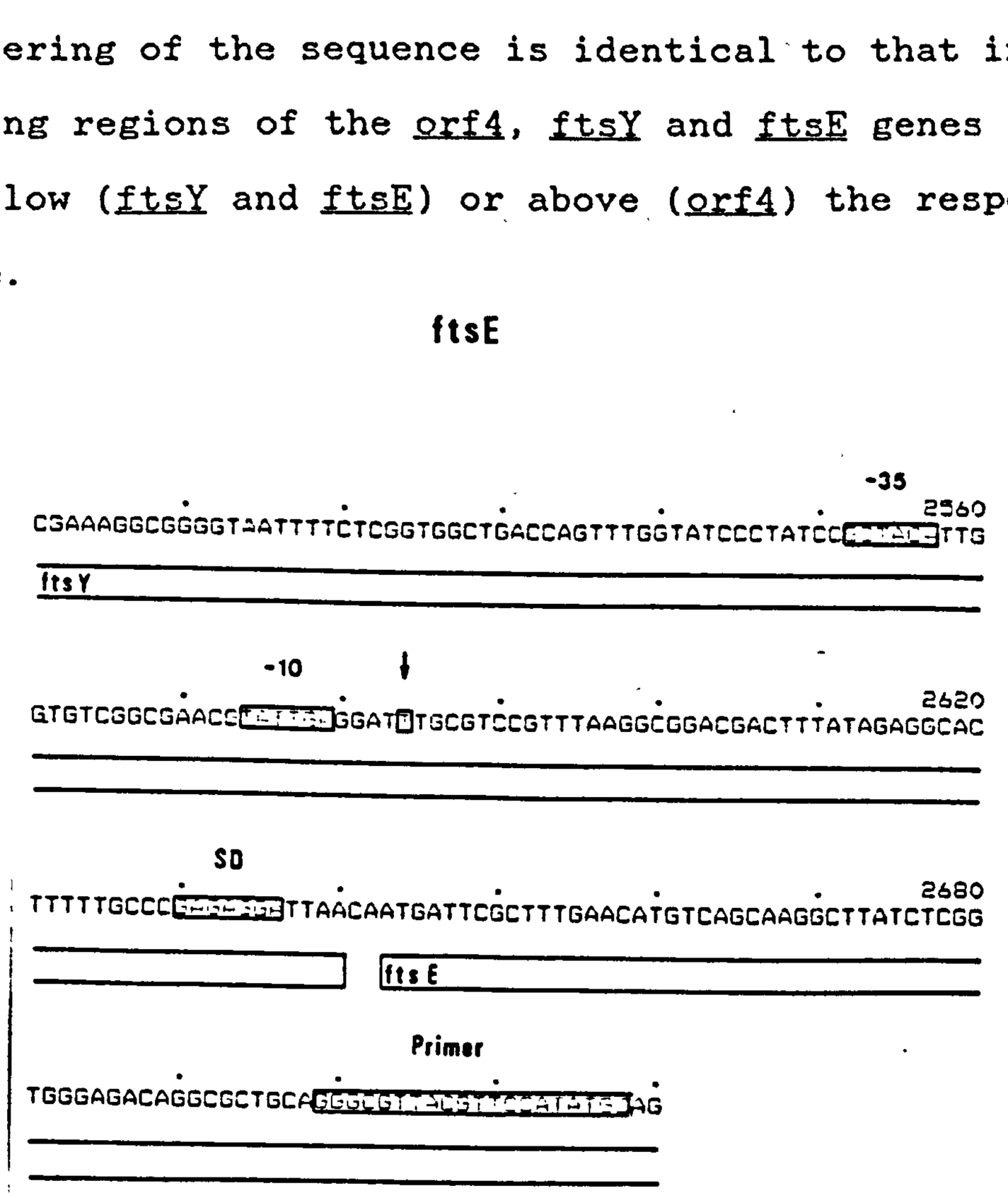
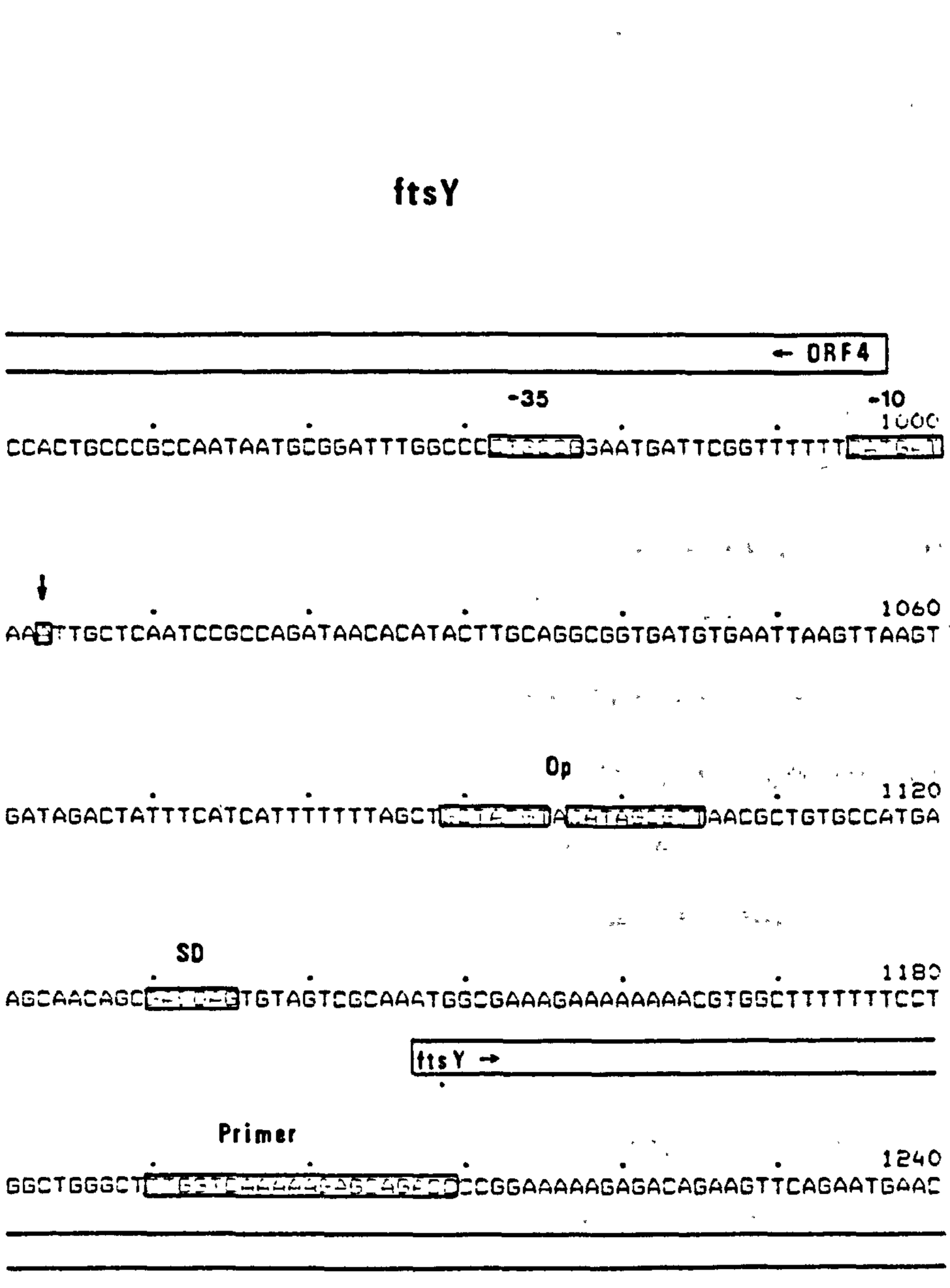


FIGURE 7.7 Continued



7.5 S1 Mapping of Transcriptional Terminators

The technique of S1 mapping used in section 7.3 to identify promoters can also be used to identify terminators. The only significant difference is that the probe is labelled at its 3' end by an end filling reaction (section 2.19). The scheme used for this experiment is shown in figure 7.8.

The resulting autoradiogram is shown in figure 7.9. Two main bands can be seen, the largest being the full length probe (2kb). The lower band presumably represents the RNA:DNA hybrid, containing sequences between the BstE2 site of ftsE and the downstream transcriptional terminator. The size of this band however was measured at only 700bp, which would map the terminator at position 3910, in the middle of ftsX. The expected termination site would be expected to be around 4400, no larger bands that would map to this site could be observed on the autoradiograph.

A similar unexpected result was found by Stuber and Bujard (1981) whilst studying the transcriptional organisation of pBR322. The tet gene in pBR322 lies between positions 86 and 1276 and the above workers found that the tet transcript terminated around position 650. They could not explain this result but noted the presence of an attenuator-like structure between positions 618 and 666. No such structure could be found around position 3910 in ftsX.

FIGURE 7.8 S1 Mapping ftsYEX Transcriptional Terminators

•—— represents the 3' ^{32}P labelled BstE2-Pst1 probe

———— represents an ftsYEX transcript, in this case originating from an ftsY promoter.

After S1 digestion the DNA:RNA hybrid was electrophoresed on a 1% agarose gel along with standard size DNA markers.

Y,E,X - ftsY, ftsE, and ftsX respectively

H - rpoH

B - BstE2

P - PstI

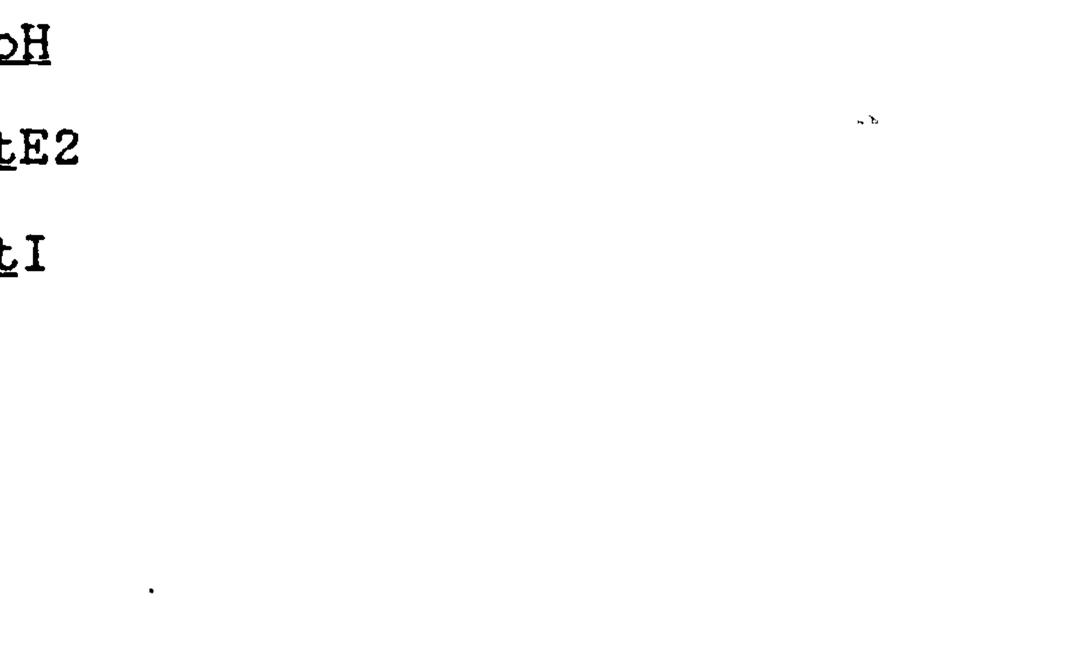


FIGURE 7.9 Location of a Transcriptional Terminator by S1 Mapping

The experimental procedure used was essentially that described for S1 mapping in section 2.29. Instead of using a 5' end labelled probe a 3' end labelled probe was prepared by an end filling reaction (section 2.19).

FLP - Full Length Probe (2kb BstE2-PstI fragment)

The major DNA:RNA hybrid is indicated by the arrow.

The size of this hybrid was calculated from the position of the standard DNA size markers (not shown).

7.6 Discussion

The aim of this work was to identify transcripts originating from the ftsYEX operon for later use in the study of gene expression under normal physiological conditions. Northern blotting provided the first evidence that a full length transcript was synthesized encoding all three operon genes, S1 mapping partially confirmed this result. A combination of S1 mapping and primer extension not only mapped the 5' end of this long transcript to within several base pairs, but also identified the presence of a second transcript. This smaller transcript was found to originate from a promoter within ftsY and confirmed the presence, at least in a multicopy clone, of an internal promoter for ftsE , predicted from other studies. The long transcript could be observed in a strain containing no cloned DNA from the operon and thus represented a physiologically active promoter. The existence of the smaller transcript under similar conditions could not be established.

The 5' end of the long transcript was mapped by primer extension to position 1003. Examination of the sequence upstream from this position identified a potential promoter which is shown in table 7.2. Although this promoter differs from the consensus sequence for E.coli promoters, it shows reasonable homology with other known promoters. A similar situation is found with the putative ftsE promoter which is also shown in Table 7.2.

TABLE 7.2 Sequence of the Putative ftsY and ftsE Promoters.

CONSENSUS SEQUENCE	-35 TTGACA	-10 TATAAT	
ftsY	CTGACA	CATGAT	
araBAD	CTGACG	CATGAT	LacI
araI	CTGGCG	CATCAT	hisA
ISlins PL	CTGCCA	CATTAT	hisB
pyrE	CTGCGG	CATAAT	λ P_o
lacP	CTTCCG	TATGAT	hisS
pBRP1	CTGACT	GATGAT	malPQ
ftsE	GCTACA	TATTGA	
rpoB	GCGACA	TATTAA	argR
aroG	TTTACA	TATTGT	fumA
galP2	GTCACA	TATTTT	araE
metA P1	TCGACA	TATCAA	argCBH
rnp	GTGACA	TATAGT	divE
tyrT16	GTAACA	TATAAA	pyrE2

MISSING

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The absence of any sequences with strong homology to the consensus sequence for the σ^{70} promoter means that there is no obvious verification of the mapping result. The result can be verified experimentally by the use of another primer displaced by some 50bp from the first primer. This method, identical in principle to that used for the S1 mapping experiments in section 7.3, was used to successfully map the transcriptional start site of the gyrA gene (Hussain et al. 1987b). This second primer was synthesized but failed to give a result when used for the primer extension reaction; time did not permit a repeat of that experiment

The assigning of a promoter on the basis of homology with both -10 and -35 regions of the promoter may also prove to be at fault. This is because there is now evidence that a consensus -35 sequence is not always required for efficient promotion (Ponnambalam et al. 1986).

If the location of the ftsY promoter does prove to be correct then an interesting situation arises. The orf4 gene, transcribed in the opposite direction to the operon genes, has a translational start site within the putative promoter. The promoters for ftsY and orf4 would then almost certainly interfere with each other, and may represent a mechanism for the control of ftsYEX expression. The function of the orf4 gene product is unknown but in theory it could code for a transcriptional repressor/activator of the operon.

8.1 Summary of the Main Results

The 76 minute region appeared to represent a major morphogene cluster in E.coli, similar to the 14 gene cluster at 2 minutes. As well as the recently identified ftsYEX and orf4 genes (Gill et al. 1986), four other genes (rpoH, fam, dnaM and ftsS), all with differing mutant phenotypes but all affecting morphogenesis, have been mapped to this region. The main aim of this project was to establish the organisation of the 76 minute cluster and to investigate the expression of the component genes. The study of this region gave the following results.

(1) The cloned DNA in λpGS22 was extended in vivo to give a phage (λS3d) that complemented mutations in both fam and rpoH.

(2) Restriction analysis of λS3d and the subcloning of fragments into pBR322 showed that the rpoH gene lay immediately downstream of the ftsYEX operon.

(3) The construction of deletions in the subcloned fragments showed that both fam and dnaM were alleles of rpoH.

(4) The fam and dnaM alleles were shown to result in identical morphologies and heat shock deficiencies to the classic rpoH mutation, htpR165.

(5) Southern blotting was used to construct a restriction map of the region clockwise of the operon in order to facilitate further analysis of this region. The two smaller fragments found associated with the 4.5kb, HindIII fragment in λpGS22 were found to be separate from it within the chromosome.

(6) The ftsS gene was found to be an allele of ftsX. Previous, confusing, results were attributable to a faulty strain.

(7) Deletions were constructed from cloned segments of the operon, and revealed the presence of two promoters. One was found to be proximal to ftsY and the other proximal to ftsE.

(8) The pKO system was used to measure promoter activity in fragments derived from the operon, and confirmed the presence of two promoters. The positions of these promoters were mapped to within several hundred base pairs.

(9) Northern blotting was used to identify mRNA transcripts originating from the operon. Evidence was found for a large transcript encoding all three genes of the operon.

(10) S1 mapping confirmed the presence of a large polycistronic transcript and also identified a smaller transcript, originating from the ftsE promoter. The start sites of these transcripts were mapped to within 100bp. S1 mapping was

attempt to
also used to identify the termination point of the ftsYEX transcripts.

(11) Primer extension was used to map the start sites of the transcripts to within several base pairs. Examination of the sequence at these points revealed potential promoter structures.

8.2 Possible Significance of the Results

The possibility of another major morphogene cluster at 76 minutes has not materialised. The unmapped genes, fam, dnaM and ftsS all proved to be alleles of previously characterised genes. The fact that fam and dnaM proved to be allelic to rpoH was somewhat surprising considering the nature of their mutant phenotypes. In particular, fam was studied by Torti and Park (1976 and 1980) as a mutation affecting the biosynthesis of murein lipoprotein. Although they showed that it was not an allele of the lipoprotein gene lpp, and observed other pleiotrophic effects it still presented a relatively clean phenotype. After the work on fam, discussed in this thesis, was completed, Tsuchido et al. (1986) also provided evidence that fam and rpoH were allelic, and considered possible reasons for the novel phenotype associated with fam. They postulated that murein lipoprotein may itself be a heat shock protein. The absence of a σ^{32} consensus sequence for lpp, and the observed decrease in lipoprotein synthesis after a temperature upshift argued against this postulate, though.

The fam allele, like other mutations in rpoH, results in a defect in cell division. Filamentation is observed at the non-permissive temperature (chapter 4). The mechanism by which rpoH can affect cell division is unknown, none of the known heat shock proteins are known to be involved in cell division, although mutations in dnaK and groEL can result in filamentation (F. Neidhardt unpublished information). The SOS response has been shown not to be induced by the growth-restricting conditions in an rpoH mutant (Tsuchido et al. 1986). It remains possible, though, that one of the other known pathways of cell division inhibition discussed in section 1.5.7 may be involved.

One of the genes in the 2 minute gene cluster, ftsZ, appears to have a central role in the control of cell division as discussed in section 1.5. Various workers have studied the transcription of ftsZ in an attempt to discover regulatory mechanisms. As discussed in chapter 6, Sullivan and Donachie (1984) proposed that efficient transcription of ftsZ requires the co-operation of at least two promoters. There is doubt though on the validity of this result (Yi et al. 1985, J. Lutkenhaus pers. comm.). Donachie et al. (1983) studied the expression of lacZ from the ftsZ promoter region and obtained the following results.

a) Transcription was increased in cya (adenylate cyclase) mutants but reduced to normal levels by the addition of cAMP.

b) Transcription was increased up to 10 fold when the cell also contained a nonsense mutation in ftsA, ftsI, ftsQ or ftsZ.

c) Transcription increased with increasing growth rate, that is with the size of the cell.

The latter two results have recently been confirmed by Dewer and Donachie (1987) who also showed that transcription was strongly repressed by a multicopy plasmid carrying the ftsQ, ftsA and ftsZ genes.

Whilst evidence exists for the transcriptional control of one of the genes found within the 2 minute cluster, there is no evidence for co-ordinated expression with any of the other genes.

This thesis has considered the transcriptional organisation of the 76 minute region. As with the 2 minute cluster internal promoters have been identified and shown to be active under certain conditions. The identification though, of a polycistronic mRNA suggests there is also some co-ordination of expression. The mapping of the promoter of the polycistronic transcript also leads to a potentially interesting regulatory situation. Gill et al. (1986) had previously identified a potential promoter/operator region upstream of ftsY. This was identified on the basis of a reasonable homology to the trp operon promoter/operator region. The homology to the actual trp promoter, though, was very poor. Primer extension mapping of the

polycistronic transcript located a putative promoter approximately 100bp upstream of this potential operator region. The location of this putative ftsY promoter places it within the transcriptional unit of orf4 and therefore presents the possibility of co-ordination between the two genes. This is currently being investigated.

A major problem with the study of the 76 minute cluster, with the exception of rpoH, is the lack of knowledge as to the function of the individual genes. This is particularly true of ftsY and orf4 since no mutations have been identified in these genes. Experiments are currently being performed to try and mutate the chromosomal copy of ftsY by either localised mutagenesis or by the insertion of an antibiotic resistance-encoding fragment.

Investigation of the subcellular locations of the gene products has revealed that ftsX is an integral inner membrane protein, whereas ftsE and ftsX are both peripherally associated with this membrane (Gill and Salmond 1987). Sequence data has revealed the presence of a strong homology between ftsE and a family of nucleotide binding proteins (Higgins et al. 1986). Many of these proteins are components of periplasmic binding protein-dependent transport systems. It is quite possible that FtsE is part of an, as yet uncharacterised, transport system. FtsY and FtsX may also be part of the same system. This is quite likely since the genes encoding components of other transport systems are often found within an operon (Higgins et al. 1982). One can postulate that

the substrate of the proposed transport system will have some regulatory role in E.coli cell division (perhaps among other processes) and that a disruption in its intracellular concentration will affect cell division. Cations such as calcium or magnesium may be candidates for such a substrate.

The fact that mutations in ftsE and ftsX result in filamentation does not necessarily invoke them as having a direct role in the cell division machinery. As discussed in this thesis the fam and dnaM alleles of rpoH also result in filamentation yet the gene apparently has no direct role in cell division. Furthermore, the previously characterised cell division gene allele ftsB, which also results in filamentation, has recently been shown to encode the B2 subunit of ribonucleoside-diphosphate reductase, an enzyme with no obvious role in cell division (Kren and Fuchs 1987).

The possibility that the ftsYEX operon genes are actually involved in cell division, perhaps encoding integral parts of the septalsome discussed by Holland and Jones (1985), can not be discounted, but needs futher investigation. Experiments are currently underway to isolate antibodies to the gene products and then to use the technique of immuno-gold labelling to identify the exact location of the proteins within the cell. The localisation of the gene products to the cell septum would provide good evidence for a role in the septalsome.

In conclusion the results presented in this study have determined the organisation of the 76 minute morphogene cluster. In doing so it has provided a solid base for a further, more detailed, study into the control of cell division in E.coli. However, at this point in time, future work must concentrate on establishing the degree of involvement, if any, of the orf4,ftsYEX genes in the process of cell division. Without this knowledge the search for complex control mechanisms is somewhat hindered.

APPENDICES

Appendix A The Complete Nucleotide Sequence of the orf4-rpoH Region

The sequence shown was derived from Gill et al. (1986) and Landick et al. (1984).

The sequence of the first 1040bp has been reversed to show the amino acid sequence of the orf4 gene product. The orf4 gene is transcribed in the opposite orientation to the operon genes and rpoH.

All three reading frames are shown under the sequence; the coding regions are shown as boxed amino acids.

All restriction sites referred to in this thesis are highlighted and labelled.

Abbreviations used:

Y,E,X - ftsY, ftsE and ftsX respectively

H - rpoH

ORF4

```
      1031        1021        1011        1001         991         981         971         961
CCGCCTGCAAGTATGTGTTATCTGGCGGATTGAGCAATTTATCATGAAAAAACCGAATCATTCCGGCAGCGGCCAAATCC
 P  P  A  S  M  C  Y  L  A  D  *  A  I  Y  H  E  K  T  E  S  F  R  Q  R  P  N  P
  R  L  D  V  C  V  I  W  R  I  E  Q  F  I  M  K  K  P  N  H  S  G  S  G  Q  I
   A  C  K  Y  V  L  S  G  G  L  S  N  L  S  *  K  N  R  I  I  P  A  A  A  K  S

       951         941         931         921         911         901         891         881
GCATTATTGGCGGGCAGTGGCGAGGCCGTAAACTCCCGGTTCCTGATAGCCCAGGTCTGCGCCCCACCACCGACCGCGTA
  H  Y  W  R  A  V  A  R  P  *  T  P  G  S  *  *  P  R  S  A  P  H  H  R  P  R
 R  I  I  G  G  Q  W  R  G  P  K  L  P  V  P  D  S  P  G  L  R  P  T  T  D  R  V
  A  L  L  A  G  S  G  E  A  V  N  S  R  F  L  I  A  Q  V  C  A  P  P  P  T  A  Y

       871         861         851         841         831         821         811         801
CGCGAAACGTTGTTTAACTGGCTGGCTCCGGTCATTGTTGACGCCCAATGTCTGGATTGCTTCGCCGGGAGCGGCGCGCT
T  R  N  V  V  *  L  A  G  S  G  H  C  *  R  P  M  S  G  L  L  R  R  E  R  R  A
 R  E  T  L  F  N  W  L  A  P  V  I  V  D  A  Q  C  L  D  C  F  A  G  S  G  A  L
  A  K  R  C  L  T  G  W  L  R  S  L  L  T  P  N  V  W  I  A  S  P  G  A  A  R

       791         781         771         761         751         741         731         721
GGGGCTGGAAGCGTTATCGCGCTACGCTGCGGGGGCAACGTTGATTGAGATGGATCGCGCGGTTTCTCAGCAGTTAATTA
 G  A  G  S  V  I  A  L  R  C  G  G  N  V  D  *  D  G  S  R  G  F  S  A  V  N  *
  G  L  E  A  L  S  R  Y  A  A  G  A  T  L  I  E  M  D  R  A  V  S  Q  Q  L  I
W  G  W  I  R  Y  R  A  T  L  R  G  Q  R  *  L  R  W  I  A  R  F  L  S  S  *  L

       711         701         691         681         671         661         651         641
AGAATCTGCCGACACTAAAAGCAGGCAATGCACGCGTGGTGAACAGCAACGCGATGTCATTCCTGGCGCAAAAAGGTACA
  E  S  G  D  T  K  S  R  Q  C  T  R  G  E  Q  Q  R  D  V  I  P  G  A  K  R  Y
 L  N  L  Q  I  L  K  A  G  N  A  R  V  V  N  S  N  A  M  S  F  L  A  Q  K  G  T
  R  I  W  R  H  *  K  Q  A  M  H  A  W  *  T  A  T  R  C  H  S  W  R  K  K  V  H

       631         621         611         601         591         581         571         561
CCGCATAATATCGTGTTTGTCGATCCACCGTTCCGCCGTGGCTTGTTAGAAGAGACGATAAATTTACTGGAAGATAACGG
T  A  *  Y  P  V  C  R  S  T  V  P  P  W  L  V  R  R  D  D  K  F  T  G  R  *  R
 P  H  N  I  V  F  V  D  P  P  F  P  R  G  L  L  E  E  T  I  N  L  L  E  D  N  G
  R  I  I  S  C  L  S  I  H  R  S  A  V  A  C  *  K  R  R  *  I  Y  W  K  I  T

       551         541         531         521         511         501         491         481
CTGGCTGGCTGACGAAGCCCTGATTTATGTCGAAAGCGAAGTCGAAAACGGTCTGCCCACTGTTCCAGCAAACTGGTCAT
 L  A  G  *  R  S  P  D  L  C  R  K  R  S  R  K  R  S  A  H  C  S  S  K  L  V  I
  W  L  A  D  E  A  L  I  Y  V  E  S  E  V  E  N  G  L  P  T  V  P  A  N  W  S
A  G  W  L  T  K  P  *  F  M  S  K  A  K  S  K  T  V  C  P  L  F  Q  Q  T  G  H

       471         461         451         441         431         421         411         401
TACATCGGGAAAAAGTGGCGGGTCAGGTGGCTTATCGGCTGTATCAACGCGAAGCACAAGGAGAAAGTGATGCTGATTAA
  T  S  G  K  S  G  G  S  G  G  L  S  A  V  S  T  R  S  T  R  R  K  *  C  *  L
 L  H  R  E  K  V  A  G  Q  V  A  Y  R  L  Y  Q  R  E  A  Q  G  E  S  D  A  D  *
  Y  I  G  K  K  W  R  V  R  W  L  I  G  C  I  N  A  K  H  K  E  K  V  M  L  I  N

       391         381         371         361         351         341         331         321
TATTGGTCGTTTGTTAATGCTCTGCGTTTGGGGATTTTTAATCCTCAACCTGGTGCATCCCTTCCCACGCCCGCTGAATA
I  L  V  V  C  *  C  S  A  F  G  D  F  *  S  S  T  W  C  I  P  S  H  A  R  *  I
 Y  W  S  F  V  N  A  L  R  L  G  I  F  N  P  Q  P  G  A  S  L  P  T  P  A  E  Y
  I  G  R  L  L  M  L  C  V  W  G  F  L  I  L  N  L  V  H  P  F  P  R  P  L  N
```

PvuII

```
      311        301        291        281        271        261        251        241
TCTTCGTTAACGTGGCGCTGATTTTTACCGTGCTGATGCATGGTATCCAGCTTGCGCCTATTGAAATCCACTTTACCGAAA
 S  S  L  T  W  R  *  F  L  P  C  *  Q  M  V  C  S  W  R  Y  *  N  P  L  Y  R  K
  L  R  *  R  G  A  D  F  Y  R  A  D  A  U  Y  A  A  G  A  I  E  I  H  F  T  E
I  F  V  N  V  A  L  I  F  T  V  L  M  H  G  M  Q  L  A  L  L  K  S  T  L  P  K

      231        221        211        201        191        181        171        161
GATGGCCCGCAGATGACCACCGCCGAAAAGGTACGGATTTCCTTTTCGGCGTGTTTGAACTGCTGGCCTGGCAGAAGAA
  M  A  P  R  *  P  P  P  K  R  Y  G  F  S  F  S  A  C  L  N  C  W  P  G  R  R
 R  W  P  A  D  D  H  P  R  F  G  T  D  F  P  F  R  R  V  *  T  A  G  L  A  E  E
  D  G  P  Q  M  T  T  A  E  I  V  R  I  F  L  F  G  V  F  E  L  L  A  W  Q  K  K

      151        141        131        121        111        101         91         81
ATTTAAAGTTAAAAAATAACCTTATTGTTCGCCTACAAAGCTGACAAAGCGCGTTCCTTTATAGCTCAGCGTACCTTTAT
N  L  K  L  K  N  N  L  I  V  R  L  D  S  *  Q  S  A  F  L  Y  S  S  A  Y  L  Y
 I  *  S  *  K  I  T  L  L  F  A  Y  K  A  D  K  A  R  S  F  I  A  Q  R  T  F  I
  F  K  V  K  K  *  P  Y  C  S  P  T  F  L  T  K  R  V  P  L  *  L  S  V  P  L

                                                                        HindIII
      71         61         51         41         31         21         11          1
CGCCCACTGTCAGGGCGTGGTACTGCTGGCGCTCGAGGCGAAACGTCTGCTCCATTCCTCCGCTTTGCGGTTTCGAGCTT
 R  P  L  S  G  R  G  T  A  G  R  R  G  E  T  S  A  P  F  L  R  F  A  V  *  S
  A  H  C  Q  G  V  V  L  L  G  V  E  A  K  R  L  L  H  S  S  A  L  R  F  E  A
S  P  T  V  R  A  W  Y  C  W  A  S  R  R  N  V  C  S  I  P  P  L  C  G  L  K  L

     1050       1060       1070       1080       1090       1100       1110       1120
TGATGTGAATTAAGTTAAGTGATAGACTATTTCATCATTTTTTTAGCTGCTATGTACATAGCGTTAACGCTGTGCCATGA
  D  V  N  *  V  K  *  *  T  I  S  S  F  F  *  L  L  C  T  *  R  *  R  C  A  M
 V  M  *  I  K  L  S  D  R  L  F  H  H  F  F  S  C  Y  V  H  S  V  N  A  V  P  *
  *  C  E  L  S  *  V  I  D  Y  F  I  I  F  L  A  A  M  Y  I  A  L  T  L  C  H  E

                                    ftsY
     1130       1140       1150       1160       1170       1180       1190       1200
AGCAACAGCCGAGGAGTGTAGTCGCAAATGGCGAAAGAAAAAAAACGTGGCTTTTTTTCCTGCCTGCGCTTTGGTCAAAAA
K  Q  Q  R  G  G  V  *  S  Q  M  A  L  E  K  I  P  G  F  F  S  W  L  G  F  G  Q  F
 S  N  S  E  E  C  S  R  K  W  R  K  K  K  N  V  A  F  F  P  G  W  A  L  V  K  K
  A  T  A  R  S  V  V  A  N  G  E  R  K  K  T  W  L  F  F  L  A  G  L  W  S  K

     1210       1220       1230       1240       1250       1260       1270       1280
GAGCAGACCCCGCAAAAAGAGACAGAAGTTCAGAATGAACAACCGGTTGTAGAAGAAATCGTTCAGGCGCAAGAGCCTGT
E  Q  T  P  E  K  E  T  E  V  Q  N  E  Q  P  V  V  E  E  I  V  Q  A  Q  E  P  V
 S  R  F  R  K  K  R  Q  K  F  R  M  N  N  R  L  *  K  K  S  F  R  R  K  S  L
R  A  D  P  G  K  R  D  R  S  S  E  *  T  T  G  C  R  R  N  R  S  G  A  R  A  C

StuI
     1290       1300       1310       1320       1330       1340       1350       1360
GCAGGCCTCGAACAAGCCGTTGAAGAGCAGCCGCAAGCGCATACTGAAGCCGAGGCGGAAACTTTTGCTGCCGACGTTG
F  Q  A  S  E  D  A  V  E  E  Q  P  Q  A  H  T  E  A  E  A  E  T  F  A  A  D  V
 *  R  P  L  N  K  P  L  I  S  S  R  K  R  I  L  K  P  R  R  K  L  L  L  P  I  L
  E  G  L  *  T  S  R  *  R  A  A  A  S  A  Y  *  S  R  G  G  N  F  C  C  R  R  C

     1370       1380       1390       1400       1410       1420       1430       1440
TGGAAGTCACTGAACAGGTTGCTGAAAGTGAAAAACCGCAGCCTGAAGCGGAAGTCGTTGCACAGCCGGAACCGGTCGTA
V  E  V  T  E  Q  V  A  E  S  E  K  A  Q  P  E  A  E  V  V  A  Q  P  E  P  V  V
 W  K  S  L  N  R  L  L  K  V  K  K  R  S  L  K  R  K  S  L  H  S  R  N  R  S  *
  G  S  H  *  T  G  C  *  K  *  K  S  A  A  *  S  G  S  R  C  T  A  G  T  G  R
```

AatII

1450 1460 1470 1480 1490 1500 1510 1520

GAAGAAACGCCGGAGCCAGTGGCTATCGAACGTGAAGAGCTGCCGTTGCCGGAACGACGTCAACGCCGAAGCGGTTTCGCC

E E T P E P V A I E R E E L P L P E D V N A E A V S P Y

K K R R S Q W L S N V K S C R C R K T S T P K R F R

R R N A G A S Q Y R T * R A A V A G R R Q R R S G F A

1530 1540 1550 1560 1570 1580 1590 1600

AGAAGAGTGGCAGGCTGAAGCGGAAACCGTAGAGATTGTCGAAGCGGCGGAAGAAGAAGCGGCTAAAGAAGAAATTACCG

E E W Q A E A E I V E I V E A A E E E A A K E L I I

Q K S G R L K R K P * R L S K R R K K K R L K K K L P

R R V A G * S G N R R D C R S G G R R S G * R R N Y R

1610 1620 1630 1640 1650 1660 1670 1680

ACGAAGAGCTGGAAACGGCGCTGGCTGCCGAAGCCGCAGAAGAGGCGGTGATGGTGGTTCCTCCGGCAGAAGAAGAGCAG

D E E L E T A L A A E E A A E E A V M V V P P A E E E Q

T K S W K R R W L P K R Q K K R R * W W F L R Q K K S S

R R A G N C A G C R S G R R G G D G G S S G R R R A

1690 1700 1710 1720 1730 1740 1750 1760

CCGGTGGAAGAAATCGCTCAGGAGCAGGAAAAACCGACCAAAGAAGGTTTTTTCGCGCGCCTGAAACGCAGCCTGTTAAA

P V E E I A Q E Q E K P T K E G F F A R L K R S L L I

R W F K S L R S R K N R P K K V F S R A * N A A C *

A G G R N R S G A G K T D Q R R F F R A P E T Q P V K

1770 1780 1790 1800 1810 1820 1830 1840

AACCAAAGAAAATCTCGGTTCCGGATTTATCAGCCTGTTCCGCGGTAAAAAAATCGACGATGATCTGTTTGAGGAGCTGG

T K E N L G S G F I S L F R G K K I D D D L F E E L

K P K K I S V P D L S A C S A V K K S T M I C L R S W

N Q R K S R F R I Y Q P V P R * K N R R * S V * G A G

1850 1860 1870 1880 1890 1900 1910 1920

AAGAGCAGCTTTTGATCGCCGATGTGGGTGTGGAAACCACACGTAAAATTATCACCAATCTGACGGAAGGCGCATCCCGC

E E Q I L I A D V G V E T T R K I I T N L T E G A S R

K S S F * S P M W V W K P H V K L S P I * R K A H P A

R A A F D R R C G C G N H T * N Y H Q S D G R R I P

1930 1940 1950 1960 1970 1980 1990 2000

AAGCAGCTTCGTGACGCCGAGGCGCTCTATGGCCTGCTGAAAGAAGAGATGGGCGAGATTCTGGCGAAAGTCGATGAGCC

K Q L R D A E A L Y G L L K E E M G E I L A K V D E P

S S F V T P R R S M A C * K K R W A R F W R K S M S

D A A S * R R G A L W P A E R R D G R D S G E S R * A

2010 2020 2030 2040 2050 2060 2070 2080

GCTGAATGTTGAAGGCAAAGCGCCGTTTGTGATCCTGATGGTGGCCGTCAACGGTGTGGGTAAAACCACGACGATTGGTA

L N V E G K A P F V I L M V G V N G V G K T T I G K

R * M L K A K R R L * S * W W A S T V W V K P R R L V

A E C * R D S A V C D P D G G R Q R C G * N H D D W *

PvuII

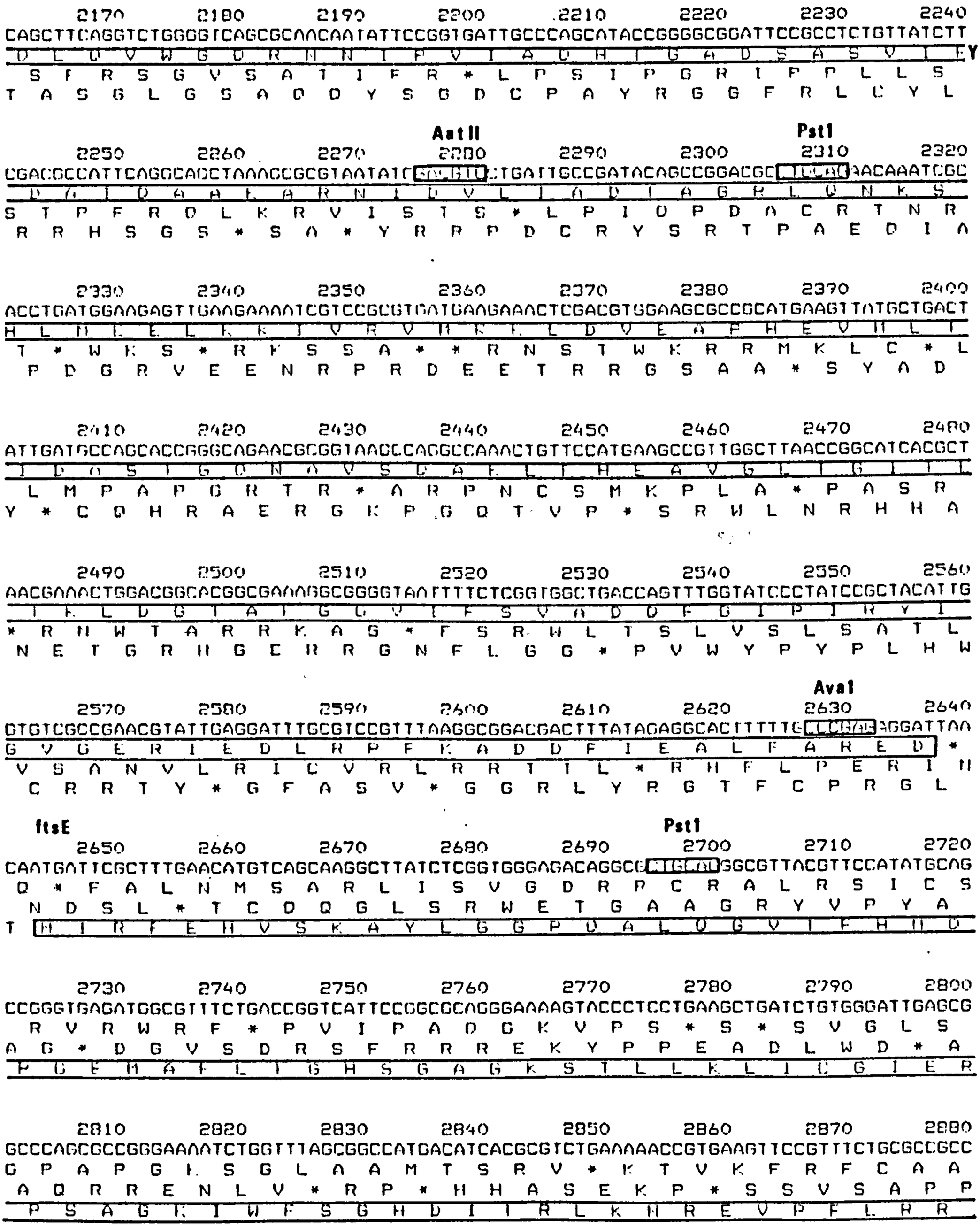
2090 2100 2110 2120 2130 2140 2150 2160

AGCTGGCGCGTCAGTTTGAGCAGCAGGGTAAATCGGTGATGCTGGCGGCGGGTGATACTTTCCGTCAAGCTGCGGTTGAA

K L A R D F E Q Q G K S V M L A A G D T F R A A A V E

S W R V S L S S R V N R * C W R R V I L S V Q L R L N

A G A S V * A A G * I G D A G G G * Y F P C S C G *


2170 2180 2190 2200 2210 2220 2230 2240
CAGCTTCAGGTCTGGGGTCAGCGCAACAATATTCCGGTGATTGCCCAGCATACCGGGGCGGATTCCGCCTCTGTTATCTT
AatII
PstI
2250 2260 2270 2280 2290 2300 2310 2320
2330 2340 2350 2360 2370 2380 2390 2400
2410 2420 2430 2440 2450 2460 2470 2480
2490 2500 2510 2520 2530 2540 2550 2560
AvaI
2570 2580 2590 2600 2610 2620 2630 2640
FtsE
PstI
2650 2660 2670 2680 2690 2700 2710 2720
2730 2740 2750 2760 2770 2780 2790 2800
2810 2820 2830 2840 2850 2860 2870 2880

AccI

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        2890        2900        2910        2920        2930        2940        2950        2960
AGATTGGCATGATTTTCCAGGATCACCATCTACTGATGGACCGTACTGTCTAGATAACGTGGCGATCCCGCTGATTATC
 R  L  A  *  F  S  R  I  T  I  Y  *  W  T  V  L  S  T  I  T  W  R  S  R  *  L  S
   D  W  H  D  F  P  G  S  P  S  T  D  G  P  V  C  L  R  *  R  G  D  P  A  D  Y
D  I  G  M  I  F  D  D  H  H  L  L  M  D  R  I  V  Y  D  N  V  A  I  P  L  I  I  E

        2970        2980        2990        3000        3010        3020        3030        3040
GCCGGTGCCAGCGGTGACGATATTCGTCGCCGGGTGTCGGCGGCGCTGGATAAAGTCGGGCTACTGGACAAAGCGAAGAA
 P  V  P  A  V  T  I  F  V  A  G  C  R  P  R  W  I  K  S  G  Y  W  T  K  R  R
R  R  C  Q  R  *  R  Y  S  S  P  G  V  G  G  A  G  *  S  R  A  T  G  D  S  E  E
 A  G  A  S  G  D  D  I  R  R  R  V  S  A  A  L  D  K  V  G  L  L  D  K  A  K  N

        3050        3060        3070        3080        3090        3100        3110        3120
CTTCCCTATTCAGCTTTCGGGCGGTGAACAACAGCGTGTTGGCATTGCCCGCGCGGTGGTGAACAAGCCCGCGGTACTGC
 T  S  L  F  S  F  R  A  V  N  N  S  V  L  A  L  P  A  R  W  *  T  S  P  R  Y  C
L  P  Y  S  A  F  G  R  *  T  T  A  C  W  H  C  P  R  G  G  E  Q  A  R  G  T  A
 F  P  I  Q  L  S  G  G  E  Q  Q  R  V  G  I  A  R  A  V  V  N  K  P  A  V  L

                BstE2
        3130        3140        3150        3160        3170        3180        3190        3200
TGGCGGACGAACCGACTGGTAACTGGACGACGCGCTGTCGGAAGGCATTTTACGTCTGTTTGAAGAGTTTAACCGCGTT
 W  R  T  N  R  L  V  T  W  T  T  R  C  R  K  A  F  Y  V  C  L  K  S  L  T  A  L
  G  G  R  T  D  W  *  P  G  R  R  A  V  G  R  H  F  T  S  V  *  R  V  *  P  R
L  A  D  E  P  T  G  N  L  D  D  A  L  S  E  G  I  L  R  L  F  E  E  F  N  R  V

BstE2                                                                   SphI
        3210        3220        3230        3240        3250        3260        3270        3280
GCGGTAACTGGTATTGATGGCAACGCACGACATCAACCTGATCTCGCGGCGTTCCTATCGCATGCTCACCCTGAGCGATGG
  G  *  P  Y  *  W  Q  R  T  T  S  T  *  S  R  G  V  P  I  A  C  S  P  *  A  M
W  G  N  R  I  D  G  N  A  R  H  Q  P  D  L  A  A  F  L  S  H  A  H  P  E  R  W
 G  V  T  V  L  M  A  I  H  D  I  N  L  I  S  R  R  S  Y  R  M  L  T  L  S  D  G

                ftsX
        3290        3300        3310        3320        3330        3340        3350        3360
TCACTTGCATGGAGGCGTGGGCCATGAATAAGCGCGATGCAATCAATCATATTCGGCAGTTTGGCGGGCGTCTTGATCGC
 V  T  C  M  E  A  W  A  M  N  K  R  D  A  I  N  H  I  R  Q  F  G  G  R  L  D  R
 S  L  A  W  R  R  G  P  *  I  S  A  M  D  S  I  I  F  G  S  L  A  G  V  L  I  A
  H  L  H  G  G  V  G  H  E  *  A  R  C  N  Q  S  Y  S  A  V  W  R  A  S  *  S

        3370        3380        3390        3400        3410        3420        3430        3440
TTCCGTAAATCGGTCGGCGGCTCAGGCGACGGCGGTCGTAACGCACCAAAACGCGCGAAATCCTCGCCAAAACCGGTAAA
 F  R  K  S  V  G  G  S  G  D  G  G  R  N  A  P  K  R  A  K  S  S  P  K  P  V  N
  S  V  N  R  S  A  A  Q  A  T  A  V  V  T  H  Q  N  A  R  N  P  R  Q  N  R  *
L  P  *  I  G  R  R  L  R  R  R  R  S  *  R  T  K  T  R  E  I  L  A  K  T  G  K

        3450        3460        3470        3480        3490        3500        3510        3520
TCGCAAAACCAACGTTTTCAACGAACAGGTGCGCTATGCCTTCCACGGCGCATTGCAGGATCTGAAAGCAAACCGTTCG
 R  K  T  N  V  F  N  E  Q  V  R  Y  A  F  H  G  A  L  Q  D  L  K  S  K  P  F
I  A  K  P  T  F  S  T  N  R  C  A  M  P  S  T  A  H  C  R  I  *  K  A  N  R  S
 S  Q  N  Q  R  F  Q  R  T  G  A  L  C  L  P  R  R  I  A  G  S  E  K  Q  T  V  R

        3530        3540        3550        3560        3570        3580        3590        3600
CCACGTTTTTAACGGTGATGGTTATCGCCATTTCTCTGACGCTGCCCAGCGTCTGTTATATGGTGTACAAAAACGTTAAC
 A  T  F  L  T  V  M  V  I  A  I  S  L  T  L  P  S  V  C  Y  M  V  Y  K  N  V  N
 P  R  F  *  R  *  W  L  S  P  F  L  *  R  C  P  A  S  V  I  W  C  T  K  T  L  T
  H  V  F  N  G  D  G  Y  R  H  F  S  D  A  A  Q  R  L  L  Y  G  V  Q  K  R  *
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      3610       3620       3630       3640       3650       3660       3670       3680
CAGGCGGCGACGCAGTATTATCCGTCACCGCAAATCACTGTTTATCTGCAAAAAACGCTGGACGATGACGCTGCTGCGGG
 Q  A  A  I  Q  Y  Y  P  S  P  Q  I  T  V  Y  L  Q  K  F  L  D  D  D  A  A  A  G X
  R  R  R  R  S  I  I  R  H  R  K  S  L  F  I  C  K  K  R  W  T  M  T  L  L  R
P  G  G  D  A  V  I  S  V  T  A  N  H  C  L  S  A  K  N  A  G  R  *  R  C  C  G

      3690       3700       3710       3720       3730       3740       3750       3760
CGTGGTGGCACAGTTGCAGGCCGAGCAAGGCGTGGAGAAAGTGAACTATCTTTCTCGTGAAGACGCACTGGGTGAGTTCC
 V  V  A  Q  L  Q  A  E  Q  G  V  E  K  V  N  Y  L  S  R  E  D  A  L  G  E  F
A  W  W  H  S  C  R  P  S  K  A  W  R  K  *  T  I  F  L  V  K  T  H  W  V  S  S
 R  G  G  T  V  A  G  R  A  R  R  G  E  S  E  L  S  F  S  *  R  R  T  G  *  V  P

      3770       3780       3790       3800       3810       3820       3830       3840
GTAACTGGTCTGGTTTTGGTGGTGCGCTGGATATGCTGGAAGAAAACCCGCTTCCGGCAGTGGCGGTGGTGATCCCGAAA
 R  N  W  S  G  F  G  G  A  L  D  M  L  E  E  N  P  L  P  A  V  A  V  V  I  P  K
 V  T  G  L  V  L  V  V  R  W  I  C  W  K  K  T  R  F  R  D  W  R  W  *  S  R  N
  *  L  V  W  F  W  W  C  A  G  Y  A  G  R  K  P  A  S  G  S  G  G  G  D  P  E

      3850       3860       3870       3880       3890       3900       3910       3920
CTCGATTTCCAGGGGACGGAATCACTGAATACGCTGCGTGATCGTATCACGCAGATTAACGGCATTGACGAAGTGCGGAT
 L  D  F  Q  G  T  E  S  L  N  T  L  R  D  R  I  T  Q  I  N  G  I  D  E  V  R  M
  S  I  S  R  G  R  N  H  *  I  R  C  V  I  V  S  R  R  L  T  A  L  T  K  C  G
T  R  F  P  G  D  G  I  T  E  Y  A  A  *  S  Y  H  A  D  *  R  H  *  R  S  A  D

PvuII
      3930       3940       3950       3960       3970       3980       3990       4000
GGATGACAGCTGGTTTGCCCGTCTGGCGGCGTTGACCGGGCTGGTCGGGCGCGTTTCGGCGATGATCGGCGTGTTGATGG
 D  D  S  W  F  A  R  L  A  A  L  T  G  L  V  G  R  V  S  A  M  I  G  V  L  M
W  M  T  A  G  L  P  V  W  R  R  *  P  G  W  S  G  A  F  R  R  *  S  A  C  *  W
 G  *  Q  L  V  C  P  S  G  G  V  D  R  A  G  R  A  R  F  G  D  D  R  R  V  D  G

      4010       4020       4030       4040       4050       4060       4070       4080
TGGCGGCCGTGTTCCTCGTCATCGGTAACAGTGTGCGTCTGAGTATCTTTGCTCGCCGTGACTCCATTAACGTACAGAAA
 V  A  A  V  F  L  V  I  G  N  S  V  R  L  S  I  F  A  R  R  D  S  I  N  V  Q  K
 W  R  P  C  S  S  S  S  S  V  T  V  C  V  *  V  S  L  L  A  V  T  P  L  T  Y  R  N
  G  G  R  V  P  R  H  R  *  Q  C  A  S  E  Y  L  C  S  P  *  L  H  *  R  T  E

      4090       4100       4110       4120       4130       4140       4150       4160
CTGATTGGTGCGACAGATGGATTCATCCTGCGCCCGTTCCTGTATGGTGGCGCACTGCTGGGATTTTCTGGCGCATTGTT
 L  I  G  A  I  D  G  F  I  L  R  P  F  L  Y  G  G  A  L  L  G  F  S  G  A  L  L
  *  L  V  R  Q  M  D  S  S  C  A  R  S  C  M  V  A  H  C  W  D  F  L  A  H  C
T  D  W  C  D  R  W  I  H  P  A  P  V  P  V  W  W  R  T  A  G  I  F  W  R  I  V

      4170       4180       4190       4200       4210       4220       4230       4240
GTCATTAATTTTGTCAGAAATTCTGGTGCTGCGATTGTCATCGGCGGTTGCGGAAGTGGCACAGGTTTTCGGAACGAAGT
 S  L  I  L  S  E  I  L  V  L  R  L  S  S  A  V  A  E  V  A  Q  V  F  G  T  K
 C  H  *  F  C  D  K  F  W  C  C  D  C  H  R  R  L  R  K  W  H  R  F  S  E  R  S
  V  I  N  F  V  R  N  S  G  A  A  I  V  I  G  G  C  G  S  G  T  G  F  R  N  E  V

EcoRV
      4250       4260       4270       4280       4290       4300       4310       4320
TTGATATCAATGGCTTATCATTCGATGAATGCCTGCTATTGCTGCTGGTATGCTCGATGATTGGCTGGGTGGCAGCGTGG
 F  D  I  N  G  L  S  F  D  E  C  L  L  L  L  L  V  C  S  M  I  G  W  V  A  A  W
 L  I  S  M  A  Y  H  S  M  N  A  C  Y  C  C  W  Y  A  R  *  L  A  G  W  Q  R  G
  *  Y  Q  W  L  I  I  R  *  M  P  A  I  A  A  G  M  L  D  D  W  L  G  G  S  V
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          4330       4340       4350       4360       4370       4380       4390       4400
CTTGCCACGGTACAACATTTACGCCACTTTACGCCTGAATAATAAAAGCGTGTTATACTCTTTCCCTGCAATGGGTTCCG
I  A  T  V  Q  H  L  R  H  F  T  P  E  *  *  K  R  V  I  L  F  P  C  N  G  F  R
  L  P  R  Y  N  I  Y  A  T  L  R  L  N  N  K  S  V  L  Y  S  F  P  A  M  G  S
A  C  H  G  T  T  F  T  P  L  Y  A  *  I  I  K  A  C  Y  T  L  S  L  Q  N  V  P

                                                                          Hind III
          4410       4420       4430       4440       4450       4460       4470       4480
TAGCAGGGAAACAGACCCCGTTGTCTCTTCCCGGTATTTCATCTCTATGTCACATTTTGTGCGTAATTTATTCAC[AAGCTT]
  S  R  E  R  D  P  V  V  S  S  R  Y  F  I  S  M  S  H  F  V  R  N  L  F  T  S
V  A  G  K  E  T  P  L  S  L  P  G  I  S  S  L  C  H  I  L  C  V  I  Y  S  Q  A
 *  Q  G  K  R  P  R  C  L  F  P  V  F  H  L  Y  V  T  F  C  A  *  F  I  H  K  L

          4490       4500       4510       4520       4530       4540       4550       4560
]GCATTGAACTTGTGGATAAAATCACGGTCTGATAAAACAGTGAATGATAACCTCGTTGCTCTTAAGCTCTGGCACAGTT
L  H  *  T  C  G  *  N  H  G  L  I  K  Q  *  M  I  T  S  L  L  L  S  S  G  T  V
 C  I  E  L  V  D  K  I  T  V  *  *  N  S  E  *  *  P  R  C  S  *  A  L  A  Q  L
  A  L  N  L  W  I  K  S  R  S  D  K  T  V  N  D  N  L  V  A  L  K  L  W  H  S

                EcoRV  ClaI                                  rpoH
          4570       4580       4590       4600       4610       4620       4630       4640
GTTGCTACCACTCAAGCGCCAGAA[GATATCATCGAT]TGGGAGGATTTGGATGGCTGACAAAATGCAAAGTTTGGCTTTGGCCC
 V  A  T  T  E  A  P  E  D  I  D  W  E  D  L  D  G  *  Q  N  A  K  F  G  F  G  P
  L  L  P  L  K  R  Q  K  I  S  I  G  R  I  W [M  A  D  K  M  Q  S  L  A  L  A]
C  C  Y  H  *  S  A  R  R  Y  R  L  G  G  F  G  W  L  T  K  C  F  V  W  L  W  P

          4650       4660       4670       4680       4690       4700       4710       4720
CAGTTGGAGGCGTAGATTCCTACATCCGGGCAGCTAACGCGTGGCCGATGTTGTCGGCTGACGAGGAGCGGGCCCTGGCT
  S  W  R  R  R  F  L  H  P  G  S  *  R  V  A  D  V  V  G  *  R  G  A  G  A  G
P  V  G  G  V  D  S  Y  I  R  A  A  N  A  W  P  M  L  S  A  D  E  E  R  A  L  A
Q  L  E  A  *  I  P  T  S  G  Q  L  T  R  G  R  C  C  R  L  T  R  S  G  R  W  L

          4730       4740       4750       4760       4770       4780       4790       4800
GAAAAGCTGCATTACCATGGCGATCTGGAAGCAGCTAAAACGCTGATCCTGTCTCACCTGCGGTTTGTTGTTCATATTGC
*  K  A  A  L  P  W  R  S  G  S  S  *  N  A  D  P  V  S  P  A  V  C  C  S  Y  C
E  K  L  H  Y  H  G  D  L  E  A  A  K  T  L  I  L  S  H  L  R  F  V  V  H  I  A
 K  S  C  I  T  M  A  I  W  K  Q  L  K  R  *  S  C  L  T  C  G  L  L  F  I  L

          4810       4820       4830       4840       4850       4860       4870       4880
TCGTAATTATGCGGGCTATGGCCTGCCACAGGCGGATTTGATTCAGGAAGGTAACATGGGCCTGATGAAAGCAGTGCGCC
S  *  L  C  G  L  W  P  A  T  G  G  F  D  S  G  R  *  H  G  P  D  E  S  S  A  P
 R  N  Y  A  G  Y  G  L  P  Q  A  D  L  I  Q  E  G  N  M  G  L  M  K  A  V  R
L  V  I  M  R  A  M  A  C  H  R  R  I  *  F  R  K  V  T  W  A  *  *  K  Q  C  A

          4890       4900       4910       4920       4930       4940       4950       4960
GTTTCAACCCGGAAGTGGGTGTGCGCCTGGTCTCCTTCGCCGTTCACTGGATCAAAGCAGAGATCCACGAATACGTTCTG
F  Q  P  G  S  G  C  A  P  G  L  L  R  R  S  L  D  Q  S  R  D  P  R  I  R  S
R  F  N  P  E  V  G  V  R  L  V  S  F  A  V  H  W  I  K  A  E  I  H  E  Y  V  L
 V  S  T  R  K  W  V  C  A  W  S  P  S  P  F  T  G  S  K  Q  R  S  T  N  T  F  C

          4970       4980       4990       5000       5010       5020       5030       5040
CGTAACTGGCGTATCGTCAAAGTTGCGACCACCAAAGCGGAGCGCAAACTGTTCTTCAACCTGCGTAAAACCAAGCAGCG
A  *  L  A  Y  R  Q  S  C  D  H  Q  S  G  A  Q  T  V  L  Q  P  A  *  N  Q  A  A
R  N  W  R  I  V  K  V  A  T  T  K  A  E  R  K  L  F  F  N  L  R  K  T  K  Q  R
 V  T  G  V  S  S  K  L  R  P  P  K  R  S  A  N  C  S  S  T  C  V  K  P  S  S
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      5050       5060       5070       5080       5090       5100       5110       5120
TCTGGGCTGGTTTAACCAGGATGAAGTCGAAATGGTGGCCCGTGAACTGGGCGTAACCAGCAAAGACGTACGTGAGATGG
 S  G  L  V  *  P  G  *  S  R  N  G  G  P  *  T  G  R  N  Q  Q  R  R  T  *  D  G
    L  G  W  F  N  Q  D  E  V  E  M  V  A  R  E  L  G  V  T  S  K  D  V  R  E  M   H
 V  W  A  G  L  T  R  M  K  S  K  W  W  P  V  N  W  A  *  P  A  K  T  Y  V  R  W

      5130       5140       5150       5160       5170       5180       5190       5200
AATCACGTATGGCGGCACAGGACATGACCTTTGACCTGTCTTCCGACGACGATTCCGACAGCCAGCCGATGGCTCCGGTG
   I  T  Y  G  G  T  G  H  D  L  *  P  V  F  R  R  R  F  R  Q  P  A  D  G  S  G
E  S  R  M  A  A  Q  D  M  T  F  D  L  S  S  D  D  D  S  D  S  Q  P  M  A  P  V
N  H  V  W  R  H  R  T  *  P  L  T  C  L  P  T  T  I  P  T  A  S  R  W  L  R  C

      PstI
      5210       5220       5230       5240       5250       5260       5270       5280
CTCTATCTGCAGGATAAATCATCTAACTTTGCCGACGGCATTGAAGATGATAACTGGGAAGAGCAGGCGGCAAACCGTCT
A  I  S  A  G  *  I  I  *  L  C  R  R  H  *  R  *  *  L  G  R  A  G  G  K  P  S
L  Y  L  Q  D  K  S  S  N  F  A  D  G  I  E  D  D  N  W  E  E  Q  A  A  N  R  L
   S  I  C  R  I  N  H  L  T  L  P  T  A  L  K  M  I  T  G  K  S  R  R  Q  T  V

      5290       5300       5310       5320       5330       5340       5350       5360
GACCGACGCGATGCAGGGTCTGGACGAACGCAGCCAGGACATCATCCGTGCGCGCTGGCTGGACGAAGACAACAAGTCCA
D  R  R  D  A  G  S  G  R  T  Q  P  G  H  H  P  C  A  L  A  G  R  R  Q  Q  V  H
   I  D  A  M  Q  G  L  D  E  R  S  Q  D  I  I  R  A  R  W  L  D  E  D  N  K  S
*  P  T  R  C  R  V  W  T  N  A  A  R  T  S  S  V  R  A  G  W  T  K  T  T  S  P

                                                    PvuII
      5370       5380       5390       5400       5410       5420       5430       5440
CGTTGCAGGAACTGGCTGACGCTTACGGCGTTTCCGCTGAGCGTGTACGCCAGCTGGAAAAGAACGCGATGAAAAAATTG
   V  A  G  T  G  *  R  L  R  R  F  R  *  A  C  T  P  A  G  K  E  R  D  E  K  I
T  L  Q  E  L  A  D  A  Y  G  V  S  A  E  R  V  R  Q  L  E  K  N  A  M  K  K  L
R  C  R  N  W  L  T  L  T  A  F  P  L  S  V  Y  A  S  W  K  R  T  R  *  K  N  C

      5450       5460       5470       5480       5490       5500       5510       5520
CGTGCTGCCATTGAAGCGTAATTTCCGCTATTAAGCAGAGAACCCTAGATGAGAGTCCGGGGTTTTTGTTTTTTGGGCCT
A  C  C  H  *  S  V  I  S  A  I  K  Q  R  T  L  D  E  S  P  G  F  L  F  F  G  P
R  A  A  I  E  A  *  F  P  L  L  S  R  E  P  *  M  R  V  R  G  F  C  F  L  G  L
   V  L  P  L  K  R  N  F  R  Y  *  A  E  N  P  R  *  E  S  G  V  F  V  F  W  A

      5530       5540       5550       5560       5570       5580       5590       5600
CTGTAATAATCAATTTCCCCTCCGGCAAAACGCCAATCCCCACGCAGATTGTTAATAAACTGTCAAAATAGCTATTCCAA
L  *  *  S  I  S  P  P  A  K  R  Q  S  P  R  R  L  L  I  N  C  Q  N  S  Y  S  N
   C  N  N  Q  F  P  L  R  Q  N  A  N  P  H  A  D  C  *  *  T  V  K  I  A  I  P
S  V  I  I  N  F  P  S  G  K  T  P  I  P  T  Q  I  V  N  K  L  S  K  *  L  F  Q

      5610       5620       5630
TATCATAAAAATCGGGATATGAAAAAGCAGAGTATGCT
   I  I  K  I  G  I  *  K  S  R  V  C
I  S  *  K  S  G  Y  E  K  A  E  Y  A
 Y  H  K  N  R  D  M  K  K  Q  S  M
```

Appendix B DNAGEL Program Listing

This program was written in BBC Basic for a BBC Master or Model B microcomputer. To obtain a hard copy of the output an assembled screen dump routine was used. This was loaded into memmory at &A00 before running the program.

If a hard copy is not required then the VDU and CALL&A00 instructions in lines 70 and 90 can be removed, and appropriate pauses introduced between PROCDISP (displays error calculations), PROCTABLE (displays calculated sizes) and PROCGRAPH (displays regression curve).

```
10 MODE4 :CLEAR:I%=0
20 NTC=20:GL=0:GLM=1000000
30 DIMWT(50),DI(50),PR(50),DW(50),DD(50),DP(50),C(50),D(50),N(20),DG(20,20)
   ,WG(20,20),M$(20,20),TT$(20),NIT(20),SWT(50),SDI(50)
40 FOR TC=1 TO 20:NIT(TC)=0:NEXT
50 CLS:VDU23,1,0;0;0;0;
60 @%=&20208:PROCSTD:PROCSUMS:PROCINPUT
 70 CLS:VDU2:PROCDISP:PROCTABLE:CALL&A00:VDU3
 80 PROCGRAPH
 90 VDU2:CALL&A00:VDU3
 95 VDU2,1,27,1,48,3
100 END
110DEF PROCENTER:CLS:VDU23,1,1;0;0;0;
120        INPUT"TRACK NUMBER    "TC
130        PRINT:PRINT:INPUT"ENTER NUMBER OF STANDARD FRAGMENTS "N(TC)
140        PRINT:FOR J%=1 TO N(TC)
150        PRINT"SIZE       OF FRAGMENT "J%+I%;:INPUTWT(J%+I%)
160        PRINT"MIGRATION OF FRAGMENT "J%+I%;:INPUTDI(J%+I%)
170        PROCPLACE(WT(J%+I%),DI(J%+I%),TC)
180        IF DI(J%+I%)>GL THEN GL=DI(J%+I%)
190        IF DI(J%+I%)<GLM THEN GLM=DI(J%+I%)
200        PRINT: NEXT
210        I%=I%+N(TC): ENDPROC
220DEF PROCSUMS
230        FORJ%=1TOI%:SW=SW+WT(J%):SI=SI+DI(J%):PR(J%)=WT(J%)*DI(J%):
           SP=SP+PR(J%):NEXT
240        MW=SW/I%:MD=SI/I%:MP=SP/I%
250        FORJ%=1TOI%:DW(J%)=WT(J%)-MW:DD(J%)=DI(J%)-MD:DP(J%)=PR(J%)-MP:NEXT
260        FORJ%=1TOI%:CS=CS+DW(J%)^2:CM=CM+DD(J%)^2:CC=CC+DW(J%)*DD(J%):
           CP=CP+DP(J%)*DW(J%):CL=CL+DP(J%)*DD(J%):NEXT
270        DT=CS*CM-CC^2:M=(CM*CP-CC*CL)/DT:LO=(-CC*CP+CS*CL)/DT
280        FORJ%=1TOI%:C(J%)=(WT(J%)-LO)*(DI(J%)-M):S=S+(C(J%)-C(1)):
           SS=SS+(C(J%)-C(1))^2:NEXT
290        CB=S/I%+C(1):SC=SQR((SS-S^2/I%)/(I%-1))
300        ENDPROC
310DEF PROCDISP
320        VDU1,27,1,69
330        PRINT:PRINT"STD LEN   DIST   PRED LEN   DEV   %DEV":PRINT
340        DEF FNA(Z)=ABS(INT(Z+0.5))
350        DEF FNB(Z)=ABS(INT(Z*10+0.5)/10)
360        FORJ%=1TOI%:PW=CB/(DI(J%)-M)+LO:WD=WT(J%)-PW:PC=100*WD/WT(J%):
           SD=SD+WD:D=D+(WD^2)
370        PRINT:PRINT;TAB(0)WT(J%);TAB(10)DI(J%);TAB(17)FNA(PW);
           TAB(27)FNA(WD);TAB(34)FNB(PC):NEXT
380        ENDPROC
390DEF PROCSTD:VDU23,1,0;0;0;0;
400        CLS: PRINT"WHICH OF THE FOLLOWING SETS OF STANDARDS"
410        PRINT"WILL YOU USE (ENTER NUMBER)":PRINT:PRINT
420        PRINT:PRINT"      1.  LAMBDA/HindIII"
430        PRINT:PRINT"      2.  LAMBDA/EcoRI"
440        PRINT:PRINT"      3.  LAMBDA/HindIII/EcoRI"
450        PRINT:PRINT"      4.  pBR322/BaeIII"
460        PRINT:PRINT"      5.  pBR322/Sau3AI"
470        PRINT:PRINT"      6.  pAT153/Sau3AI"
480        PRINT:PRINT"      7.  pBR322/Hinf1/Hinf1+EcoR1"
490        PRINT:PRINT"      8.  Any other standards"
500        X$=GET$: IF VAL(X$)<1 OR VAL(X$)>8 THEN 500
510        SN=VAL(X$)
520        ON VAL(X$) GOTO 530, 540, 550, 560, 570, 580, 590, 600
530        V%=8:  RESTORE 1900:GOTO610
540        V%=6:  RESTORE 1910:GOTO610
550        V%=13: RESTORE 1920:GOTO610
560        V%=18: RESTORE 1930:GOTO610
570        V%=12: RESTORE 1940:GOTO610
```

```
580       V%=17: RESTORE 1950:GOTO610
590       V%=11: RESTORE 1960:GOTO610
600       PROCENTER:GOTO 760
610       CLS:VDU23,1,1;0;0;0;:INPUT"TRACK NUMBER    "TC
620       IF TC<1 THEN CLS:GOTO 610
630       CLS: PRINT:PRINT"ENTER MIGRATION OF STANDARD FRAGMENTS"
640       PRINT:PRINT"TO OMIT ANY FRAGMENT JUST PRESS RETURN"
650       PRINT:PRINT;TAB(2)"FRAGMENT";TAB(15)"SIZE";TAB(28)"MIGRATION"
660       K%=1: FOR J%=1 TO V% :READ X%
670       PRINT:PRINT;TAB(2);K%;TAB(15);X%;:INPUTTAB(29);Y$
680       IF ASC(Y$)=-1 THEN 740
690       WT(K%+I%)=X%: DI(K%+I%)=VAL(Y$)
700       IF VAL(Y$)>GL THEN GL=VAL(Y$)
710       IF VAL(Y$)<GLM THEN GLM=VAL(Y$)
720       PROCPLACE(WT(K%+I%),DI(K%+I%),TC)
730       K%=K%+1
740       NEXT
750       I%=I%+K%-1
760       VDU23,1,0;0;0;0;:PRINT : PRINT"ANY MORE STANDARDS (Y/N) ? ";
770       IF GET$="Y" THEN 400
780       CLS:  VDU14: PRINT TAB(2);"FRAGMENT";TAB(16)"SIZE";TAB(29)
          "MIGRATION":PRINT: FOR J%=1TO I%:PRINTJ%;TAB(16)WT(J%)TAB(29)DI(J%)
          : PRINT:NEXT:VDU15:ELSE CLS:GOTO890
790       VDU10,10: PRINT"EDIT DATA ? (Y/N)"
800       IF GET$<>"Y" THEN 870
810       PRINT:INPUT"TRACK NUMBER     ";TC
820       PRINT:INPUT"FRAGMENT NUMBER ";J%
830       PRINT:PRINT"FRAGMENT SIZE     ";WG(TC,J%)
840       PRINT:INPUT"MIGRATION         "; DI(J%)
850       TS=NIT(TC):NIT(TC)=J%-1:PROCPLACE(WT(J%),DI(J%),TC):NIT(TC)=TS
860       GOTO 780
870       GL=GL+GL/5
880       FOR K%=1 TO I%:SWT(K%)=WT(K%):SDI(K%)=DI(K%):NEXT
890       ENDPROC
900DEF PROCBAND(TC,M,GL)
910       MOVE  60*TC,1023-10*M*75/GL
920       DRAW 60*TC+40,1023-10*M*75/GL
930       ENDPROC
940DEF PROCGELDRAW(NTC)
950       X=60:Y=1021
960       FOR T=1 TO NTC
970       MOVEX,Y:DRAWX+40,Y:DRAWX+40,Y-10
980       DRAWX,Y-10:DRAWX,Y:X=X+60
990       NEXT:ENDPROC
1000DEF PROCINPUT:VDU15
1010      VDU23,1,1;0;0;0;:VDU28,0,31,39,24:CLS:CLG:TC=0
1040      PROCGELDRAW(NTC):PROCFILLIN(NTC)
1050      N(TC)=1:NTC=0
1060      CLS:INPUT"TRACK NUMBER TO BE READ "TC
1070      IF TC>NTC THEN NTC=TC
1080      CLS
1090      PRINTTAB(0,6);"OR PRESS:          T  FOR NEW TRACK"
1100      PRINTTAB(0,7);"                   F  TO  FINISH"
1110      PRINTTAB(0,8);"                   E  TO EDIT"
1120      INPUT TAB(0,2)"MOBILITY OF BAND ";M$(TC,N(TC))
1130      IF M$(TC,N(TC))="T" THEN 1060
1140      IF M$(TC,N(TC))="F" THEN VDU28,0,31,39,24:GOTO1250
1150      IF M$(TC,N(TC))="E" THEN PROCEDIT:PRINT:GOTO1120
1160      PW=CB/(VAL(M$(TC,N(TC)))-M)+LO
1170      PRINT TAB(18,0)"                          "
1180      PRINTTAB(18,0);M$(TC,N(TC))
1190      PRINTTAB(18,2);"                          "
1200      IF VAL(M$(TC,N(TC)))>(5*GL/6) OR VAL(M$(TC,N(TC)))<GLM
          THEN PRINTTAB(25,2)"OUT OF RANGE !!"
```

```
1210        PRINT TAB(25,0);"SIZE = "FNA(PW)
1220        PRINT: PROCBAND(TC,VAL(M$(TC,N(TC))),GL)
1230        PROCPLACE(FNA(PW),VAL(M$(TC,N(TC))),TC)
1240        GOTO 1120
1250        ENDPROC
1260DEF PROCPLACE(W,D,TC)
1270        NIT(TC)=NIT(TC)+1
1280        WG(TC,NIT(TC))=W
1290        DG(TC,NIT(TC))=D
1300        ENDPROC
1310DEF PROCFILLIN(NTC)
1320        FOR T=1 TO NTC :J%=1
1330        REPEAT
1340        PROCBAND(T,DG(T,J%),GL)
1350        J%=J%+1
1360        UNTIL DG(T,J%)=0
1370        NEXT
1380        ENDPROC
1390DEF PROCTABLE
1400        @%=10:PRINT:PRINT:PRINT:J%=0
1430        FOR K%=1 TO 5
1440        PRINTTAB(13*K%+(13-LEN(TT$(K%+J%)))/2);J%+K%;:NEXT:PRINT
1460        PRINT" BAND SIZES  "
1470        FOR K%=1 TO 15
1480        FOR L%=1 TO 5
1490        IF WG(J%+L%,K%)=0 THEN 1510
1500        PRINT TAB(13*L%)WG(J%+L%,K%);
1510        NEXT:PRINT:NEXT
1520        J%=J%+5:IFJ%>=NTC THEN ENDPROC ELSE 1430
1530DEF PROCEDIT :TCS=TC:CLS
1540        INPUT"TRACK    NUMBER ";TC
1550        INPUT"FRAGMENT NUMBER ";J%
1560        GCOL4,0:PROCBAND(TC,DG(TC,J%),GL):GCOL0,7
1570        WG(TC,J%)=0:DG(TC,J%)=0
1580        CLS:TC=TCS
1590        ENDPROC
1670DEF PROCGRAPH
1680        CLS:CLG:MOVE0,1000:DRAW 0,0:DRAW 1000,0
1701        FOR K%=200 TO 1000 STEP 200:MOVEK%,0:DRAWK%,30:NEXT
1710        IF SN=1 OR SN=2 OR SN=3 THEN AX=25
1720        IF SN=5 OR SN=7 OR SN=8 THEN AX=2
1730        IF SN=4 OR SN=6         THEN AX=1
1735        @%=&10:VDU5:MOVE570,30:PRINTAX;" kb"
1740        MOVE 0,(LO*-1)/AX:DRAW 1000,(AX*1000-LO)/AX
1760        FOR K%=1 TO I%
1770        X=SWT(K%)/AX:Y=(CB/(SDI(K%)-M))/AX
1790        MOVE X,Y+10:DRAW X,Y-10:MOVE X+10,Y:DRAW X-10,Y
1800        MOVE X+10,Y:DRAW X-10,Y
1810        NEXT
1820        ENDPROC
1900DATA 23130, 9416, 6557, 4361, 2322, 2027, 564, 125
1910DATA 21226, 7421, 5804, 5643, 4878, 3530
1920DATA 21226, 5148, 4973, 4268, 3530, 2027, 1904, 1584, 1375, 947, 831, 564,
    125
1930DATA 587, 540, 504, 458, 434, 267, 234, 213, 192, 184, 124, 123, 104, 89,
    80, 64, 57, 51
1940DATA 1374, 665, 358, 341, 317, 272, 258, 207, 105, 91, 78, 75
1950DATA 587, 458, 434, 339, 267, 234, 213, 192, 184, 124, 123, 104, 89, 80,
    64, 57, 51
1960DATA 1632,1000,632,517,506,396,334,298,221,154,75
```

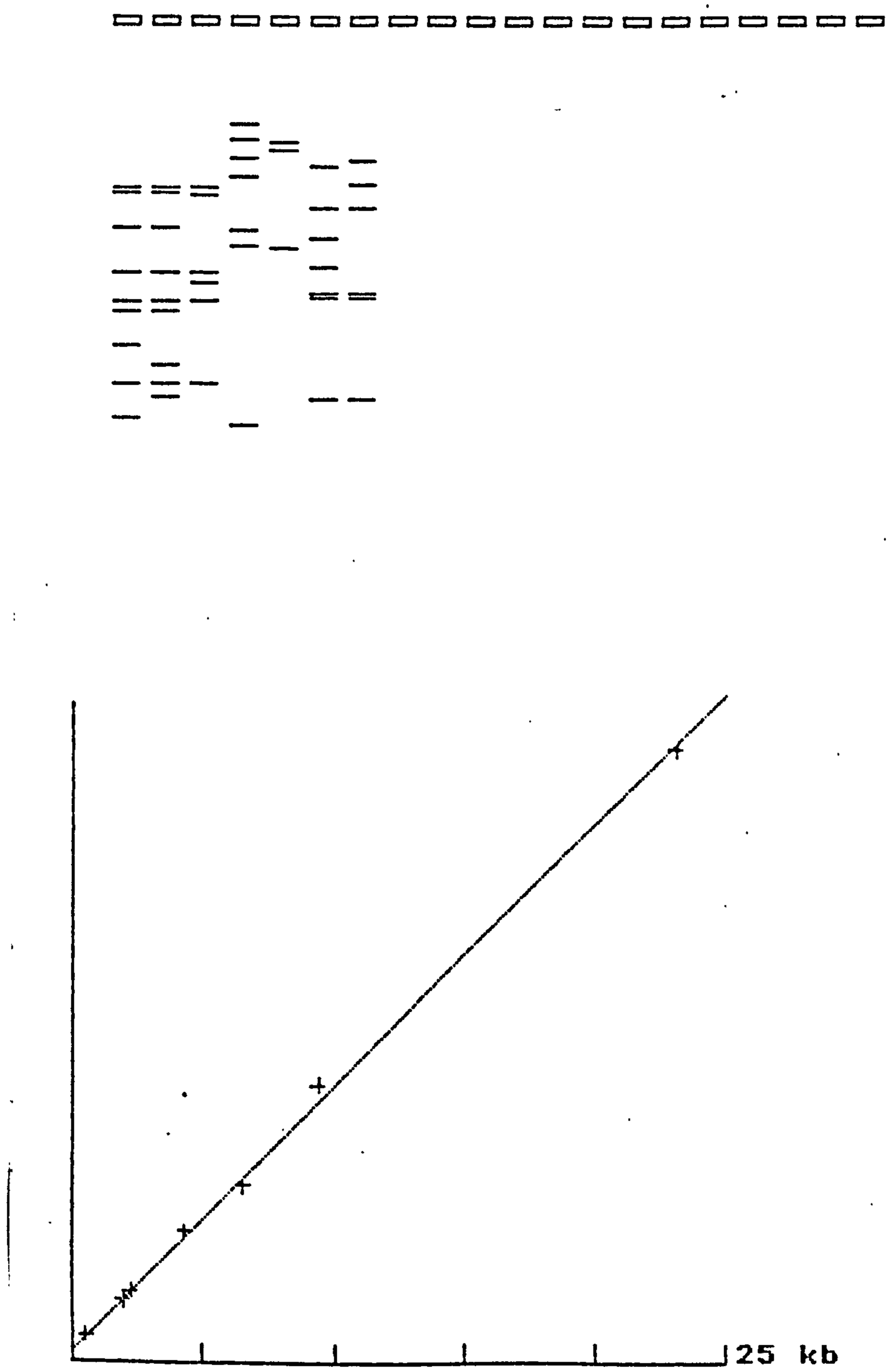
Appendix C Sample Output from the DNAGEL Program

The output resulted from measurements taken from the agarose gel shown in figure 3.4 and was used in the restriction mapping of pSPHd

STD LEN	DIST	PRED LEN	DEV	%DEV
23130.00	12.00	22848.00	282.00	1.20
9416.00	14.00	9975.00	559.00	5.90
6557.00	16.00	6256.00	301.00	4.60
4361.00	17.90	4555.00	194.00	4.40
2322.00	24.10	2279.00	43.00	1.90
2027.00	26.00	1943.00	84.00	4.20
564.00	46.00	580.00	16.00	2.90

BAND SIZES	1	2	3	4	5
	3812	3812	3812	23130	9428
	3497	3497	3455	9416	7374
	2299	2299	1552	6557	1897
	1552	1552	1448	4361	
	1261	1261	1261	2322	
	1170	1170	742	2027	
	924	827		564	
	742	742			
	605	682			
	4138				

BAND SIZES	6	7	8	9	10
	5327	6025			
	2894	3912			
	2091	2894			
	1643	1328			
	1328	1269			
	1269	678			
	678				


25 kb

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