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THE EFFECTS OF THE ENVIRONMENT ON THE STABILITY AND EXPRESSION OF GENETICALLY ENGINEERED PLASMIDS

Submitted by

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TO SARAH, WITH LOVE

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The effects of the environment on the stability and expression of genetically engineered plasmids

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The lac promoter is widely used in plasmid expression systems, even though it is prone to catabolite repression. As a consequence glycerol is often used as an alternative carbon source. Three plasmids containing various sizes of the staphylococcal protein A (SPA) gene, which are under the control of the lac promoter were investigated in continuous culture, to evaluate the effects of nutrient limitation on their stability and expression.

The fears of catabolite repression were dispelled as a low expression plasmid (pPA16) produced a greater amount of truncated SPA under glucose limiting conditions (11 μg mg^{-1} cell protein) when compared to that using glycerol (8 μg mg^{-1} cell protein). Segregational instability was also observed under glycerol limiting conditions at all the dilution rates investigated. Whereas pPA16 was relatively stable under glucose limiting conditions, with SPA production being continuous. Experiments using excess glycerol with limited ammonium increased the stability of pPA16, (when compared to limited glycerol) with expression of SPA being continuous but reduced (6 μg mg^{-1} cell protein). With excess glucose and limited ammonium the copy numbers remained high but expression of SPA paralled that produced under glucose limiting conditions. This might indicate that the higher levels of glucose are reducing expression (catabolite repression) or that the low level of ammonium is affecting protein production.

A high expression plasmid (pPA31) produced nearly 100 μg full length SPA mg^{-1} cell protein, while another high expression plasmid (pPA34) producing truncated SPA proved to be very unstable. An ELISA was developed to detect the SPA produced by these experiments, which could be adapted for western blotting or immunogold probing using electron microscopy. SPA was localised in electron lucent areas present in the periplasmic space of the *E. coli* host harbouring pPA16. While in the same host containing pPA31, SPA was localised not only in electron lucent areas but also around the whole of the outer-membrane.

Key words: Catabolite repression, continuous culture, plasmid stability, protein translocation, staphylococcal protein A.

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ABBREVIATIONS

APS	Ammonium persulphate
bp	Base pairs
BSA	Bovine serum albumin
CAMR	Centre for Applied Microbiology and Research
CAP	Catabolite gene activator protein
CDM	Chemically Defined Medium
CIC	Circulating immune complexes
D	Dilution rate
EDTA	Ethylenediaminetetraacetic acid
F	Flow rate
GARP	Goat anti-rabbit peroxidase
IPTG	Isopropyl-thiogalactoside
Kb	Kilobase pairs
Kd	Kilodaltons
L	Litres
μ	Specific growth rate
μ^{MAX}	Maximum specific growth rate
PAGE	Polyacrylamide gel electrophoresis
par	Partitioning function
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline with Tween 20
Rom	Repressor of primer
SDS	Sodium Lauryl Sulphate
SPA	Staphylococcal Protein A
TEMED	Tetramethylethylenediamine
TSB	Tryptone soya broth
TSBA	Tryptone soya agar
x-gal	5-bromo-4-chloro-indolyl-B-D-galactoside

1. INTRODUCTION

Biotechnology is not a new field in biological sciences: its basic techniques were first used to produce alcoholic drinks and bread by ancient civilisations. However over the past 15 years there have been dramatic breakthroughs with both the introduction of genetic engineering techniques and the development of monoclonal antibody technology. Which have been augmented by the advances in computing and biophysics. These developments have not only lead to a greater understanding of molecular biology but also have brought about a breakdown of barriers between the different disciplines within biological sciences.

1.1 PLASMID TECHNOLOGY

With the advent of DNA technology, vectors for recombinant DNA were soon constructed. One of the first documented cases was in 1977 when Itakura *et al.* cloned the somatostatin gene into a pBR322 based plasmid and obtained expression of the peptide in *E. coli*. This was then followed by the cloning of a range of foreign genes into microorganisms, resulting ultimately in the large scale production of recombinant gene products. Interest in maximising gene expression has stemmed primarily from the involvment of industry in the production of recombinant proteins. Although the genetic construction of plasmid vectors and their effects on gene expression has been described in great detail (down to the last base pair), little is known about the effects of host cell physiology on the expression of recombinant genes.

Initial vectors were designed and constructed from a genetic viewpoint, with little consideration being paid to the physiological consequences of the recombinant product on the host. Problems can arise, for example when constitutive promoters are used to express cloned genes. In some cases the high levels of expression have been found to be deleterious to the host, resulting in an overall loss of production (Caulcott *et al.*, 1985). The attention then paid to the consequences of maximising gene expression resulted in the development of a new generation of vectors which were both controllable and well defined. However, remarkably little is known about the effects on host cell physiology resulting from the expression of recombinant plasmids.

1.2 Categorising plasmids

Plasmids are autonomously replicating circular genetic elements which are found in most Gram-positive and Gram-negative bacteria, and also found in some yeasts and higher eukaryotes (reviewed by Clewell and Helinski, 1969 and Helinski, 1979). They sometimes confer new phenotypes on their hosts and vary in size from 1 to over 200 megadaltons. They can be easily classified by three main properties, replication, conjugation and copy number.

If an attempt was made to construct a strain with two plasmids of identical replication systems, then the two plasmids would rapidly segregate into different progeny. As a consequence plasmids with identical replication systems will not exist in the same cell; such plasmids are said to

be incompatible. Plasmids with different replication systems are able to co-exist and are termed compatible (Novick *et al.* 1976; Cesareni and Banner, 1985).

A second type of classification is whether the plasmids are conjugative or not. This depends upon the presence of the tra or mob genes and also if the plasmid has a bom site. The tra genes promote growth of pili for the transfer of linear plasmid DNA (Williams and Skurray, 1980), while the mobilising (mob) genes are involved in the nicking of the plasmid DNA producing linear forms which can then be transferred to recipient hosts (Willetts and Crowthers 1981; Bagdasarian *et al.* 1981). The bom site is an area on the plasmid which allows the DNA to be nicked and linearised. The loss of the bom site or the mob genes from plasmids would therefore result in the loss of transfer as nicking cannot occur. The loss of the tra genes from plasmids may not have this effect as recipient hosts may be able to form conjugation tubules allowing the transfer of plasmid DNA (Young and Pollis, 1978). Most new plasmid vectors for genetic manipulation are now non-mobilisable to satisfy new legislation on containment.

A third property of plasmids by which they can be categorised, is on the basis of their copy number, which may be high (relaxed plasmids) or low (stringent plasmids). The replication of stringent plasmids is of necessity linked to chromosomal replication, hence their low copy number. Generally conjugative plasmids are of a relatively high molecular weight and are present in low copy numbers per host chromosome, whereas non-conjugative plasmids are of low

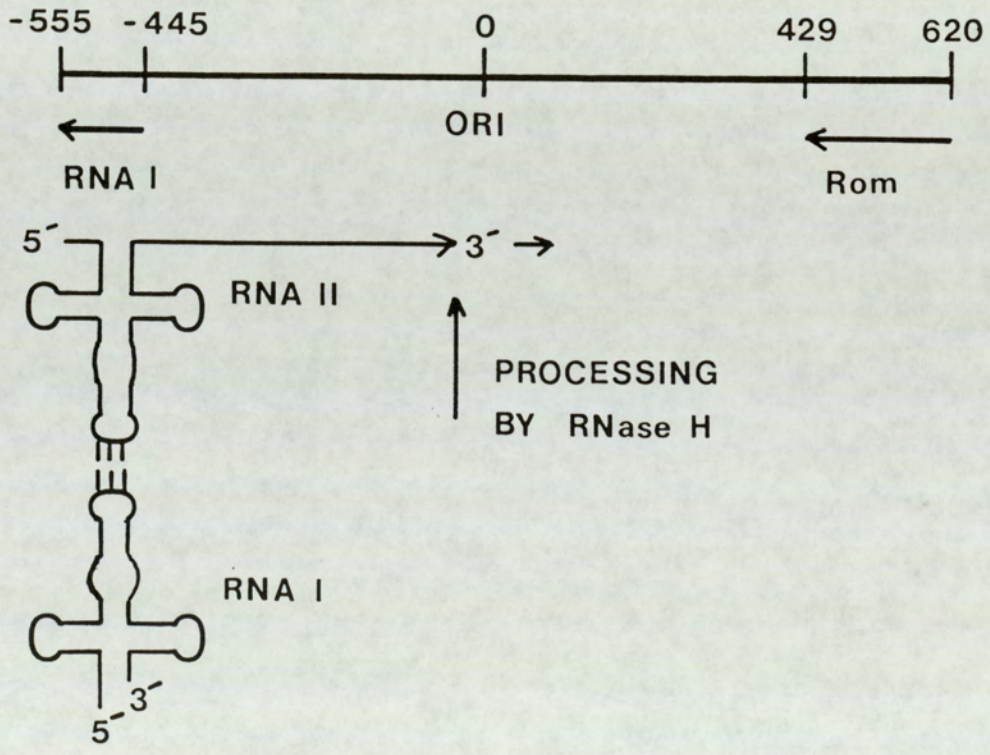
molecular weight and are present as multicopies in the cell (Primrose *et al.* 1983).

1.3 PLASMID REPLICATION

A plasmid can be maintained in the population of a growing bacterial culture only if it is replicated before cell division and partitioned so that each daughter cell receives at least one copy. Most research on plasmid replication has centred on the small, colicin-bearing plasmid of *E. coli* ColE1, which is also the parent of many plasmids including the pUC series, which are used in this study. The replication of ColE1 type plasmids has been shown to be unidirectional from a fixed origin, by Cairns type replication (Figure 1; Inselburg, 1974). The origin of replication spans several hundred nucleotides and codes for a RNA molecule called RNA II, which acts as a primer for DNA replication. The transcription of RNA II is initiated 555 nucleotides upstream from the origin of DNA replication (Itoh and Tomizawa, 1980). As RNAII serves as a primer for DNA replication, it is trimmed by RNAase H, so as to provide a 3' end which lies exactly at the initiation site for DNA replication. In addition a smaller RNA (RNA I) of 108 nucleotides, is transcribed at the opposite site to RNA II and is complementary as termination occurs at the site of initiation of RNA II. On the formation of stem-looped structures the smaller RNA I molecule can bind with RNA II at these complementary points (Tomizawa, 1984). RNA I when complexed to the nascent RNA II transcript, represses primer formation by preventing RNA II from adopting the secondary

Fig. 1 The replication control region of ColE1

The replication region spans several hundred base pairs and codes for RNA II, which often replicates beyond the point of the origin of DNA replication. As RNA II serves as a primer for DNA replication it is trimmed by RNase H to give a 3' end. Rom helps in the binding of RNA I to RNA II which results in the formation of a repressor complex which inhibits processing by RNase H (Cesareni and Banner, 1985).



structure required for primer activity (Wong and Polisky, 1985). This prevents initiation of DNA replication (Lacatena and Cesarini, 1981). The transcription of RNA II is also subject to negative control by a 63 amino acid peptide (Cesareni *et al.*, 1982). This peptide is coded for by a gene 600 nucleotides downstream of the origin called Rom, which when deleted results in an increase in plasmid copy number (Twigg and Sherratt, 1980).

1.4 PLASMIDS USED FOR CLONING IN *E. COLI*

The use of plasmids is now well documented in molecular biology/biotechnology with a wide range now available for cloning, screening and expressing recombinant DNA (Pouwels *et al*, 1985). The ideal properties of a cloning plasmid should be:

1) The ability to replicate autonomously within the host.

2) Their size should be as small as possible, so as to maximise the size of the clonable fragments without affecting plasmid stability (Warnes and Stephenson, 1986).

3) They must possess at least one selectable marker, which confers a new phenotype on the host.

4) A range of single restriction sites should be present with some of these in a marker gene which would be inactivated by the insertion of a cloned fragment.

5) Safety considerations require that plasmid vectors be neither transmissible nor mobilisable thus increasing their containment (see 1.2).

6) Finally, multicopy plasmids are preferable to avoid problems with stability and the subsequent loss of the

plasmid in the absence of selective pressure.

As naturally occurring plasmids do not fulfil all the requirements listed above, artificial plasmids have been constructed, with pBR322 being the first widley used cloning vector (Bolivar *et al.*, 1977). pBR322 is based on the naturally occurring plasmid ColE1 (Kahn *et al*, 1979) and has a copy number of approximately fifty (Hashimoto-Goto and Timmis, 1981). The absence of a partitioning function results in the plasmid being segregated randomly (see 1.7.1). This can cause segregational instability especially when the host is grown under nutrient limitations (Jones *et al.*, 1980) or when used in RecA strains of *E. coli* (Bedbrook *et al.*, 1979). The two plasmid genes cer (Summers and Sherratt, 1984) and Rom (Twigg and Sherratt, 1980; Helmer-Citterich *et al.*, 1988) also affect plasmid stability, with cer causing a recombination event which reduces plasmid multimers to monomers. As pBR322 lacks the cer gene it suffers from multimerisation which effectively reduces the copy number of the plasmid. The Rom gene (see 1.3 and Figure 1) in pBR322, negatively controls plasmid replication to produce a copy number of approximately fifty (Hashimoto and Timmis, 1981). Consequently pBR322 has been shown to suffer segregational instability (Jones and Melling, 1984); this may be attributed to the absence of a partitioning function, to multimerisation and to the fact that the copy number is regulated. Successive pBR plasmids have included the insertion of partitioning functions to help stabilise the plasmid, others have incorporated chloramphenicol resistance markers, the loss of the mob genes and

additional restriction sites including those for blunt end ligation (for a review see Balbas *et al*, 1986). pAT153 is a well known derivative of pBR322 (Twigg and Sherratt, 1980), with a 705 base pair deletion resulting in the loss of the Rom and mob genes making it comparatively stable (Jones and Melling, 1984) and non-mobilisable.

A new generation of plasmids were the pUC vectors (Vieria and Messing, 1982), which like many others they were derived from the pBR family. They are non-mobilisable and have a *B*-lactamase gene conferring ampicillin resistance. They also contain part of the lac operon, which includes the operator, promoter and part of the lac Z structural gene. There are a number of restriction sites in the structural gene, which allows the cloning of DNA fragments. Expression of any cloned material is therefore under the control of the strong lac promoter, with the production of a fusion protein and the subsequent loss of *B*-galactosidase activity. If the host organism is also deficient in *B*-galactosidase activity (i.e. *E. coli* strains JM101 and JM83), then growth on media containing the substrate 5-bromo-4-chloro-indolyl-B-D-galacto- side (X-gal) and inducer isopropyl-thiogalactoside (IPTG) result in clear colonies. In contrast those plasmids without inserts produce blue colonies, due to the action of the B-galactosidase on the substrate (Miller 1972). This then allows positive screening of the pUC vectors for the insertion of any cloned DNA.

1.5 PLASMIDS USED FOR EXPRESSION IN *E. COLI*

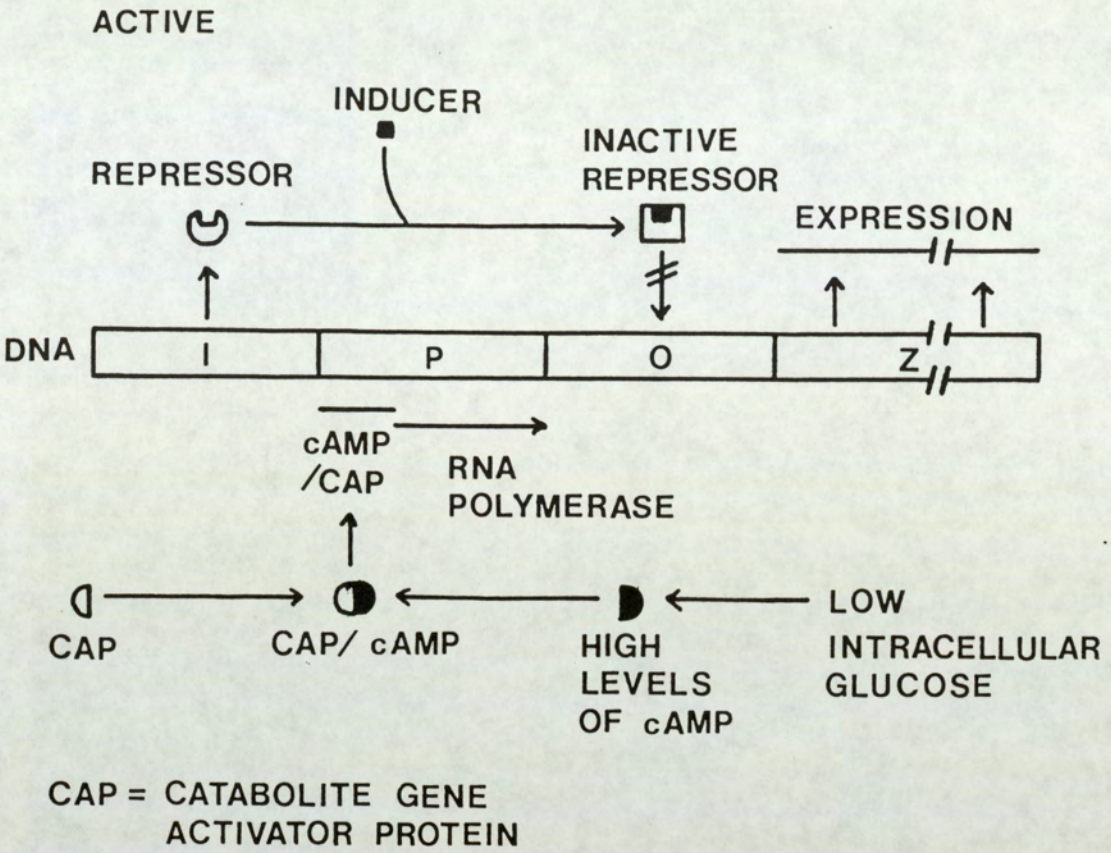
Initially no expression vectors were available and as a consequence marker promoters of cloning vectors were unintentionally used for expression (e.g. Tol genes in pBR322, Inouye *et al.* 1981; proinsulin in pBR322, Villa-Komaroff *et al.* 1978). However these promoters are unregulated and as a result expression may be deleterious to the host. The pUC plasmids have also been employed as expression vectors, producing lac Z fusion peptides when in phase with the reading frame. The promoter can also be regulated by induction with lactose or IPTG or repressed by the lac I gene product. Over the last five years a range of expression vectors have been developed with many being derived from pBR322 (for a review see Balbas *et al.* 1986). Due to the number of expression vectors available they are classified according to their main promoter, as described below.

1.5.1 lac promoter

The lac promoter was one of the first promoters used in expression vectors and is also present in the pUC family. These plasmids allow the expression of fusion proteins, provide a positive selection of recombinants (Norrander *et al.* 1983), have the potential for nucleotide sequencing (Hanna *et al.* 1984) and site directed mutagenesis of DNA fragments (Yanisch-Perron *et al.* 1985). There are a number of other vectors using the lac promoter which are also described by Pouwles *et al.* (1985). A major drawback when using the lac promoter in expression vectors is the effects

of catabolite repression. This results in loss of expression due to excess intracellular glucose which causes the levels of cAMP to fall (Pastan and Perlman, 1969; Figure 2). The low levels of cAMP results in the loss of formation of a catabolite gene activator protein (CAP)/AMP complex which is used to bind the promoter aiding binding of RNA polymerase which is required for transcription. As a consequence glycerol is often used as an alternative carbon source to avoid repression. Totally independent of catabolite repression are the effects of the lac I regulator gene, which produces a repressor protein. The repressor protein prevents readthrough by binding to the operator gene (Miller and Reznikoff, 1980). Total repression is unobtainable in high copy number plasmids as there are not enough repressor molecules produced to switch off the numerous lac genes (Windlass *et al.* 1982). The lac I gene is missing from *E. coli* JM83 and also from the pUC vectors, this leaves expression switched on, although the operon is still vunerable to catabolite repression. An alternative is to use the lac UV5 promoter which is widely quoted to be free from repression. Silverstone (1970) first isolated the lac UV5 promoter, from the wild type *E. coli* strain 800. Using a scale of one to a hundred with regards to *B*-galactosidase activity, the values obtained from the wild type were 100 when using glycerol as the carbon source and 17 when using glucose as the carbon source. The lac UV5 mutation gave a value of 56 when using glycerol as the carbon source and 55 using glucose. Further work showed that even glycerol can cause catabolite repression albeit

Fig. 2 REPRESSION OF THE LAC Z GENE



CAP = CATABOLITE GENE
ACTIVATOR PROTEIN

REPRESSED FORM

In the presence of glucose the levels of intracellular cAMP will fall, producing a loss of formation of the cAMP/CAP complex which helps initiate RNA polymerase binding, resulting in loss of expression. If the lac I gene is present and an inducer is absent then the repressor protein will bind to the operator thus reducing expression.

five times less than glucose (Wanner, 1978). To summarise the lac UV5 promoter is often used and quoted as a promoter which does not suffer catabolite repression. Whereas Silverstones original work shows that the lac UV5 is about fifty percent efficient when grown in a medium with glucose compared to the wild type grown in glycerol. As glycerol itself causes repression then the full extent of maximal expression from the lac promoters may not have been realised.

1.5.2 trp promoter

The *trp* promoter is also controllable with many vectors producing fusion proteins. There are five structural genes (A-E) which are responsible for the biosynthesis of tryptophan (Yanofsky *et al.* 1981). They are negatively controlled by the trp R gene, the product of which binds to the operator region in the presence of high levels of tryptophan, resulting in repression. When grown in low levels of tryptophan or in the presence of inducers (Squires *et al.* 1975) expression will be high. A way to increase repression would be to clone the repressor on the plasmids (Gonsalus and Yanofsky, 1980). A series of trp vectors with three possible reading frames were constructed (Tacon *et al.* 1980), which were widely used and included the expression of influenza surface antigens (Emtage *et al.* 1980) and urogastrone (Smith *et al.* 1982). Another series of trp plasmids called pWT were then produced which allowed further use of restriction sites in the correct orientation (Tacon *et al.* 1983).

1.5.3 tac promoter

The tac promoter is a hybrid constructed from the -35 region of the trp and the -10 region of the lac promoter. The resulting promoter has a transcriptional strength ten times higher than the parent lac promoter (DeBoer *et al.* 1983). The distance between the two conserved regions may also be responsible for the increase in expression, as the tac has 16 base pairs which not only differs from the trp (17) and the lac promoter (18) but is also the distance found in strong ribosomal RNA promoters. As the lac operator region is also present then expression can be controlled as described in section 1.5.1. This is of particular importance as extremely high expression requires regulation so as to avoid problems with toxicity which may lead to the loss of the plasmid and a fall in production (Brosius, 1984). The first vectors employing the tac promoter were used to express the phage λ cI repressor protein (Amann *et al.* 1983). The most efficient over-producers contained the lac Z ribosome binding site fused to the ATG of the cI gene. This proved to be important in the initiation of translation (see 1.6.3) and was consequently developed into a new plasmid series, which was used to express the Herpes simplex virus type I glycoprotein D gene (Amann and Brosius, 1985), human coagulation factor XIII and human anti-coagulant protein PP4 (Amman *et al.* 1988).

1.5.4 λP_L promoter

The λP_L promoter unlike those mentioned above is

regulated by temperature rather than by the addition of specific inducers or inhibitors. To switch off the promoter the bacterial host must contain a mutated repressor (cI) gene which renders the repressor thermolabile. At 32°C the repressor functions normally, blocking transcription, whereas at 41°C the repressor is inactivated allowing transcription. A temperature shift is looked upon more favourably by biotechnologists as there is minimal interference with the culture in the control of expression (Remaut *et al.* 1981).

1.6 FACTORS AFFECTING EXPRESSION

1.6.1 Promoters

Promoters are required for the initiation of transcription for the expression of any gene and DNA sequencing of *E. coli* promoters has revealed structural similarities which are now regarded as characteristic features (Rosenburg and Court, 1979; Hawley and McClure, 1983). There are two highly conserved regions at the -35 5'-TTGACA- (Schaller *et al.* 1975) and -10 5'-TATAAT- (Pribnow, 1975) positions upstream of the initiation of replication. To measure their strength various promoters were cloned upstream of the structural gene for galactokinase (Russell and Bennett, 1982; DeBoer *et al.* 1983). The levels of galactokinase expression indicated that promoter strength is directly proportional to the degree of similarity between the concensus sequences and the distance between them. The promoters most frequently used in expression systems are the

lac, trp, tac, and λP_L promoters, all of which can be regulated so as to minimise the effects of toxicity on the host. For optimal levels of expression the selection of a promoter depends not only on the strength but the type of regulation required, the availability if using a series of plasmids and also the host organism. The use of promoters in various hosts is of particular importance as certain plasmids are required as part of a particular screening system (i.e the pUC vectors).

1.6.2 Transcriptional terminators

Transcriptional terminators are required to prevent readthrough, especially when the gene is under the control of a strong promoter (Stueber and Bujard, 1982). The precise termination at the end of a cloned gene also prevents long transcripts being made which may interfere with the folding and structure of the protein (Gentz *et al.* 1981).

1.6.3 Translation

Protein translation involves the binding of mRNA to the ribosome which is the rate limiting step in protein synthesis. This binding can be affected either by increasing the levels of mRNA or by raising its binding capacity for the 16s ribosomal subunit. The levels of mRNA transcripts can easily be increased simply by using high copy number plasmids. Although many plasmids have high copy numbers this could produce a heavy energy drain on the host which may lead to plasmid instability. An alternative

expression system could use temperature sensitive mutants which when switched on increase the copy number by several hundred fold (Uhlin and Nordstom, 1977; Sninsky *et al.* 1981). This expression system would be of use in batch or fed-batch systems as well as in coupled continuous culture systems. The binding of mRNA to the 16S ribosomal subunit is dependent upon a number factors, including the comparison of the recognition or Shine-Dalgarno site (Shine and Dalgarno, 1975) to the 5' ACCUCC 3'sequence located at the 3' end of the 16S subunit (DeBoer *et al.* 1983). Other factors are important, including the initiation site for translation which is usually AUG in *E. coli*, with GUG being less common. The distance between the Shine-Dalgarno and initiation site is also crucial, with 7-9 base pairs being the optimal distance. Small changes in this distance can lead to a hundred fold increase in translational efficiency (Shepard *et al.* 1982). These increases in efficiency have been related to changes in the mRNA secondary structure, which may allow the ribosome easier access to the Shine-Dalgarno and AUG regions (Wood *et al.* 1984). Finally an initiation complex is required which includes an initiation tRNA, mRNA and the ribosomal subunits to form a peptide bond with the incoming aminoacyl-tRNA during the elongation phase of protein synthesis (for a review see Maitra *et al.* 1982).

1.6.4 Codon selection

Codon selection is also important as the genetic code comprises 64 codons, 61 of which specify 20 amino acids

while three codons (UAA, UAG and UGA) are used as termination signals. Only two amino acids are coded for by a single triplet, the remaining eighteen being specified by at least two codons. With the corresponding tRNA's having different specificities for each codon in different hosts. A codon change resulting in the same amino acid may then have differing effects on the production of a protein depending on the host used (reviewed by Grosjean and Fiers, 1982; Gouy and Cautier, 1982).

1.6.5 Copy number

Another factor affecting expression of a recombinant protein is the copy number of the plasmid. Raised copy numbers will increase the levels of the gene and consequently the levels of expression. This has been used in runaway replication vectors, where at low temperatures the copy numbers are small while above 35°C the levels can reach 2000 copies per chromosome (Uhlin *et al.* 1979; Sninsky *et al.* 1981). One major problem with the measurement of copy numbers is the inaccuracy of the methods avialable, the problems arising with these methods are highlighted by Projan *et al.* (1983). However, the method used in this thesis (see 2.2.11.9) was chosen because of the ease with which large numbers of samples could be handled. Factors affecting copy number are also discussed in section 1.7.4.

1.6.6 Plasmid stability

Plasmid stability is not usually a problem in research as antibiotic resistance conferred by selection markers

should ensure no plasmid free cells arise in the population (although toxicity of the product can be a problem). In industry, however, plasmid stability can be a serious problem as antibiotics may not be as effective in large scale and are also not desirable due to the costs involved in removal and disposal. There are two types of plasmid instability; segregational which is the loss of the plasmid on cell division, which is discussed in section 1.7.1 and structural instability which may be caused by deletion, addition or rearrangment of the genetic material and is discussed in section 1.7.5.

1.6.7 Protein stability

Protein stability is a problem as degradation of the recombinant products can occur, especially when nitrogen or carbon sources are limited. Eight different protease activities have been discovered although they may vary from host to host (Swamy and Goldberg, 1982). One of the proteases la is coded by the lon gene, and hosts with mutations in this gene have been used to protect recombinant proteins from proteolysis (Buell *et al.* 1985). The use of fusion peptides may also prevent degradation, as demonstrated with somatostatin when linked to *B*-galactosidase (Simmons *et al.* 1984; Schoner *et al.* 1985). Although the formation of inclusion bodies may have afforded some protection, this is discussed further in section 1.12.

1.6.8 Host cell physiology

Once expression has been attained host cell physiology is often the last area to be investigated. Factors such as the choice of nutrients, temperature, pH or oxygen tension can greatly influence the levels of expression and should not be overlooked (Carrier *et al.* 1983). This area has also been discussed in sections 1.7.3.

1.6.9 Selection markers

The use of selection markers, especially those conferring resistance to antibiotics are routinely used in both cloning and expression vectors. Their use however may be detrimental to the production of recombinant proteins in a number of ways. The use of the tetracycline resistance gene has been shown to be deleterious to the host under certain growth conditions (Lee and Edlin, 1985). *B*-lactamase markers may also cause problems especially when the recombinant protein is localised in the periplasmic space. This may be due to competition for transfer into the periplasmic space, resulting in loss of production. Indeed Chambers *et al.* (1988) have shown that removal of the *B*-lactamase gene can result in a 30% increase in carboxy-peptidase production. Pridmore (1987) has also shown that substitution of the ampicillin resistance gene in pUC18 with a kanamycin resistance gene results in an elevated copy number. The use of minimal antibiotic-resistance markers have also been used by Panayotatos (1988).

1.7 PLASMID INSTABILITY

Even on obtaining expression the genetic load imposed by the plasmid may well lead to plasmid free cells arising. If a mutant arises which has either lost the recombinant plasmid or has undergone structural rearrangement so that the recombinant gene is no longer expressed or has a reduced copy number, then this will have a faster growth rate and may quickly take over and become the predominant organism. There are two main areas of plasmid instability, segregational which is due to defective partitioning and structural which may arise by deletion, insertion or rearrangement of the plasmid DNA.

1.7.1 Segregational instability

Segregational instability can occur due to the lack of a par function, a reduction in copy number or toxicity of the product. In naturally occuring plasmids with low copy numbers (e.g. IncFII, Timmis, *et al*. 1981 and pSC101 Meacock and Cohen, 1980) the presence of a par function usually ensures that they are actively segregated at cell division, thus avoiding segregational instabiltiy. The higher copy number plasmid, CoLE1, and many other genetically engineered plasmids do not contain a par function and are segregated randomly. It has been sugested that random partitioning may be sufficient to ensure the stability of multicopy vectors and that active partitioning may not be essential (Summers and Sherratt, 1984). Obviously a reduction in the copy number of a plasmid without par could lead to segregational instability. A range of factors can influence the copy

number of a plasmid including, nutrient limitation, an increase in the hosts growth rate, an increase in the genetic load imposed upon the hosts replication system, an increase in plasmid multimers or the protein produced on expression.

1.7.2 Partitioning functions

Partitioning functions help to ensure each daughter cell retains a plasmid. Those without a partitioning function are therefore segregated randomly and the probability P(o) of either daugher cell failing to inherit a plasmid is given by a binomial distribution:

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

Where c is the number of plasmid molecules per cell (copy number) at division. In fact Cooper *et al.* (1986) have taken the mathematical modelling a stage further, to produce a formula which will indicate whether the instability of a plasmid is due either to the loss of the plasmid on segregation or to differences in the growth rates between cells with or without the plasmid. This may be of limited value in predicting the area of instability of particularly unstable plasmids, although far greater efforts may be needed to cure instability. Although the copy number of pBR322 is known to be high (approximately 50 per host chromosome in *E. coli* HB101; Hasimoto-Goto and Timmis, 1981), there is a probability of plasmid free cells arising under certain conditions such as nutrient limitations or during rapid cell growth (Jones *et al.* 1980; Nugent, 1983). This problem can be prevented by continually selecting for the phenotypic

traits exhibited by the plasmid. However this may not be a desirable solution for large-scale cultures because of the costs of producing a product free from contamination and the problems involved in waste disposal. Partitioning regions have been cloned into unstable plasmids such as pBR322 (Primrose *et al.* 1983) thus enhancing their stability. Although par functions are often used to overcome problems with instability, the extent of curing may be variable (Nilsson and Skogman, 1986). Even though par is commonly used in plasmid constructions, little is known about its mode of action. It does not code for any proteins or contain any transcription or translation start signals. There are areas of the par locus which are capable of forming regions of intrastrand secondary structures and these may play some role in partitioning (Miller *et al.* 1983).

1.7.3 Effects of nutrient depletion

Melling *et al.* (1977) were one of the first groups to investigate the effects of nutrient limitation on plasmid stability while working on the RP1 plasmid in *E. coli* W3110. The results showed the plasmid to be stable at various dilution rates (0.05 to 1.0 hr-*1*) under nutrient limitations of carbon, magnesium and phosphate. These results were later confirmed showing there was no loss of antibiotic resistance under prolonged chemostat runs (Jones *et al.* 1980). As the copy number of RP1 was only 1-2 per cell then a par function was thought to be involved in the plasmids replication (Nordstrom *et al.* 1980). However, Klemperer *et al.* (1979)

found plasmid free cells arose after three months storage at 37°C. They also discovered that the specific growth rate of cells without the plasmid (R-) was double that of cells containing the plasmid (R+) under low phosphate concentrations in batch culture. Similar results were shown in competition experiments where R+ cells were out-grown by a 1% inoculum of R- cells in phosphate limited chemostats (Melling *et al.* 1977). Converse experiments always showed R- cells came to predominance under phosphate limiting conditions, whereas the outcome of other limitation studies relied only on the size of the inoculum of R+ cells.

Further studies showed plasmid free segregants of pBR322 (par minus) arose under glucose or phosphate limiting studies in continuous culture, which correlated with a drop in copy number after 30 generations (Jones *et al.* 1980; Wouters *et al.* 1980). An increase in the stability of pBR322 (to 240 generations) was seen with an increase in dilution rate. Since nutrient restriction is less severe at higher dilution rates then it may take many more generations before plasmid free segregants arise. Similar results were obtained with decreases in temperature, the plasmid being far more stable at 30°C than 37° or 42°C. In contrast results by Noak *et al.* (1981) found no loss of pBR322 from *E. coli* grown in glucose or nitrogen limiting conditions. The reasons for these conflicting results could be due to variations in the genetic backgrounds of the host organisms used by the different workers. In fact, variations in strains of bacteria have been observed at CAMR (Sharp *et al.* 1988) in the large scale production of genetically

engineered products. Recently Sayadi *et al.* (1988) have shown an increase in the stability of pBR322 to 240 generations when the host was immobilised. However, the abstract fails to note which nutrient was limiting, with the medium consisting of tryptone yeast extract and 0.1 M KCl.

1.7.4 Copy number and plasmid size

Plasmid stability can be affected by changes in copy number as shown by the stability of pAT153, which is a derivative of an unstable plasmid pBR322 (Jones and Melling, 1984). The reason for this increased stability may be due to the increase in copy number of pAT153 to pBR322 (Twigg and Sherratt, 1980), or the fact that too few generations had elapsed before segregants could be detected. Other workers have also found pAT153 to be stable (for 90 generations; Caulcott *et al.* 1985), although Chew *et al.* (1986) found pAT153 to be unstable (30 generations) at low dilution rates under all nutrient limitations, but stably maintained (90 generations) at high dilution rates (0.3 - 0.5 hr^{-1}) in glucose limiting media. In fact if the tetracycline resistance gene of pAT153 was inactivated by the introduction of an insertion element (IS1) from the chromosome the plasmid could then be stably maintained at low dilution rates under glucose limitation. This is in agreement with Warnes and Stephenson, (1986) who investigating the effect of various sizes of cytomegalovirus DNA inserted into the tetracycline resistance gene of pAT153, found the plasmid with the smallest insert to be stably maintained for 80 generations at varying dilution rates (0.1

- 0.4 hr^{-1}). The plasmids with the larger inserts of DNA became increasingly unstable and the maximum specific growth rate of the host organism was also greatly reduced with this increase in the size of the plasmid. This indicates that an increased load imposed upon the cell could instigate plasmid instability. The plasmid ClODF13 was shown to be stably maintained at a copy number of 10 in *E. coli* by a competitive process at the level of transcription, with segregation being influenced by plasmid size and copy number (Hakkaart *et al.* 1983). They also showed that the effect of copy number was dominant over plasmid size, in causing instability.

Finally a sequence of DNA was discovered in CoLE1 called cer which causes recombination to occur which reduces plasmid dimers or multimers to monomers (See 1.3; Summers and Sherratt, 1984). This is extremely important in plasmids lacking a partitioning function as an increase in multimers effectively reduces the copy number of the plasmid which could then affect stability. The cer region has also been cloned into pBR322 thus increasing stability. The same workers also discovered a region in the plasmid DNA called Rom (See 1.3), which reduces plasmid copy number this could again lead to plasmid instability.

1.7.5 Structural instability

The second type of instability is called structural instability and may be caused by deletion, addition or rearrangment of the genetic material. There are numerous examples in the literature of structural instability of

recombinant plasmids (Cohen *et al.* 1977; Timmis *et al.* 1978; Imamaka *et al.* 1980) but few reports of these changes in continuous culture (Goodwin and Slater, 1979). Although pAT153 has been shown to be prone to structural instability in continuous culture, with deletions and insertions occuring in the tetracycline resistance gene with host grown under a number of nutrient limitations and at various dilution rates (Chew *et al*, 1988; Brownlie *et al.* personal communications).

1.8 CONTINUOUS CULTURE

The chemostat is an invaluable tool in studying the stability of recombinant plasmids. Batch cultures have many disadvantages including shifts in nutrient depletion, and the accumulation of toxic biproducts, which lead to changes in cell numbers. With bacteria in batch culture subjected to a constantly changing environment the kinetic analysis of plasmid loss would be impossible to interpret. As chemostat culture does not suffer these disadvantages, the steady state environment allows the study of segregational instability. Chemostat culture comprises an open system where fresh media is constantly supplied, with excess culture constantly removed by a weir (Herbert, 1956), establishing a so called "Steady State" (Van Hemert, 1974). The theoretical basis for continuous culture was first described by Monod, (1950) and by Novick and Slizard (1950), with later accounts by Herbert, (1956) and Tempest, (1974). Where a culture is at a constant volume (V) in steady state, the dilution rate (D) will be equal to the specific growth

rate (μ).

$\mu = D = F/V$ Where F is the flow rate

The importance of defining a synthetic medium becomes apparent as changes in the limiting nutrient will affect the growth rate of the organism. If the bacterial concentration increases then the limiting nutrient will decrease resulting in a decrease in growth rate. This will decrease the population until a new steady state is achieved. The growth rate can then be calculated from the following equation:

$\mu = D = \ln 2/t_d$ where t_d is the doubling time

Increasing the dilution rate will increase the specific growth rate of the organisms, up to a point at which the growth rate will no longer keep pace with the dilution rate. This is termed the maximum specific growth rate (μ^{max}) at which point the organism will be "washed out".

1.9 EXPRESSION OF RECOMBINANT PLASMIDS IN CONTINUOUS CULTURE

Although gene cloning techniques involving the expression of prokaryotic and eukaryotic genes in bacteria are now commonly used, the results of expression are quite varied. Few workers comment on the lack of expression of their vectors and little data has been published on continuous culture experiments. Total lack of expression may occur (the expression of pertussis antigens in *E. coli*: Dr. Duggleby, CAMR, personal communications). In plasmid vectors, the reasons for this have been explained earlier (See 1.6). Some workers have experienced great instability with plasmids, (e.g. the loss of pUC8 based

plasmids expressing Lassa fever glycoproteins: Dr. Clegg CAMR, personal communications) with loss occurring after two or three subcultures, even with the aid of selection. Nevertheless, enough protein may be produced for further research work to continue. High level translation of the recombinant gene, calf prochymosin was also shown to cause plasmid instability (Caulcott *et al.* 1985). This was because low copy number plasmids with high expression and high copy number plasmids with a defect in translation were both stably maintained. Nilsson and Skogman (1986) found that high copy number plasmids expressing tryptophan were unstable and they developed strategies to stabilise these production vectors. The use of a par locus RP1 did not prevent loss of the plasmid (60% loss in 50 generations) neither did the presence of the par locus from pSC101 (95% loss in 60 generations), however, both recombinant plasmids did reduce instability compared to the initial construct. The use of the valyl-tRNA synthetase gene (which selects for plasmid containing cells) did produce stability (100% stability after 150 generations) and also produced segregational stability. The use of the valyl-tRNA transferase gene should be applicable in other *E. coli* plasmid vector systems and thereby be of considerable advantage for stability in large scale cultures.

1.10 DETECTION OF RECOMBINANT PROTEINS

The detection of recombinant products is dependent on the protein and its level of expression. The most straightforward method available, is to visualise the protein on a SDS-PAGE gel, according to their apparent molecular weight. However, *E. coli* proteins may interfere with the detection of recombinant gene products. This interference may be reduced by western blotting or radioimmune precipitation which involves probing the gel or the proteins transferred to nitrocellulose with specific labelled antibodies. Other methods available for reducing interference include pulse labelling with ^{35}S- methionine (following expression of the recombinant gene by an inducible promoter), or by using the mini- or maxi-cell techniques (Reeve, 1979; Sancar, *et al.* 1981). The former involves the production of mini-cells which contain plasmid DNA but no chromosomal DNA. This results in translation depletion of normal host proteins leaving only those encoded by the plasmid. The latter uses specific hosts with mutated DNA repair mechanisms, which in conjunction with ultra-violet light inactivates the chromosomal DNA, leaving the high levels of plasmid DNA relatively intact. Both of these methods reduces the proteins encoded by the chromosomal DNA resulting in a reduction of background interference.

Although these methods give an indication of size, they cannot detect low levels of expression, or determine whether the protein is in its native form. Immunological techniques are important in confirming the identity of the expressed

proteins. Epitopes can be be detected using either monoclonal or polyclonal antibodies, which can indicate any major changes in structural organisation. A variety of methods can be used to monitor expression such as ELISA, radioimmune assays, immunofluoresence, immunoblotting and immunodiffusion. The immunological assays are also employed to screen large numbers of colonies for the expression of recombinant proteins, and are also particularly useful for epitope mapping (Broome and Gilbert, 1978). The methods mentioned above may be redundant if a biochemical assay is available to measure the activity of the recombinant product, such as *B*-lactamase (Sutcliffe, 1979). Complementation of mutants in the host chromosome is another method of detecting expressed proteins as shown with *B*-galactosidase. However, in the detection of recombinant proteins these last two methods are rarely available.

1.11 STAPHYLOCOCCAL PROTEIN A

1.11.1 The structure and function of native SPA

Forsgren (1966) first described the strucuture and function of native SPA when he demonstrated SPA's ability to bind certain immunoglobulins at the Fc-region. Its use has increased dramatically, playing a crucial role in the advancement of both immunology and immunochemistry. Hjelm *et al.* (1972) then isolated protein A from *S. aureus* using IgG-sepharose 4B, and went on to use SPA coupled to sepharose as an immunosorbent. With a series of localisation studies it was concluded that SPA was covalently bound to the peptidoglycan moiety of the cell wall of *S. aureus* (Sojquist, 1972), while sedimentation analysis and gel chromatography on SPA revealed a molecular weight of 42000 (Bjork *et al.* 1972). Hydrodynamic studies gave a frictional ratio of 2.1-2.2 and an intrinsic viscosity of 29 ml/gm. The combination of these parameters suggest that SPA is not a typical globular protein, but has a markedly extended shape which indicates it exists in a rod form. Further SDS-PAGE analysis of protein A from various strains of staphylococci also revealed molecular weights of 45 - 57 Kd (Cheung *et al.* 1987). A number of IgG binding regions were discovered which were arranged consecutively from the N-terminal end of the protein. These IgG binding regions were highly homologous and contained 52-62 amino acid residues, which could be cleaved with trypsin. Figure 3A shows the envisaged structure of SPA as attached to the cell wall of

Fig. 3A

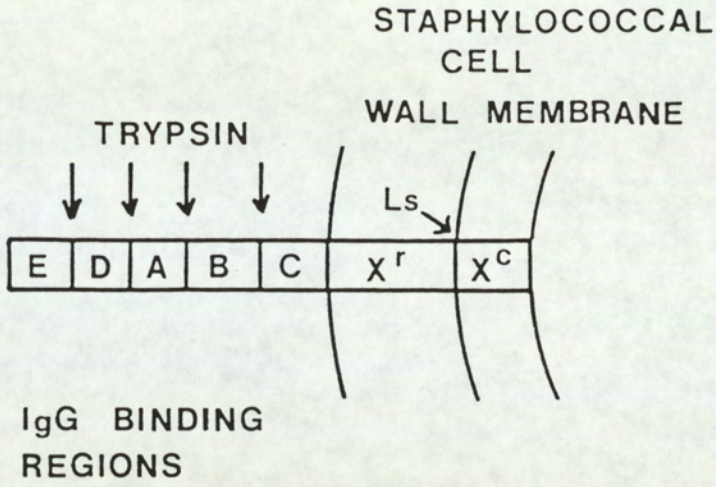

STAPHYLOCOCCAL
CELL
WALL MEMBRANE
TRYPSIN
Ls
E
D
A
B
C
X^r
X^c
IgG BINDING
REGIONS

Fig. 3B

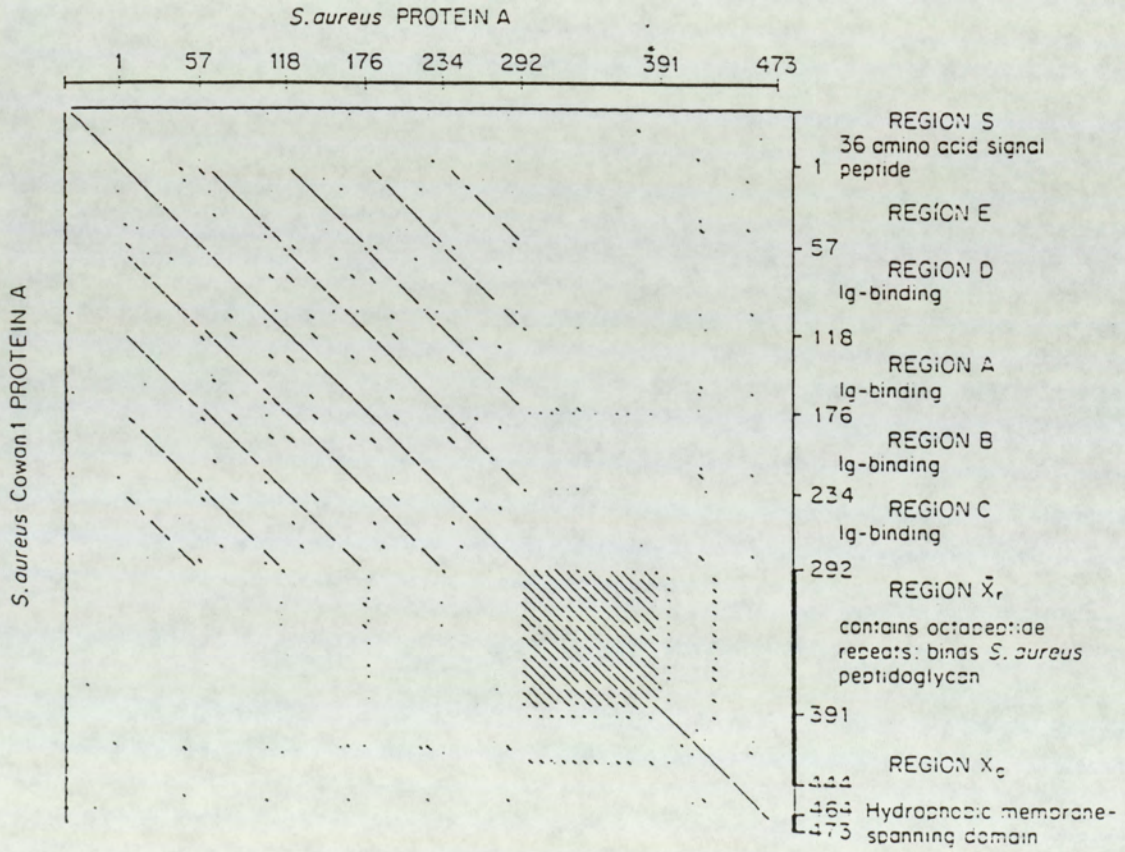

S. aureus PROTEIN A
1
57
118
176
234
292
391
473
S. aureus Cowan 1 PROTEIN A
REGION S
36 amino acid signal peptide
1
REGION E
57
REGION D
Ig-binding
118
REGION A
Ig-binding
176
REGION B
Ig-binding
234
REGION C
Ig-binding
292
REGION $\bar{X}_r$
contains octapeptide repeats: binds S. aureus peptidoglycan
391
REGION X_c
464
473
Hydrophobic membrane-spanning domain

Fig. 3A Binding of SPA to *S. aureus*

SPA binds the staphylococcal cell wall and membrane at the peptidoglycan (Xc) and membrane (Xr) binding regions, leaving the IgG binding regions (A-E) protruding into the surrounding environment. Cleavage of SPA with trypsin and lysostaphin (Ls) are also indicated.

Fig. 3B Dot matrix analysis SPA

The dot matrix analysis is of Cowan I SPA at both axes. Five amino acids of the horizontal axis were sequentially compared with segments of the vertical axis and stored using a matrix. In the above plot a point was plotted when three or more amino acids matched with a span of five.

S. aureus, with the appropriate cleavage sites. Due to the high homology of the IgG Fc binding regions, separation proved difficult. Because of this and studies using N-terminal amino acid sequencing (Edman degradation) it appears that there were four Fc binding regions (A-D) at the N-terminal end of the protein. The residual C-terminal end of the protein (region X) was found to be 150 amino acids long and was suggested to be the peptidoglycan binding region.

In 1983 the gene coding for SPA was cloned into plasmid vectors and expression obtained in an *E. coli* host (Lofdahl *et al.* 1983; Duggleby and Jones, 1983). The work by Lofdahl proved to be crucial in that they were able to perform partial DNA sequence analysis of the cloned gene, which revealed some important facts. Initially a region with the general features of a prokaryotic signal peptide (region S) was revealed. Although this was larger than the *E. coli* signal peptides by some ten amino acids it was still recognised by the membrane, as localisation experiments showed the presence of SPA in the periplasmic space. A promoter region was discovered which appeared before the signal peptide as shown in Figure 4. Little is known about this region although it is recognised by *E. coli* as well as *S. aureus*. A fifth structural region was also discovered at the N-terminal end of the protein which comprised of 50 amino acids and was referred to as region E. The full structure of SPA was therefore revealed using DNA sequencing on the cloned gene (Uhlen *et al.* 1984). The DNA sequencing not only indicated the presence of region E and S, but also

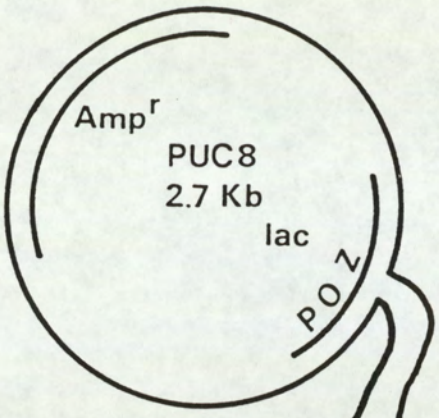
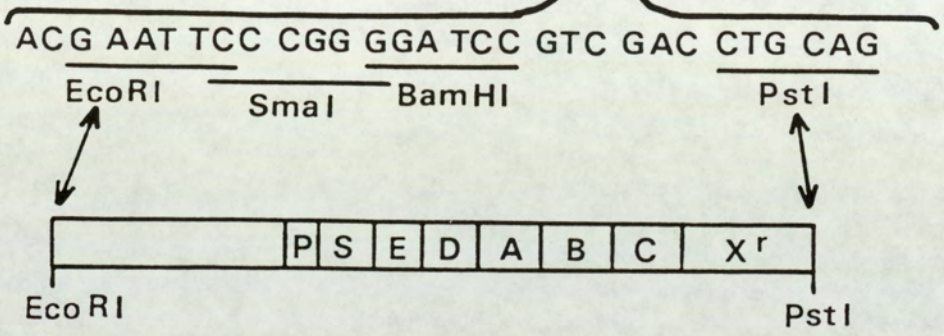
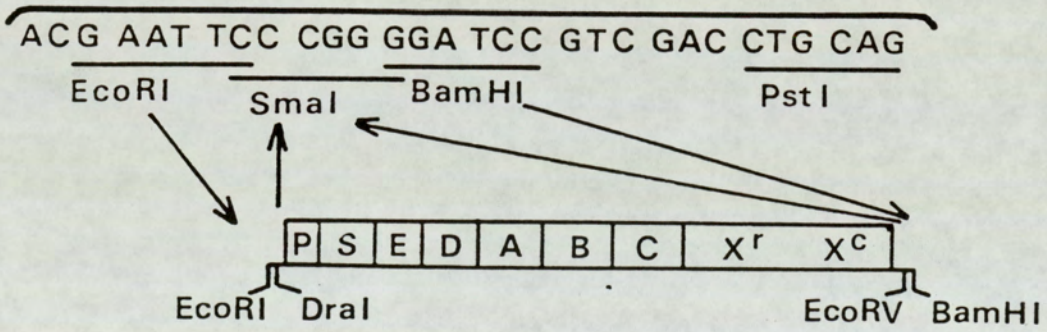

Amp r
PUC8
2.7 Kb
lac
P O Z

Fig. 4A


ACG AAT TCC CGG GGA TCC GTC GAC CTG CAG
EcoRI
SmaI
BamHI
PstI
pPA 16
P S E D A B C X r
EcoRI
PstI

4B


ACG AAT TCC CGG GGA TCC GTC GAC CTG CAG
EcoRI
SmaI
BamHI
PstI
pPA 31
P S E D A B C X r X c
EcoRI
DraI
EcoRV
BamHI

4C

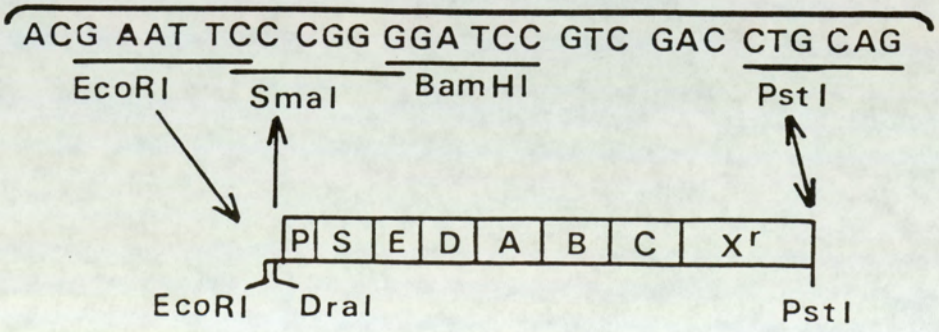

ACG AAT TCC CGG GGA TCC GTC GAC CTG CAG
EcoRI
SmaI
BamHI
PstI
pPA 34
P S E D A B C X r
EcoRI
DraI
PstI

Fig. 4A Construct pPA16

The SPA gene including 600 base pairs prior to the promoter and signal peptide have been cloned into pUC8 at the EcoRI and PstI restriction endonuclease sites: resulting in the loss of the membrane binding region (pPA16).

Fig. 4B Construct pPA31

The construct pPA31 is where the SPA gene has been blunt end ligated into the SmaI site, to remove the unwanted 600 base pairs of chromosomal DNA and to include the membrane binding region. The inserted DNA can then be removed by using the restriction enzymes EcoRI and BamHI.

Fig. 4C Construct pPA34

The construct pPA34 has lost the membrane binding region by cloning into the PstI site as in pPA16, but without the extra 600 base pairs of chromosomal DNA as in pPA31.

showed the internal homology of SPA. This is clearly seen in the dot matrix homology plot of SPA (figure 3B) where regions A-E showed high homology, though the binding capacity for IgG varied, with A-B showing the greatest affinity, down to E which showed no binding activity for human IgG. This loss of affinity for human IgG by region E could be due to the reduction in size of the sequence from 60 to 50 amino acids.

The C-terminal domain also exhibits high homology within the Xr region, which consists of a single sequence repeated twelve times. Further work by Guss *et al.* (1984) on the Xr region revealed that the sequence of the Xr portion was an octapeptide repeat (Lys-Glu-Asp-Gly or Asn-Asn-Lys-Pro-Gly) which covalently binds to the peptidoglycan. The Xc region possessing the unique DNA sequence is thought to be the membrane binding region. The polypeptide exists in an extended form although it is stable over a wide pH range (0.99 - 11.8) and resists denaturing on boiling. The molecule is highly hydrophilic with a reduced binding to IgG from regions A-E, although they are highly homologous.

1.11.2 Construction of the SPA expression clones

Due to the pathogenicity of *S. aureus* Cowan I and to increase expression, the gene coding for SPA was first cloned by Lofdahl *et al.* (1983) and Duggleby and Jones (1983), improved expression vectors were later produced by Shuttleworth *et al.* (1987). The constructs used in this thesis are shown in Figure 4A-C where the gene coding for SPA was cloned into the pUC8 plasmid. The main features of

these plasmids are that they contain an ampicillin resistance marker, with the SPA gene under the control of the lac promoter, with expression switched on permanently. In each case the COOH-terminal end of the SPA gene is out of frame with 42 base pairs of the galactosidase gene, resulting in the translation of the SPA protein and not a fusion peptide. pPA16 was cloned directly into the EcoRI/ PstI restriction sites and has 600 bp of *S. aureus* chromosomal DNA in front of the SPA promoter, resulting in weaker readthrough from the lac promoter (Shuttleworth, personal communications). Due to the cloning of the gene the last 69 amino acids have been deleted resulting in the loss of the membrane binding site. A production strain pPA31 was cloned into the SmaI restriction site using the DraI/EcoRV sites with blunt end ligation resulting in the loss of the extra 600 base pairs of chromosomal DNA. The SPA gene can be retrieved by using the restriction enzymes EcoRI and BamHI. Compared to pPA16 there is higher expression due to strong readthrough form the lac promoter (Shuttleworth *et.al.* 1987). The membrane binding site has also been included to give the full length protein. pPA34 is another production strain which does not have the extra 600 base pairs in front the SPA promoter, but the membrane binding site or hydrophobic region is missing as in pPA16. The gene can be retrived from the plasmid by using the restriction enzymes EcoRI and PstI.

1.11.3 The application of SPA

The application of SPA has increased greatly over the

past decade, especially in the area of immunology and immunochemistry (Goding, 1978; Langone, 1982a; Surolia *et al.* 1982). SPA's ability to bind more than one IgG molecule allows it to be used in ELISAs using labelled SPA, to detect either monoclonal antibodies or antigens to specific monoclones. By linking SPA to other substances such as conjugates, heavy metals or sephadex, a whole range of uses can be introduced (Lagone 1982b). Pharmacia have also produced a bulletin sheet, Protein A-sepharose CL-4B (Jan 1982) which lists the following applications of sepharose bound SPA:

1) Interaction with immunoglobulins which includes the binding properties of SPA to species, class and subclass.

2) SPA as a mitogen involved in the activation of human T and B lymphocytes.

3) Preparation of immunoglobulins and immunoglobulin sub-classes.

4) Isolation of immune complexes and antigens.

5) SPA in clinical and microbiological applications.

6) Applications to radioimmunoassay.

7) Enzyme-immuno techniques.

A new clinical use, is the treatment of cancer patients using immobilised SPA. Research work initially indicated that the removal of circulating immune complexes (CIC) from the blood, by perfusing over SPA columns can produce a positive response against cancer tissue (Daskal *et al.* 1984; Salinas, 1985). Although the results in animal tests appeared to be good, the response in human experiments have varied (for a review see Solal-Geligny *et al.* 1985).

Further research has shown that perfusing small volumes of serum still produces a positive effect against cancer cells. This would therefore indicate that it is not the removal of CIC's but rather a stimulatory effect which could well be the activation of the B or T lymphocytes. However the treatment is in its infancy and further research is required in this area (Bertram, 1985). Finally Pharmacia are now marketing plasmid vectors which contain the SPA gene which can be used in the production of fusion peptides (Nilsson *et al.* 1985; Hellebust *et al.* 1988), from which the SPA can easily be removed by binding to an IgG matrix and removing the required peptide by proteolytic cleavage (Lowenadler *et al.* 1987).

1.11.4 The quantitation of SPA

The estimation of recombinant SPA was originally performed at CAMR by using Rocket electrophoresis techniques (Duggleby and Jones, 1983). Due to the sensitivity of the assay system only quantities greater than 2 μg/ml could be measured. Although this was adequate for the production strains, the assays were tedious to perform with a large error on measurement of peak results. The rocket assay was therefore not suitable for the detection of low levels of SPA which may be produced by low expression vectors or that secreted or released into the environment by the host organism. The need for a simple, quick, reliable and sensitive assay was therefore essential for the work being investigated in this thesis. Several sensitive ELISA methods for assaying SPA have been published. Those

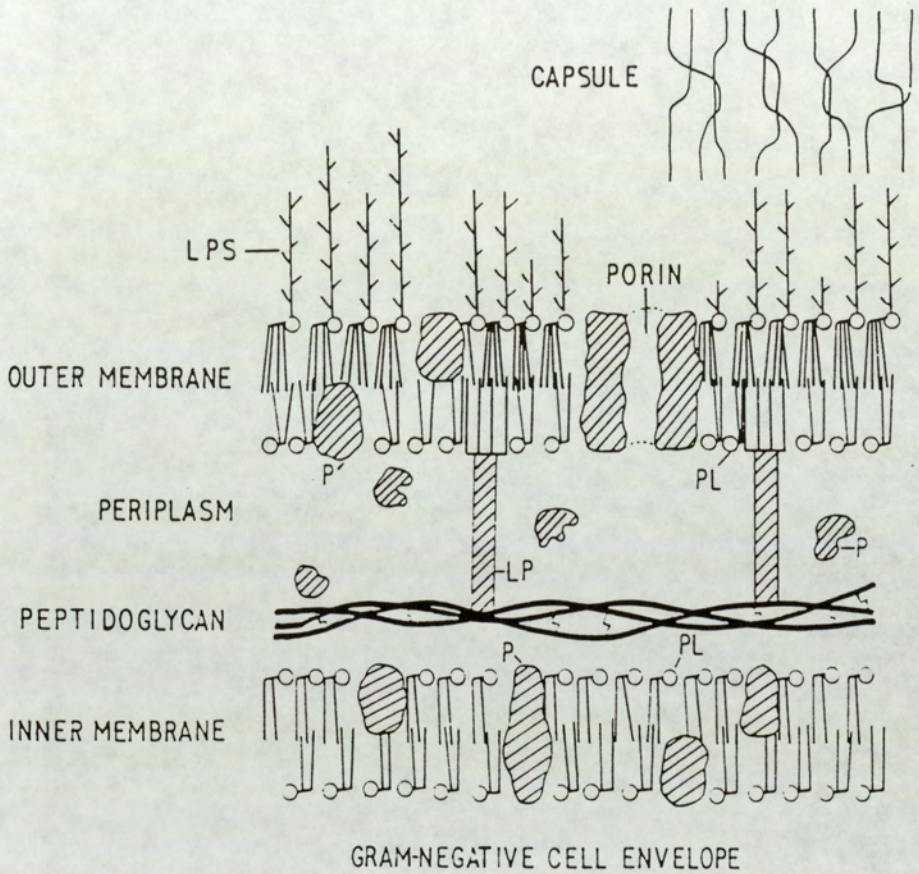
1.12 CELL STRUCTURE AND THE LOCALISATION OF RECOMBINANT GENE PRODUCTS

The structure of a typical Gram-negative bacterium is shown in Figure 5. The cytosol contains the nuclear material which unlike eukaryotes is not surrounded by a membrane, there are also ribosomes, mesosomes and granuoles present. The cytoplasm is encapsulated by three structural layers, the inner– or cytoplasmic-membrane, the peptidoglycan layer and the outer – membrane (Lugtenberg and Van Alpen, 1983). The periplasmic space lies between the two membranes and contains the peptidoglycan layer as well as periplasmic proteins, porins, lipoproteins and binding proteins. Specific enzymes such as alkaline phosphatase are also present, which can be used in localisation studies (Costerton and Cheng, 1975). The inner membrane contains phospholipids and proteins and is associated with the transport of nutrients, oxidative phosphorylation, synthesis of phospholipids, peptidoglycan and lipopolysaccharides and may also be an anchorage point for DNA in replication. The hydrophobic outer membrane, as well as containing phospholipids and proteins, also contains lipoproteins which are covalently linked to the peptidoglycan layer (Inouye, 1974).

In Gram-negative bacteria there are three regions where the protein may be localised, these are the cytoplasm, periplasmic space or the environment. By utilising genetic engineering techniques it is now possible to direct a recombinant protein to any one of these areas. Localisation of a recombinant protein in the cytosol may be attained

Fig. 5 The structure of a gram-negative bacterium

LP: Lipoprotein; LPS: lipopolysaccharide; P: protein; PL: phospholipid.



by using proteins without signal peptides which direct the protein across the cytoplasmic membrane. Depending upon the signal peptide the protein would be transported across the inner membrane, cleaved, and released as a mature protein into the periplasmic space or bound to one of the membranes (Duffaud *et al.* 1982; Randall *et al.* 1987). Recombinant proteins may have their own signal peptide as shown with protein A (Abrahamsen, *et al.* 1985), or they may be placed downstream of a chosen signal which is present on the parent plasmid (Grey *et al.* 1985). To excrete a recombinant protein into the environment is a difficult task though this has been achieved by Nagahari *et al.* (1985), in the expression of human *B*-endorphin as a fused protein with the outer membrane protein (omp F). However, success may have been related to the small size of the protein and its ability to pass through the porin structures.

In downstream processing the choice of location may vary depending upon two main factors. The first being ease of purification and the second susceptability to degradative enzymes. When located in the environment the protein is freely available and as there are no extracellular proteases relatively free from proteolysis. As large scale batch cultures can involve using volumes of over 1000 litres, then the environment maybe an undesirable location because of handling problems. The use of small volumes involves using cell pastes which means localising the recombinant protein in either the cytosol or the periplasmic space. The periplasmic space is often the location of choice as fewer proteases exist compared to the cytosol (Swarmy and

Goldberg, 1982). Extraction procedures are also easier as only the outer-membrane has to be removed, whereas lysis of the whole cell will increase purification difficulties. Other problems arise when the protein is incorrectly folded or post-translational processing such as acylation or cleavage do not occur.

Localisation of recombinant proteins is by no means the end of purification problems as high level expression can result in the formation of inclusion bodies (Williams, *et al.* 1982; Kane and Hartley, 1988). Inclusion bodies are denatured aggregates of proteins which are clearly visible as electron dense layers using an electron microscope. In downstream processing the extraction and purification of inclusion bodies can be relatively easy, with a single centrifugation step (Marston *et al.* 1984). Due to the proteins denatured state some protection against intra-cellular proteases is provided (Cheng *et al.* 1981). There are however, problems with renaturation, which is dependent upon the folding and structure of the protein involved. Other reports have also documented the formation of inclusion bodies (Emtage *et al.* 1983), though the basis for this phenomenon has not yet been explained in both biochemical or physiological terms. While this process is likely to be a dynamic interaction of recombinantly expressed polypeptide and components within the host cell, more information is needed to define the features of the recombinant protein necessary for the formation of inclusion bodies. Though certain factors have been implicated in their formation including the size, hydrophobicity or the

number of disulphide bonds of the protein as well as the pH or the host strain used (Kane and Hartley, 1988).

Obviously it is important to demonstrate the location of the recombinant protein so as to minimise the loss of product whether it be from a positional error or due to inclusion body formation. Inclusion bodies can easily be demonstrated by electron or even phase contrast microscope techniques Kenealy *et al.* (1987). Localisation of soluble proteins has relied on traditional lysis and centrifugation procedures, using host enzymes as purification markers (Osborn and Munson, 1974). Problems arise in the purification of the different fractions especially when high levels of recombinant proteins are involved (Randall *et al.* 1987). The recent development of immunoelectron microscopy using colloidal gold has added a new dimension to localisation (Horisberger, 1979), as the technique does not suffer the same misinterpretations as traditional studies. It does have its own limitations however as large amounts are required for detection and the protein must be in an antigenic state. The antigenic sites of the protein may be altered when translated, or by the fixation and dehydration treatment which may also alter the cells structure in the process.

1.13 AIMS OF RESEARCH

Staphylococcal protein A was originally produced at CAMR from the pathogenic host *S. aureus* Cowan I. Due to the increasing use of SPA in medicine and the reluctance to work with large volumes of *S. aureus* the gene was cloned into the plasmids pBR322 (Duggleby and Jones, 1983) and pUC8 (Shuttleworth *et al.* 1987). This allowed the large batch scale production of recombinant SPA which although was free from *S. aureus* and its contaminating toxins, did contain *E. coli* lipoproteins.

The main aim of the research was to investigate the stability and expression of the recombinant SPA plasmids in *E. coli*, when grown in continuous culture. The effects of nutrient limitation especially the carbon source, were to be investigated, as glycerol is used in the large scale production process instead of glucose. This is because the SPA gene is under the control of the lac promoter and therefore may be prone to catabolite repression.

Before commencing with continuous culture two areas of work had to be investigated. The first of these was the development of an assay system for recombinant protein A. This was essential to the main theme of the work, as the only assay system available was a rocket electrophoresis technique which only measures concentrations down to two microgrammes. It is laborious to perform and is not suitable for measuring the large numbers of samples which would be generated by continuous culture. The second area to be investigated was the development of a chemically defined medium for the growth of the host in continuous

culture. This area is often neglected by workers although it is an essential part of the research, to establish the concentration of the nutrients which will give a specific value of growth. Elucidating the effects of the environment would therefore be the main aim of the investigation with attention also being paid to the location of the recombinant SPA. Identifying the location of the SPA would verify the work of Lofdahl *et al.* 1983 showing the Staphylococcal signal peptide could work in an *E. coli* host and also help in the downstream purification processes.

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Organisms and plasmids

The host strain *Escherichia coli* strain JM83 (lac pro strA thi 080d lacZ M15) (Vieira and Messing 1982), was used throughout the experiments, both with and without the relevant plasmids. With initial experiments involving *Staphylococcus aureus* Cowan I NCTC 8530 and *Staphylococcus aureus* Wood 46 NCTC 7121 as positive and negative contols.

The organisms were all stored at -20 °C in tryptone soya broth with 15% glycerol and prior to each experiment *E. coli* JM83 was grown at 37°C for 16 hr on chemically-defined media agar (CDMA) plates and if harbouring a plasmid 50 μg ml^{-1} of ampicillin (Sigma Chemical Co.) was added. A single colony was then inoculated into 25 ml of chemically-defined media (CDM) in 100 ml fluted flasks and grown for 16 hours at 37°C in an orbital shaker (New Brunswick) at 150 rpm.

The host plasmid used in this study was pUC8 (Vieria and Messing 1982), which contained various sizes of the SPA gene (pPA16, pPA31 and pPA34 Figures4A, B and C) as described by Shuttleworth, *et al* (1987).

2.1.2 Media

Tryptone soya broth (TSB) and tryptone soya agar (TSBA) were purchased from Oxoid Ltd. and prepared according to the manufacturer's instructions. The chemically defined media used in both batch and continuous culture experiments was based on that reported by Klemperer *et al.* (1979) and

includes the substrates shown in Table 1. The media used in the batch experiments was filter sterilised using a 0.2 μm Acrodisc filter (Gelman Sciences, Prod. No. 6224192), whereas the medium used in the continuous culture experiments was filter sterilised into 19 L of sterile distilled water using a 0.22 μm Sterivex-GS filter unit (Millipore Co., Prod. No. SVGS01015).

2.1.3 Continuous culture

E. coli JM83 containing one of the protein A plasmids was grown in continuous culture in LH 500 series chemostats (LH Fermentation Ltd., Stoke Poges) at various dilution rates (D). Where 1L glass vessels were used with a working pot volume of 650 ml (V). The pH was monitored using an autoclavable pH electrode (Russell pH Ltd., Auchtermuchty, Fife) and kept at a constant value of 7.2 pH with the addition of 1M NaOH. Aeration was maintained at 1ml air ml^{-1} culture min^{-1} with difusion kept at a maximum by air passsed through a sterile Gelman filter down the direct drive stirring mechanism which enhances bubble breakage. The stirrer speed was maintained at 500 rpm and the medium flow rate (F) was measured using a burette under the control of a low gear peristaltic pump.

2.2 METHODS

2.2.1 Preparation of cell free lysates for detection of SPA by ELISA and western blotting techniques

A single colony was used as an inoculum in 10 ml (TSB)

Table 1 Concentration of nutrients for batch culture analysis

Nutrient	Concentration (mM)
Glucose	50.0
Glycerol	50.0
$MgSO_4 . 7H_2O$	0.2
KCl	0.2
NH_4Cl	10.0
$NH_4Fe(SO_4)_2 . 12H_2O$	0.005
MOPS	12.5
Phosphate buffer	11.0
Proline	7.5
Thiamine	0.1

with 25 μg ml^{-1} ampicillin and grown for 16 hr at 37°C in a shaking incubator at 200 rpm. The organisms were harvested by centrifugation at 2800xg at 4°C for 10 min and processed further by either lysozyme or lysostaphin treatment, or by sonication.

2.2.2 Lysozyme and lysostaphin treatment

The cells were resuspended in 1 ml 50 mM Tris-HCl, pH 8.0 containing 25% sucrose. For cultures of *E. coli*, 0.15ml of lysozyme solution (20 mg ml^{-1} in 250 mM EDTA, pH 8.0) were added and left on ice for 20 min. In the case of *S. aureus* cultures 0.15 ml of lysostaphin solution (200 μg ml^{-1} in sterile distilled water) were added and incubated at 37°C for 20 min. Aliquots of 1.0 ml of EDTA solution (250 mM, pH 8.0) were then added and the lysates were then left on ice for 5 min while swirling occasionally. Further aliquots of 1.5 ml of a 1.0% Brij 58, 0.04% sodium deoxycholate solution in 10 mM EDTA were rapidly added and the lysates left on ice for 30 min or until lysis had occured. The lysates were then centifuged at 17500xg at 4°C for 45 min and the supernatants used as the cell-free lysates.

2.2.3 Sonication

The bacteria were resuspended in 3 ml Tris-Hcl buffer and sonicated twice for 15 sec at amplitude 2 on an MSE soniprep while kept on ice. All lysates were stored at -20°C.

2.2.4 Assay for total soluble protein

An aliquot of 1 ml was taken from the sonicated samples and centrifuged for 5 min at ambient temperature in a minifuge (Micro centaur, MSE Ltd.) to remove insoluble proteins and the supernatant was assayed using a BCA kit (Pierce U.K. Ltd Cambridge). Bovine serum albumin (BSA) was used as the standard and a new calibration curve was performed for each of the continuous culture experiments.

2.2.5 ELISA

This assay used human IgG bound to the plate as the capture antibody to which any SPA in the sample should bind. The concentration of human IgG used in the test was arrived at empirically (see section 3.1.2). Internal standards containing 100 ng ml^{-1} SPA (Sigma) were run on every plate. Other samples contained lysate buffer or lysates of *E. coli* or *S. aureus* strains, both those expressing and those not expressing SPA. Known concentrations of SPA were added to these samples to detect interference by bacterial constituents.

The first detection antibody was a commercial rabbit anti-SPA serum; the inclusion of this stage greatly improved the sensitivity of the assay. The second detection antibody was a commercial goat anti-rabbit-IgG peroxidase conjugate. 5-Amino salicylic acid was used as the chromogenic agent. ELISA Plates (Dynatech M129A) were coated with 100 μl $well^{-1}$ of capture antibody (Commercial human IgG (Dako)) at optimal dilution in 15 mM Na_2CO_3, 35 mM $NaHCO_3$, 0.02% NaN_3 (pH 9.6) at room temperature overnight. After each stage of the

assay the plates were washed four times in phosphate buffered saline (PBS) containing 0.1% Tween 20 (PBS-T). This solution was found to be adequate to block all nonspecific adsorption of protein to the plates (see results section). PBS-T was used as the diluent for all stages of the ELISA procedure. Internal standards and other samples were diluted appropriately, and 2 fold dilution series were set up in 100 µl volumes in sterilin M25A plates which had been presoaked in PBS-T for 2 min to block adsorption of protein. 90 µl samples of these dilution series were transfered to the ELISA plates and incubated for 90 min at room temperature. Bound SPA was detected by incubation with rabbit anti-SPA serum (Sigma) (see results section for details) for 90 min then with goat (anti-rabbit IgG) IgG-horse radish peroxidase conjugate (Bio-Yeda) (GARP) 90 min both at room temperature. The bound conjugate was then incubated for 15 min at room temperature with 0.1% 5-amino salicylic acid, 0.005% H_2O_2, in 50 mM Na_2HPO_4/NaH_2PO_4, pH6.0 and the optical density of the coloured product was determined at 450 nm, using a Titertek Multiskan MCC automatic plate reader linked to an Acorn BBC B microcomputer running data capture reduction software written in this laboratory. The endpoint dilution was defined as the dilution producing a given O.D. above background and was transformed into SPA concentration by comparison with internal standards run on every plate.

2.2.6 Polyacrylamide slab gel electrophoresis (PAGE)

The PAGE buffer system was based on that of Laemli

(1970) and used both gradient and standard gels. The gradient gels used 50% (w/v) acrylamide and 0.8% N,N'-bis-methylene acrylamide stock solution diluted with buffer (final concentration 0.1% SDS; 375 mM Tris-HCl, pH 8.8) and polymerised by the addition of 0.042% tetramethylethylene-diamine (TEMED) and 0.7% ammonium persulphate (APS). Gradient gels (7.5 to 20%) were produced using a gradient maker (BRL, Cambridge), with a 99:1 (w/w) proportion of acrylamide to bisacrylamide throughout. The standard gels used a running gel of final concentration 10% (w/v) acryl-amide, 0.17% (w/v) N,N'-bis-methylene acrylamide, 0.15% SDS, 500 mM Tris-HCl, pH 8.8 and polymerised by the addition of 0.1% (APS) and 0.01% (TEMED). The stacking gels both had a final concentration of 5% (w/v) acrylamide and 0.17% (w/v) N,N-bis'-methylene acrylamide, 0.1% SDS, 125mM Tris-HCl pH 6.5 and polymerised by 0.1% APS and 0.01% TEMED. The samples were prepared for PAGE under denaturing conditions in 0.1% (w/v) SDS; 0.1M Tris-HCl pH 6.8 containing a trace bromophenol blue and heated to 100 °C for 1 min. The gel was then run in running buffer (Tris-HCl 6.32g; glycine 4.0g and 1.0g SDS made upto 1L in distilled water) at 10v/cm.

2.2.7 Western blotting

Polyacrylamide slab gels were equilibriated in two changes of blotting buffer (7.5 mM $Na_2HPO_4.12H_2O$, 17.5 mM NaH_2PO_4 $2H_2O$) for 30 min each at room temperature. A nitrocellulose membrane (Bio-Rad) was soaked in the same buffer for 30 min then proteins were electrophoretically transferred from the polyacrylamide gel to the nitrocellulose

by applying a transverse electric field of 15 V (2.5 V cm^{-1}) for 2 hr. Portions of the membrane were either blocked overnight at 4°C in ELISA diluent (PBS-T) containing 3% gelatin and 0.02% NaN_3, to prevent non-specific binding, or stained in 0.1% Coomasie blue in 50% methanol, 45% distilled water, 5% acetic acid, and destained in the same solvent. For immunological probing, the blot was washed in diluent and exposed to antibodies (90 min each) by sealing it an polythene bag contaning 10 ml of serum or conjugate at appropriate dilutions in PBS-T + 1% gelatin. After final washing in diluent, it was washed in substrate buffer (50 mM sodium acetate buffer pH 5.0) and rocked in substrate solution (0.16 mg ml^{-1} amino-ethyl carbazole, 0.03% H_2O_2) until developed (usually 5-10 min at room temperature). The reaction was stopped by washing the blot in water and leaving it to dry. Problems with excessive background colour could be eliminated by using gold labelled goat anti-rabbit IgG with a silver enhancer (Jennsen Products, ICN Biomedicals Ltd., High Wycombe. Auroprobe BLplus GAR and IntenSE II) and used as specified by the manufacturer's instructions.

2.2.8 Viable counts

Viable colony counts were performed by the spread plate method (Crone, 1948) which consists of spreading known volumes of the diluted culture on overdried TSB plates. Serial dilutions of ten or one hundred fold were made in PBS so that 0.1 ml volumes gave 30 - 300 colonies per plate, assuming that one colony is formed from by the growth of one

organism.

2.2.9 Measurement of bacterial cell concentration

The method used to measure cell concentration in this study was to measure the optical density with a spectrophotometer, as access to the equipment for total counts was limited. In optical density measurements where a parallel beam of monochromatic light passes through a suspension of bacterial cells, part of the light will be scattered due to the difference in the refractive index between the cell surface and the medium. The light scattered by the cells is directly proportional to the concentration and is related to the Lambert-Beer law which can be expressed as the following:

$$e\ l\ c = \log_{10}\ (I_o/I)$$

Where

e = The extinction coeffient

l = Path length

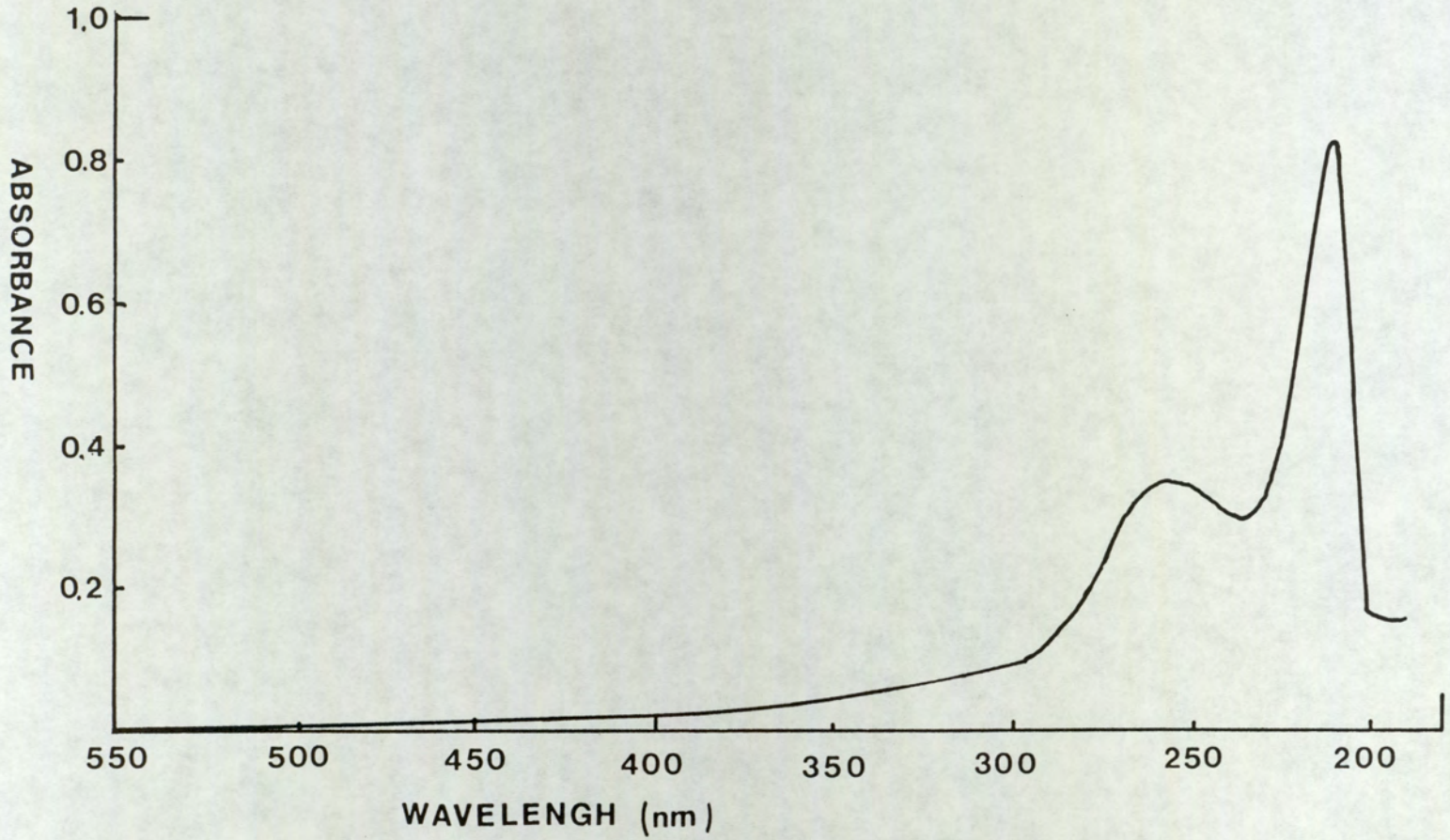
c = Concentration of the bacteria in the suspension

Io= Intensity of the incident light

I = Intensity of the transmitted light

This relationship only holds at low bacterial concentrations, as high concentrations may produce secondary scattering by the cells thus giving an underestimated value of the suspension (Meynell and Meynell, 1970). As the wavelength below 380 nm is not practical due to the absorbtion of proteins and nucleic acids, 470 nm was used as the wavelength for all the experiments in this study (Figure 6).

Fig. 6 Scan of wavelengths of a supernatant from *E. coli* JM83 with pPA16 grown in glucose limited medium



2.2.9.1 Relationship between optical density (absorbance) and cell concentration

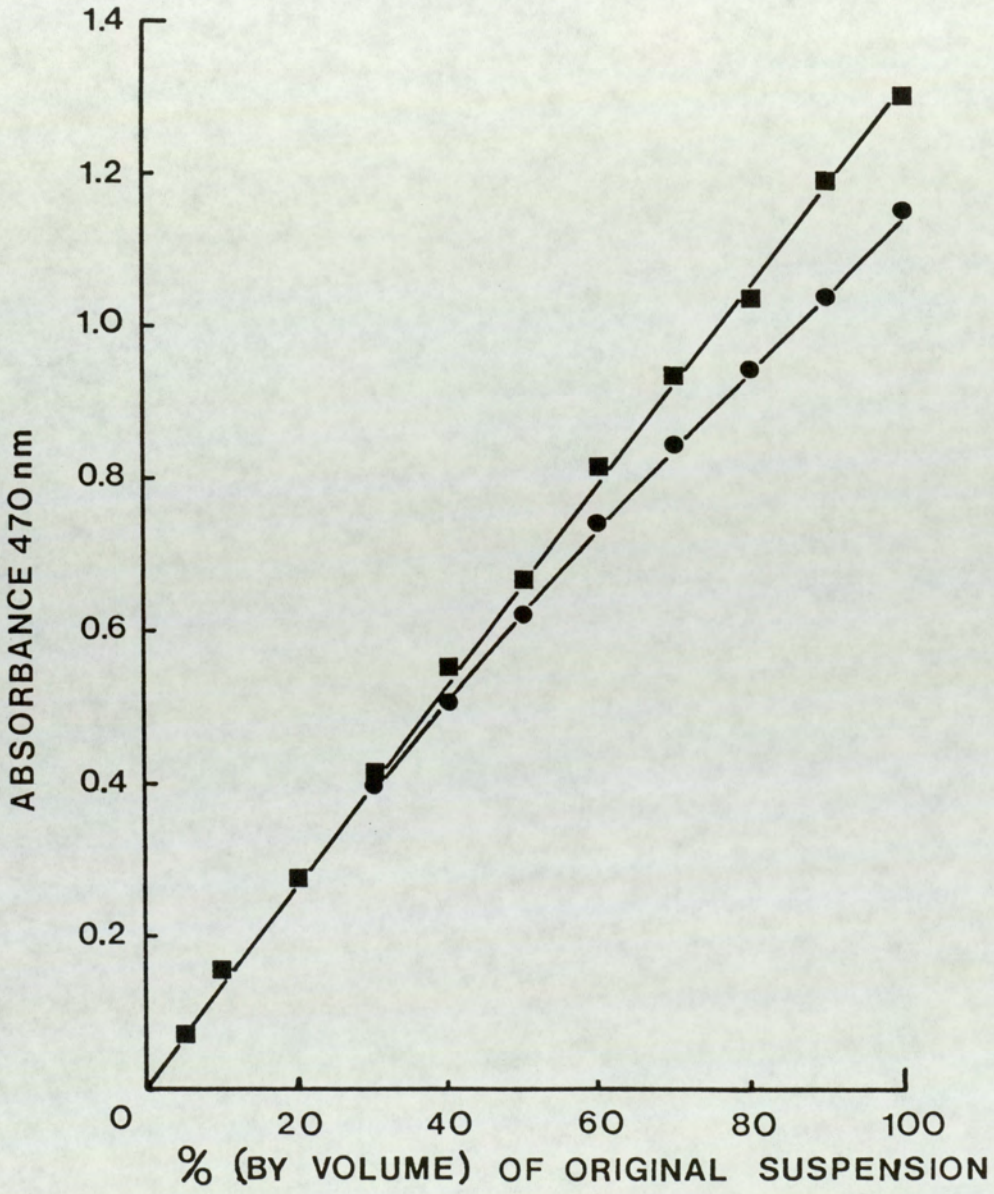
Kenward (1975) found that the relationship between optical density and cell concentration only obeyed the Lambert-Beer law up to an optical density of 0.3 with *Pseudomonas aeruginosa*, above which the optical density increased less than was predicted with regards to the increase in the cell concentration (which corresponds to the result expected by Meynell and Meynell). To test to see if the same relationship existed with *E. coli*, it was grown in basic CDM overnight, then centrifuged at 8000 x g for 10 min, washed twice and resuspended in PBS. From this suspension a series of dilutions were prepared in PBS and the optical density measured at 470 nm (Figure 7). *E. coli* was found to behave similarly to *Pseudomonas aeruginosa*, therefore all measurements of cultures above an optical density of 0.3, should be diluted appropriately.

2.2.9.2 Relationship between optical density and dry weight at different stages of the growth curve

This was performed with 1.0 L of CDM in a 3 L flask where growth of the organism *E. coli* JM83 pPA16 would be restricted by the concentration of the glucose present (3 mM) allowing the organism to grow exponentially to an optical density of 1.2. The culture was shaken at 100 rpm in an orbital shaker at 37°C. 10 ml volumes were centrifuged (8000 x g for 20 min) at different stages of growth, then washed once with PBS and filtered through a pre-weighed

Fig. 7 Relationship between optical density and cell concentration using *E. coli* JM83

Diluted suspension (●); undiluted suspension (■).



Gelman 0.20 μm filter. The bacteria were again washed while on the filter which was then dried under a infra-red lamp for 30 min and reweighed. The results are presented in Figure 8 and show a linear relationship between dry weight and absorbance to an optical density of 1.2.

2.2.9.3 Relationship between optical density and viable counts at different stages of the growth curve

This was performed as in 2.2.9.2 and the results are shown in Figure 9 these again show a linear relationship as in the previous experiment. These results were similar to those produced by Kenward(1975) with *Pseudomonas aeruginosa*.

2.2.10 Batch cultures used for the study of growth

Growth experiments were performed using an inoculum of *E. coli* JM83 both with and without the plasmid (pPA16) which was grown in 25 ml of complete CDM (Table 1) at 37°C for 16 hr in an orbital shaker at 150 rpm. The culture were washed twice in a medium lacking the nutrient under study and resuspended in the same medium to give an optical density of 2.5. Aliquots of 0.25 ml of this cell suspension was then used to inoculate 24.75 ml of the appropriate medium in 100 ml ribbed flasks.

2.2.11 The use of continuous culture for the study of plasmid stability and expression

The chemostats were inoculated with a culture of *E. coli* JM83 containing the appropriate plasmid as prepared in 2.2.10 and run using the conditions described in 2.1.3. To

Fig. 8 Relationship between optical density and dry weight at different stages of growth of *E. coli* JM83 with pPA16 grown in batch culture in glucose limiting conditions

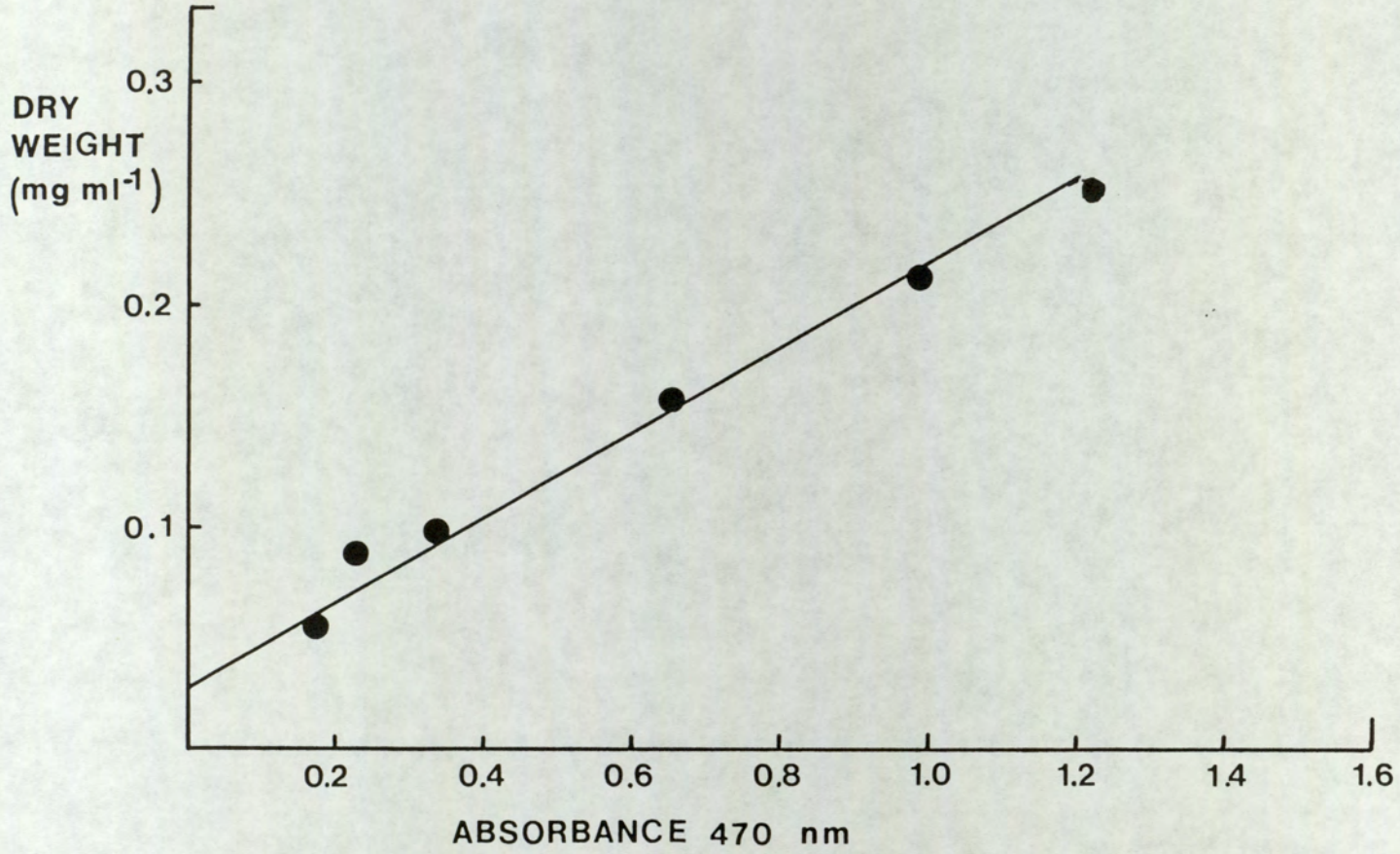
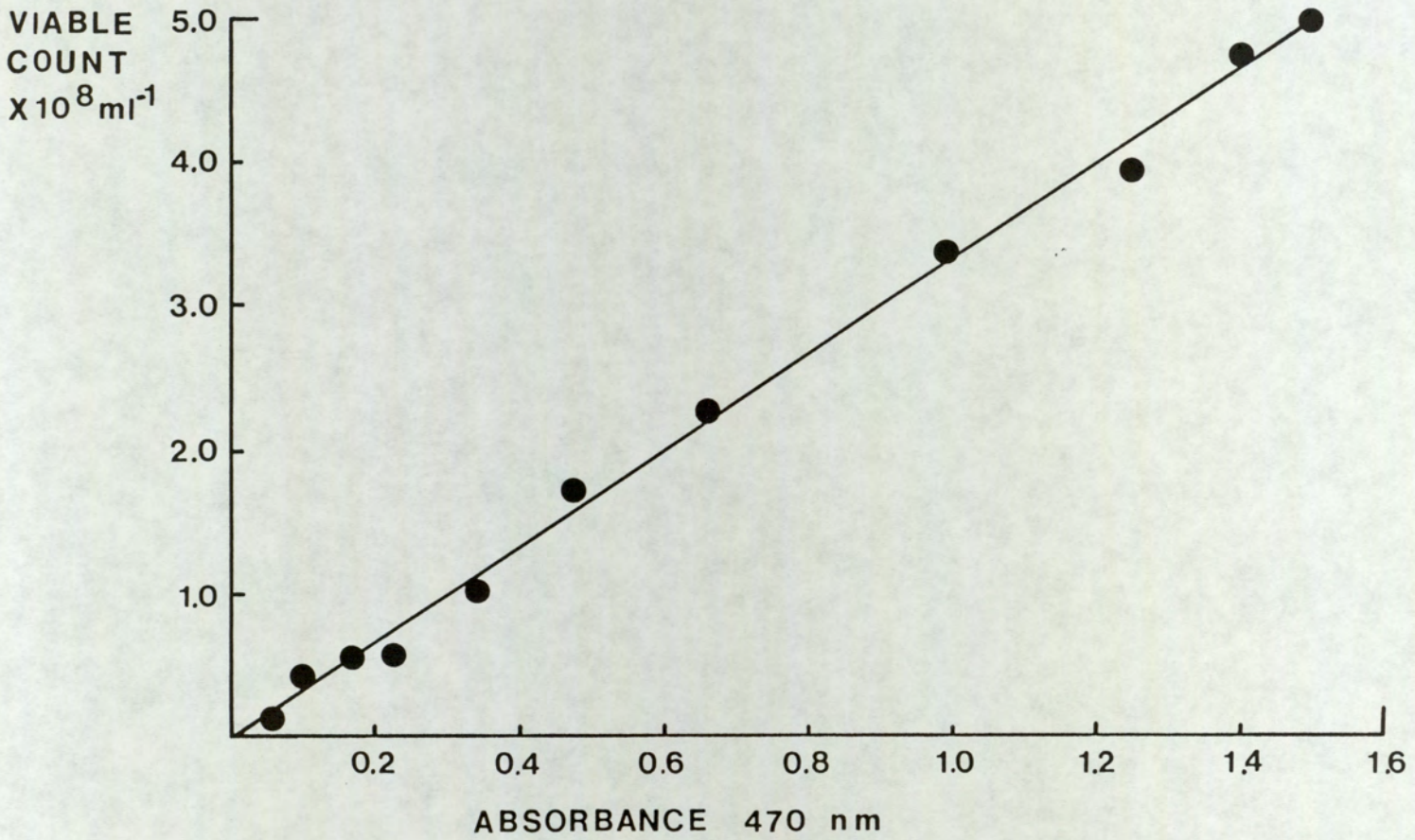


Fig. 9 Relationship between optical density and viable counts at different stages of growth of *E. coli* JM83 with pPA16 grown in batch culture in glucose limiting conditions



investigate the stability and expression of the SPA clones, 20 ml samples were taken from the fermenter at various times throughout the experiment and processed as follows.

2.2.11.1 **Optical density** see 2.2.9

2.2.11.2 **Viable counts** see 2.2.8

2.2.11.3 **Screening for antibiotic resistance**

One hundred colonies from each viable count were picked onto TSBA plates using sterile toothpicks and incubated for 16 hr at 37°C. The colonies were then replicated (using sterile velvet discs) onto TSBA plates both with and without ampicillin (50 μg ml^{-1}) and screened for growth after incubation for 16 hr at 37°C. The results of which are expressed as the percentage of ampicillin resistant cells in the population which is related to the amount of cells harbouring the plasmid.

2.2.11.4 **Physical detection of plasmids**

Each sample was assayed for the prescence of plasmid DNA by a microscale technique based on Birnboim and Doly (1979) where 1.5 ml of culture was centrifuged at room temperature in a minifuge (12000 rpm) for 5 min. The pellet was resuspended in 100 ul of lysis solution (20 mg ml^{-1} lysozyme (Sigma) 25 mM Tris-Hcl pH 8.0; 10 mM EDTA; 50 mM glucose) vortexed and left on ice for 30 min. An aliquot of 200 μl of 0.2 M NaOH/1.0% SDS was then added and incubated for a further 5 min until the mix cleared. A

further aliquot of 150 µl 3M sodium acetate pH 4.8 was then added and left for 1 hr on ice with gentle mixing. The preparation was centrifuged for 5 min in a minifuge and 400 µl of the supernatant removed avoiding any precipitate and added to 1.0 ml of cold ethanol, vortexed and left at -20°C for 30 min. The supernatant-ethanol mix was then centrifuged for 5 min and the pellet redissolved in 0.1 ml 50 mM Tris-HCl, 100 mM sodium acetate (pH 6.0). A further aliquote of 0.2 ml of cold ethanol was added and the mix left at -20°C for 15 min. The mix was again centrifuged and the pellet dried in a vacuum desicater before being resuspended in 75 µl sterile TE buffer (50 mM Tris-Hcl pH 7.2; 20 mM EDTA).

2.2.11.5 **Restriction analysis of the plasmid DNA**

The purified plasmid DNA was cut according to the manufacturers instructions (BCL) using the restriction enzymes EcoRI and PstI. An aliquote of 17 µl of plasmid DNA was added to 1 unit of PstI and 2 µl of 3M sodium acetate (pH 4.8) high salt buffer (x10 conc.) and incubated at 37°C for 1.5 hr. The mix was then heated to 65°C to denature the first enzyme and the same procedure repeated for EcoRI. Agarose gels (1.0% (w/v)) in B buffer (90 mM Tris pH 8.2, 90mM boric acid, 2.5mM EDTA) were then used to separate the DNA fragments. The agarose was disolved by boiling in B buffer and poured at 50-60°C using a horizontal gel apparatus. Samples of 8 µl of the the plasmid mix were then added to 2 µl of loading buffer (40% w/v sucrose, 0.1M EDTA and 0.25% Bromophenol blue) and the gel run with molecular

weight markers for 3 hr at 100 mA. The gel was then stained with 1 μg ml^{-1} ethidium bromide for 30 min and destained in distilled water for 30 min and photographed using polaroid type 55 film producing a positive and negative print. The size of the resulting fragments were then calculated and compared to those produced by pPA16 (2.1 and 1.9 Kb) which correspond to the host plasmid (pUC8) and the protein A gene.

2.2.11.6 **Protein assay**(see 2.2.4)

Because of the interference produced by lysozyme the protein assays were only performed on the sonicated samples.

2.2.11.7 **Quantitative assay for SPA**(see 2.2.5)

Assays were performed on both the sonicated and supernatant samples as described in 2.2.3.

2.2.11.8 **Qualitative assay for SPA**(see 2.2.6-7)

Western blots were only performed on the sonicated samples taken from the fermenter, as contamination of lysozyme in the lysed samples may interfere with the interpretation of results.

2.2.11.9 **Determination of copy number**

Direct visualisation of the plasmid content was obtained by using a modification of the method of Twigg and Sherratt (1980). A 0.5 ml sample of culture was centrifuged (12000 rpm) at room temperature in a minifuge for 5 min and the supernatant removed. The pellet was resuspended in 100 μl

of lysis solution (20 mM Tris-Hcl pH 8.0, 10 mM EDTA, 100 mM NaCl, 20% w/v sucrose, 2 mg/ml lysozyme and 5 units/ml pancratic RNase) and incubated at 37°C for 30 min. Aliquots of 100 µl 2% SDS was then added and the sample vortexed for 1 min before freeze-thawing twice in liquid nitrogen to 37°C. Aliquots of 10 µg ml^{-1} proteinase K were then added and incubated at 37°C for 30 min. An equal amount of sample to loading buffer (50% glycerol, 0.02% xylene cyanol, 0.02% bromophenol blue) was then run on a 1.0% agarose gel (made up as above) for 4 hr at 100 mV. The gel was stained and photographed as in 2.2.11.15 and the negative scanned using a Joyce-Loebl scanning densitometer and the areas under the peaks integrated. Using the following formulae as described by Projan *et al.* (1983), the copy number per host chromosome can be calculated:

$$Cp = Dp \times I \times Mc / Dc \times Mp$$

Where

Cp = Plasmid copies per host chromosome

Dp = Amount of plasmid DNA in the gel

Dc = Amount of chromosomal DNA in the gel

Mc = The total chromosomal DNA per cell (3.8×10^6)

Mp = Mol. wt. of the plasmid (pBR322= 50)

I = Difference in the binding of ethidium bromide to chromosomal or plasmid DNA (1.36)

The copy number per host chromosome of the SPA vectors can then be calculated using pBR322 in *E. coli* HB101 as a known standard of 50 (Hashimoto-Goto and Timmis, 1981).

2.2.11.10 Assay for *B*-lactamase

B-lactamase activity was estimated using the hydrolysis of nitrocefin as described by O'Callaghan *et al.* (1972). A stock solution of nitrocefin was first prepared by disolving 5 mg in 0.5 ml dimethyl sulphoxide which was then added to 9.5 ml of phosphate buffer (pH 7.0). A 4 ml aliquote of this solution was added to 36 ml of phosphate buffer (pH 7.0) and used as the substrate for the assay. Dilutions of samples would then be mixed with the substrate and monitored for changes in absorbance at 482 nm.

$$\text{Activity } (\mu\text{mol min}^{-1}) = \frac{\text{change in O.D.(482 nm)} \times V \times D}{\text{Time} \times \text{EmM}}$$

Where V = Volume of the sample

D = Dilution of the sample

EmM = 15.9 (micro-molar absorbance of hydrolysed nitrocefin at 482 nm)

B-lactamase was only assayed in the samples prepared for electron microscopy, so that levels of the enzyme could be compared directly to that of the SPA produced at that particular time.

2.2.12 The use of microscopy in the detection of SPA and the analysis of cell structure

2.2.12.1 Light microscopy

Samples were taken from exponentially growing cultures of *E. coli* JM83 containing each of the three pPA plasmids. Gram stains (Jensens modification 1984) were performed on

each of the host/vector systems using *E. coli* JM83 as a negative control. Photographs were taken using a Leitz Dialux 20 microscope, with an automatic Wild MPS51 film holder.

2.2.12.2 **Electron microscopy**

A) **Preparation of sections of host organisms for structural analysis**

A sample from the continuous culture experiments was centrifuged (8000 xg) and fixed by resuspension in 2.5% glutaraldehyde in 0.1 M phosphate buffer for 2 hr at 4°C. This was followed by a buffer wash before post-fixation in 1% osmium tetroxide in phosphate buffer for 2 hr at 4°C. The organisms were then pelleted (8000 xg), the supernatant discarded and the pellet gently mixed with molten agar (Oxoid No.1). When set, the agar was cut into approximately 1 mm cubes which were dehydrated in graded ethanol series (30%, 50% for 10 min. each; 70%, 90% for 30 min each) through to absolute ethanol (2 changes of 30 min) followed by 20 min (2x10 min changes) in 100% propylene oxide.

Embedding was carried out by infiltrating the agar cubes with 50:50 propylene oxide : araldite CY212 resin mixture for 4 hr followed by 24 hr in 100% araldite mixture. All of the above procedures were carried out at room temperature. The agar cubes were then placed in BEEM embeding capsules (size 00), covered with fresh araldite mixture and allowed to stand for a further 24 hr at room temperature before being cured at 60°C for 72 hr.

Thin sections (80 nm) were cut from the resulting blocks on a Reichert OMU2 ultramicrotone using a diamond knife and were picked up on 400 mesh copper EM specimen grids. After staining in aqueous uranyl acetate (2% for 30 min) followed by Reynolds lead citrate (10 min) (Reynolds 1963), the sections were stabilised by carbon cutting in an Edwards 12E6 vaccum coating unit prior to examination in a Philips EM400T electron microscope operated at 80 Kv.

B) **Preparation of sections of host organisms for the detection of SPA**

Organisms were pelleted from the continuous culture medium by centrifugation (8000xg) and fixed by resuspension in 0.1% glutaraldehyde, in 0.1 M phosphate buffer for two hours at 4°C. The following procedures were then as in section A while omitting the post-fixation of osmium tetroxide.

C) **Preparation of whole cells for the detection of SPA**

A sample from the continuous culture medium was cenrtifuged (8000 xg) then resuspended and fixed in 0.1% formalin in 0.1 M phosphate buffer. The cells were then adhered to formvar/carbon filmed E.M. grids by leaving a drop on the surface for four minutes. The cells could then be probed for SPA as in 2.2.12.2D

D) Assay system for the detection of SPA using electron microscopy

The grids were first resuspended on a 150 µl droplet of new born calf serum for 30 min so as to inhibit any non-specific binding. They were then washed x3 in PBS (pH 7.6) for 5 min and incubated for 1 hr on a 150 µl droplet of 1:500 (in PBS pH 7.6) of rabbit anti-SPA for sections and 1:5000 for whole cells. The grids were then washed x3 for 5 min in PBS and blocked with new born calf serum 30 min. The grids were again washed in PBS (pH 8.2) then placed on 150 µl droplets of 1:10 (in PBS pH 8.2) goat anti-rabbit labelled with gold (Biocell ltd.) for 1 hr. Finally the grids were washed x3 in PBS (pH 8.2) and dried on filter paper. All the above incubations were carried out at room temperature.

3. RESULTS

3.1 ELISA TO MEASURE SPA

3.1.1 Demonstration of adequate blocking of ELISA plates by PBS-T

An ELISA plate was set up with combinations of reagents in each column as shown in Table 2, where a reagent was absent it was substituted with diluent. Thus column 1 was a substrate-only blank, and the ELISA reader was zeroed on this column. Any colour development in column 2 would indicate binding of GARP to the plate; column 3 would indicate binding of rabbit anti-SPA serum; column 4 would indicate the binding of SPA. The results demonstrate that there was no significant binding of these reagents to ELISA plates blocked with PBS-T only.

3.1.2 Optimisation of concentration of capture antibody

Rows of wells were coated with capture antibody (commercial human IgG: Sigma) at concentrations between 0 and 2 μg ml^{-1}, and 100 μl samples containing 100 μl ml^{-1} were applied. The results are shown in Figure 10, and demonstrate that 1.5 μg ml^{-1} was a suitable concentration of capture IgG and was used routinely.

3.1.3 Optimisation of concentration of rabbit anti-SPA and the effect of using normal rabbit serum

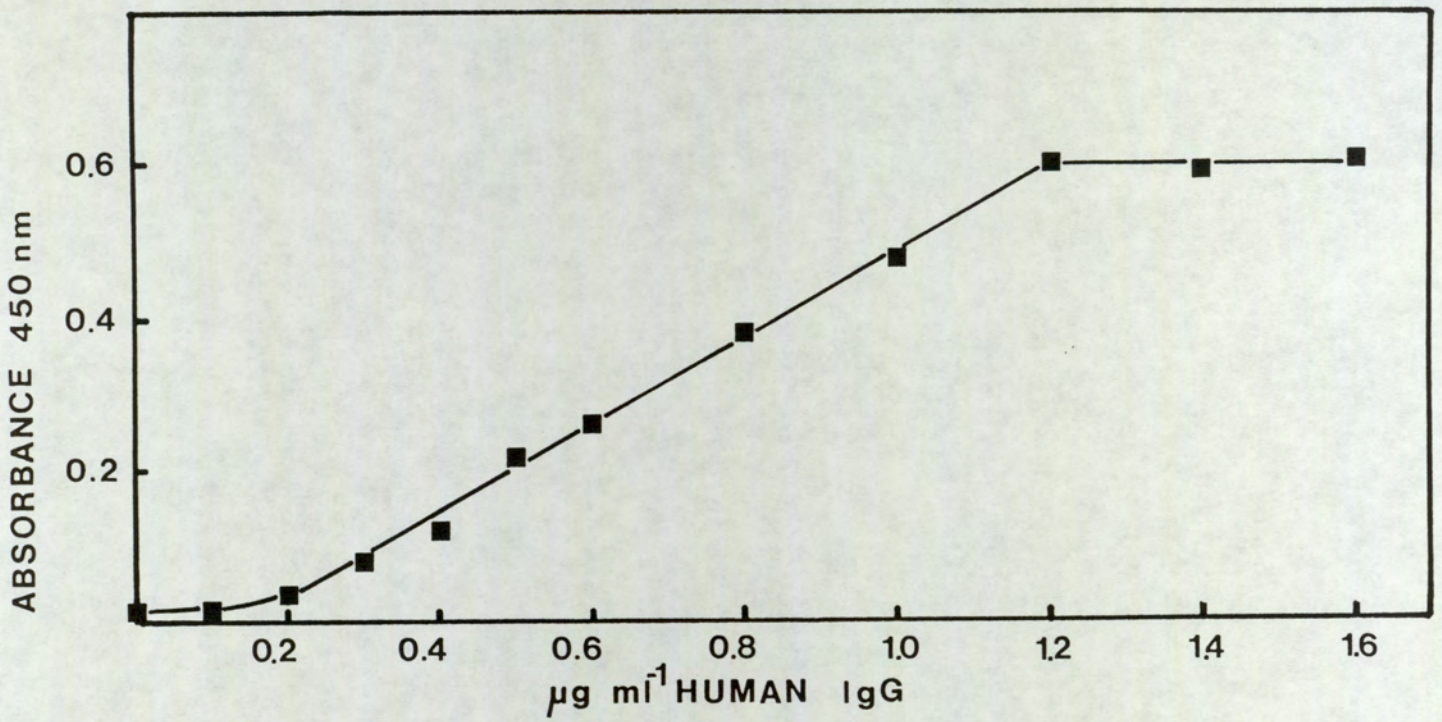
Samples containing 100 ng ml^{-1} SPA were assayed following the protocol given in section 2.2.5, but using a wide

TABLE 2 Efficiency of blocking of non-specific absorption by PBS-T

Wells recieved four different combinations of either PBS-T (shown as -) or the appropriate ELISA reagent (+). Results are expressed as mean O.D. at 450nm with a range of 8 wells.

Treatment	1	2	3	4
Coated with capture IgG (1 μg ml^{-1})	-	-	-	-
SPA (50 ng ml^{-1})	-	-	-	+
Rabbit anti-SPA (1 in 4000)	-	-	+	+
GARP (1 in 2000)	-	+	+	+
Results:				
mean O.D.	0.000	-0.001	0.001	0.005
range over 8 wells	+0.000	+0.000	+0.003	+0.008
	-0.002	-0.004	-0.001	-0.001

Fig. 10 Optimisation of human IgG concentration for ELISA



range of concentrations of rabbit anti-SPA serum. The results shown in Figure 11 indicate that a concentration of 1:2000 was suitable for routine use (although lower concentrations could be used without reducing the sensitivity of the assay significantly, it may be impractical to accurately measure small volumes of stock sera).

To demonstrate the effect on the assay sensitivity of using normal rabbit serum or PBS-T instead of rabbit anti-SPA, samples containing 10 $\mu g\ ml^{-1}$ or 100 $ng\ ml^{-1}$ SPA were assayed, using either normal rabbit serum at 1:2000 or 1:20, or PBS-T in the first detection antibody stage. Whereas the assay would normally detect down to 1-3 $ng\ ml^{-1}$ SPA at the endpoint dilution (0.1-0.3 $ng\ ml^{-1}$ in the assay), substituting normal rabbit serum at even 1:20 increased the detection limit to only 5-10 $\mu g\ ml^{-1}$ and omitting the rabbit serum altogether gave no response above background with 10 $\mu g\ ml^{-1}$ (Figure 12).

3.1.4 Concentration of enzyme conjugate

Conjugate was tested in the assay of 100 $ng\ ml^{-1}$ SPA at dilutions between 1:250 and 1:16000. The results in Figure 13 show that the assay was very sensitive to the dilution of GARP above 1:1000. Thus this batch of GARP was used at 1:1000 routinely although this dilution should be individually determined for each fresh batch of commercial conjugate.

Fig. 11 Optimisation of rabbit anti-SPA serum concentration for ELISA

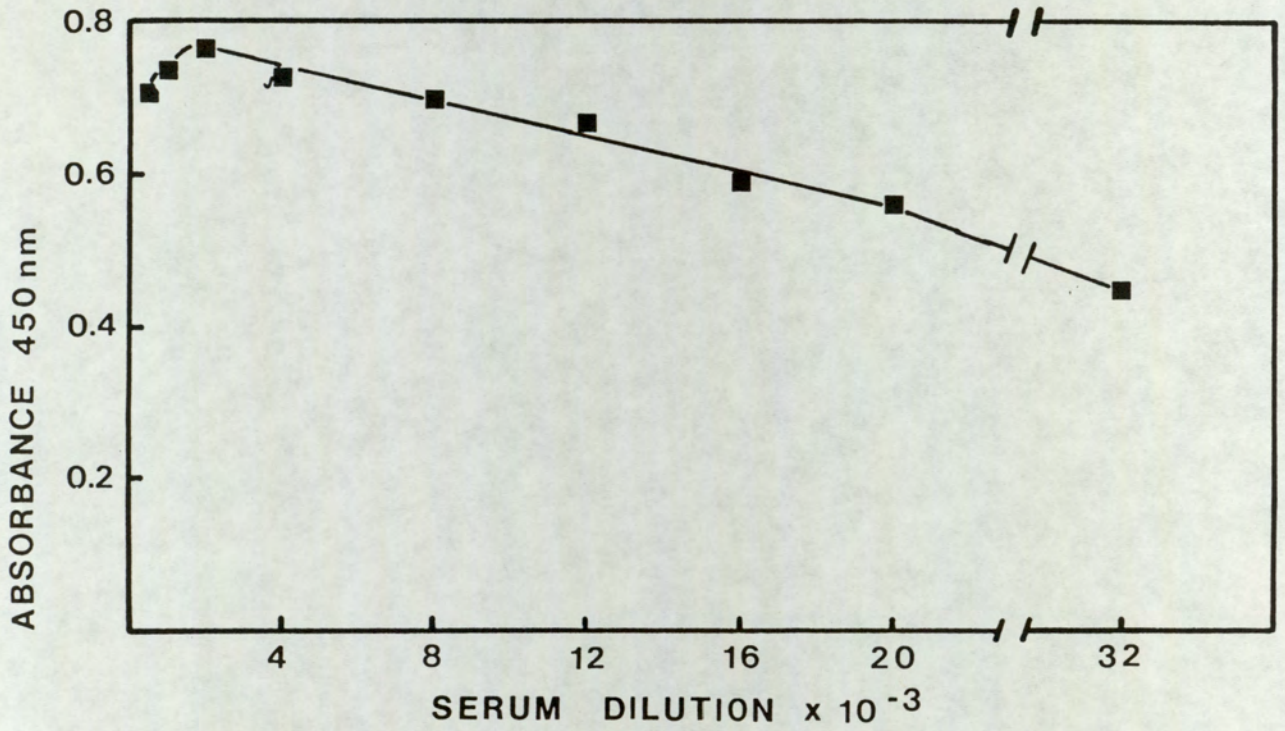


Fig. 12 Effect of omitting or substituting rabbit anti-SPA serum

Elisa's were performed as in section 2.2.5: (△) rabbit anti-SPA serum at 1:2000 dilution with 100 ng ml^{-1} SPA; (◇) rabbit anti-SPA serum at 1:2000 dilution with 10 μg ml^{-1} SPA; (▲) normal rabbit serum at 1: 2000 dilution with 10 μg ml^{-1} SPA; (○) normal rabbit serum at 1:20 dilution with 10 μg ml^{-1} SPA; (□) PBS-T with 10 μg ml^{-1} SPA.

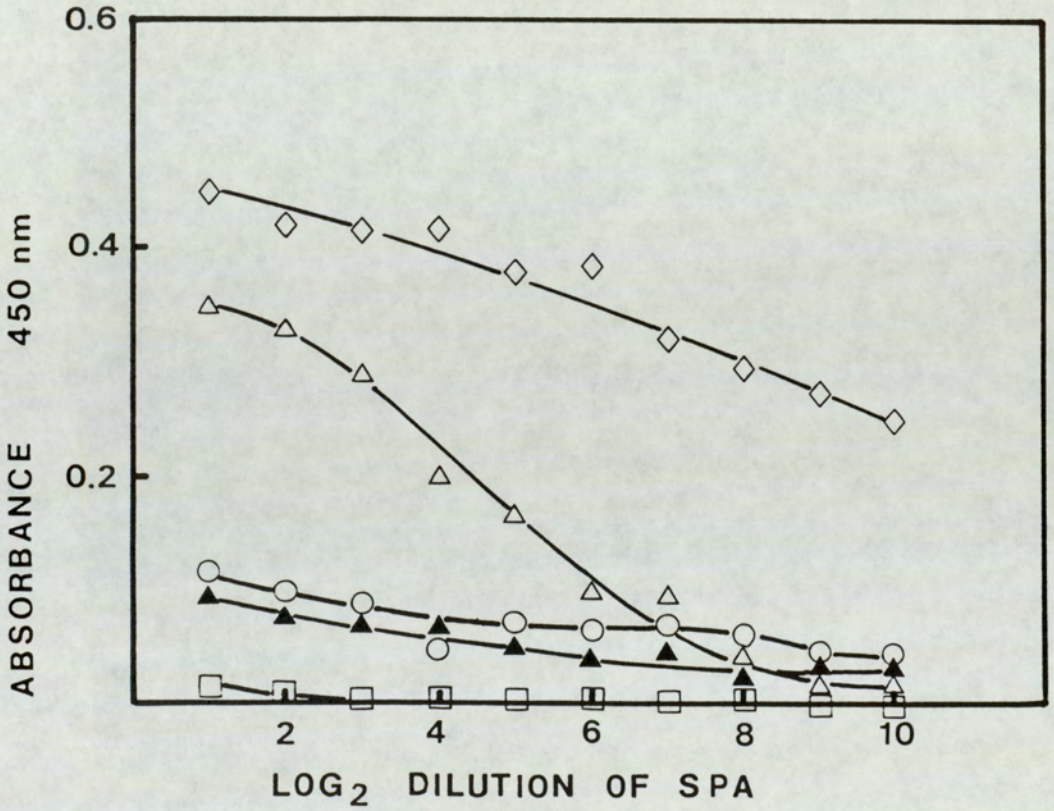
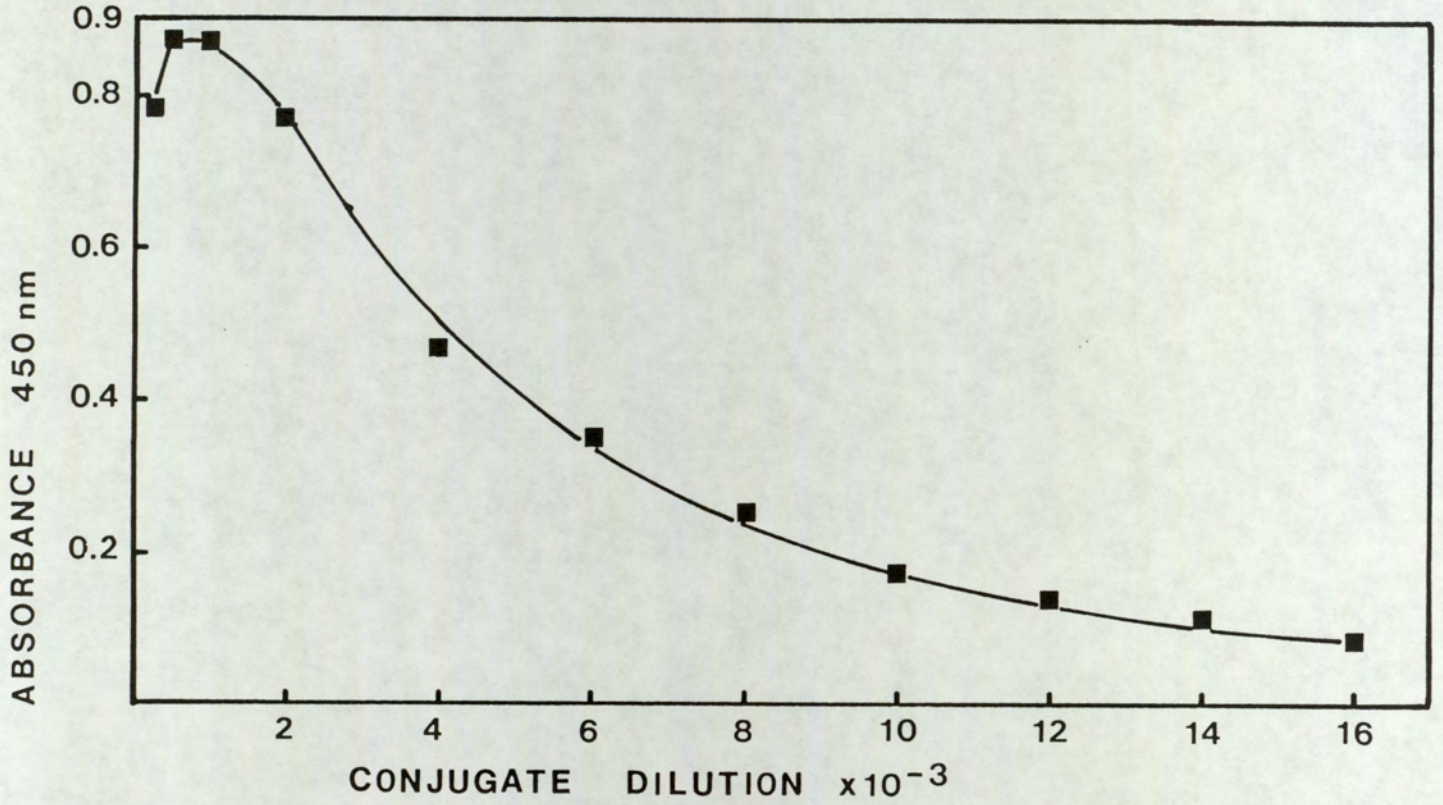


Fig. 13 Response of the ELISA using various concentrations of goat anti-rabbit IgG-peroxidase conjugate



3.1.5 Effect of time of incubation at each stage of the assay

In order to determine the optimum incubation times for the assay, samples of SPA (100 ng ml^{-1}) were assayed according to the standard protocol, but the time of incubation with SPA, rabbit anti-SPA, GARP or substrate were varied. These experiments demonstrated that the reactions with SPA, rabbit anti-SPA and GARP had reached equilibrium after 60 min (Figure 14) and that with the substrate after 25 min (Figure 15). Therefore incubations with SPA, rabbit anti-SPA and GARP were continued for 90 min and that with the substrate for 30 min. Since an internal standard was run on every plate the assay should be little affected by variations in incubation time.

3.1.6 Response of the ELISA to variations in concentration of staphylococcal protein A

Staphylococcal protein A was tested in the assay at concentrations ranging from 0.2-100 ng ml^{-1} on different occasions. The results demonstrate that saturation was achieved at 50 ng ml^{-1} and that the assay was sensitive down to 0.5 ng ml^{-1} (Figure 16). Assays performed on 10 separate occasions highlight the errors involved, which were + or - 13% at the higher values and + or - 10% at the lower values.

3.1.7 Effect of coating human IgG capture antibody in coating buffer or PBS

Plates were coated with human IgG at 1 μg ml^{-1} in either standard coating buffer or PBS containing 0.02% sodium

Fig. 14 Response of the ELISA to variations in time

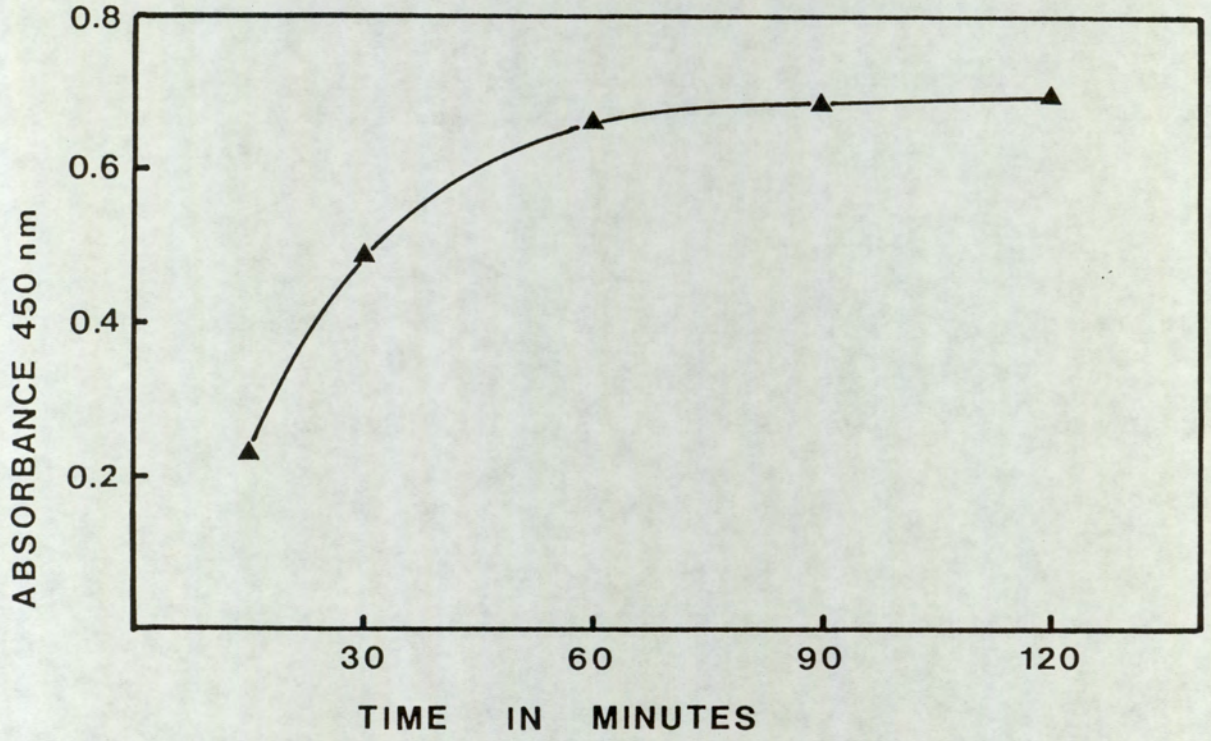


Fig. 15 Effect of the substrate incubation time on the ELISA

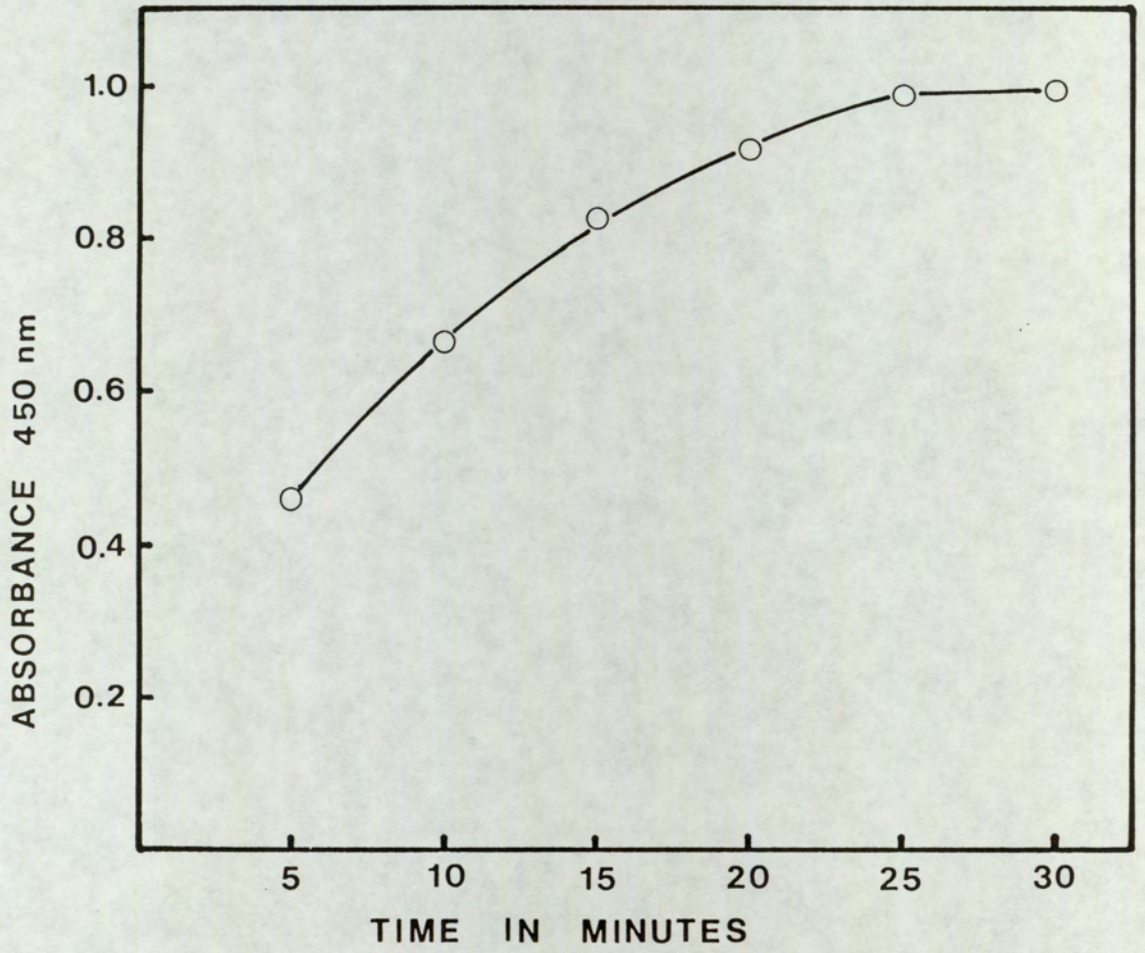
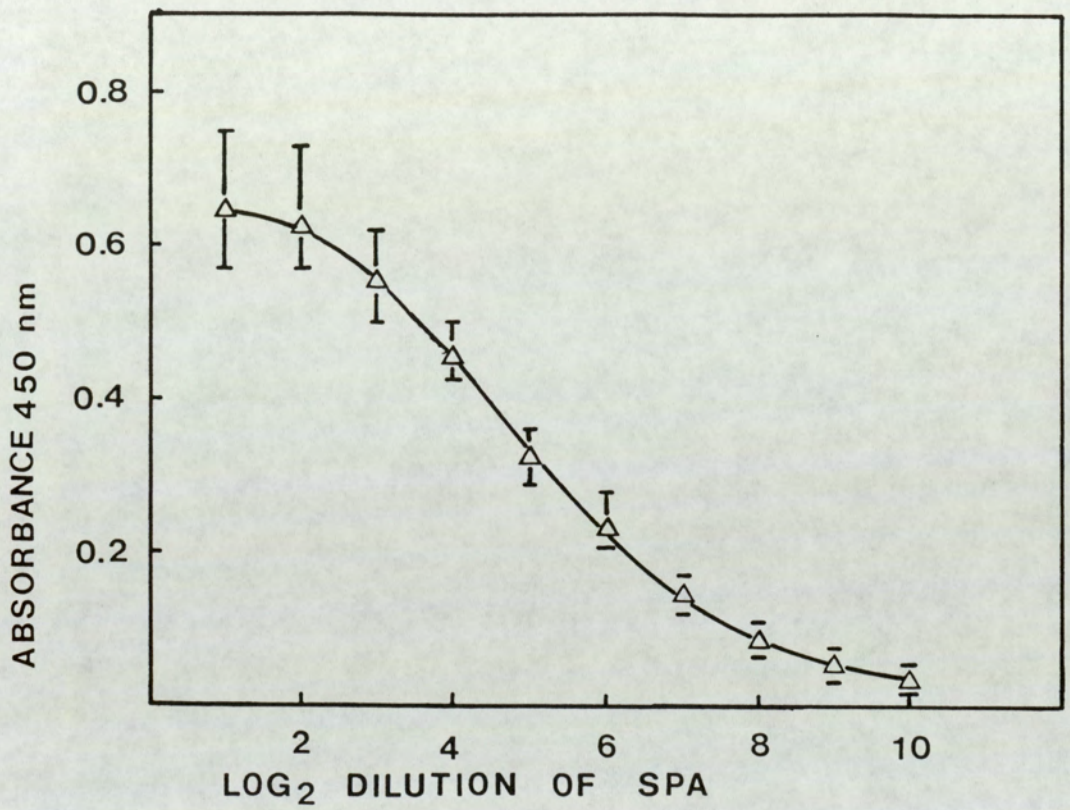


Fig. 16 Response of the ELISA to various concentrations of SPA

Each point on the curve represents the mean value from ten separate assays performed on different occasions, the error bars represent the difference in the values observed.



azide, for 16 hr at room temperature. The response to 100 ng ml^{-1} SPA was 20% lower when PBS was used as a coating buffer with these ELISA plates

3.1.8 Effect of interfering agents

A) Lysozyme, lysostaphin and other constituents of the lysis mix

Samples containing either 0,5,or 10 ng ml^{-1} SPA in either PBS-T or *E. coli* lysis buffer were diluted in PBS-T in the normal assay procedure. There was no difference in the response between the two diluents (results not shown). Lysozyme was present in the *E. coli* lysates at 1 mg ml^{-1}. To test for any interfering effects of lysozyme in the assay, it was included in samples at up to 10 mg ml^{-1}. No interference was observed (Figure 17). This result is apparently not in agreement with Esink *et al.* (1985) who sugested that lysozyme may cause spurious positive responses in assays involving antibody binding. Identical results were obtained with lysostaphin.

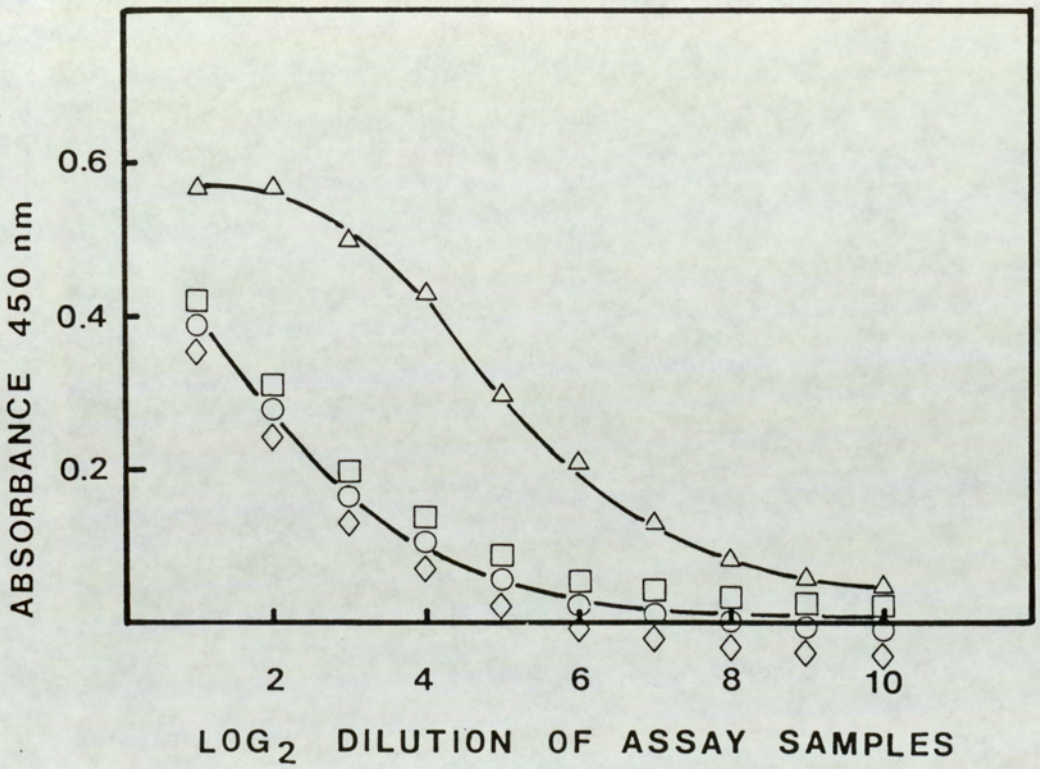
B) Bacterial lysates

The possible existence of peroxidase activity in bacterial cells was investigated by running bacterial samples, but omitting the peroxidase conjugate stage of the assay. In this situation any colour development must be due to peroxidase activity in the sample. Optical density values between +0.004 and -0.004 were obtained indicating the absence of interfering peroxidases from the bacterial lysates.

E. coli is an inconvenient source of material which is

Fig. 17 Insensitivity of the ELISA to interference from *E. coli* lysates and lysozyme

(△) SPA with 100 ng ml^{-1}; (□) *E. coli* lysate (1:4 dilution) with 10 ng ml^{-1} SPA; (○) as (□) with 10 mg ml^{-1} lysozyme; (◇) PBS-T plus 10 ng ml^{-1} SPA.



to be assayed immunologically because of the presence in all human, rabbit and goat sera of anti-*E. coli* antibodies. To establish the background due to cross-reaction with bacterial antigens, the following experiments were performed.

SPA at 0,5, or 10 ng ml^{-1} was diluted in either PBS-T; negative *E. coli* lysates or negative *S. aureus* lysates and assayed by ELISA. The *E. coli* lysates contributed a background activity which was only significant when the lysates were at a concentration of more than 1:10 (Figure 18). Identical results were obtained with the *S. aureus* lysates. Since the assay detected less than 0.5 ng ml^{-1} SPA, this background was insignificant in practice.

3.1.9 Analysis of the ELISA using western blotting techniques

ELISA data reflects the overall content of antigens in a complex mixture. PAGE and Western blotting shows the contributions of individual proteins. Figure 19A shows that several *E. coli* proteins can be recognised by the rabbit anti-SPA serum (tracks **A** and **B**), some of which are also found in both SPA-positive and SPA-negative *S. aureus* strains. The SPA in the *E. coli* lysates appear in several forms (43-48 kilo daltons), all of which are smaller than the forms of SPA found in the *S. aureus* lysates. However, although SPA is not the only protein detected by the rabbit anti-SPA serum it is clearly the major antigen in both *E. coli* and *S. aureus* lysates. Commercially available SPA was also compared (Fig. 19B), as well as a small difference in molecular weight all samples contained multiple bands

Fig. 18 Insensitivity of the ELISA to interference from bacterial antigens

(△) 100 ng ml^{-1} SPA; (□) negative *E. coli* lysate (1:4 dilution) plus PBS-T; (○) as (□) with 5 ng ml^{-1} SPA; (◇) as (□) with 10 ng ml^{-1} SPA.

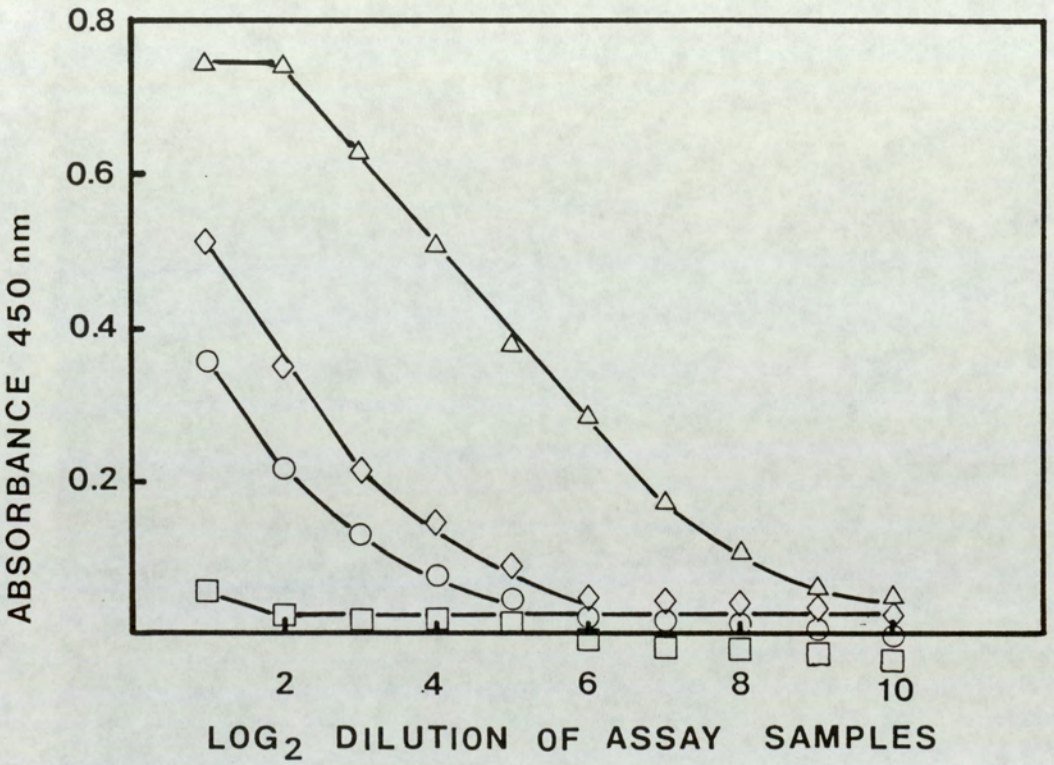


Fig. 19A PAGE analysis of antigens in bacterial lysates

Samples of bacterial lysates or purified proteins were run under partially denaturing conditions and subjected to Western blot analysis as described in section 2.2.7 and lanes A, C, E, G and I contained 2 µl of sample and lanes B, D, F, H and J 14 µl. Lanes A and B: *E. coli* lysates from JM83; C and D: *E.coli* lysates from JM83 containing pPA16; E and F: purified protein A (Sigma); G and H: *S. aureus* Cowan I lysates (positive control); I and J: *S. aureus* Wood 46 lysates (negative control). The position of various low molecular weight markers (Pharmacia) are shown on the right hand side.

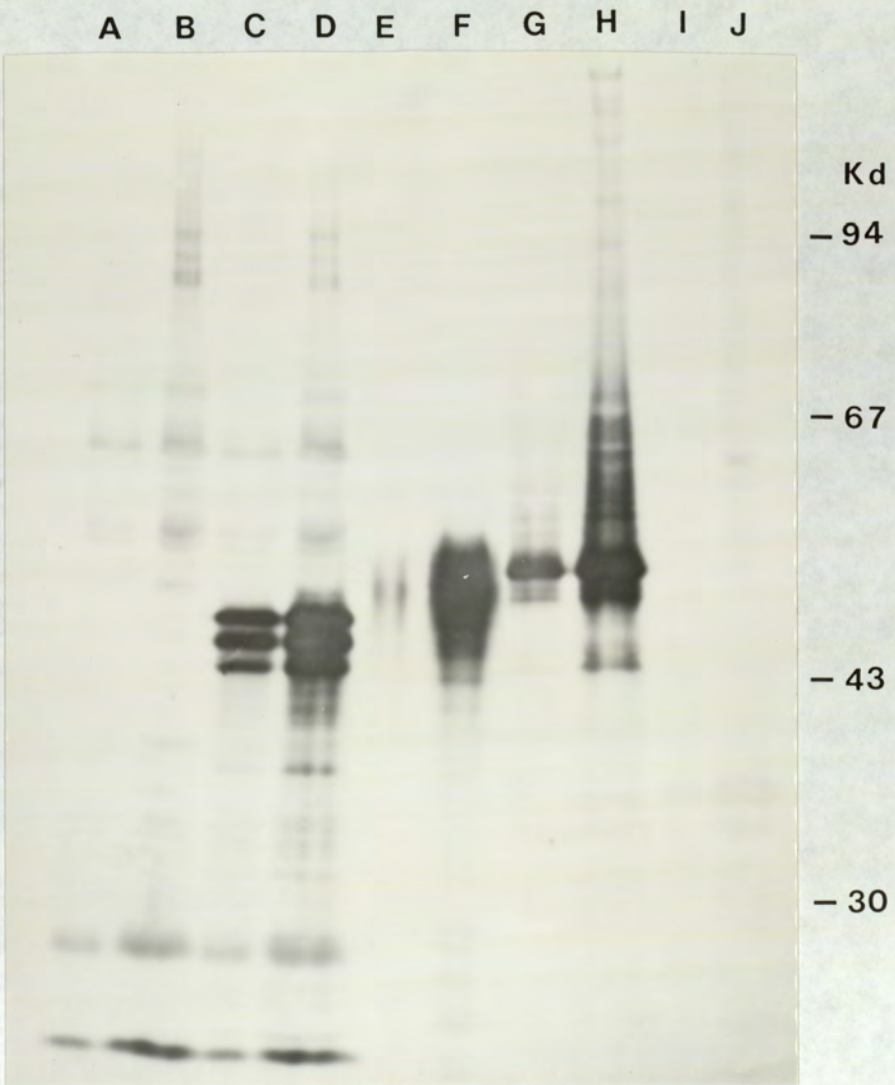
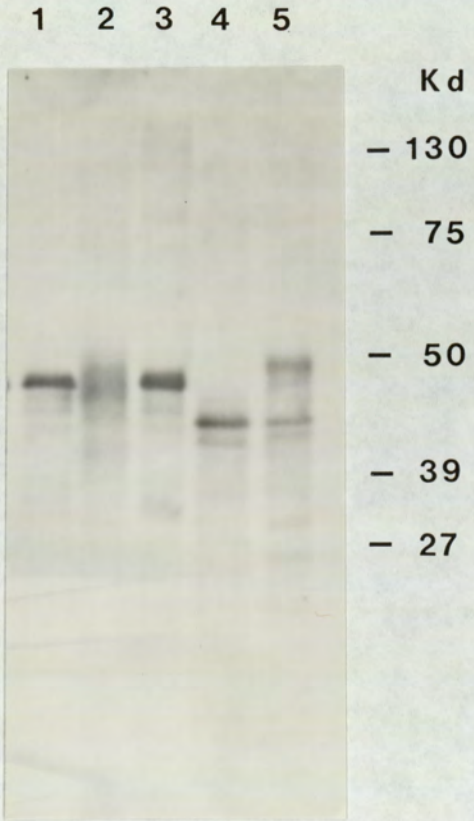


Fig. 19B PAGE analysis of commercially purified protein A Samples of purified protein A from various commercial sources were run under partially denaturing conditions and subjected to western blot analysis as in 2.2.7. Track 1, purified SPA produced by Fermentech; Track 2, purified SPA produced by Sigma; Track 3, purified SPA produced by Pharmacia; Track 4, purified tailess SPA produced by CAMR; Track 5, purified full length SPA produced by CAMR. Molecular weight markers are shown on the right hand side of the western blot.



which bound IgG.

3.1.10 Use of the ELISA to determine the amount of SPA in sonicates and lysates from *E. coli* JM83 with pPA16 when grown in batch culture

Quantitation of SPA in sonicates and lysates from *E. coli* JM83 (negative control) and the same host containing the plasmid pPA16 were performed as in 2.2.5 except that *E. coli* JM83 was grown in ampicillin free medium. The results indicate that there is increased recovery by 10% of SPA in sonicated samples compared to the lysozyme-treated cells, with a total recovery of 50µg SPA ml^{-1} culture or 4.0% of the total soluble protein (Table 3).

Table 3 Recovery of SPA from bacterial lysates

	E. coli JM83 +pPA16	*E. coli* JM83
Dry weight (mg ml^{-1})	1.78	1.8
Total soluble protein (ng ml^{-1})	1.14	1.13
Sonicated samples SPA concentration (µg ml^{-1})	47.7	<0.0004
Lysozyme treated samples SPA concentration (µg ml^{-1})	43.0	<0.0004
Supernatant SPA concentration (µg ml^{-1})	5.3	<0.0004
% SPA of total soluble protein	4.1	0
%SPA of dry weight	2.6	0

Positive and negative SPA producing *E. coli* strains were assayed for the presence of SPA by the ELISA method. Both sonicated and lysozyme treated samples were assayed and the values from the former expressed as a percentage of the total soluble protein or the dry weight.

3.2 NUTRIENT DEPLETION STUDIES OF *E. COLI* STRAIN JM83 BOTH WITH AND WITHOUT THE PLASMID pPA16 IN BATCH CULTURE

The requirement of certain nutrients in *E. coli* W3110 with and without the R plasmid RP1 has already been studied by Klemperer *et al.* (1979). They concluded that although the plasmids remained stable R+ cells had a greater requirement compared to R- cells for several nutrients, though the maximum specific growth rates were the same. The final cell density reached by a culture of *E. coli* was dependent, below certain levels, on the initial concentration of a growth limiting nutrient in the medium. The object of this study was to investigate the nutritional requirements of *E. coli* strain JM83 using either glucose or glycerol as the carbon source and to achieve a suitable medium for its growth in continuous culture.

3.2.1 **Medium**

The medium used for the study of glucose, glycerol, magnesium, ammonium, phosphate and proline is that described in Table 1. Experimental details have already been presented in section 2.1.2

3.2.2 **Carbon depletion studies**

Because of the possible effects of catabolite repression as described in 1.51 two alternative carbon sources were used. Throughout the investigations glycerol was used as the sole carbon source, while glucose was used only as an alternative to glycerol when investigating carbon depletion.

In glucose depletion studies, low levels of carbon in the medium resulted in rapid cessation of growth in the cultures of both JM83 (Figure 20) and JM83 with the plasmid pPA16 (Figure 21). At higher concentrations there was a decline in the growth rate indicating that glucose was no longer the limiting factor. The relationship between glucose concentration and the loss of linear growth for plasmid-free cells is shown in Figure 22 and Figure 23 for plasmid-containing cells. The relationship was linear to an absorbance of 1.9 at 470 nm under the conditions used in this study for both the plasmid containing and plasmid free cells.

In glycerol depletion studies, low levels of carbon in the medium resulted in a decline of growth both for plasmid-free cells (Figure 24) and plasmid containing-cells (Figure 25). At higher concentrations both investigations demonstrated a small decline in growth rate indicating glycerol was no longer limited. The onset of glycerol depletion was therefore taken to occur when growth ceased to be exponential. There is a general difference in growth rates between each study, with plasmid-free cells being approximately double that of the plasmid-containing cells. A similar result is also shown under glucose depletion where both cells with and without the plasmid have a growth rate similar to the plasmid-free cells grown under glycerol depletion. The relationship between glycerol concentration and the loss of linear growth for plasmid free cells is shown in Figure 26 and Figure 27 for plasmid containing cells. The relationship was linear to an absorbance 1.8 at

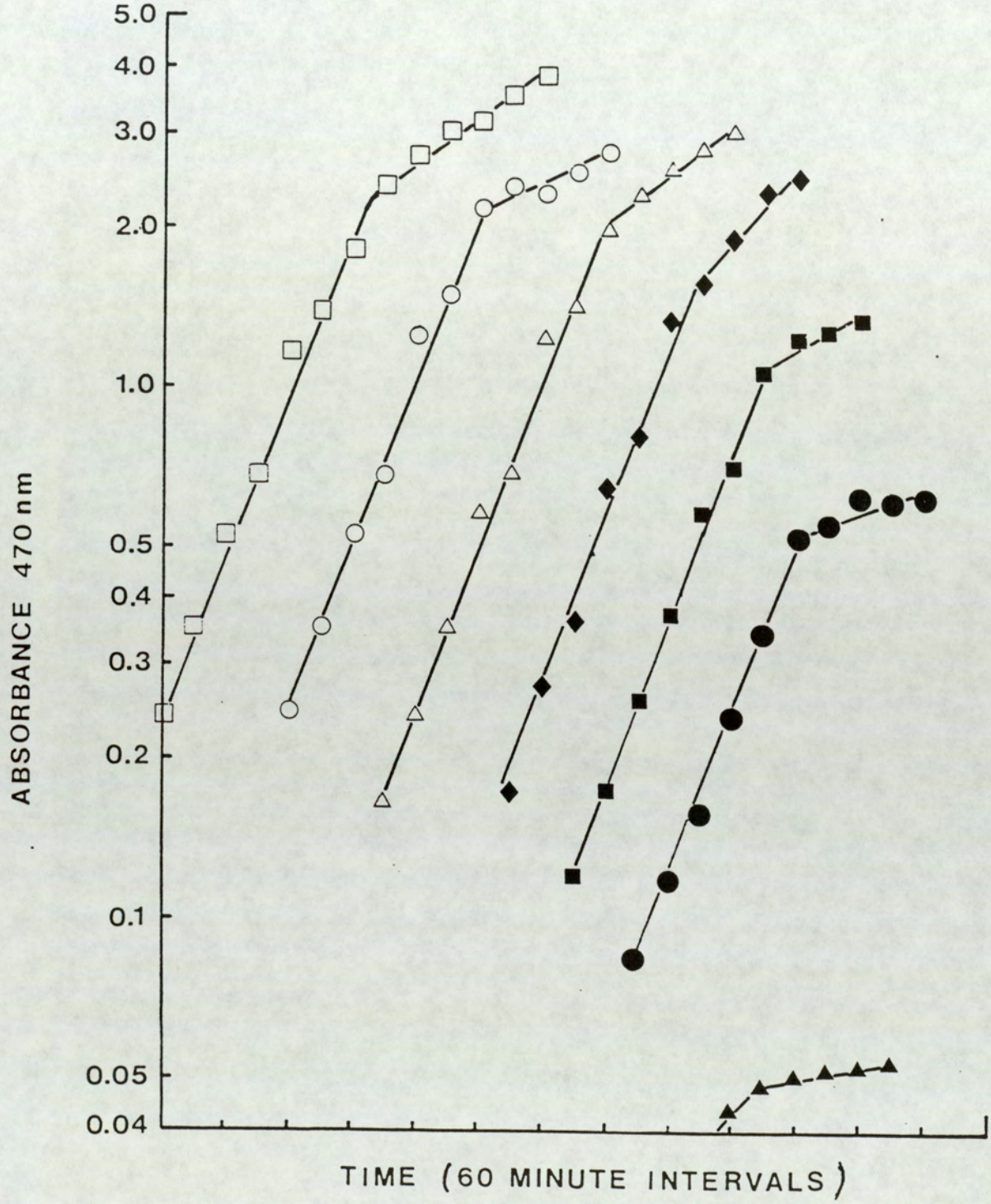
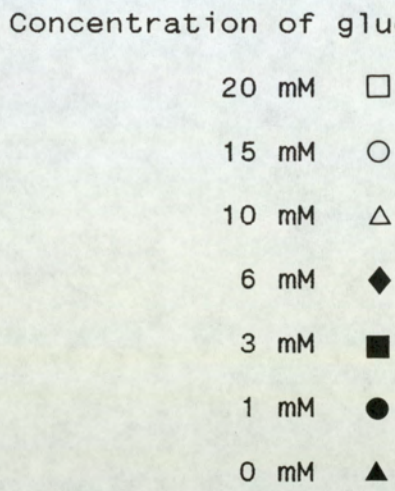

5.0
4.0
3.0
2.0
1.0
0.5
0.4
0.3
0.2
0.1
0.05
0.04
ABSORBANCE 470 nm
TIME (60 MINUTE INTERVALS)

Fig. 20 Growth of *E. coli* JM83 in CDM with graded concentrations of glucose



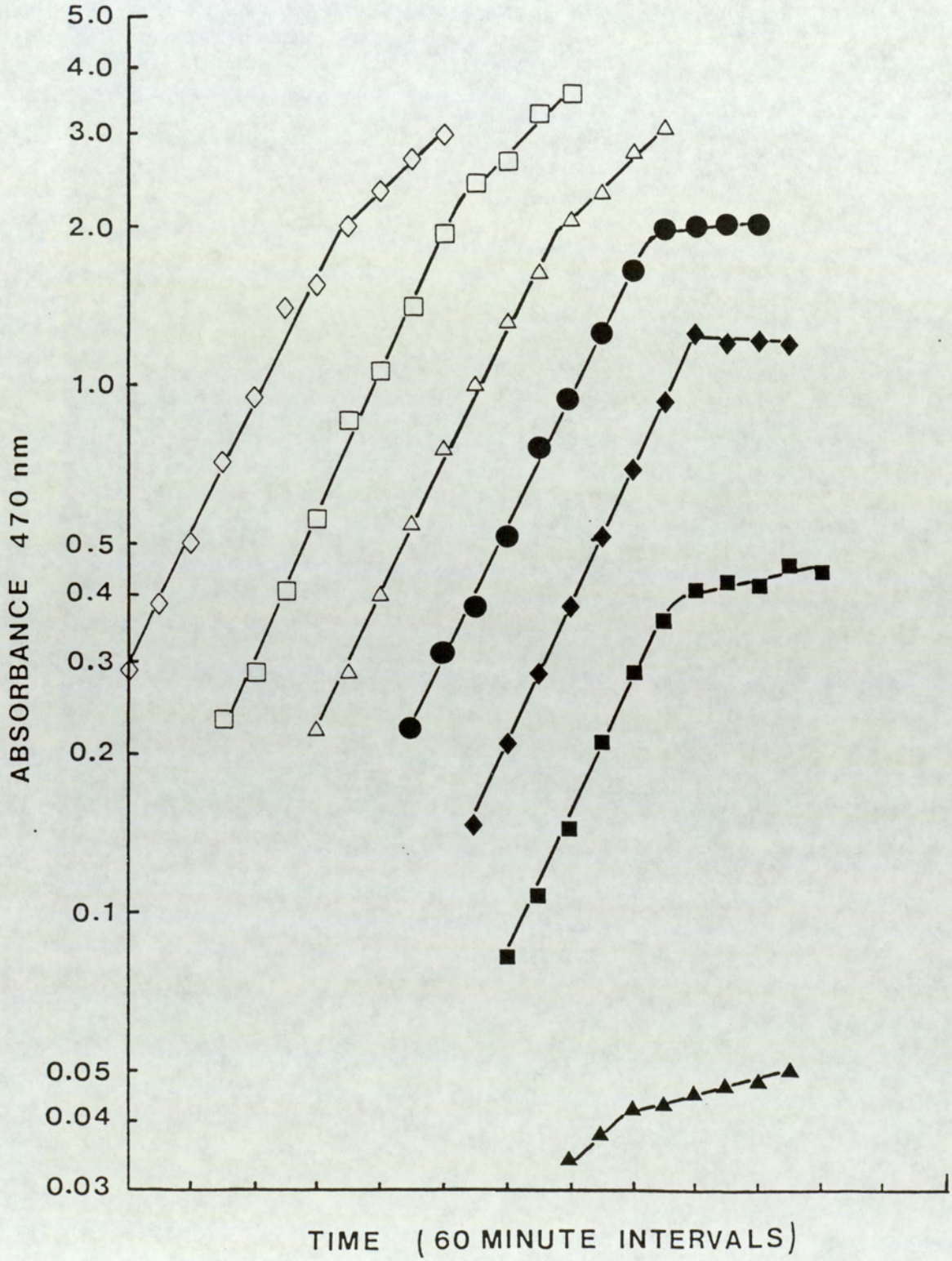

5.0
4.0
3.0
2.0
1.0
0.5
0.4
0.3
0.2
0.1
0.05
0.04
0.03
ABSORBANCE 470 nm
TIME (60 MINUTE INTERVALS)

Fig. 22 Relationship between the onset of non-linear growth of *E. coli* JM83 and initial glucose concentration

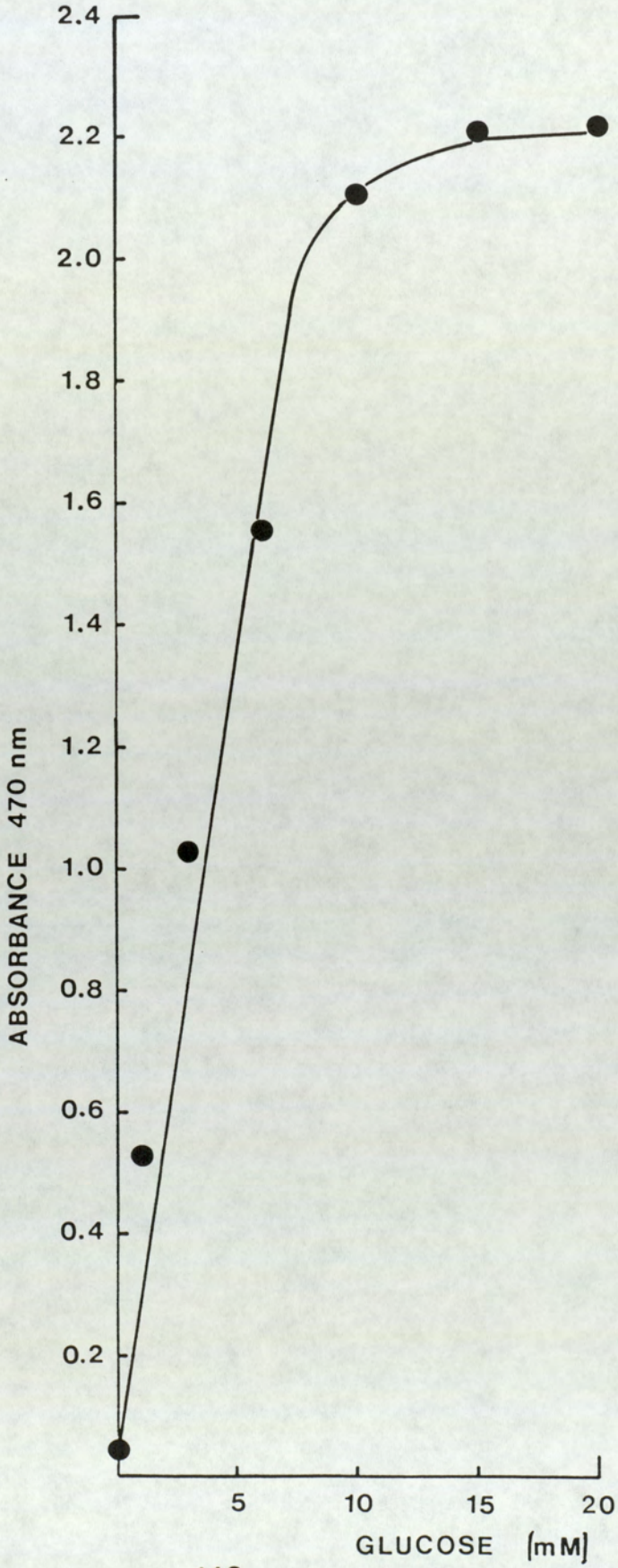
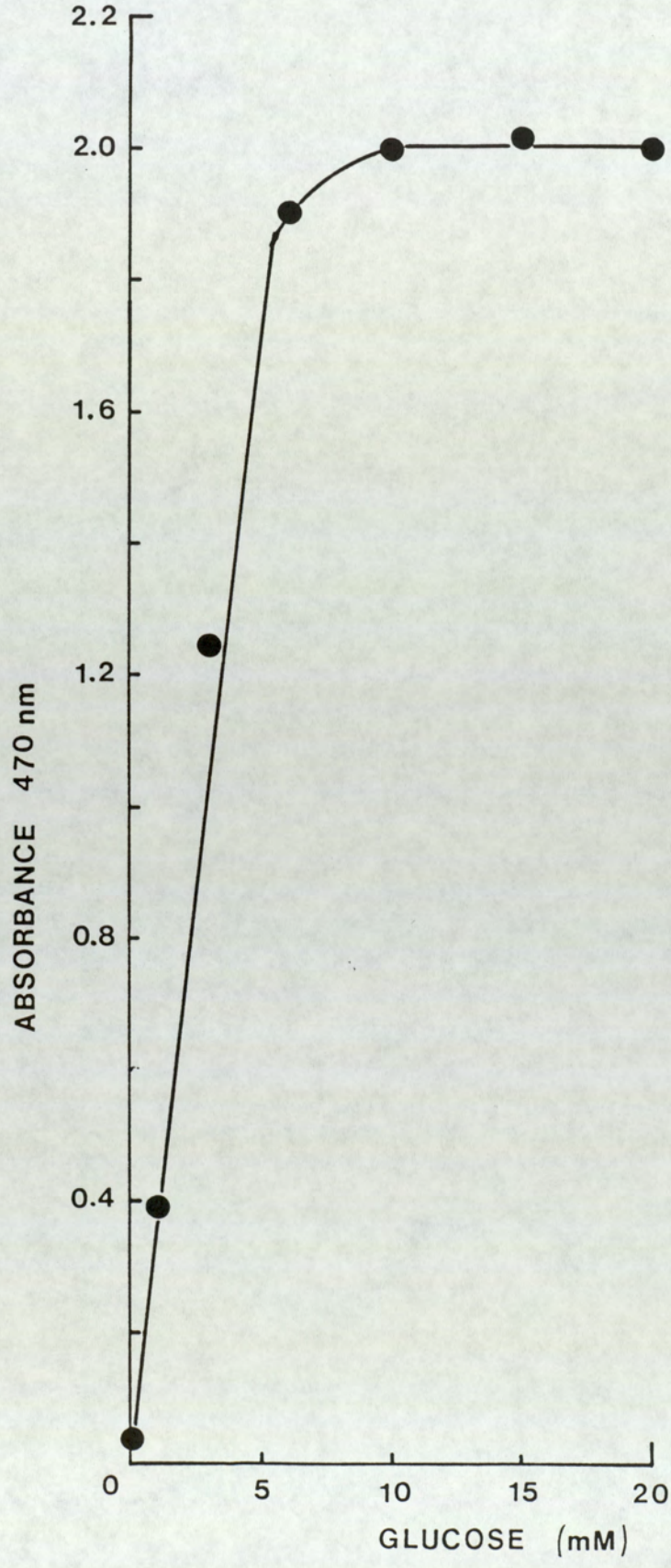


Fig. 23 Relationship between the onset of non-linear growth of *E. coli* JM83 with pPA16 and initial glucose concentration



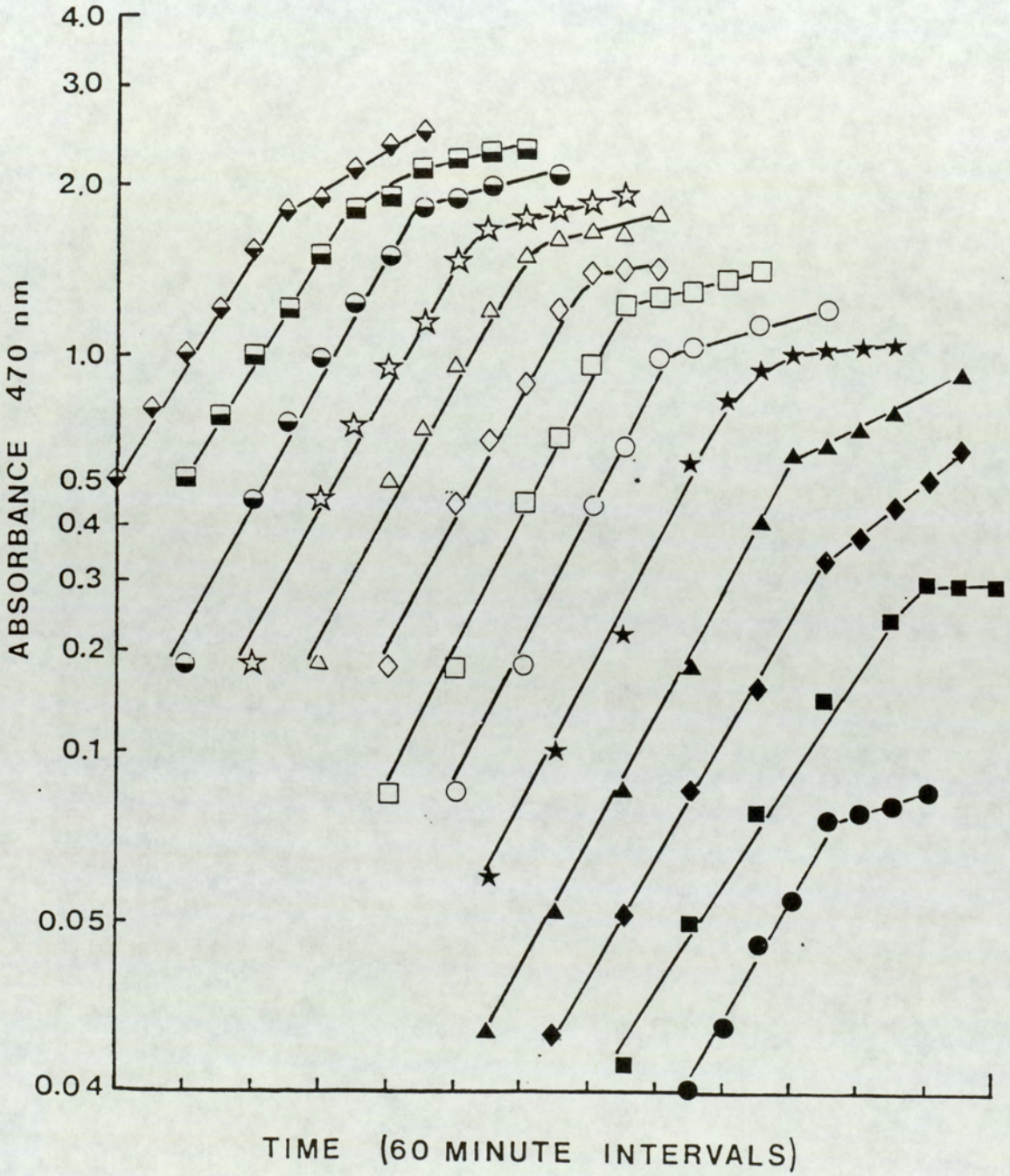
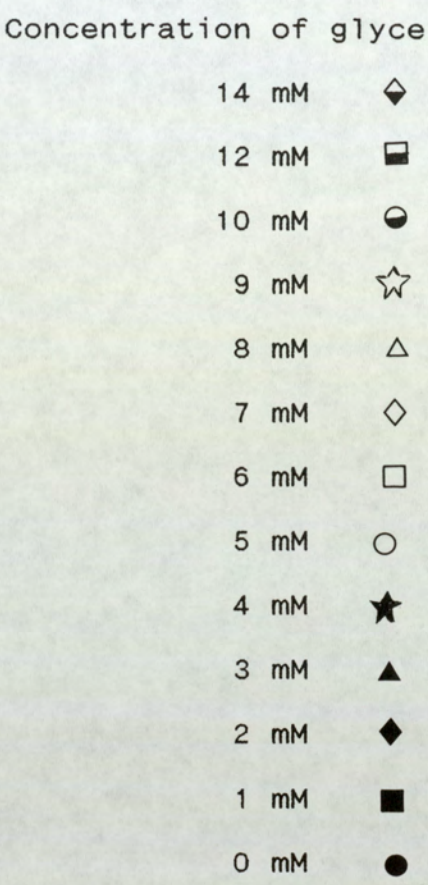

4.0
3.0
2.0
1.0
0.5
0.4
0.3
0.2
0.1
0.05
0.04
ABSORBANCE 470 nm
TIME (60 MINUTE INTERVALS)

Fig. 24 Growth of *E. coli* JM83 in CDM with graded concentrations of glycerol



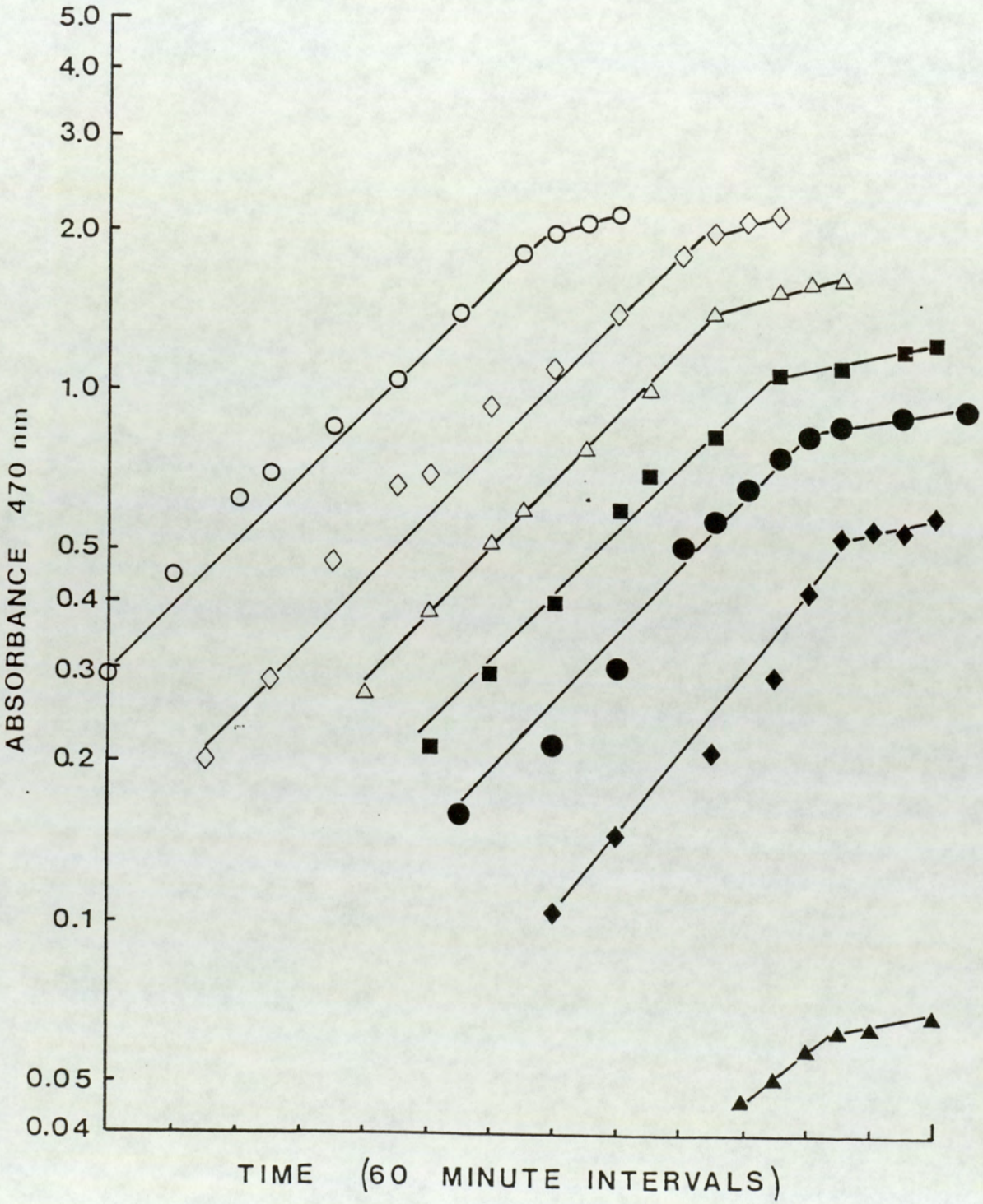

5.0
4.0
3.0
2.0
1.0
0.5
0.4
0.3
0.2
0.1
0.05
0.04
ABSORBANCE 470 nm
TIME (60 MINUTE INTERVALS)

Fig. 25 Growth of *E. coli* JM83 with pPA16 in CDM with graded concentrations of glycerol

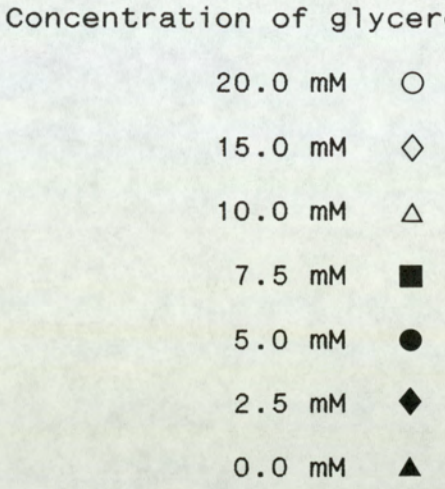


Fig. 26 Relationship between the onset of non-linear growth of *E. coli* JM83 and initial glycerol concentration

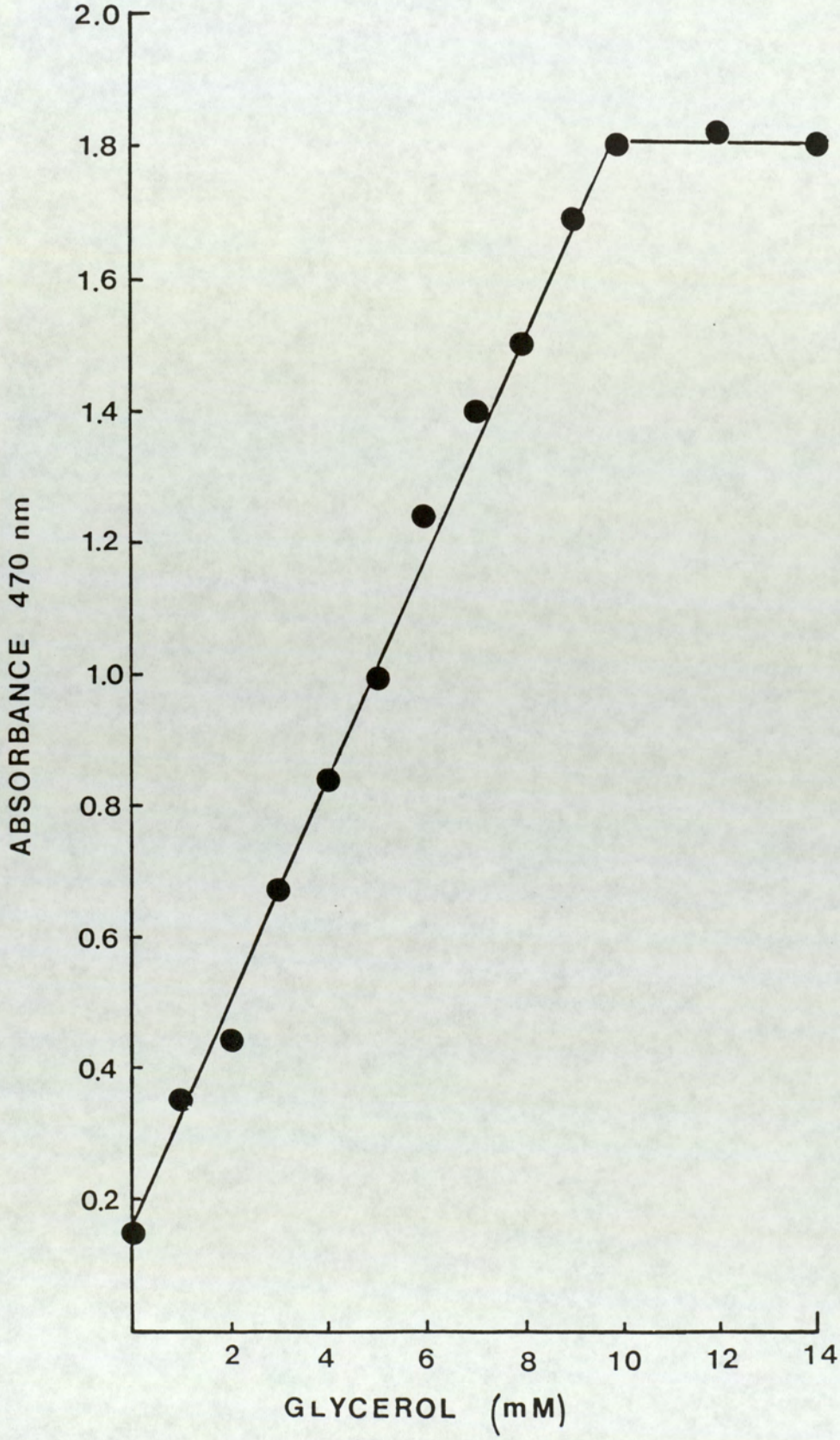
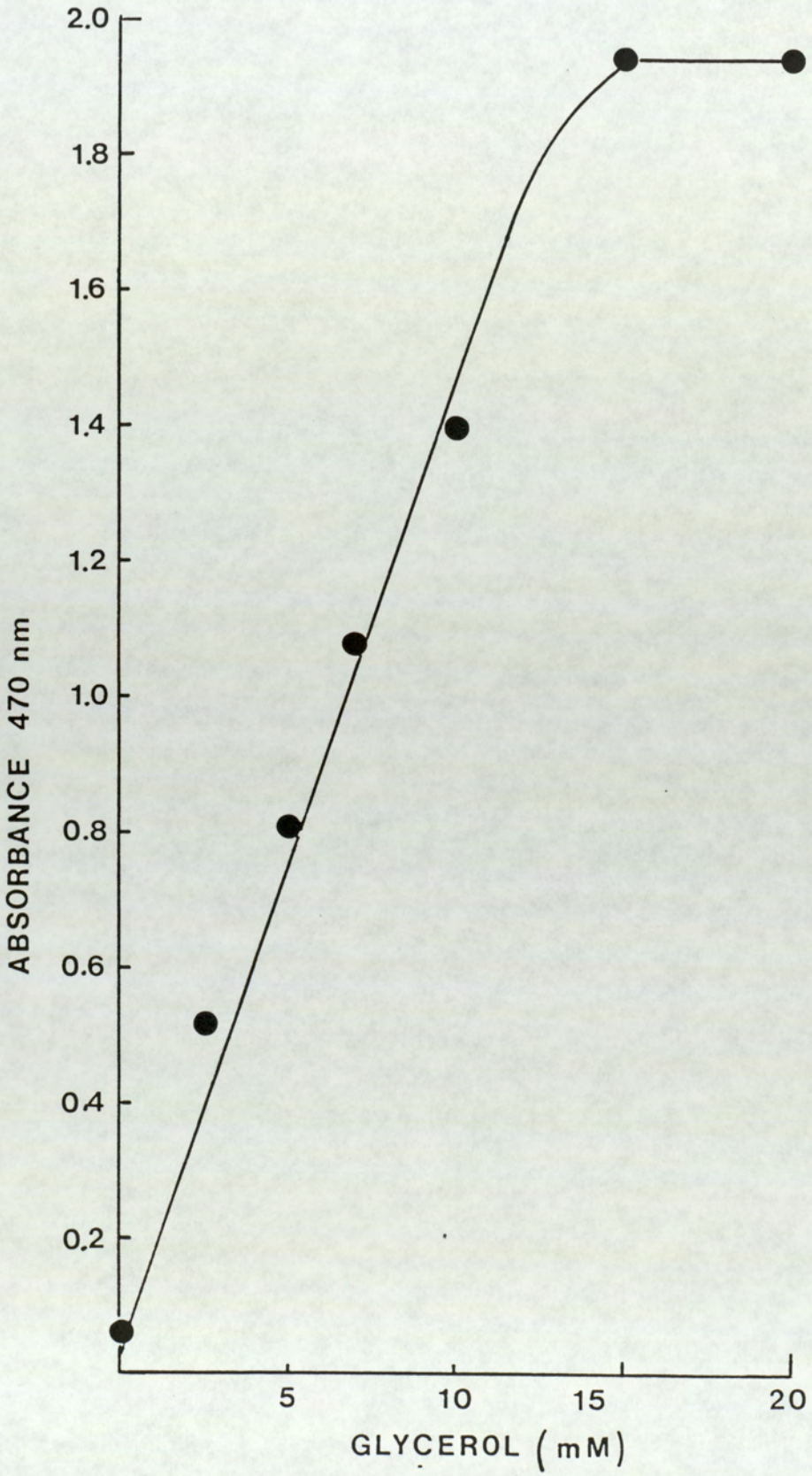


Fig. 27 Relationship between the onset of non-linear growth of *E. coli* JM83 with pPA16 and initial glycerol concentration



470 nm under the conditions used in this study both for the plasmid-containing and plasmid-free cells.

3.2.3 Other nutrient depletion studies

Nutrient depletion studies were also performed with ammonium, magnesium, phosphate and proline with both JM83 and JM83 containing pPA16 as described in section 3.2.2. The results of the growth experiments using graded concentrations of each nutrient (results not shown) allowed an estimate of the maximum specific growth rate (μ^{MAX}) in batch to be obtained (Table 4; see 1.8). Although the results are not a true estimate of the μ^{MAX} they give a clear indication of the hosts growth rate both with or without the plasmid pPA16, for all the nutrients investigated. The results indicate that μ^{MAX} values were generally lower when the host contained the plasmid compared to plasmid free cells. This was demonstrated by the high μ^{MAX} value obtained with JM83 when using glucose as a carbon source when compared to the reduced value of JM83 with pPA16. A similar result was seen when glycerol was used as the carbon source, with the μ^{MAX} for the host JM83 being greater than that containing the plasmid, although both results were greatly reduced when compared to their counterparts grown with glucose. Results with ammonium, magnesium and proline gave values similar to those obtained with glycerol when considering plasmid-free cells, while those with plasmid produced lower values. Finally the results with graded concentrations of phosphate gave the lowest μ^{MAX} values for cells both with and without the plasmid. The reduction in

TABLE 4 Batch μ^{MAX} for *E. coli* JM83 both with and without the plasmid pPA16

Substrate	*E. coli* JM83	*E. coli* JM83/pPA16
Glucose	0.72	0.57
Glycerol	0.49	0.30
Ammonium	0.52	0.24
Phosphate	0.46	0.21
Proline	0.48	0.24
Magnesium	0.52	0.24

growth under phosphate depleted conditions may be due to the wide ranging uses of phosphate in the host, including; DNA and RNA replication, protein synthesis and other metobolic functions.

3.2.4 Analysis of batch culture studies

From the results of the batch culture experiments involving nutrient depletion, the concentration of each nutrient can be calculated to produce growth of the organism to an optical density of 1.0 at 470 nm. The results for cells both with and without the plasmid pPA16 are shown in Table 5 and are also compared to the results obtained by Klemperer *et al.* (1979) from which this chemically defined medium is based. The concentrations produced from these experiments could then be used in the CDM for further investigations using continuous culture. Nutrients which would not be limited were in ten-fold excess.

3.2.5 The effect of subculturing *E. coli* JM83 with pPA16

To give an indication of the stability of the plasmid, five flasks each containing 24.75 ml of CDM were prepared with each of the following limited media: ammonium, glycerol, magnesium, phosphate and proline. The flasks were then inoculated with the host strain (*E. coli* JM83) containing pPA16 from a 16 hr culture grown in unlimited CDM, to give a final optical density of 1.0 at 470 nm after 24 hr incubation at 37°C and shaken at 150 rpm. The host organism was then subcultured every 24 hr for 5 days. JM83 with pPA16 was also subcultured into glucose limited CDM and TSB

Table 5 Concentration of added nutrients which permit growth to an optical density of 1.0 at 470 nm

Nutrient	*E. coli JM83*	*E. coli* JM83/pPA16	*E. coli W3110	*E. coli W3110/RP1
Glucose	3.5	2.5	2.5	2.8
Glycerol	5.0	6.5	-	-
$MgSO_4$	0.04	0.01	0.013	0.025
KCl	0.03	0.04	0.026	0.042
NH_4Cl	1.1	1.25	1.85	1.75
Phosphate	0.45	0.65	0.17	0.56
Proline	1.4	1.45	-	-

The symbol * denotes the results obtained by Klemperer *et al.* (1979) on whose media this was based. Trace amounts of thiamine and $NH_4Fe(SO_4)_2.12H_2O$ were also added.

(positive control) but because of differences in the growth rates the latter two were subcultured every 12 hr for 2.5 days. The final subcultures for each of the flasks were then plated onto TSBA and incubated for 18 hr at 37°C. 100 colonies were then replicated as in section 2.2.11.3. The results (Table 6) indicated that limited glucose would provide a stable environment for the plasmid as would TSB the positive control. The remaining limiting nutrients gave varying results ranging from ammonium and proline which showed little loss (2-3%) of the plasmid to magnesium and glycerol which gave a loss of 15 and 27%. Finally phosphate limiting conditions produced high instability with 77% of the population showing plasmid loss.

Table 6 The effects of subculturing *E. coli* JM83 with pPA16

Nutrient depleted	The percentage of ampicillin resistant cells after 5 subcultures
TSB (positive control)	100
Glucose	100
Ammonium	98
Proline	97
Magnesium	85
Glycerol	73
Phosphate	23

3.3 THE EXPRESSION AND STABILITY OF PLASMIDS CONTAINING THE SPA GENE IN *E. COLI* WHEN GROWN IN CONTINUOUS CULTURE

3.3.1 Glucose limiting conditions

Due to the unknown effects of catabolite repression on the stability and expression of the SPA clones, glucose and glycerol were used as alternative carbon sources. The results shown in Figure 28 depict the stability and expression of pPA16 in *E. coli* JM83 grown in continuous culture under glucose limitation at a dilution rate of 0.1 hr^{-1}. The X-axis has been broken down into three areas representing the growth in batch culture (B), the transitional growth phase (T; which includes the time when the nutrient pump is switched on to the attainment of steady state with regards to optical density) and the number of generations in continuous culture, with the changes in the nutrient reservoir denoted by the symbol (Δ). The Y-axes indicate the percentage of ampicillin resistant cells in the population as described in 2.2.11.3, the copy number of the plasmid per host chromosome in JM83 as described in section 2.2.11.9. The amount of protein A present mg^{-1} of cell protein (which not only allows comparisons to be made throughout the experiment but also with other fermenter runs) and finally the amount of SPA present in the culture supernatant. The percentage of ampicillin resistant cells in the population indicated that the plasmid was stable for up to 12 generations, although the copy number had fallen from an initial value of 350 to 170 per host chromosome. This was in agreement with SPA production, which rose initially in both batch and the transitional phase to give a

PERCENTAGE AMPICILLIN RESISTANT CELLS
COPY NUMBER x 10²
µg SPA mg⁻¹ CELL PROTEIN
µg SPA ml⁻¹ CULTURE SUPERNATANT
GENERATIONS IN CONTINUOUS CULTURE
B
T

Fig. 28 The stability and expression of pPA16 in *E. coli* JM83 when grown in glucose limited media at a dilution rate of 0.1 hr^{-1}; The X-axis has been broken down into the batch phase (B); transitional phase (T; growth from switching on the nutrient pump to "steady state"); the number of generations in continuous culture. The Y-axis depicts the percentage of ampicillin resistant cells in the population (●); Copy number of the plasmid per host chromosome (■); amount of SPA mg^{-1} of cell protein (▲); amount of SPA ml^{-1} culture supernatant (◆). With changes in the nutrient reservoir denoted by the symbol (△).

peak value of 11 μg mg^{-1} cell protein and then fell to 3 μg mg^{-1} at the end of the run. The levels of SPA in the supernatant was consistently 10% of that seen in the cell paste.

Figure 29 is a gel showing the restriction pattern of the plasmid taken from the samples when cut with the restriction enzymes EcoRI and PstI (see 2.2.11.5). As can be seen the molecular weight for the two bands are approximately 2.6 Kb and 1.9 Kb which corresponds to that of the pUC8 vector and the inserted SPA gene. This indicates that no major deletions or rearrangements had occurred although this would not detect the presence of multimers.

Figure 30 is a gel of whole cell lysates from each of the samples which have been stained with ethidium bromide. The resulting bands are shown as the chromosomal and plasmid DNA. Scanning densitometry can then be performed on photographic negatives to calculate the copy number per host chromosome (section 2.2.11.9).

Figure 31 is a western blot showing the qualitative analysis of SPA produced at the particular sample times (section 2.2.7). As can be seen there are a small number of proteins which cross react and correspond to those found in the negative control. The main bands are of molecular weights approximating 43, 45 and 48 Kd and represent the SPA produced by the host. There also appears to be no total loss of production which corresponds to the quantitative analysis of SPA.

The results from Figure 32 show the stability and expression of JM83/pPA16 when grown in glucose limiting

Fig. 29 Restriction analysis of pPA16 in *E. coli* JM83 when grown in glucose limited media at a dilution rate of 0.1 hr^{-1}

Tracks 3-14 are samples taken from the fermenter run at the following time intervals: B, 7; T, 17, 24 and 42; Generations in continuous culture, 4, 7, 9, 10, 12, 15, 17 and 18; Tracks 2 and 15 are blanks; Tracks 1 and 16 are molecular weight markers of 23, 9.4, 6.5, 4.3, 2.3 and 2.0 Kilobases.

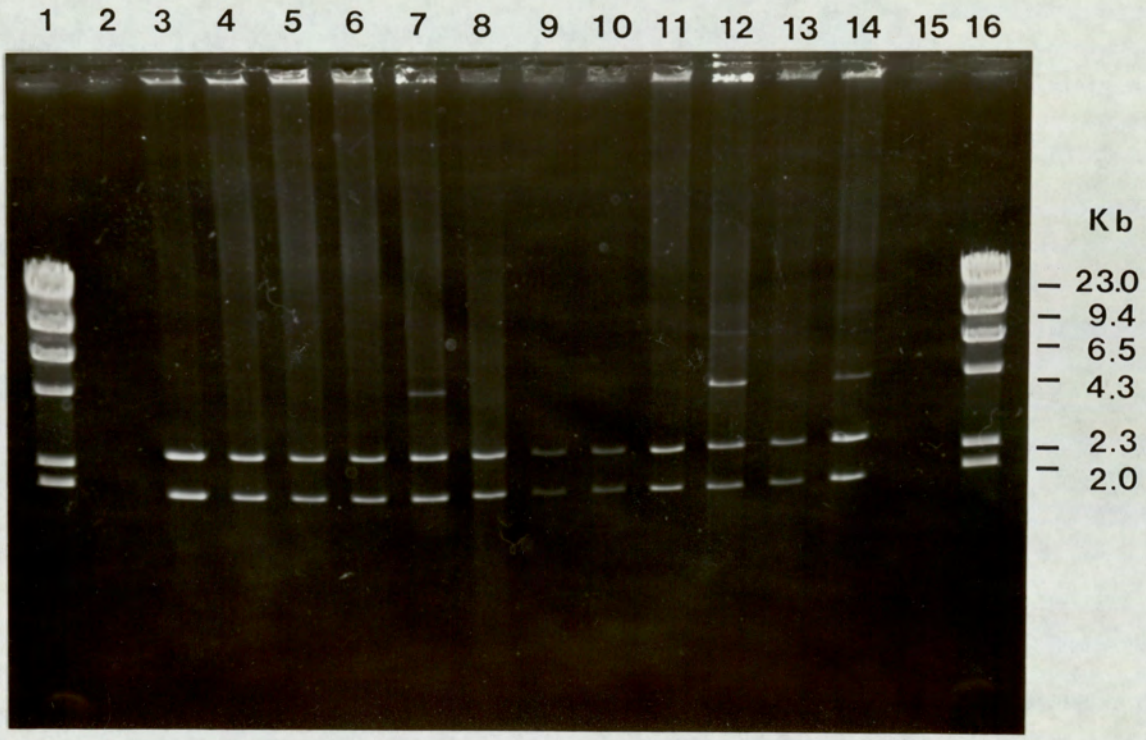


Fig. 30 Copy number analysis of pPA16 in *E. coli* JM83 when grown in glucose limited media at a dilution rate of 0.1 hr^{-1}

Tracks 1-11 are samples taken from the fermenter run as described in Fig. 29. Track 12 is the control pBR322 grown in *E. coli* HB101.

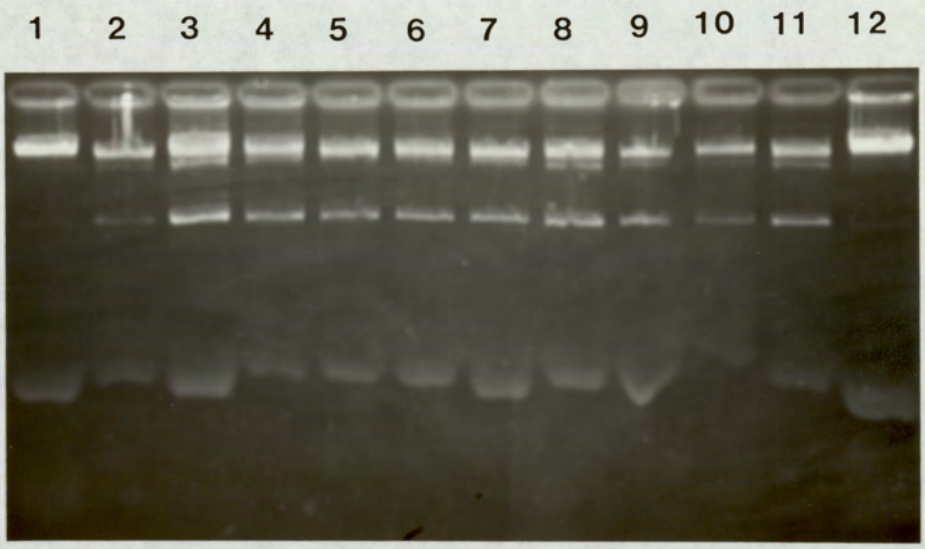


Fig. 31 Western blot analysis of *E. coli* JM83 containing pPA16 when grown in glucose limited media at a dilution rate of 0.1 hr^{-1}

Tracks 2-12 are samples taken from the fermenter run as described in Fig. 29; Track 1 is *E. coli* JM83 (negative control); The markers are shown on the right hand side of the gel.

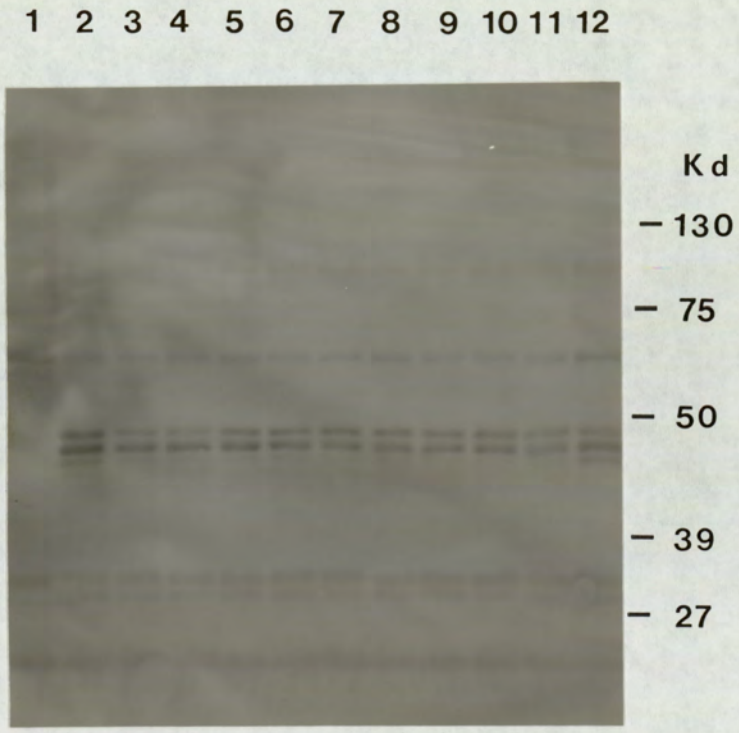
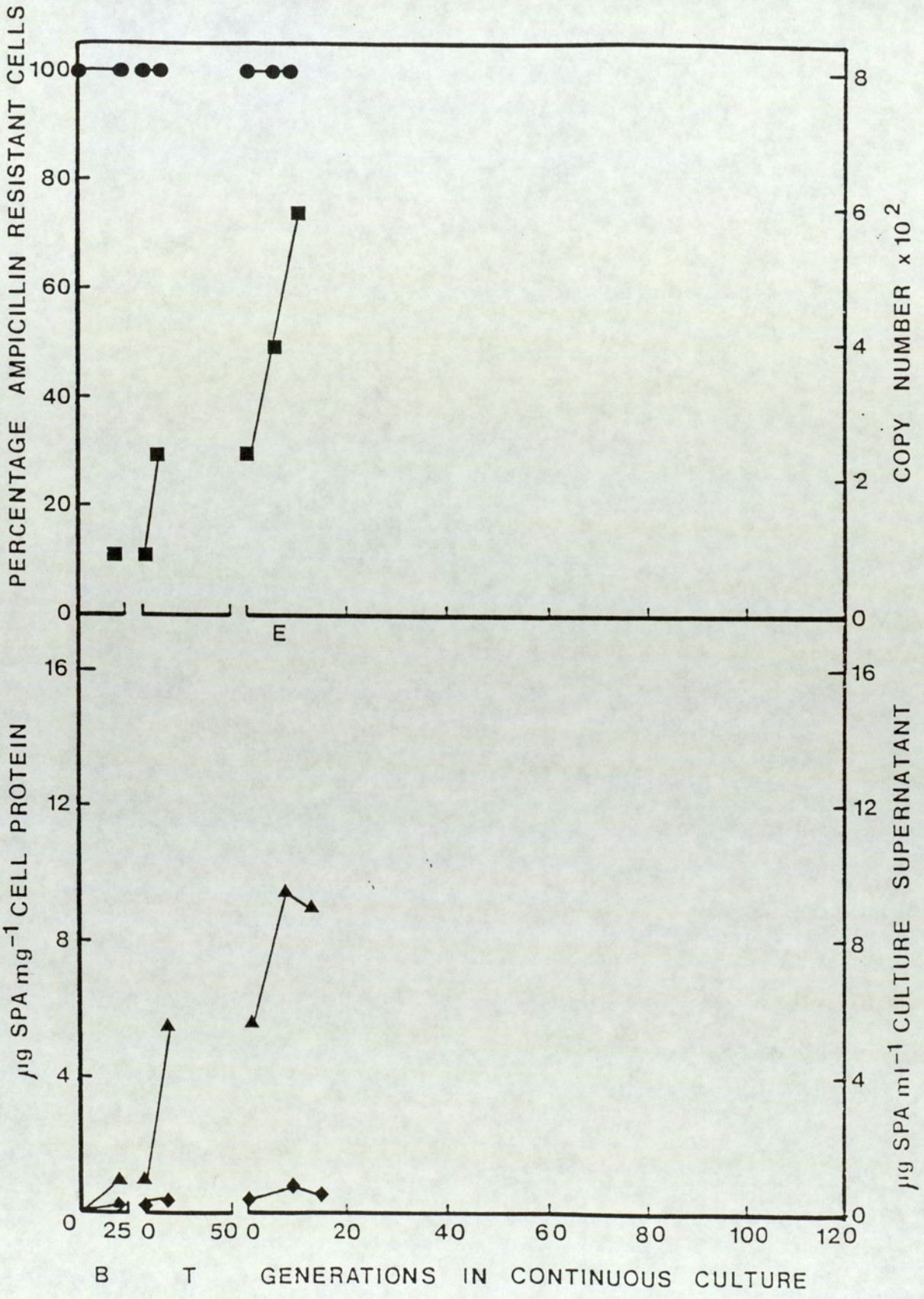


Fig. 32 The stability and expression of pPA16 in *E. coli* JM83 when grown in glucose limited media at a dilution rate of 0.2 hr^{-1}; The nomenclature is that described in Fig. 28, except for the sample taken for E. M. analysis (E).



medium at a dilution rate of 0.2 hr^{-1}. Unfortunately this experiment had to be curtailed due to problems with the pH although samples for E.M. analysis were taken before the problem arose as denoted by the symbol (E). The results obtained showed the plasmid to be stable, with the copy number reaching 600 per host chromosome. The level of SPA rose to 9 $\mu g\ mg^{-1}$ cell protein, with 1 $\mu g\ ml^{-1}$ in the culture supernatant. All subsequent E.M. work was performed on fermenter runs at dilution rates of 0.2 hr^{-1} and taken at a similar time. The same experiment was repeated and ampicillin resistance was retained at 100% for 60 generations.

Results for the experiment at a dilution rate of 0.3 hr^{-1} under glucose limiting conditions show there was a period of transient instability as detected by the percentage ampicillin resistant cells in the population (Figure 33). The copy number of the culture fell from 450 to 50 copies after 40 generations whereupon stability was attained. A similar pattern was also seen in the production of SPA with an initial peak of 6 $\mu g\ mg^{-1}$ of cell protein which dropped to 2 $\mu g\ ml^{-1}$. The SPA content in the supernatant was again 10% of that produced by the cells.

The final experiment using glucose limiting medium was run at a dilution rate of 0.4 hr^{-1} (Figure 34). There was a loss of ampicillin resistant cells from the population once steady state had been achieved, which culminated in a 35% loss after 120 generations. The copy number of the culture remained relatively stable throughout the run at 70 copies per host chromosome. The SPA levels showed a slow reduction

Fig. 33 The stability and expression of pPA16 in *E. coli* JM83 when grown in glucose limited media at a dilution rate of 0.3 hr^{-1}; The nomenclature is that described in Fig. 28.

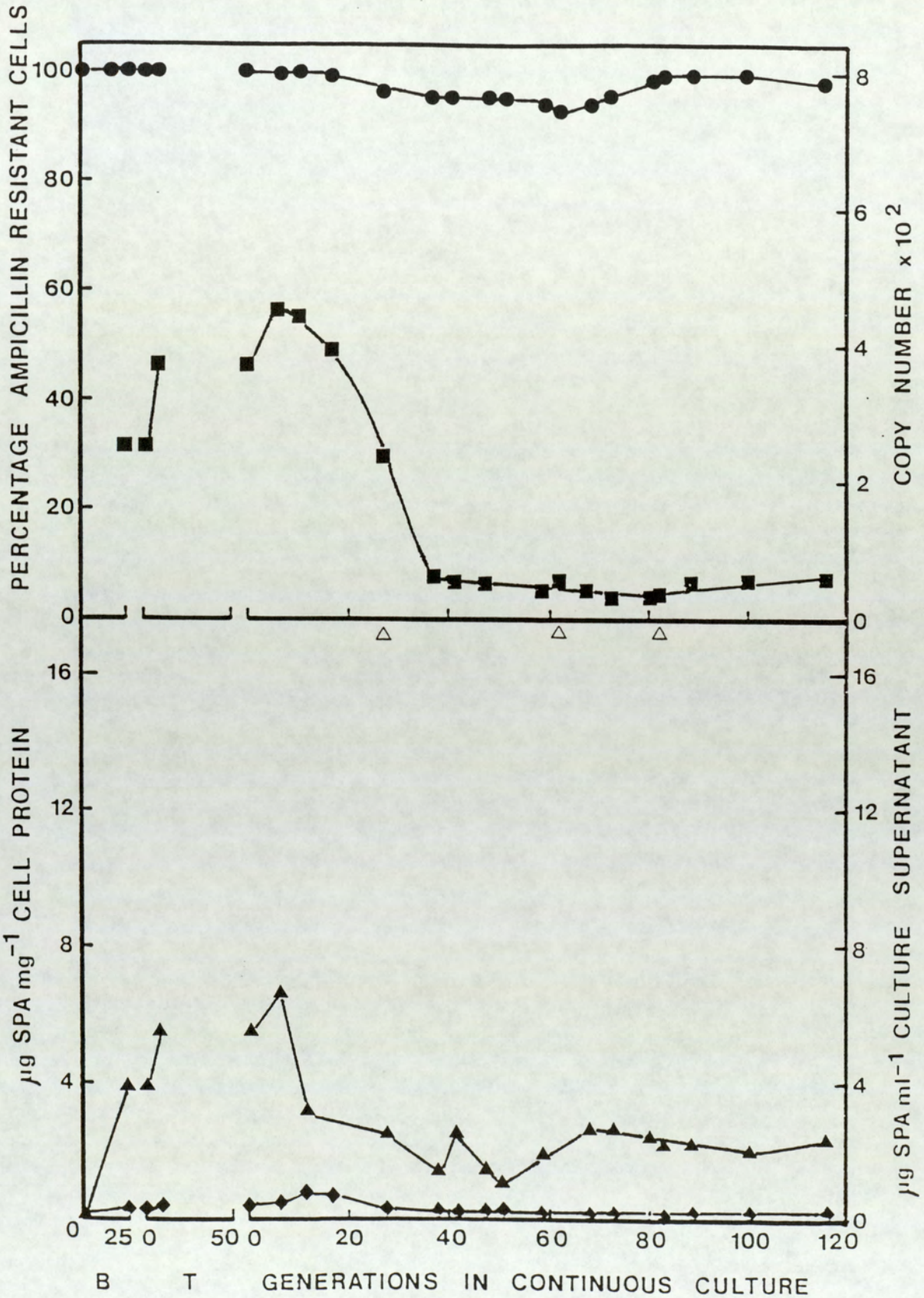
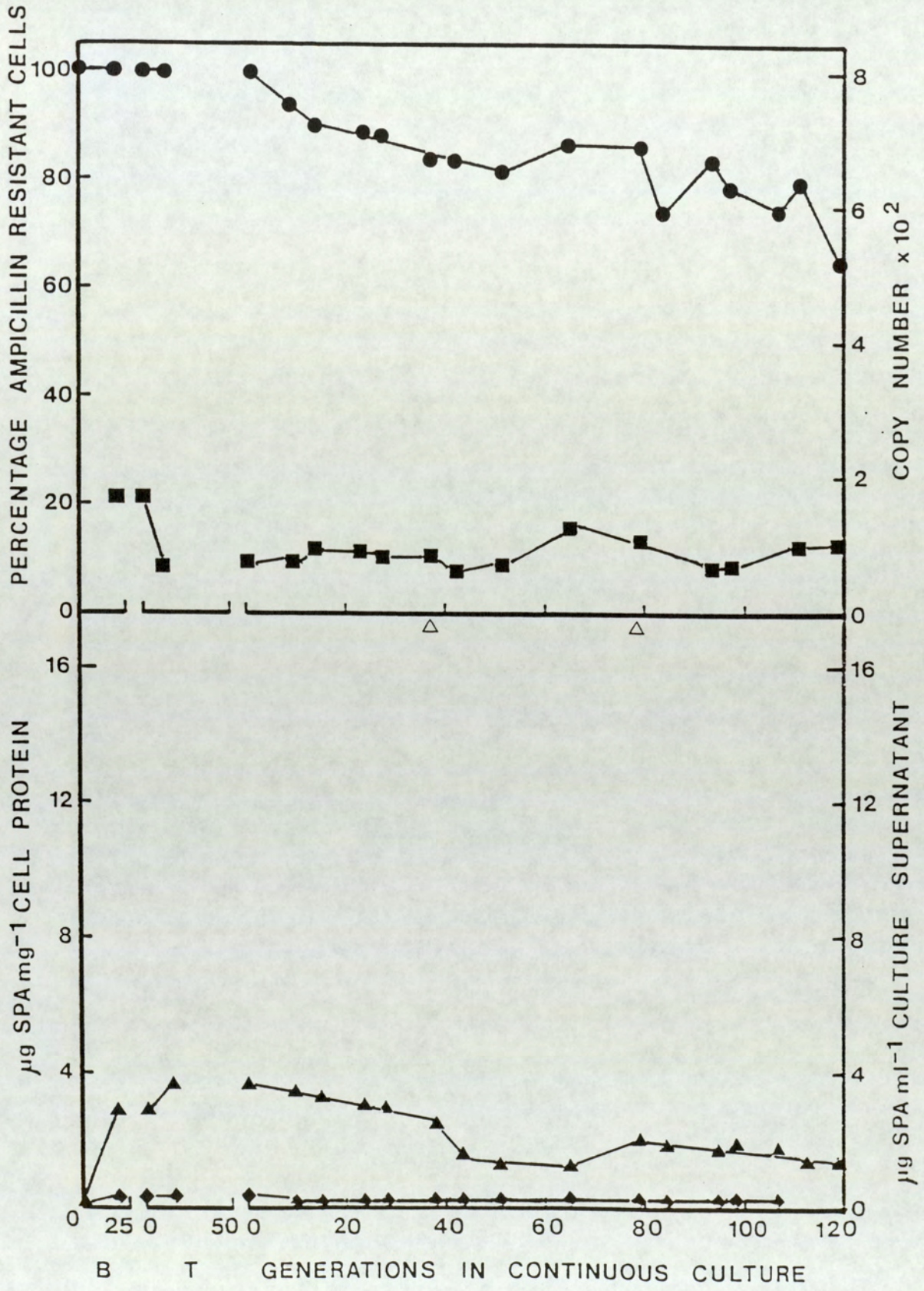


Fig. 34 The stability and expression of pPA16 in *E. coli* JM83 when grown in glucose limited media at a dilution rate of 0.4 hr^{-1}; The nomenclature is that described in Fig. 28.



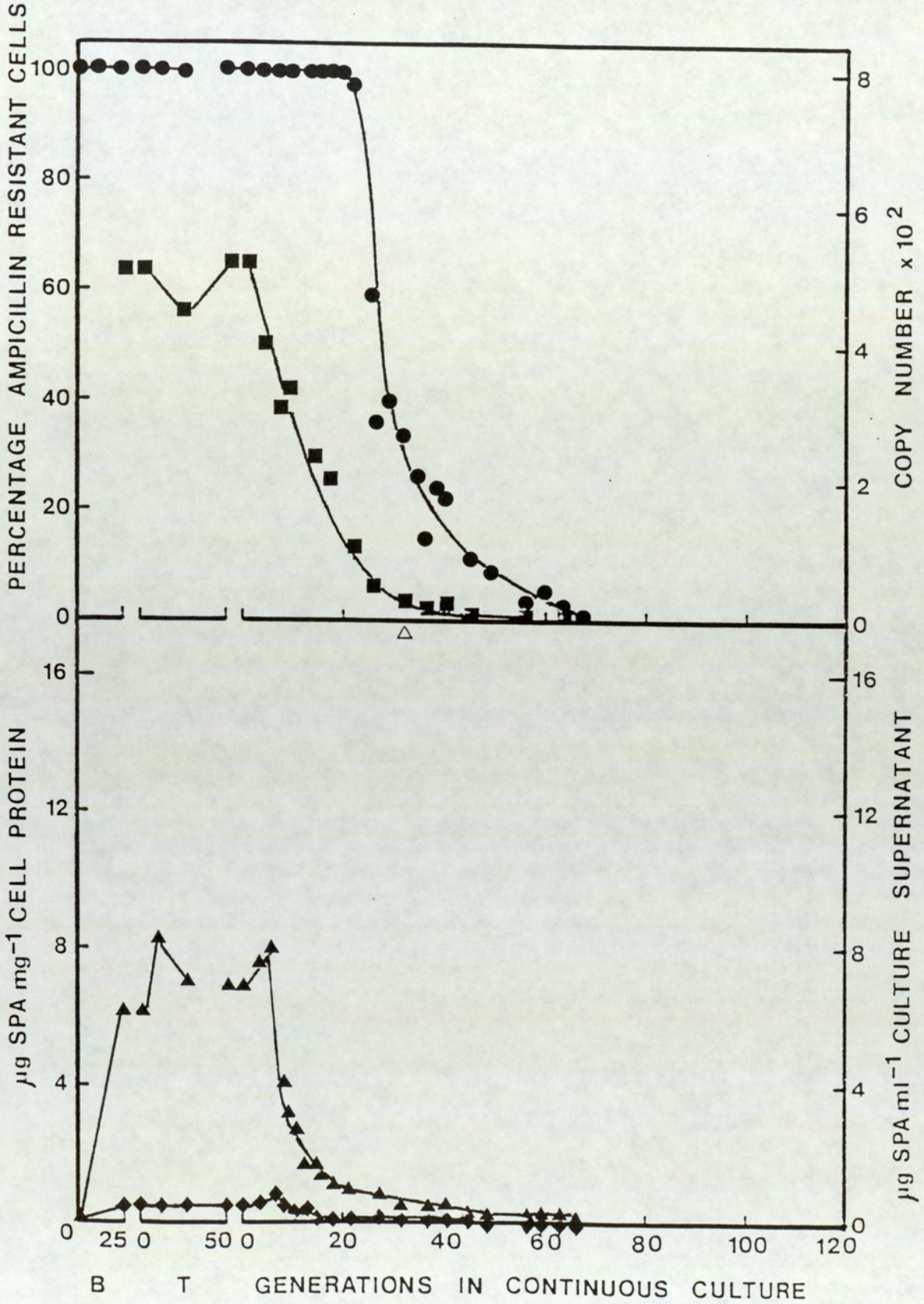
from 4 to 1.5 μg mg^{-1} cell protein which corresponded to the fall of ampicillin resistant cells in the population even though the copy number remained constant. The SPA content in the supernatant was again 10% of that found in the cells.

The results for the continuous culture experiments involving limited glucose produced various trends with increases in dilution rate. Generally the plasmid remained stable throughout the experiments, although increases in dilution rates brought an increase in instability, which at a higher D of 0.4 hr^{-1} resulted in a 20% loss of stabilty after 50 generations. The copy numbers after an initial peak in the batch or transient phase stabilised after a period of adjustment. The copy number at a D of 0.4 hr^{-1} gave the lowest peak values although once stabilised was no different to the other runs. The production of SPA again followed in a similar vein with the highest production seen in the transient phase of growth. Increases in dilution rate showed reductions in the peak production of SPA, showing some similarity to the results produced by the copy numbers. The levels of SPA in the supernatant were always approximately 10% of that produced in the cell pellet.

3.3.2 Glycerol limiting conditions

The second stage of the continuous culture experiments involved repeating the above experiments while substituting glucose with glycerol. The results at the dilution rate of 0.1 hr^{-1} (Figure 35) showed stability up to 25 generations, with a subsequent 100% loss after 65 generations. The loss of ampicillin resistance was preceded by a fall in copy

Fig. 35 The stability and expression of pPA16 in *E. coli* JM83 when grown in glycerol limited media at a dilution rate of 0.1 hr^{-1}; The nomenclature is that described in Fig. 28.



number from 500 to 0 copies per host chromosome. The SPA production also followed a similar trend with production reaching a peak of 8 $\mu g\ mg^{-1}$ of cell protein then falling to zero. The SPA found in the culture supernatant was approximately 10% of that found in the cells.

Figure 36 is a gel showing the restriction pattern of the plasmid taken from the samples when cut with EcoRI and pstI. The results give a correct value of molecular weight for the plasmid DNA, although the concentration is in decline. This is in agreement with the results from both the copy number and ampicillin resistance values which also showed a loss.

Figure 37 is a gel of whole cell lysates from each of the fermenter samples which have been stained with ethidium bromide. The resulting bands show the plasmid DNA being lost with time when compared to the chromosomal DNA. Scanning densitometry can then be used to calculate the values of the copy numbers (section 2.2.11.9).

Figure 38 is a western blot showing the qualitative analysis of SPA produced at the particular sample times (see 2.2.7). The results correspond to that found in glucose limiting conditions where 3 forms of SPA were seen with molecular weights 43, 45 and 48 Kd. The loss of SPA is obviously related to the loss of all the other parameters described in this experiment.

The results from Figure 39 show the stability and expression of JM83/pPA16 when grown in glycerol limiting medium at a dilution rate of 0.2 hr^{-1}. Ampicillin resistance was reduced to only 10% after 45 generations after

Fig. 36 Restriction analysis of pPA16 in *E. coli* JM83 when grown in glycerol limited media at a dilution rate of 0.1 hr^{-1}

Tracks 1-12 are samples taken from the fermenter run (Fig. 35) at the following intervals: B, 24; T, 24 and 50; Generations in continuous culture, 8, 15, 21, 30, 36, 40, 43, 45 and 60; Track 13 is a molecular weight marker.

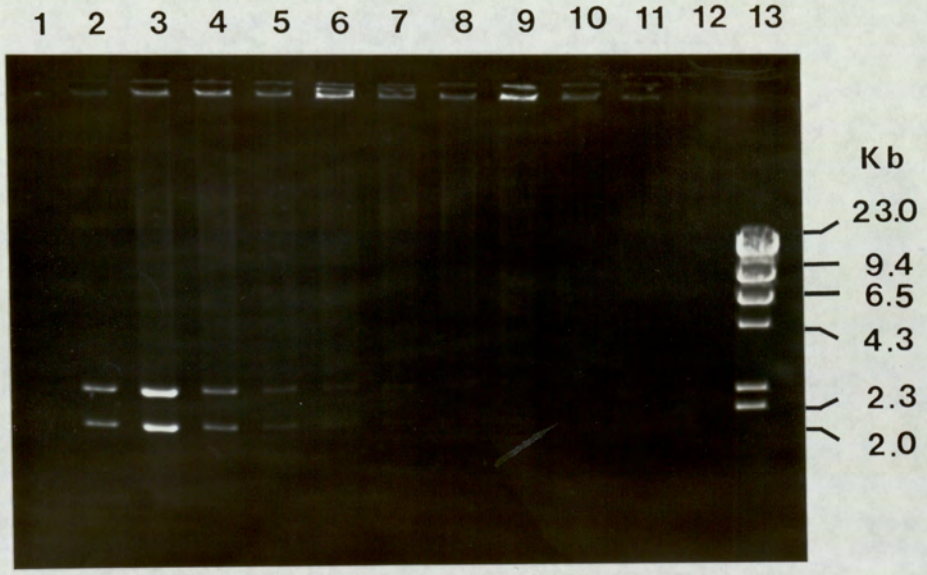


Fig. 37 Copy number analysis of pPA16 in *E. coli* JM83 when grown in glycerol limiting media at a dilution rate of 0.1 hr^{-1}

Tracks 2-11 are samples taken from the fermenter run as described in Fig. 36; Track 1 is the control pBR322 in *E. coli* HB101.

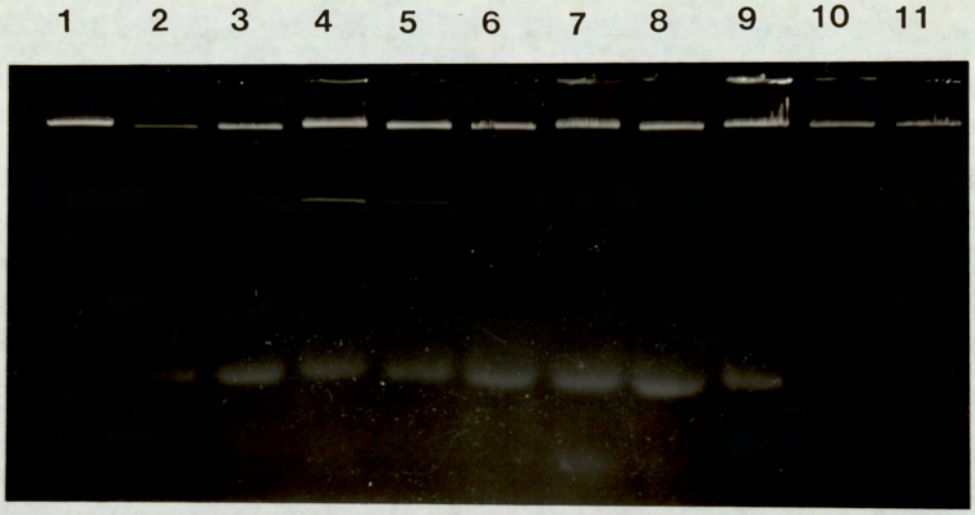


Fig. 38 Western blot analysis of *E. coli* JM83 containing pPA16 when grown in glycerol limited media at a dilution rate of 0.1 hr^{-1}

Tracks 2-13 are samples taken from the fermenter as described in Fig. 36; Track 1 is *E. coli* JM83 (negative control); The markers are shown on the right hand side of the gel.

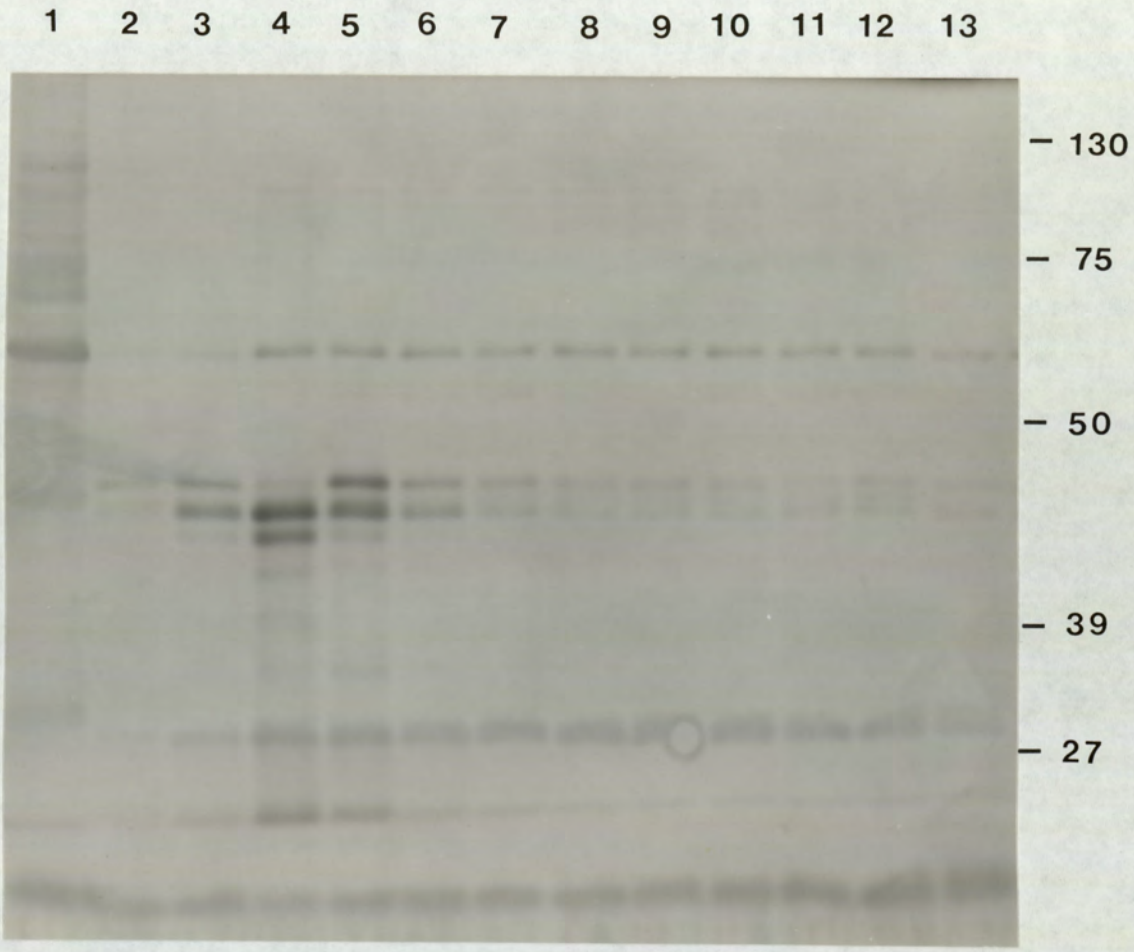
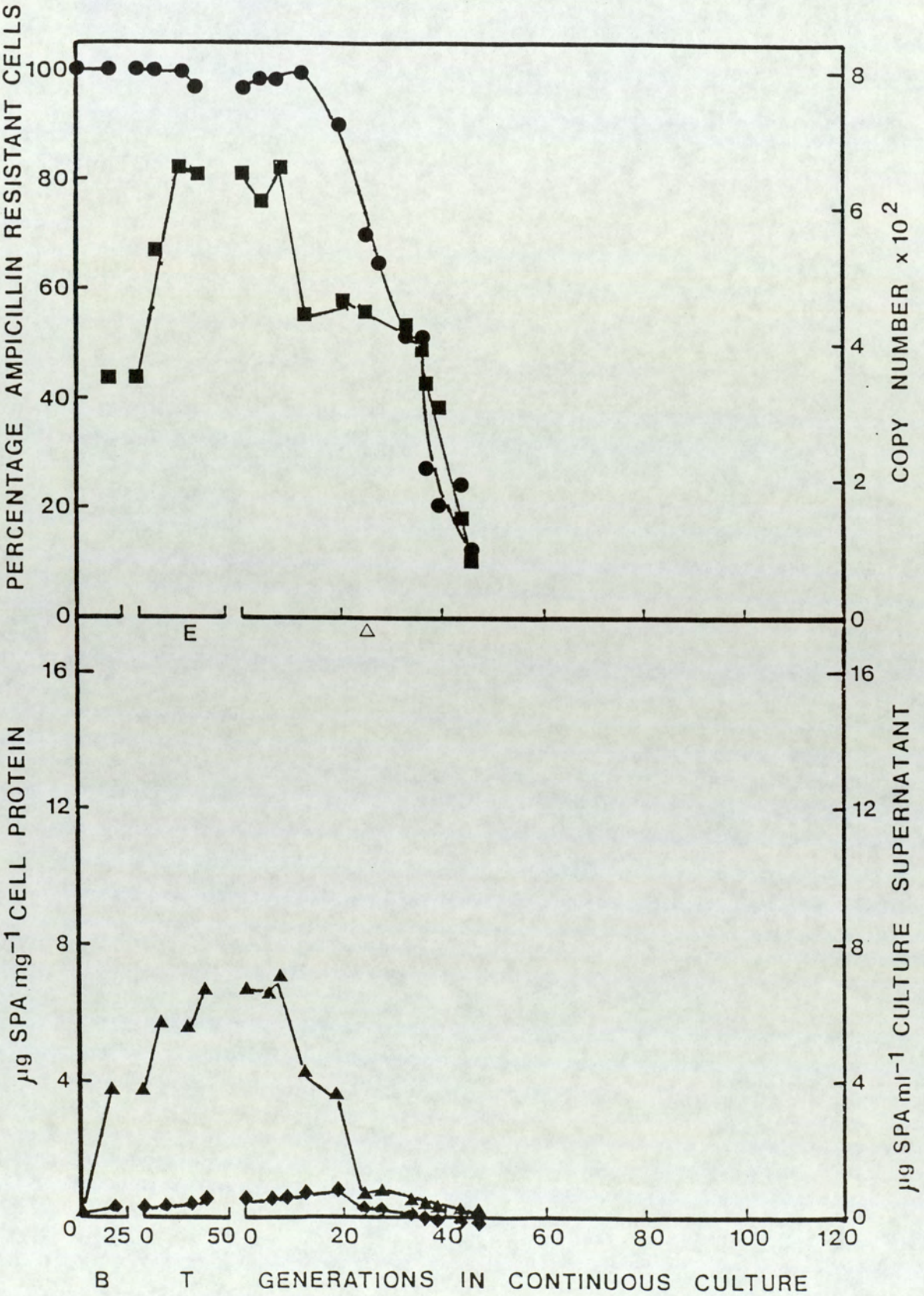


Fig. 39 The stability and expression of pPA16 in *E. coli* JM83 when grown in glycerol limited media at a dilution rate of 0.2 hr^{-1}; The nomenclature is that described in Fig. 28, except for the sample taken for E. M. analysis (E).



following the trend set by the copy numbers. SPA production was also lost after gaining a peak value of 7 $\mu g\ mg^{-1}$ cell protein which corresponded to the high copy number values. The values of the SPA found in the culture supernatant were 10% of that found in the cells with no detection after 30 generations.

The results from figure 40 show at a dilution rate of 0.3 hr^{-1} the plasmid was lost from the population and at an even faster time than that of 0.1 or 0.2 hr^{-1}. The copy numbers were also reduced at a similar rate to that of ampicillin resistance. The production of SPA although up to 8 $\mu g\ mg^{-1}$ cell protein was soon lost from the culture. The production of SPA in the supernatant was again 10% of that seen in the cells, with total loss seen after 40 generations.

The final experiment using glycerol limiting medium was run at a dilution rate of 0.4 hr^{-1} (Figure 41). Once the nutrient pumps had been switched on there was an immediate loss of ampicillin resistance resulting in total loss at 65 generations. The copy numbers preceded this trend as did the values for SPA although production was half that of the other runs.

The results for the continuous culture experiments using glycerol limited medium indicated that the plasmid was unstable at all the dilution rates investigated. This was in contrast to that found when using glucose limiting medium, were the plasmid was relatively stable. The instability of pPA16 appeared to increase with increase in dilution rate as seen by the time for a 50% drop in

Fig. 40 The stability and expression of pPA16 in *E. coli* JM83 when grown in glycerol limited media at a dilution rate of 0.3 hr^{-1}; The nomenclature is that described in Fig. 28.

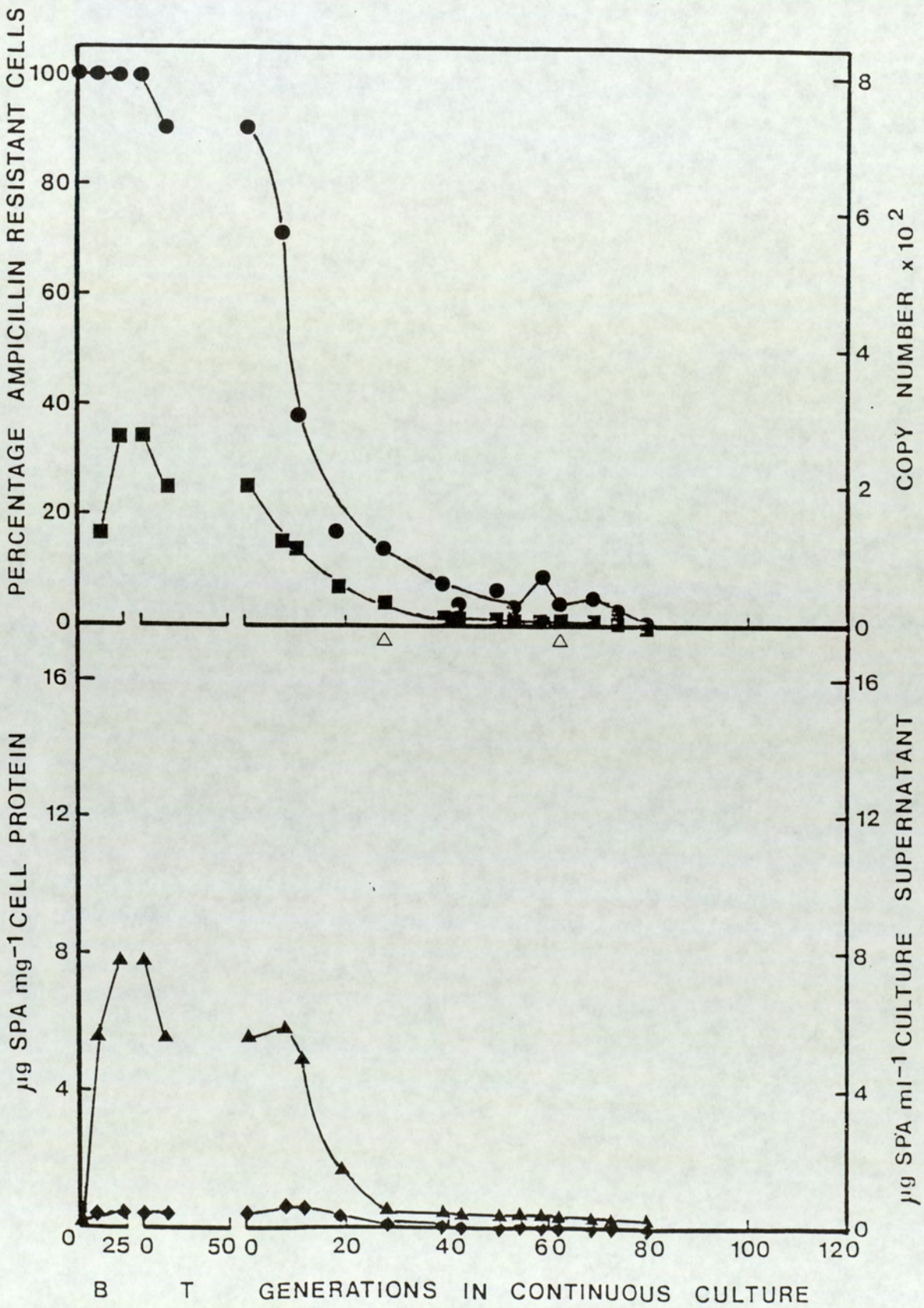
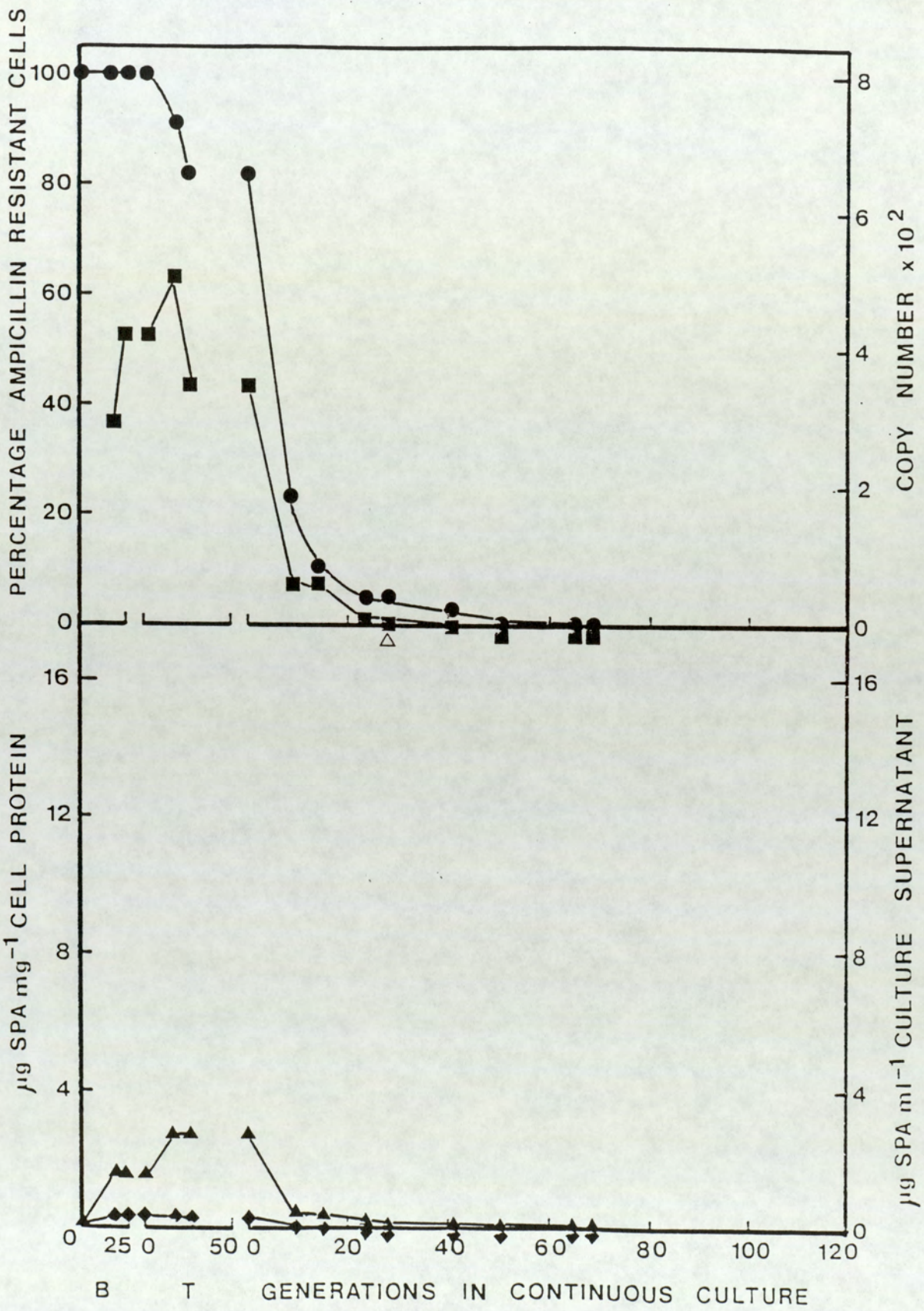


Fig. 41 The stability and expression of pPA16 in *E. coli* JM83 when grown in glycerol limited media at a dilution rate of 0.4 hr^{-1}; The nomenclature is that described in Fig. 28.



ampicillin resistance. Although the production of SPA was also lost, peak levels were similar up to a D of 0.4 hr^{-1} where the value was more than halved. The levels of SPA in the supernatants were similar at all dilution rates as well as to those seen in the glucose limiting experiments.

3.3.3 Ammonium limiting conditions

Due to the instability of the plasmid under glycerol limiting conditions it was important to show that glycerol itself was not having any adverse effects on the stability of the plasmid. As the batch subculture experiments in section 3.2.5 indicated that the plasmid was stable under ammonium limiting conditions, it was decided to investigate the stability of pPA16 under ammonium limited conditions with either glucose or glycerol as the excess carbon source. Figure 42 shows the stability and expression of pPA16 in ammonium limited cultures with excess glucose at a dilution rate of 0.3 hr^{-1}. As expected the plasmid was stable for upto 60 generations without any loss of ampicillin resistance. The copy number of the sample remained constant but at a much higher rate than seen previously. SPA production gave an initial peak of 6 $\mu g\ mg^{-1}$ cell protein which was slowly reduced to half that value by the end of the experiment. The amount of SPA found in the supernatant was approximately 10% of that found in the cells.

The next fermenter run involved using excess glycerol instead of glucose, with limited ammonium at a dilution rate of 0.3 hr^{-1} (Figure 43). The percentage of ampicillin resistant cells in the population showed a small loss after

Fig. 42 The stability and expression of pPA16 in *E. coli* JM83 when grown in ammonium limited media with excess glucose, at a dilution rate of 0.3 hr^{-1}; The nomenclature is that described in fig. 28.

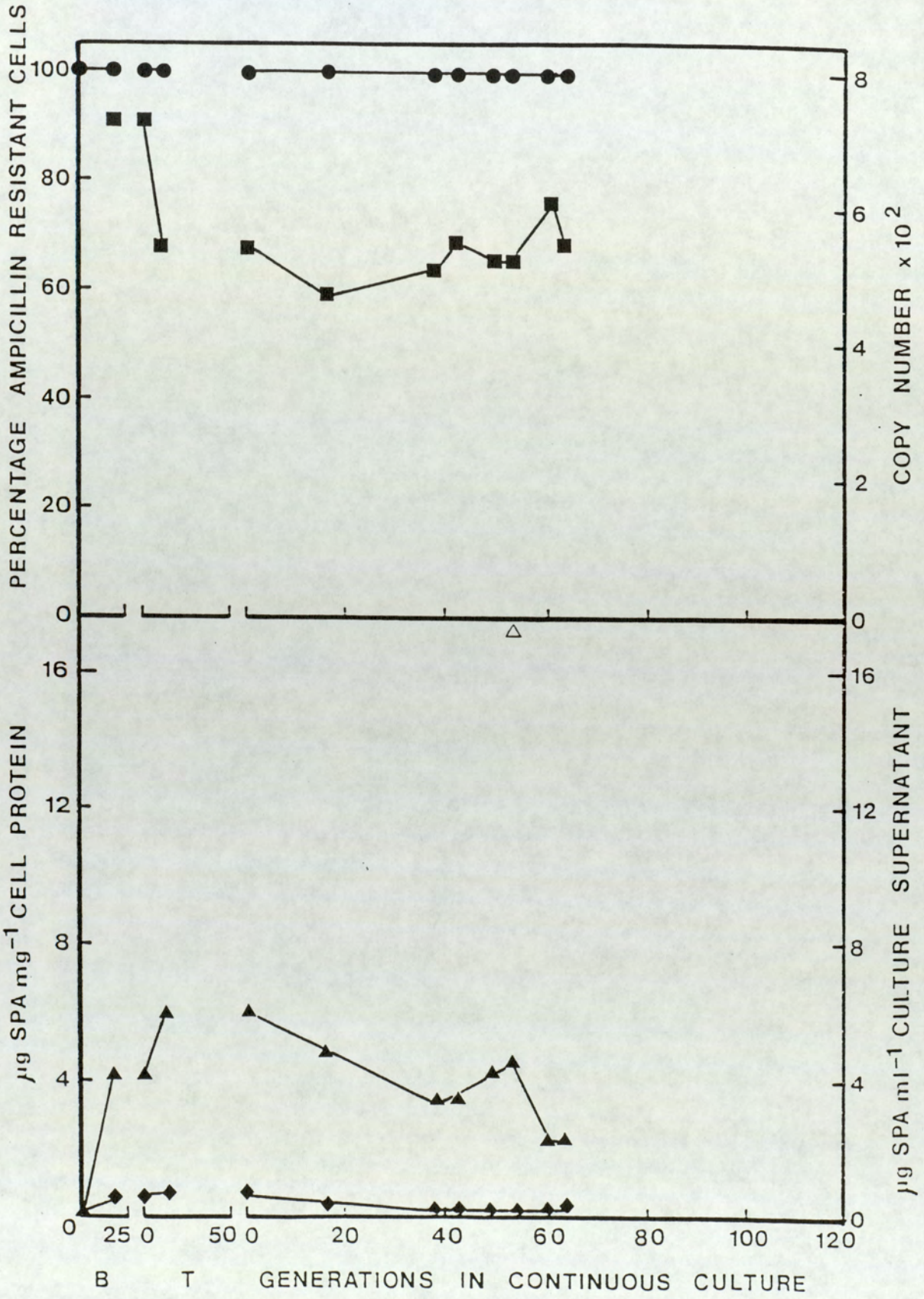
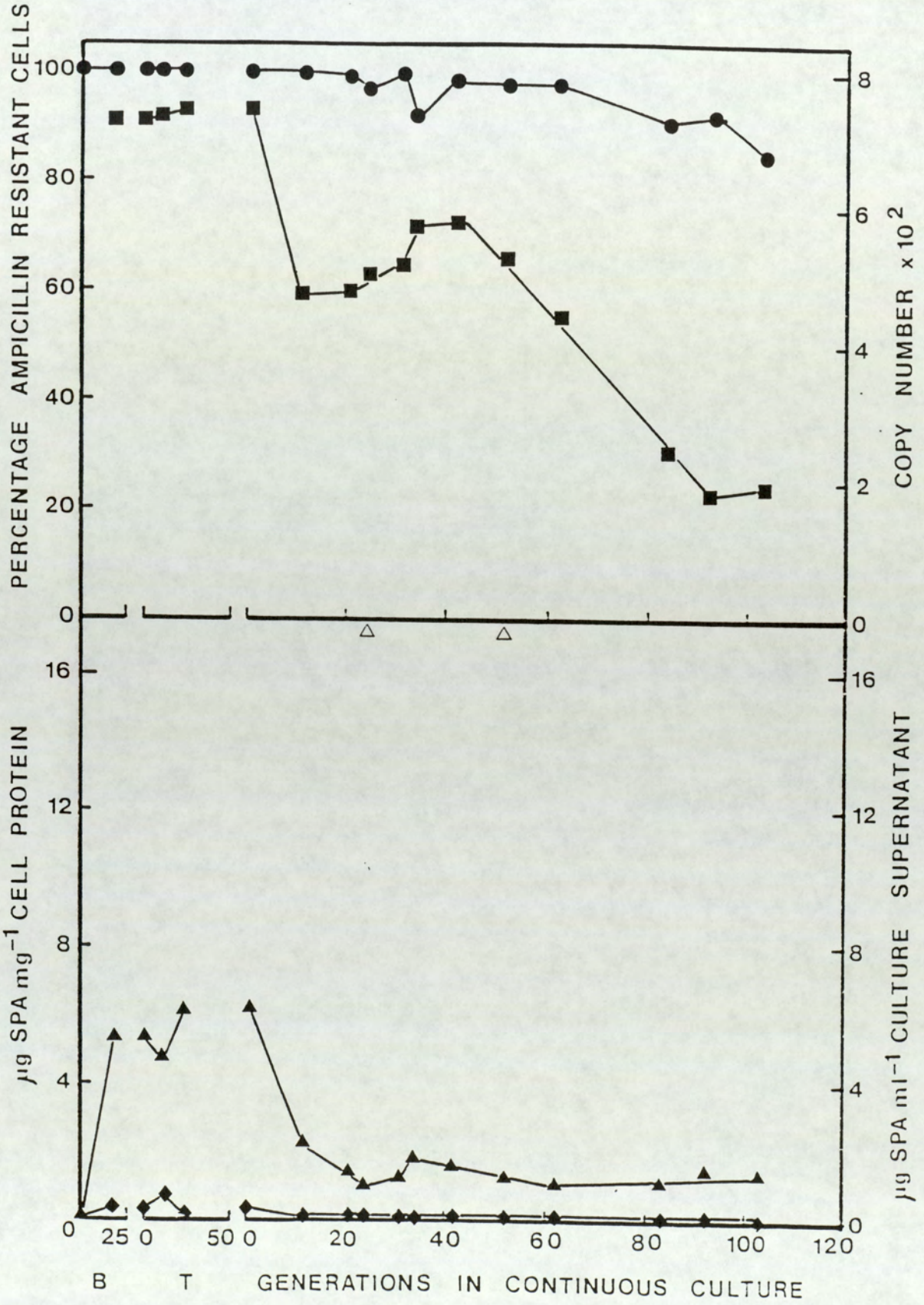


Fig. 43 The stability and expression of pPA16 in *E. coli* JM83 when grown in ammonium limited media with excess glycerol at a dilution rate of 0.3 hr^{-1}; The nomenclature is that described in Fig. 28.



60 generations, which increased to 15% after 100 generations. Initially the copy number values were high (750) but unlike Figure 43 showed a dramatic loss after the transient phase of growth. SPA production gave a peak value of 6 $\mu g\ mg^{-1}$ of cell protein in the transitional phase, this then fell to 2 $\mu g\ mg^{-1}$, which corresponded to the fall in copy number. The levels of SPA found in the supernatant although only 10% of that found in the cells, followed a similar pattern. The results indicated that although excess glycerol was not responsible for the total loss of the plasmid from the culture, it was responsible for a dramatic loss of copy number.

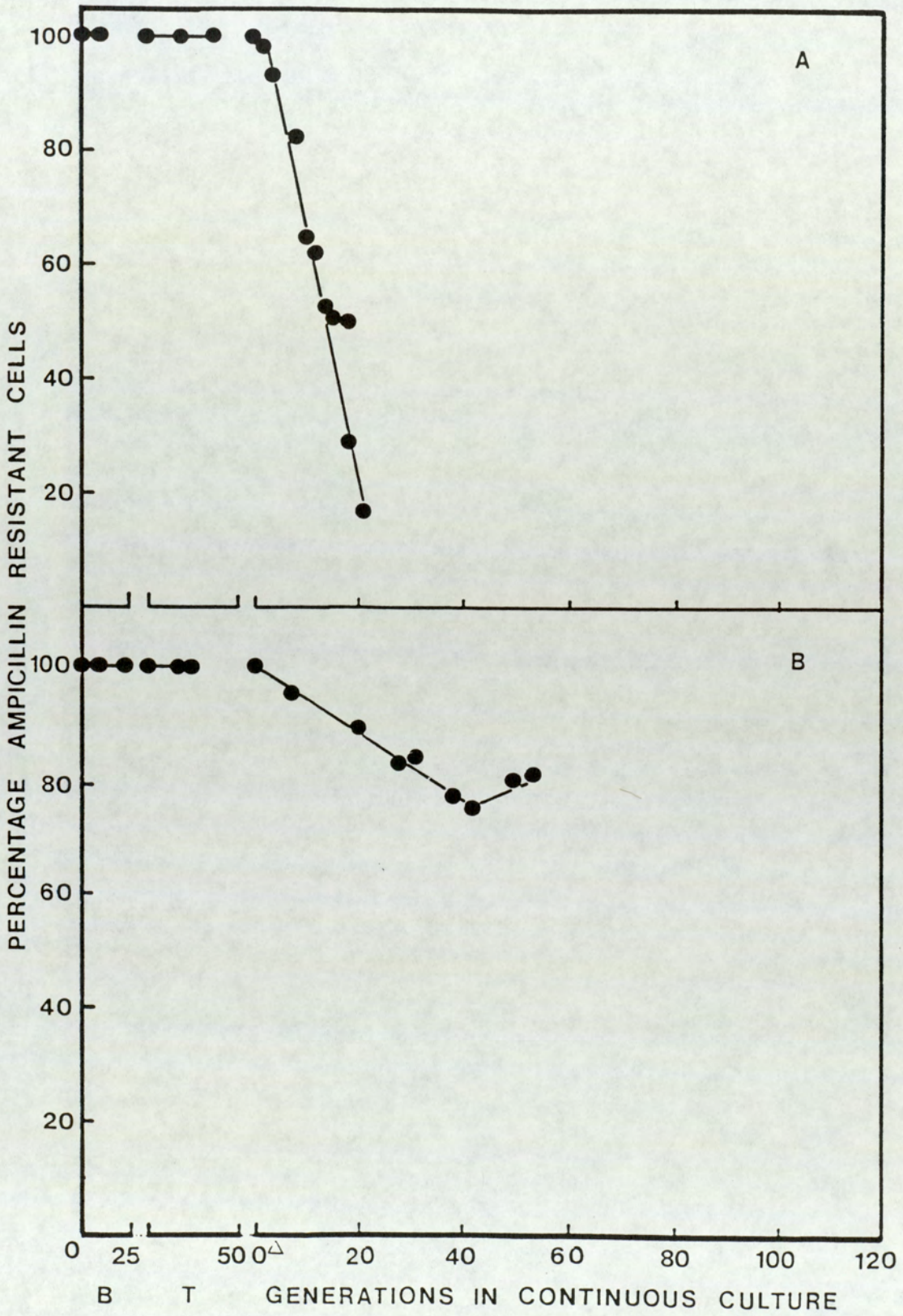
3.3.4 Phosphate limiting conditions

The effects of limited phosphate on the stability of pPA16 was also investigated at dilution rates 0.1 and 0.3 hr^{-1} (Figure 44A and B). The results at the lower dilution rate (Figure 44A) show the plasmid being lost shortly after the nutrient pump had been switched on. These results corresponded to that found in the batch subculture (see 3.2.5) experiments which also showed the plasmid to be unstable. At the higher dilution rate of 0.3 hr^{-1} (Figure 44B) the plasmid was less unstable, showing a 20% loss of ampicllin resistance after 60 generations.

3.3.5 The stability of pUC8 in *E. coli*

Due to the instability of pPA16 under glycerol limiting conditions the effects on the parent plasmid pUC8 were also investigated. A dilution rate of 0.2 hr^{-1} was used so that

Fig. 44A The stabilty of pPA16 in *E. coli* JM83 when grown in phosphate limited media at a dilution rate of 0.1 hr^{-1}
Fig. 44B The stability of pPA16 in *E. coli* JM83 when grown in phosphate limited media at a dilution rate of 0.3 hr^{-1}; The nomenclature is that described in Fig. 28.



samples could be taken for E.M. analysis and compared to the samples taken for pPA16 under both glucose and glycerol limitng conditions. The results indicate pUC8 to be stable for up to 60 generations in continuous culture, showing only a 2% loss of ampicillin resistance after 65 generations (Figure 45). The copy number of the samples rose initially to over 600 copies per host chromosome, but then fell to 500 by the end of the fermentation run. Assays for SPA were performed and as expected none was detected.

3.3.6 The stability of pPA31 in *E. coli*

As mentioned in the introduction (1.11.2) pPA31 codes for full length protein A and is currently used as a production clone in JM83. Due to the loss of the extra 600 base pairs of staphylococcus chromosomal DNA, production in batch culture was shown to be seven times higher than pPA16 (Shuttleworth *et al.* 1987). It was therefore considered appropriate to monitor the stability and expression of pPA31 in continuous culture. As the host was grown in glycerol limiting medium at a dilution rate of 0.2 hr^{-1} then E.M. analysis could also be performed and the results compared to the other host vector systems. The results are shown in Figure 46 and show the plasmid to be unstable, with the cells losing their ampicillin reistance after 50 generations. There were problems with the serial dilutions and contrary to the ampicillin results the copy number did not fall although it was rather erratic. The production of SPA nearly reached 100 $\mu g\ mg^{-1}$ of cell protein or 10% of the total soluble protein at the begining of steady state. There

Fig. 45 The stability and expression of pUC8 in *E. coli* JM83 when grown in glycerol limited media at a dilution rate of 0.2 hr^{-1}; The nomenclature is that described in Fig. 28, except for the sample taken for E. M. analysis.

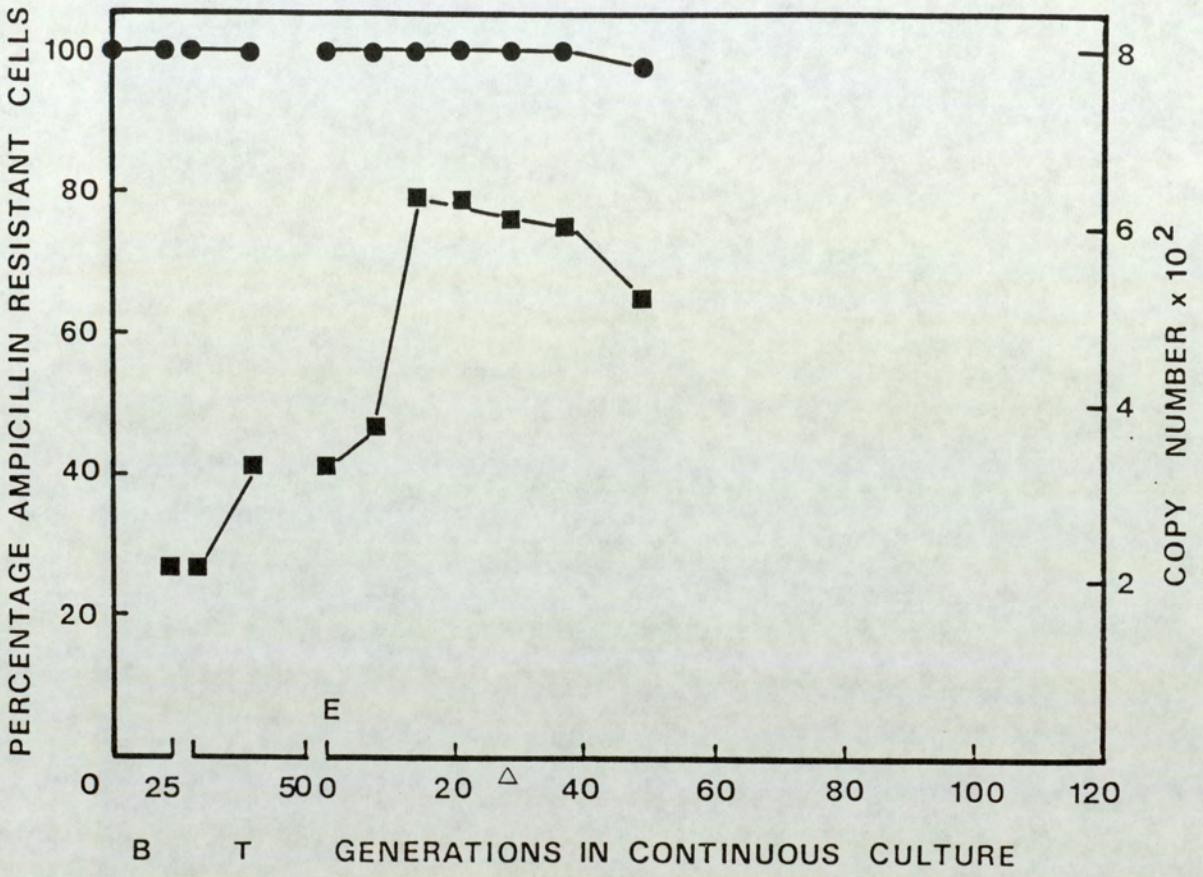
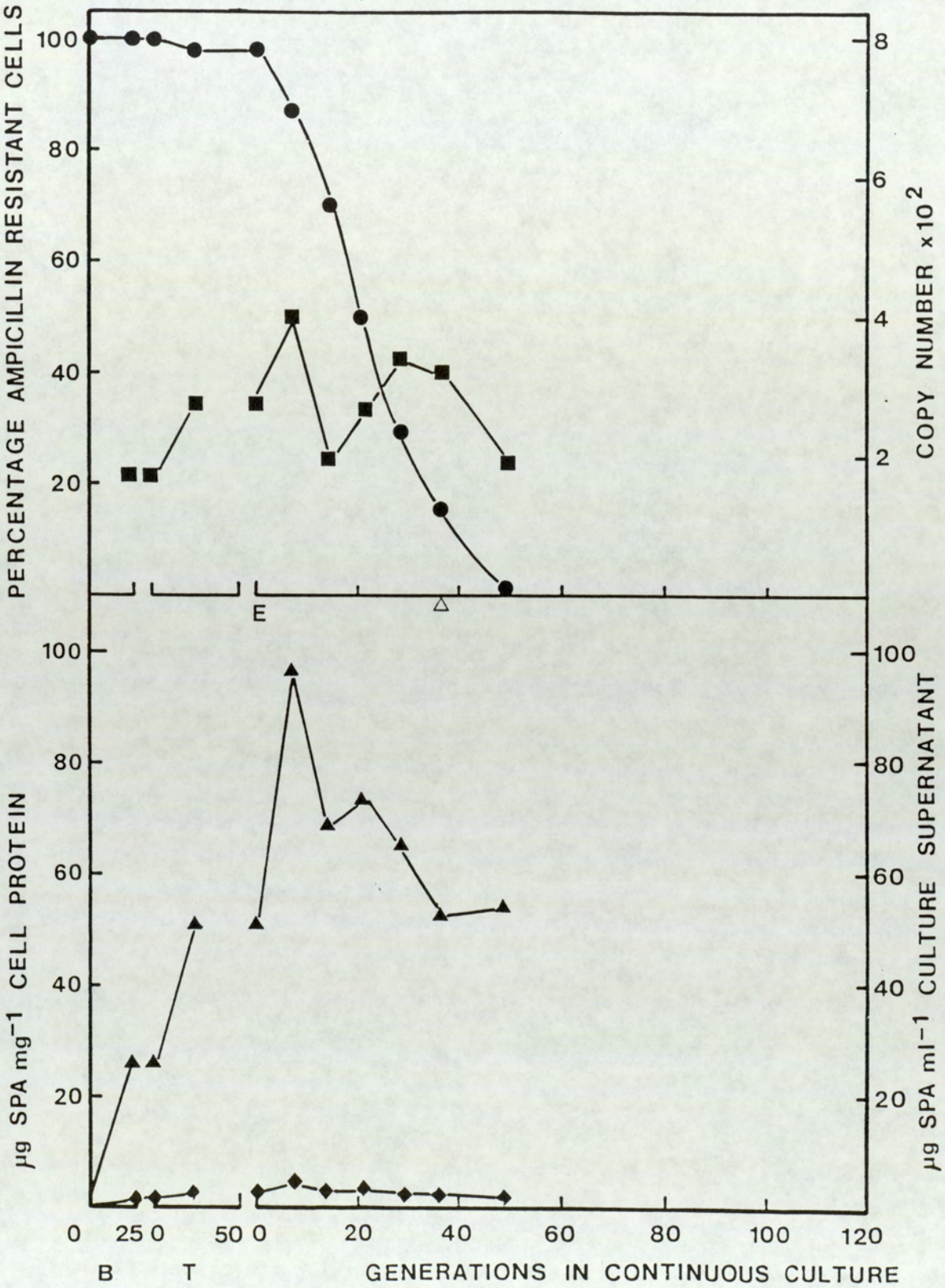


Fig. 46 The stability and expression of pPA31 in *E. coli* JM83 when grown in glycerol limited media at a dilution rate of 0.2 hr^{-1}; The nomenclature is that described in Fig. 28, except for the sample taken for E. M. analysis.

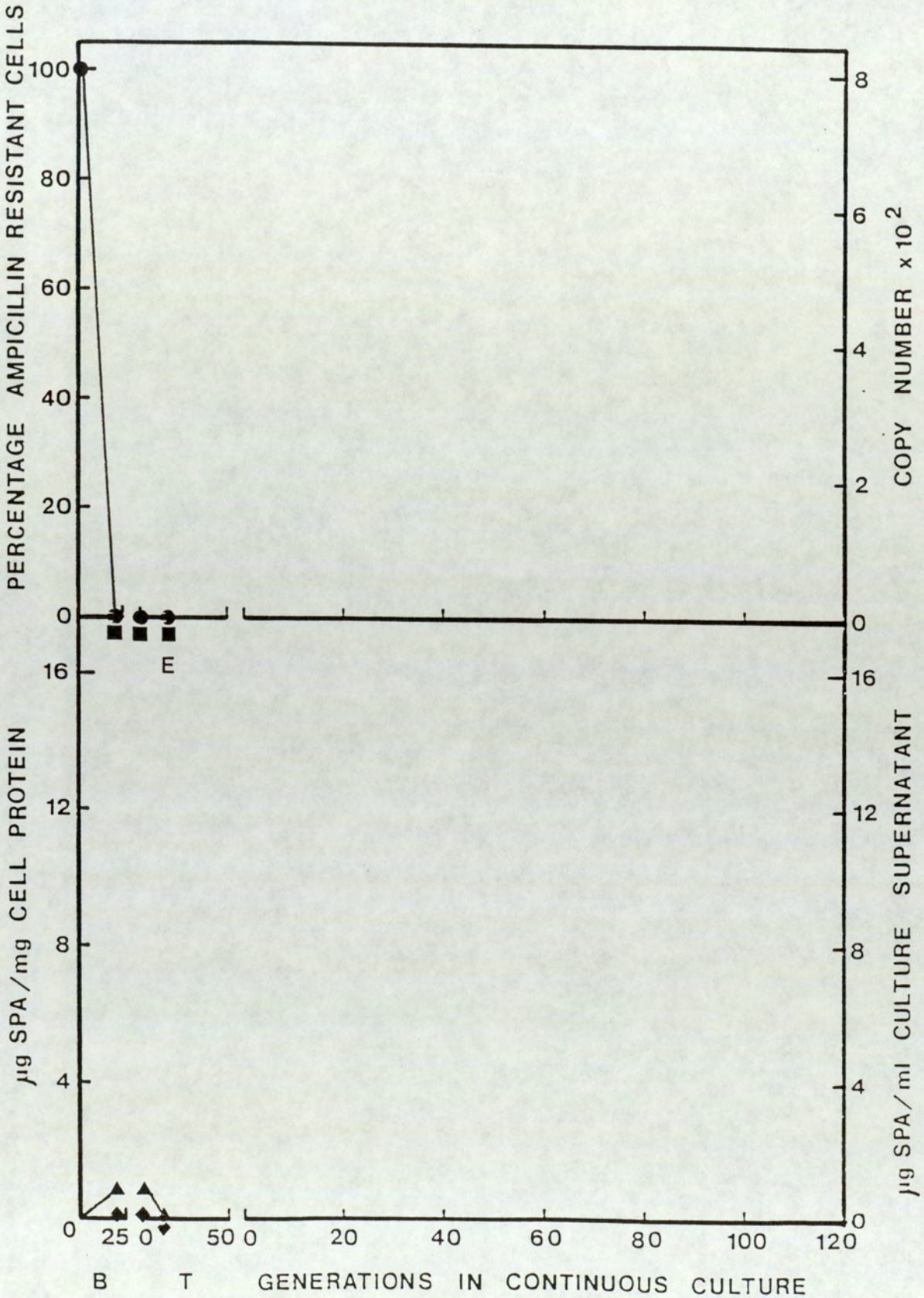


was however, no cessation of production which corresponded to the results shown by the copy numbers but contrary to those given for the ampicillin resistance of the culture. The levels of SPA in the supernatant were again 10% of that produced by the cells. An explanation for these conflicting results are given in 4.4.

3.3.7 The stability of pPA34 in *E. coli*

As mentioned in the introduction (1.11.2) pPA34 is currently used as a production clone which codes for the tailess protein A, without the extra 600 base pairs of staphylococcus chromosomal DNA. Batch results indicated that production was 10 times higher than that shown by pPA16 (Shuttleworth *et al.* 1987). This host/vector system was then grown in continuous culture as in 3.3.5. Unfortunately the plasmid was lost at a very early stage before E.M. samples could be taken. The results however, show pPA34 to be extremely unstable (Figure 47), as 100% ampicillin resistance was lost after the batch phase of growth, which corresponded to the loss of copy number and SPA production.

Fig. 47 The stability and expression of pPA34 in *E. coli* JM83 when grown in glycerol limited media at a dilution rate of 0.2 hr^{-1}; The nomenclature is that described in Fig. 28, except for the sample taken for E. M. analysis.



3.4 CELL STRUCTURE AND THE LOCALISATION OF RECOMBINANT PROTEIN A

3.4.1 Effects of the SPA clones on the size of their bacterial hosts

The bacterial host (*E. coli* JM83) both with and without the various SPA clones were grown in 25 ml TSB in 100 ml flasks at 37°C for 6 hr. Samples were removed, Gram-stained and examined under a light microscope. The host without a plasmid (negative control) showed the sample to be Gram-negative rods as one would expect for *E. coli* (Figure 48). The Gram-stain for pPA16 showed a Gram-negative diplococci rather than rods (Figure 49). The results for pPA31 again showed Gram-negative rods but this time they were at least twenty times greater than that shown by JM83 the negative control (Figure 50). Finally the results for pPA34 show the host as being Gram-negative but diplococci in shape as was pPA16 (Figure 51).

3.4.2 Analysis of host cell structure using electron microscopy

Samples for E.M. analysis were taken from the continuous culture experiments (3.3), which were denoted by the letter E. Initially the structure of JM83 containing pPA16 was investigated where the host had been grown at a dilution rate of 0.2 hr^{-1} in both glucose and glycerol limiting media. The results for JM83/pPA16 grown in glucose limiting medium are shown in Figure 52 and show sections of the host at a magnification of 37700. As can be seen the cells are longer than normal *E. coli* (even when compared to batch

Fig. 48 Gram-stain of batch grown *E. coli* JM83

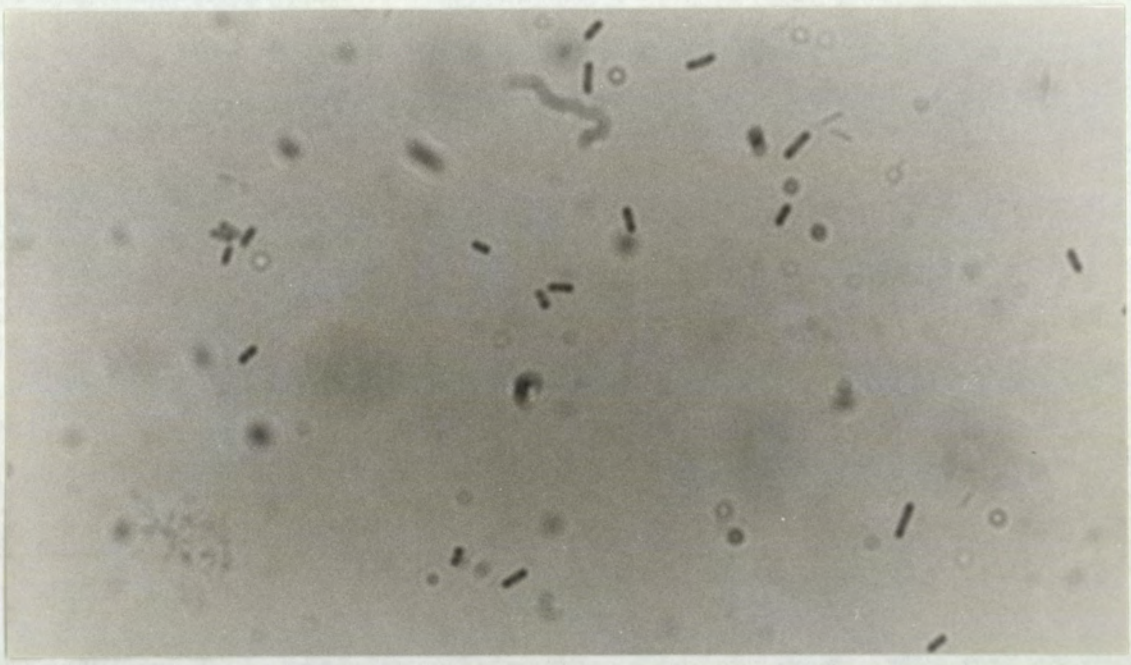


Fig. 49 Gram-stain of batch grown *E. coli* JM83 containing pPA16

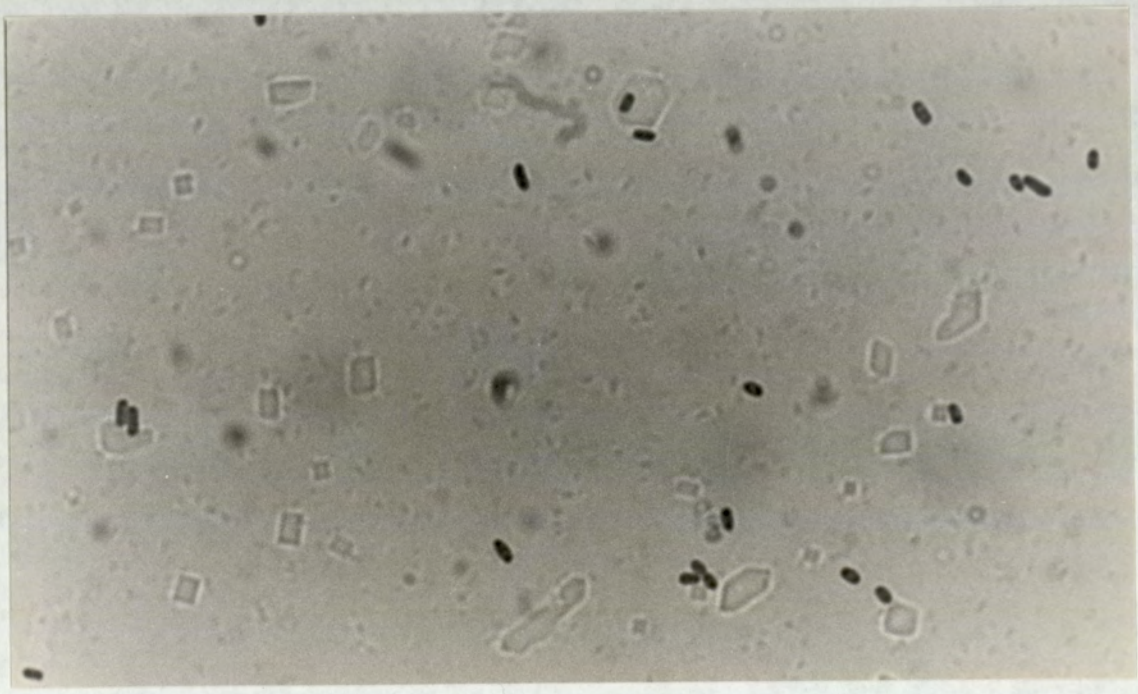


Fig. 50 Gram-stain of batch grown *E. coli* JM83 containing pPA31

Fig. 51 Gram-stain of batch grown *E. coli* JM83 containing pPA34

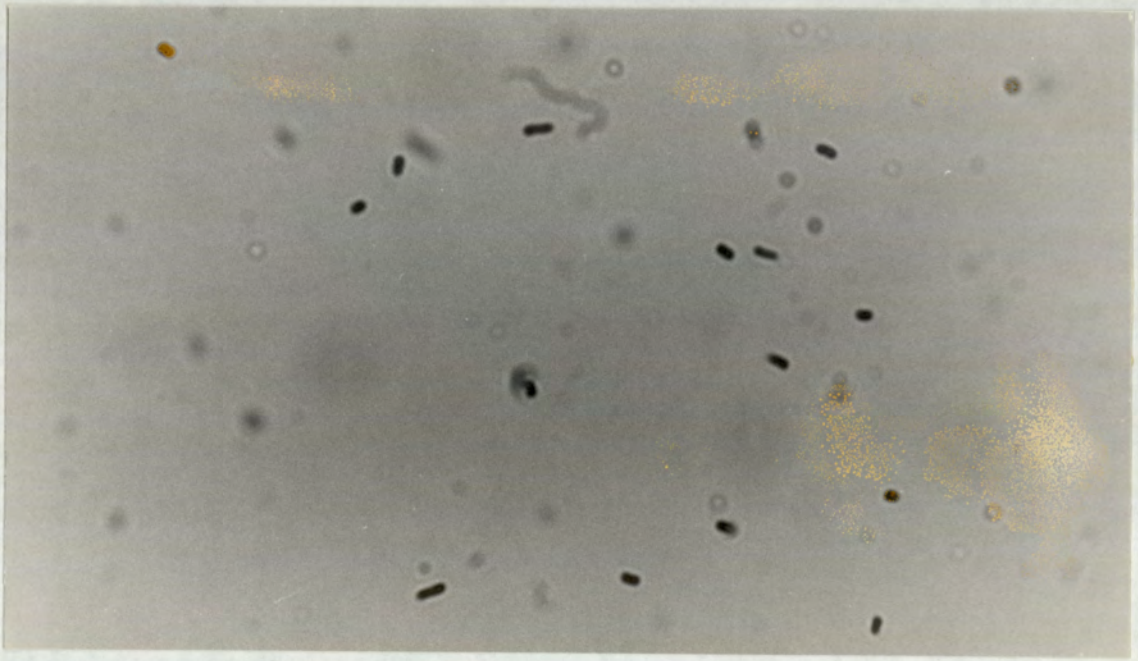
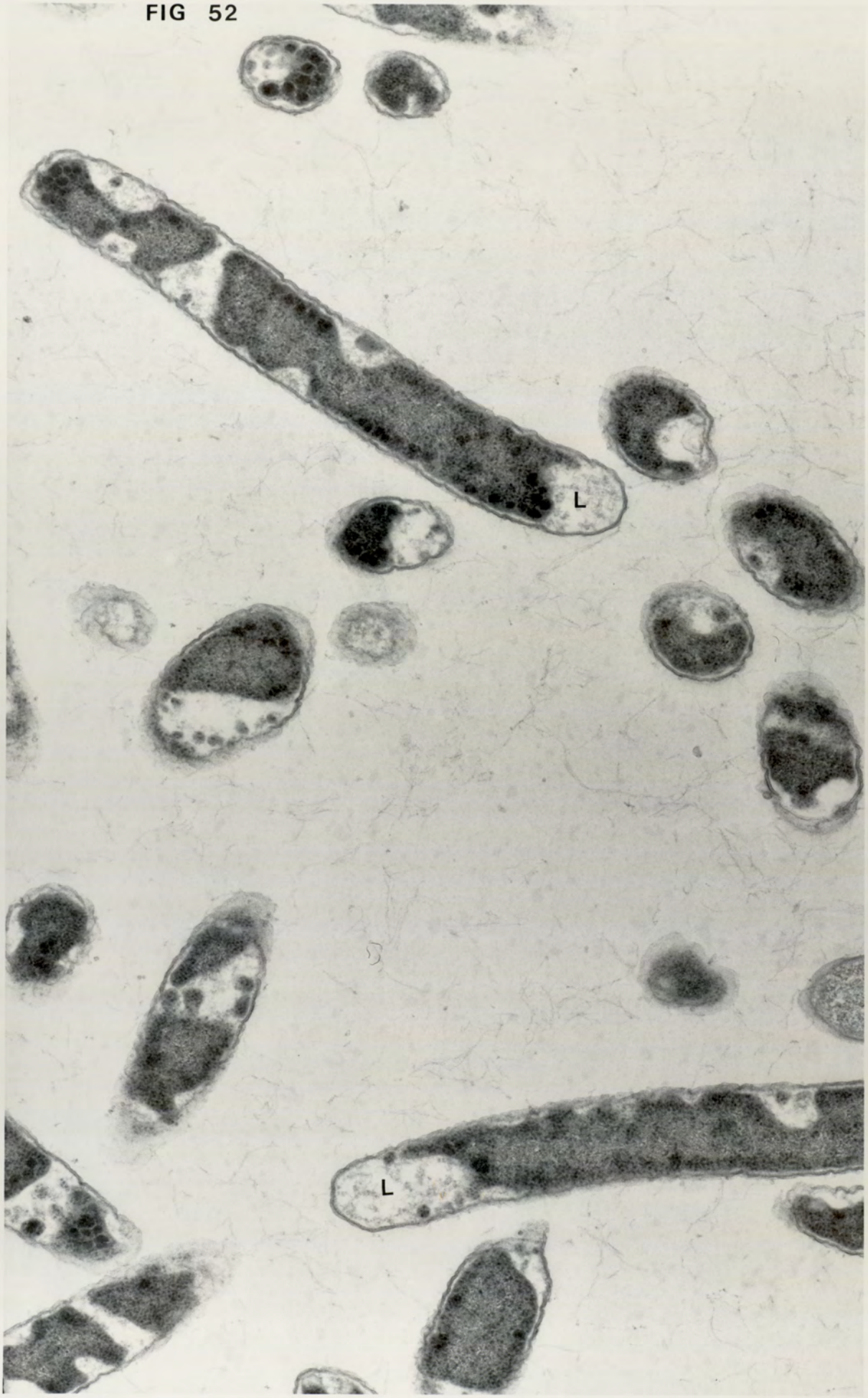


Fig. 52 Structural analysis of *E. coli* JM83 containing pPA16 when grown in glucose limited media at a dilution rate of 0.2 hr^{-1};

The photograph has a magnification of 37700 and the electron lucent areas are designated L.

FIG 52



grown cells 3.4.1: Figure 49). The other unusual factor was the presence of electron lucent areas at the ends and along the length of the cells giving the appearence of the inner-membrane being pushed into the cytoplasm. The results for JM83/pPA16 when grown in glycerol limiting medium at a dilution rate of 0.2 hr^{-1}, clearly show at a magnification of 151800 the separation of the inner-membrane from the cell wall by an electron lucent area (Figure 53). A photograph of a wider field gave similar results to that seen in Figure 52.

Because of the unusual structure of the hosts seen in Figures 52 and 53, JM83 containing pUC8 was then used as a negative control. The results are shown in Figure 54 and show the cells to be in a slightly better condition than those containing pPA16, although some electron lucent areas were still present.

Structural analysis of JM83/pPA31 when grown in glycerol limiting medium at a dilution rate of 0.2 hr^{-1} was then investigated. The results gave a similar picture to that found under the light microscope (3.4.1; Figure 50), where elongated cells were often seen with no cell septum (Figure 55). The photograph shown in Figure 56 also shows the beginnings of inclusion body formation.

Finally E.M. analysis was performed on JM83/pPA34 when grown in glycerol limited medium at a dilution rate of $0.2hr^{-1}$. Unfortunately the plasmid had been lost from the culture at this stage and the photograph (Figure 57) shows that the bacteria appear more healthy with fewer electron lucent areas present.

Fig. 53 Structural analysis of *E. coli* JM83 containing pPA16 when grown in glycerol limited media at a dilution rate of 0.2 hr^{-1}; The photograph has a magnification 151800, electron lucent areas are present (L), the inner- and outer-membranes are arrowed.

Fig. 54 Structural analysis of *E. coli* JM83 containing pUC8 when grown in glycerol limited media at a dilution rate of 0.2 hr^{-1}; The photograph has a magnification of 49400 and electron lucent areas are present (L).

Fig. 55 Structural analysis of *E. coli* JM83 containing pPA31 when grown in glycerol limited media at a dilution rate of 0.2 hr^{-1}; The photograph has a magnification of 33000 and electron lucent areas are present (L).

Fig. 56 Structural analysis of *E. coli* JM83 containing pPA31 when grown in glycerol limited media at a dilution rate of 0.2 hr^{-1}; The photograph has a magnification of 56000 and as well as electron lucent areas (L) inclusion bodies are also present (IB).

Fig. 57 Structural analysis of *E. coli* JM83 containing pPA34 when grown in glycerol limited media at a dilution rate of 0.2 hr^{-1}; The photograph has a magnification of 72000 and electon lucent areas are present (L).

FIG 53

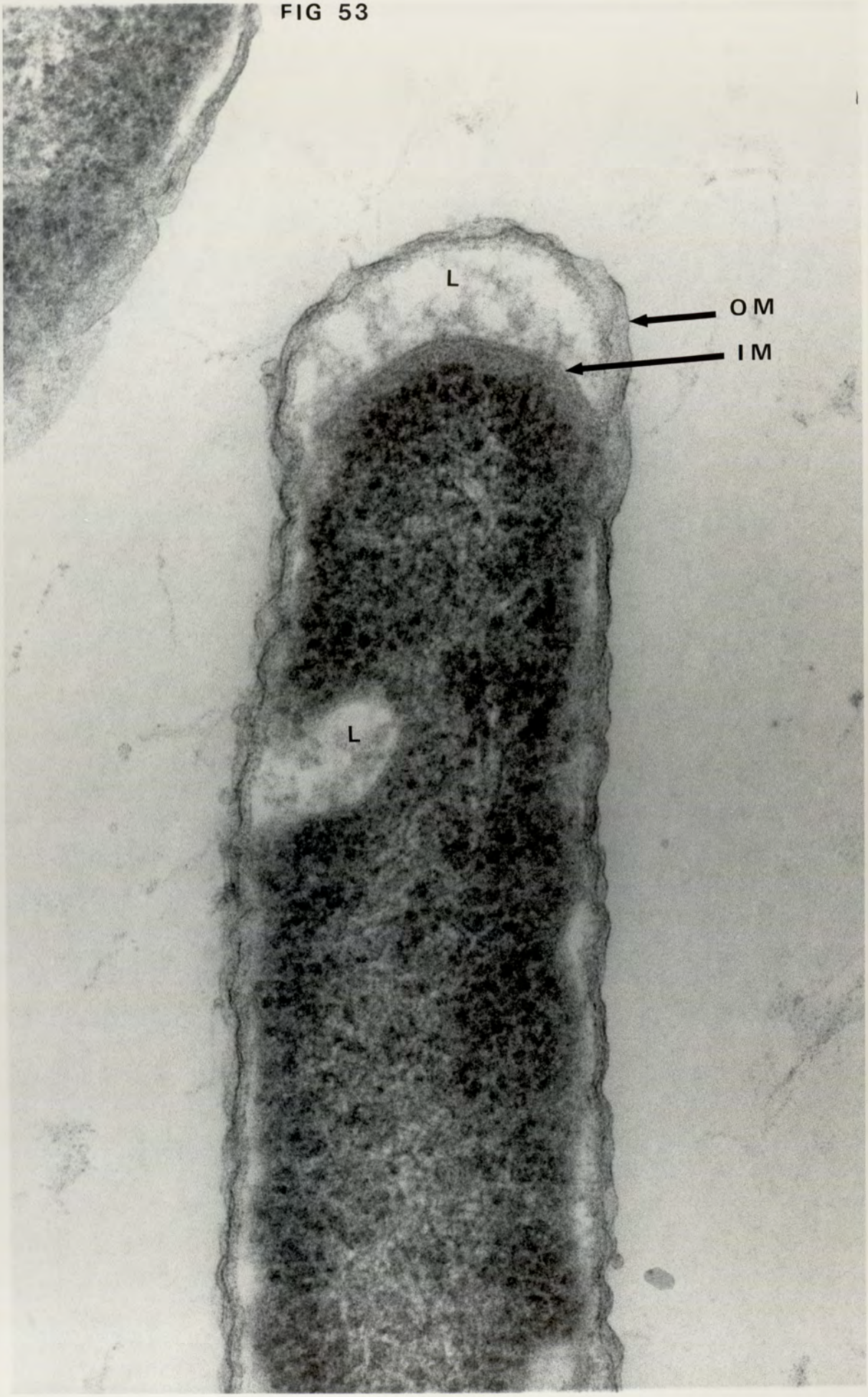


FIG 54

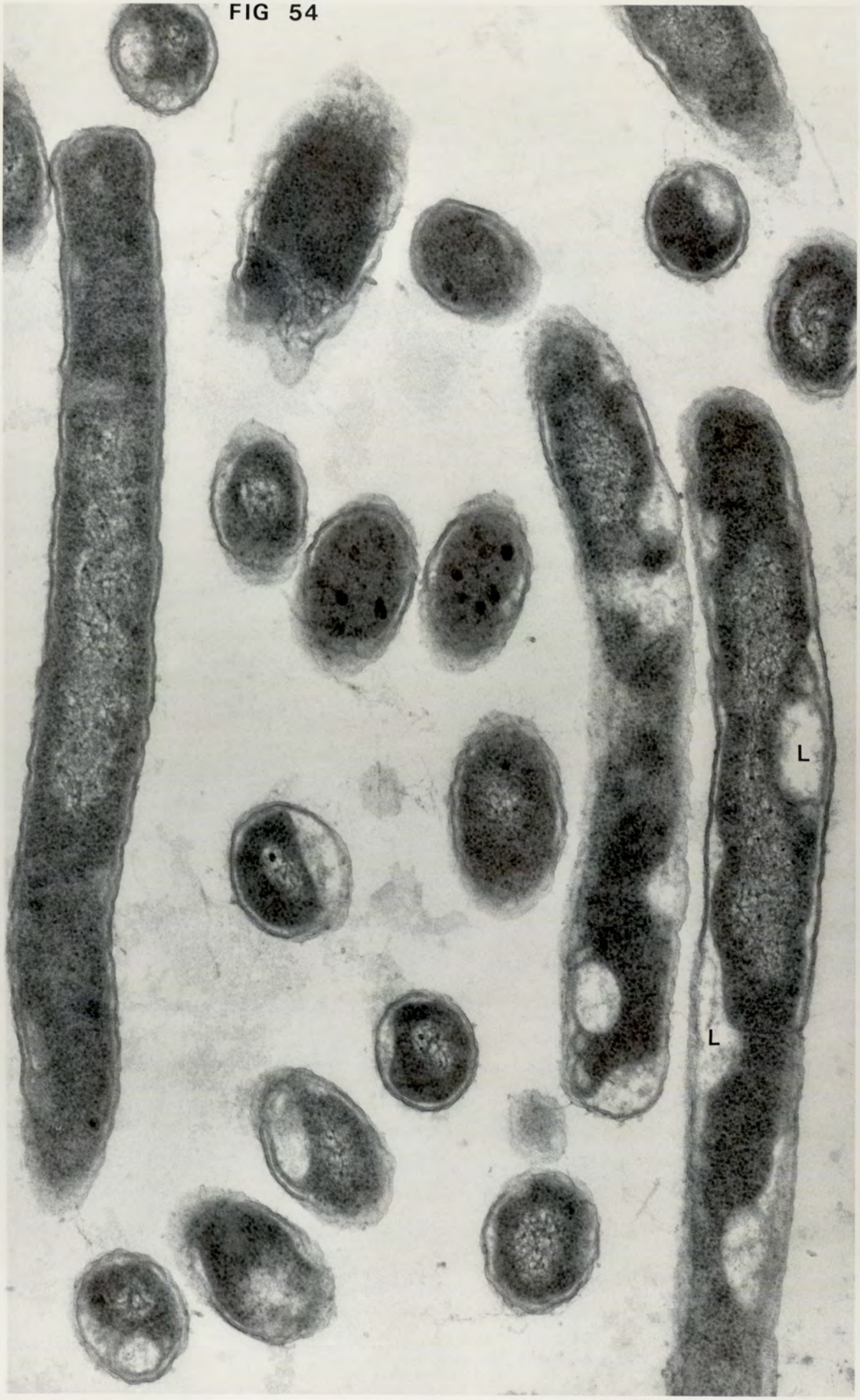


FIG 55

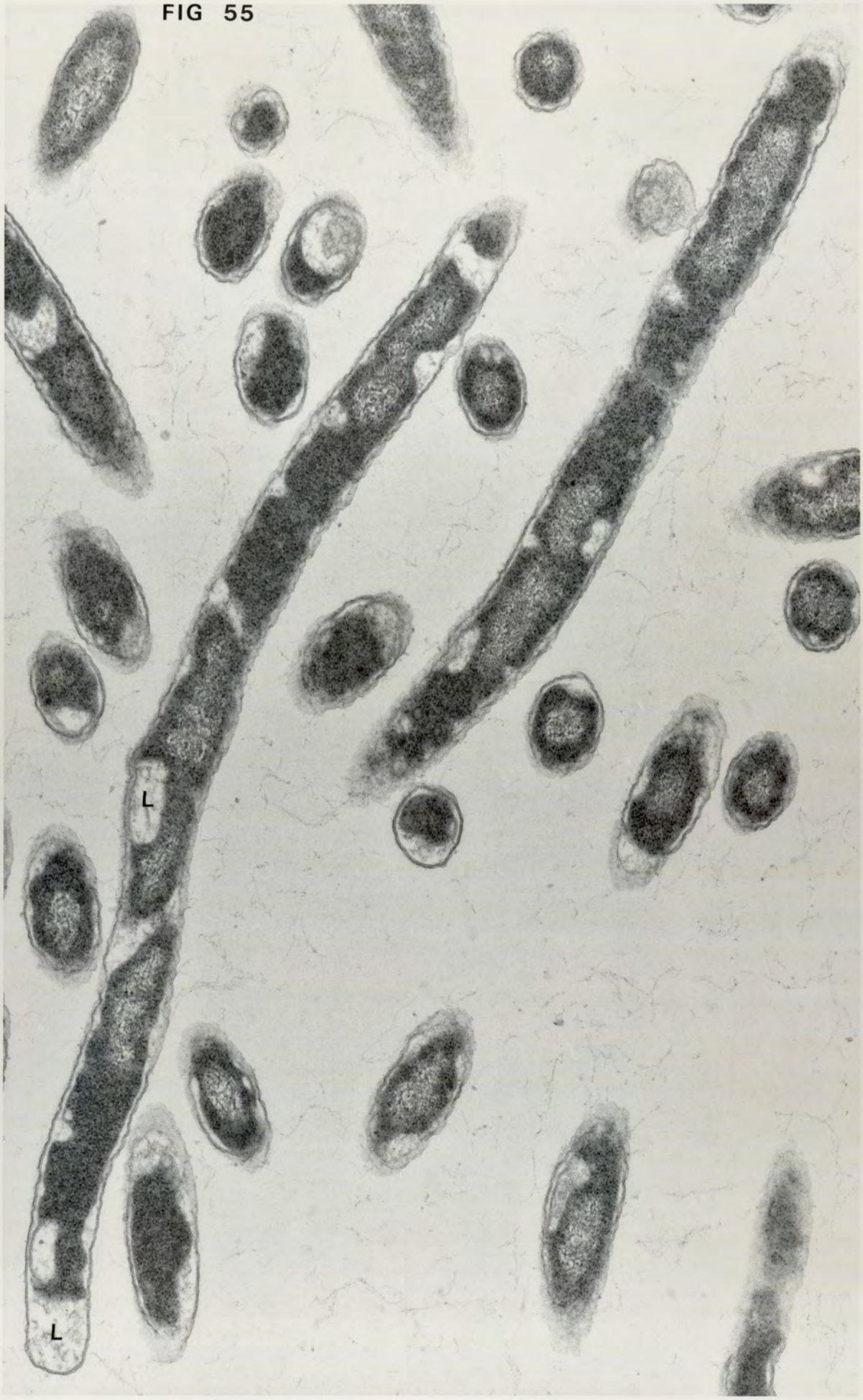


FIG 56

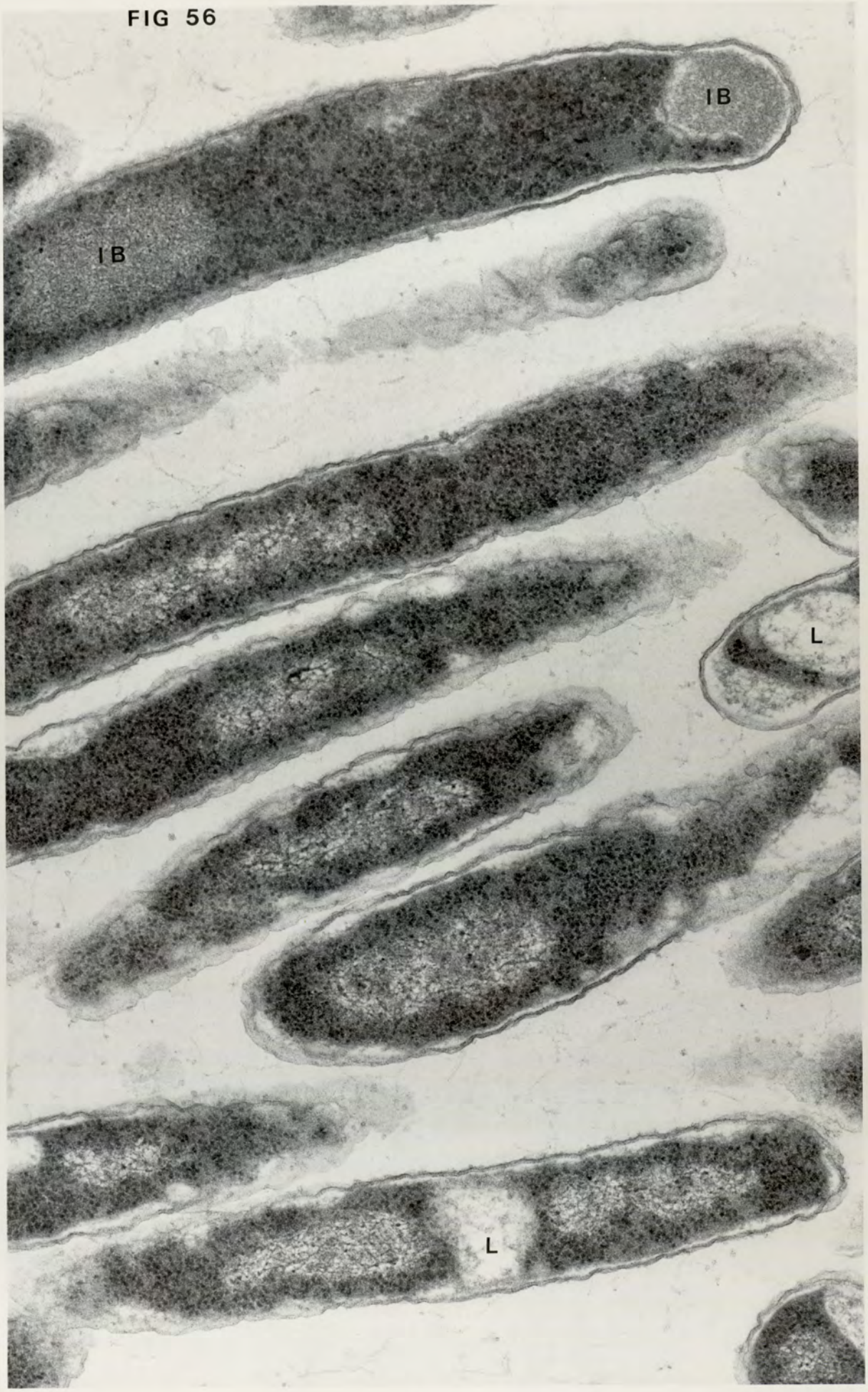
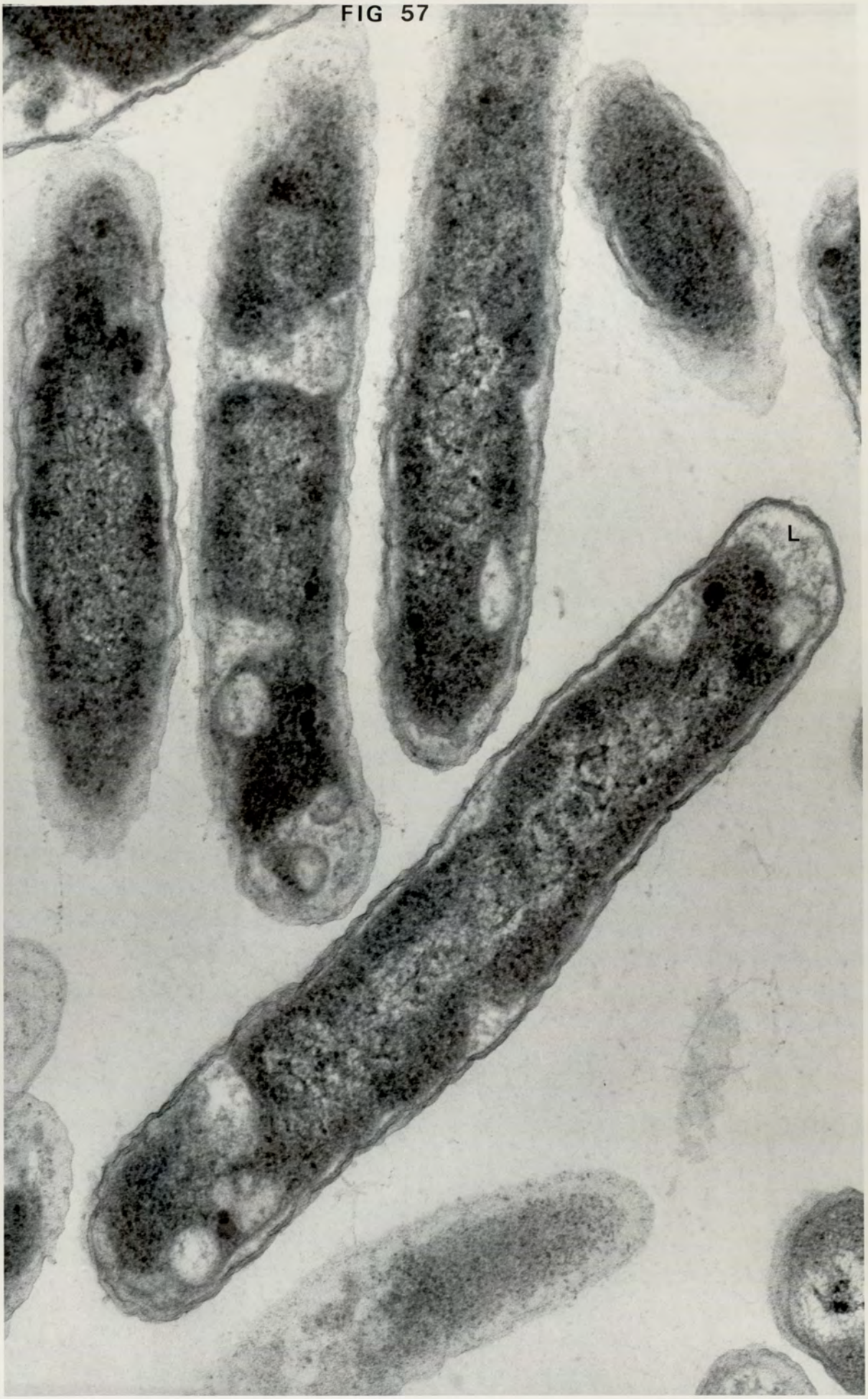


FIG 57



3.4.3 Localisation of recombinant protein A using immuno-gold probing

To study the efficacy of probing for recombinant SPA in cell sections with gold labelled antibodies, whole cells of *S. aureus* Cowan I and *E. coli* JM83 were first studied. The results for *S. aureus* Cowan I gave a good positive response (Figure 58) indicating the presence of protein A on the cell surface. There was a ten fold increase in binding to the cell (120 gold paritcles) to that seen in the background (11 gold paricles in a mean of 5 similar areas). The results for JM83/pPA16 indicated that there could be small amounts of SPA on the surface of the cell (35 gold particles), although this was little above the background level (9 gold paricles were counted in a mean of 4 similar areas; Figure 59).

The samples that were analysed for cell structure were also processed to allow for the probing of recombinant protein A (2.2.12B). The results for JM83/pUC8 (Figure 60) showed a good negative control with little immunogold present and none specifically binding the cells.

The samples for JM83/pPA16 grown in both glucose and glycerol limiting media produced similar results. When grown in glucose limiting medium the results clearly show the immunogold particles in the electron lucent areas with a small amount of non-specific binding occuring in the background (Figure 61). Results for JM83/pPA16 when grown in glycerol limiting medium are seen in Figure 62 and again clearly show the presence of the immunogold in the electron

Fig. 58 Immunogold labelling of protein A on the surface of S. aureus Cowan I; at a magnification of 111600.

Fig. 59 Immunogold labelling of protein A on the surface of E. coli JM83 containing pPA16; at a magnification of 72600.

Fig. 60 Immunogold labelling of protein A on sections of E. coli JM83 containing pUC8, when grown in glycerol limited media at a dilution rate of 0.2 hr-1; at a magnification of 68200.

Fig. 61 Immunogold labelling of protein A on sections of E. coli JM83 containing pPA16, when grown in glucose limited media at a dilution rate of 0.2 hr-1; at a magnification of 49300.

Fig. 62 Immunogold labelling of protein A on sections of E. coli JM83 containing pPA16, when grown in glycerol limited media at a dilution rate of 0.2 hr-1; at a magnification of 61600.

Fig. 63 Immunogold labelling of protein A on sections of E. coli JM83 containing pPA31, when grown in glycerol limited media at a dilution rate of 0.2 hr-1; at a magnification of 77000.

FIG 58

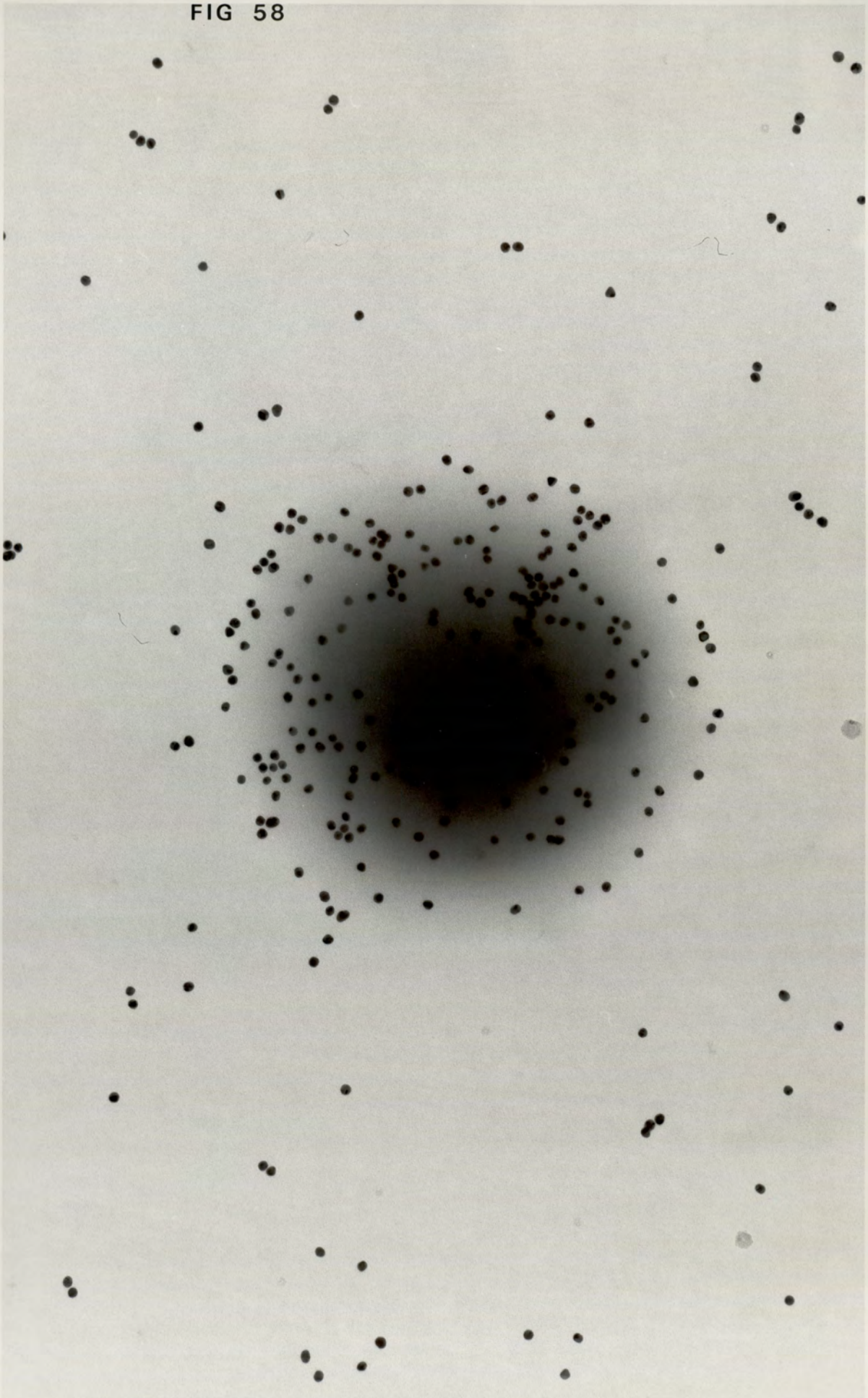


FIG 59

FIG 60

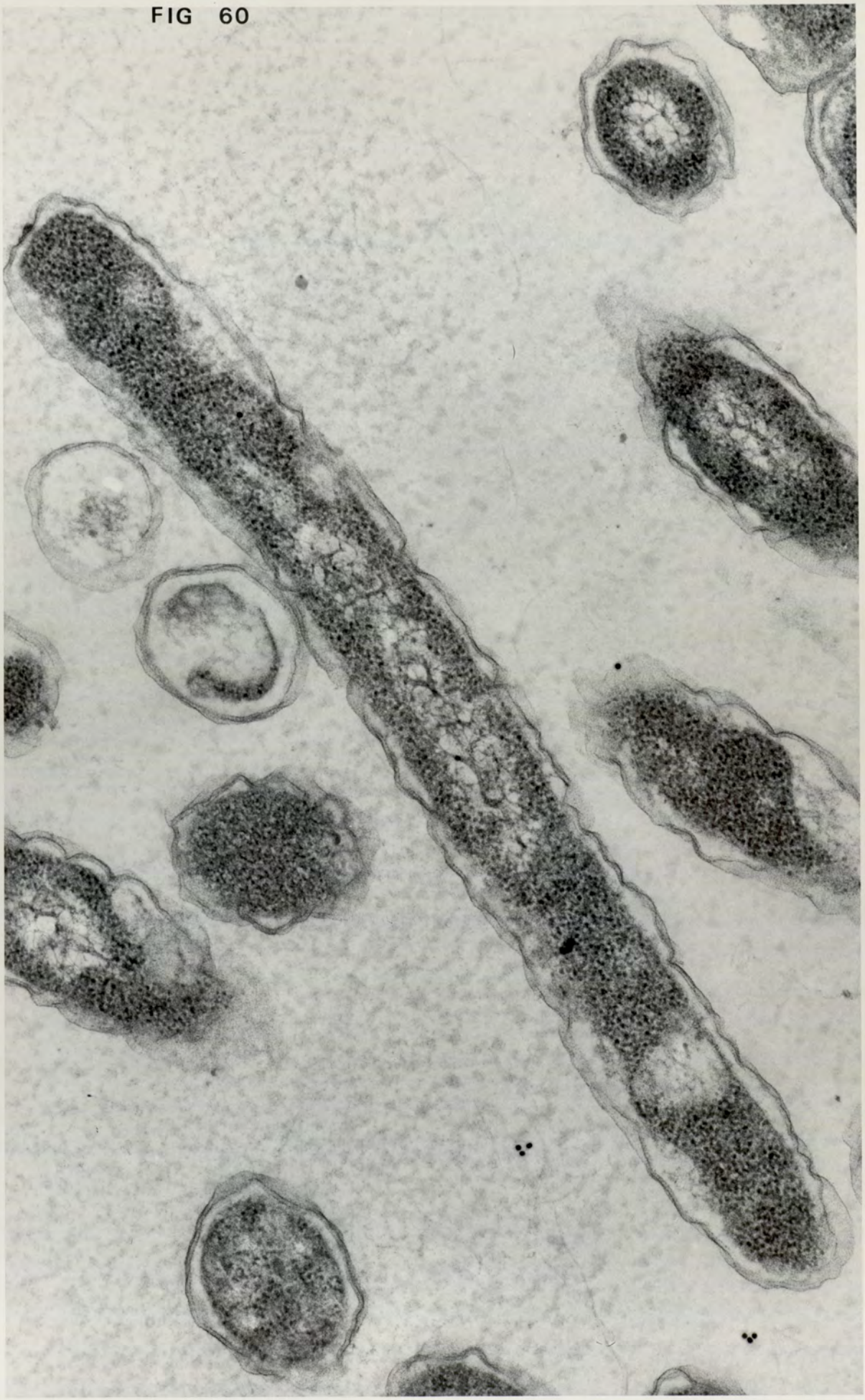


FIG 61

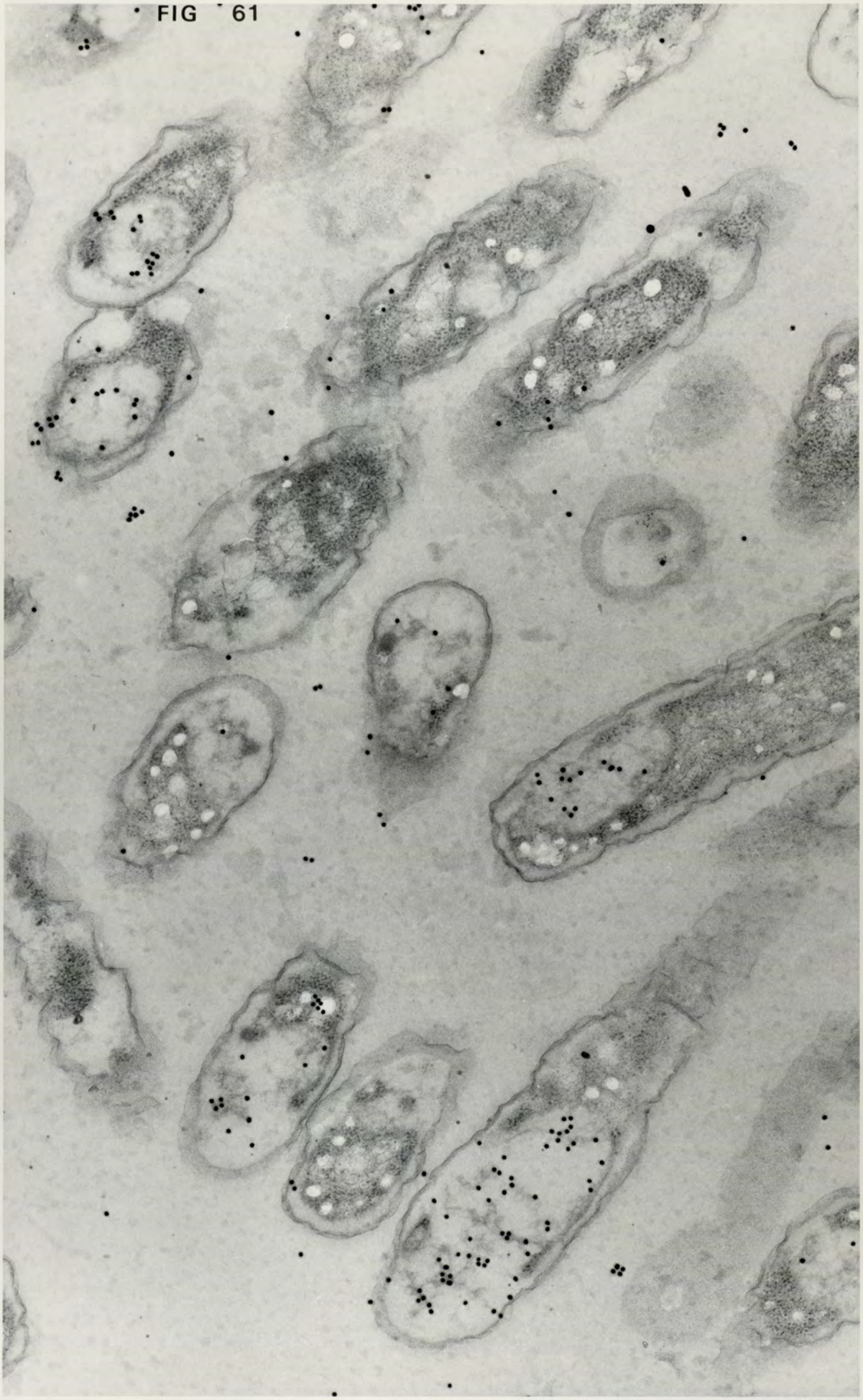


FIG 62

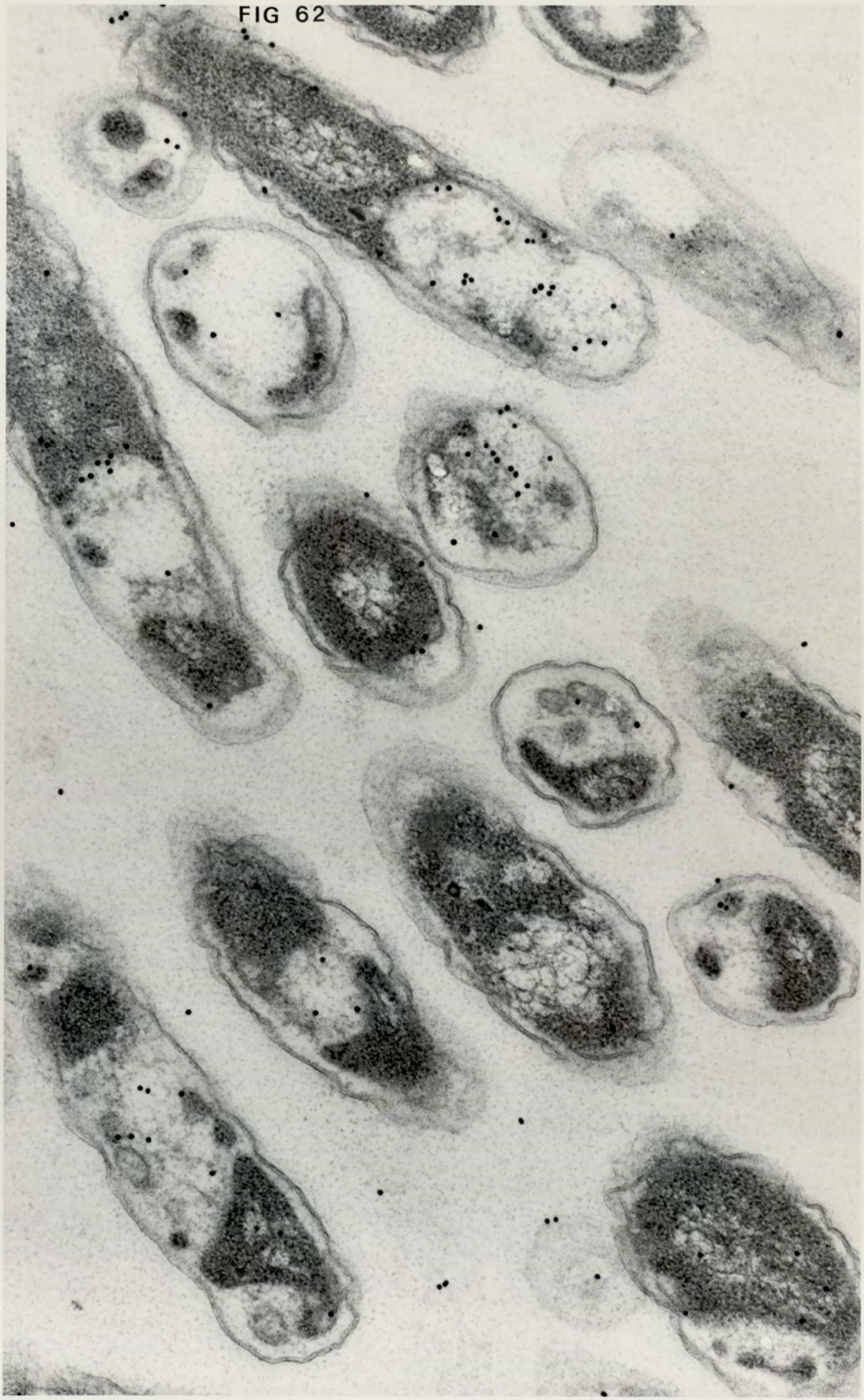
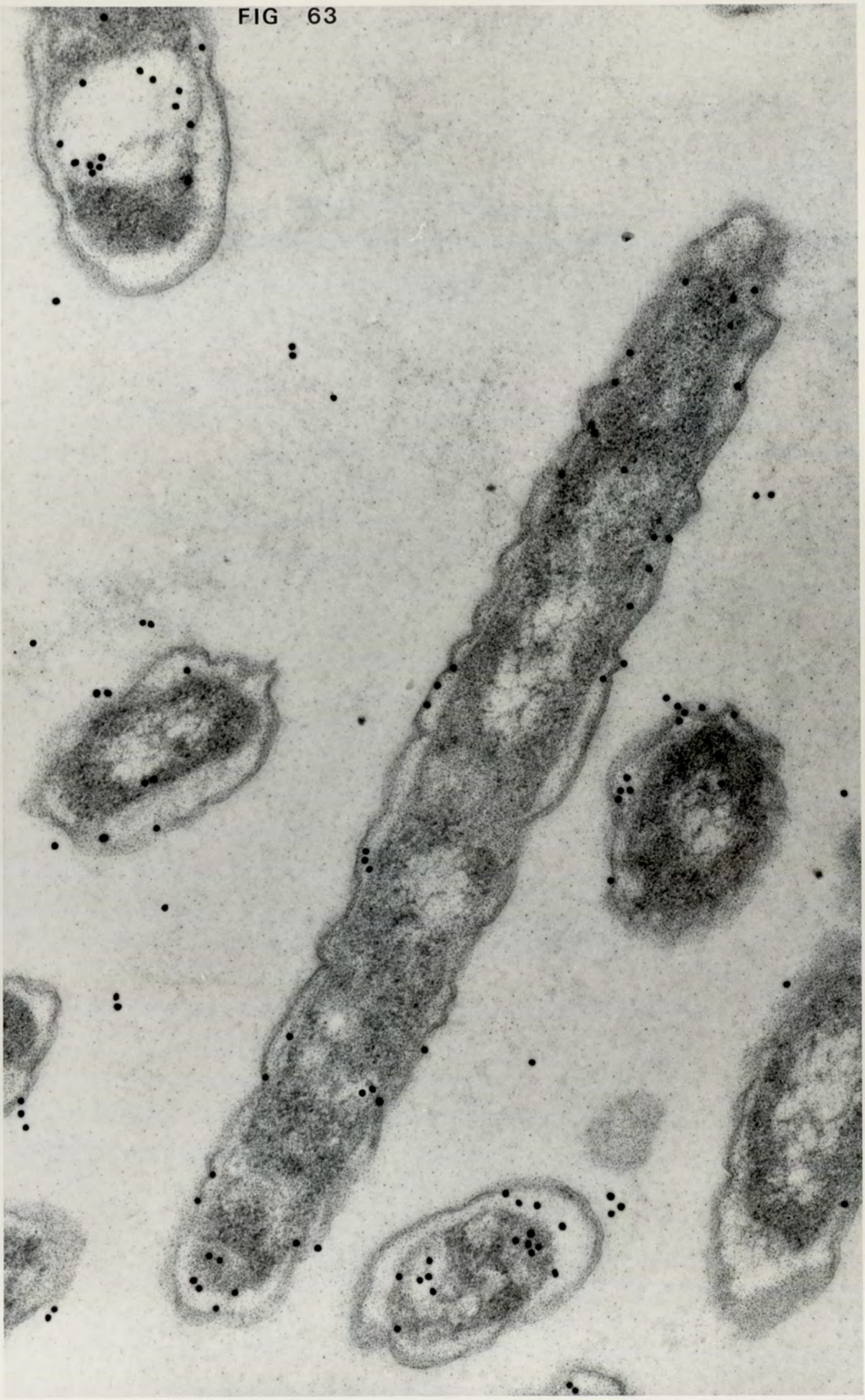


FIG 63



lucent areas.

The results of the sample involving JM83/pPA31 are shown in Figure 64 and not only show the presence of the immunogold in the electron lucent areas but also the protein A associated with the cell membrane.

The results obtained for JM83/pPA34 are not illustrated although they were similar to the negative control JM83/pUC8. This confirmed that no SPA was present in the culture at the time of sampling (Figure 47). 3.4.4.

3.4.4 Estimation of *B*-lactamase activity

Due to the presence of electron lucent areas in the negative control (i.e. JM83 containing pUC8), it was decided to assay all the samples for *B*-lactamase activity as the, production of this enzyme may alone be responsible for these changes (Table 7). The results indicated that JM83/pUC8 had the highest levels of *B*-lactamase activity, with JM83/pPA16 half that value. The levels of activity in the JM83/pPA16 host vector systems were a tenth of that seen in the control system. Due to the loss of pPA34 at the time of the sample, levels of *B*-lactamase were vastly reduced. The levels of SPA production are also included in the table, to give the full details of plasmid expression at the time of sampling.

Table 7 SPA content and *B*-lactamase activity of samples taken for electron microscopy

Plasmid	limited media	SPA ($\mu g\ mg^{-1}$)	*B*-lactamase activity ($\mu mol\ min^{-1}$)
pUC8	glycerol	0.0	10.6
pPA16	glucose	9.5	1.2
pPA16	glycerol	6.2	1.4
pPA31	glycerol	50.0	5.6
pPA34	glycerol	0.0	0.01

4. DISCUSSION

The stability of plasmids expressing recombinant SPA (Shuttleworth *et al.* 1987; pPA16, pPA31 and pPA34), have been investigated using continuous culture. Factors influencing both stability and expression include the genetic construction of the plasmids, external changes such as nutrients, pH, oxygen, temperature and changes within the host such as filamentation, structural defects or the formation of inclusion bodies. As the influence of genetic changes on the stability and expression have been widely documented (see 1.6) this work has concentrated on the effects of nutrient limitation. This was important as expression of the SPA gene is under the control of the lac promoter and as a consequence glycerol is used as an alternative to glucose to avoid the effects of catabolite repression. The work discussed here also details the development of an ELISA system capable of measuring SPA, the defining of a synthetic medium used in the continuous culture experiments and finally localising the recombinant SPA within the host *E. coli* and noting any structural changes.

4.1 ELISA FOR THE DETECTION OF SPA

Due to the large number of samples being produced in this work it was desirable to develop an assay system capable of measuring small quantities of recombinant protein A. Although a rocket system was available it was extremely laborious to perform and accurate only to 2 $\mu g\ ml^{-1}$ SPA. An

ELISA was available to measure SPA (Olsvik and Berdahl, 1981) but the sensitivity was no greater than that produced by the rocket system.

The assay system developed in this thesis avoids several of the disadvantages of those previously published in that it avoids the use of custom-conjugated SPA in competitive ELISAs (Fey and Berkhard, 1981; Lofdahl *et al.* 1983, Dertzbaugh *et al.* 1985). It also avoids relying on the non-specific cross-linking of IgGs employed in other systems (Olsvik and Berdahl, 1981). This assay system employs only commercially available reagents, is very reproducible and is very rapid, requiring only 4 hr to get a titre at maximum sensitivity or 2 hr if a lower sensitivity can be tolerated. A smooth pseudo-sigmoidal response curve is produced at concentrations of SPA from 50 - 0.1 ng ml^{-1} giving the assay a working sensitivity of 1.0 ng ml^{-1}, about 10 fold better than that produced by Dertzbaugh *et al.* (1985). This increase in sensitivity is probably the result of using human IgG as the capture antibody which binds only to the Fc receptors of SPA and using rabbit anti-SPA hyperimmune serum as the detection antibody which can not only bind the remaining Fc receptors but also at several antigenic sites along the molecule. Furthermore by including GARP as an additional amplification step, several GARP molecules can bind each rabbit anti-SPA Ig, further increasing the sensitivity of the assay. Thus attempts to replace the rabbit anti-SPA serum with normal rabbit serum (as used by Olsvik and Berdahl, 1981) resulted in severe loss of sensitivity. As mentioned in the introduction (see 1.11.3)

there has been an increase in the use of SPA, especially when linked to various substances. This includes the use of SPA-sepharose in the perfusion treatment of patients. Due to problems associated with the toxicity of SPA it has become essential to detect any leaching in the presence of high levels of human IgG. This is one aspect, which was not investigated in this thesis. However, Bloom *et al.* (1989) developed an ELISA using a biotin labelled detection antibody. They showed that by using rabbit anti-SPA for capture and anti-SPA-biotin for detection that the working sensitivity of the assay in the presence of human IgG was in the subnanogram range. The assay may be improved even further by replacing anti-SPA-biotin with anti-SPA followed by goat anti-rabbit IgG-biotin as demonstrated by the assay system mentioned here.

As other workers had reported that lysozyme can cause unreliable results in ELISA's (Esink *et al.* 1985), we tested our procedure in the presence of high concentrations of both lysozyme and lysostaphin. Unlike the above authors no interference was detected from either lysozyme or lysostaphin treated cells. It is possible that any crosslinking between IgG molecules has been avoided as Igs from different species have been used for capture and detection. In addition the peroxidase conjugate is removed a stage further from the capture antibody and this may be reflected by the lack of interference in the system. In a similar manner the system was tested to ensure there was no endogenous peroxidase present in the bacterial lysates to produce false positives. Probably the major cause of interference in

detecting SPA in bacterial lysates is the fact that all rabbits will have encountered *E. coli* or even *S. aureus* at some time in their lives and thus their sera would contain antibodies against several bacterial proteins in addition to the SPA with which they were hyperimmunised. Indeed the analysis of western blots from PAGE analysis of lysates from both SPA-positive and negative strains show that the anti-SPA sera do contain antibodies against a variety of bacterial proteins. Although these bacterial antigens are readily detected by such analysis, antibodies to them do not constitute a significant proportion of the total spectrum of anti-bacterial antibodies in the rabbit sera used here. That is to say, the backgrounds achieved in the ELISAs from the negative *E. coli* strains only became significant at dilutions of 1:10 or less, i.e. far removed from the conditions used in practice.

Experiments to determine the optimum method of releasing SPA from bacterial lysates revealed that sonication was 10% more efficient than lysozyme. This could be due to the fact that the lysozyme treatment involves a centrifugation step. This could spin down any SPA attatched to the cell walls or membranes of the host which would then be lost on assaying. Whereas sonication treatment involves assaying the whole cell lysate, the resulting difference in SPA content could therefore be due to the physical seperation step.

As shown in Figure 19A western blot analysis showed background binding of the hosts antigens with the anti-SPA used in the ELISA. Furthermore, the blots also show that the protein A produced by any host commercially or otherwise

is prone to the formation of multiple bands. The blot in Figure 19A indicates that the protein A produced by pPA16 in JM83 exists in three major forms of 43, 45 and 48 Kd. The absence of the 3' coding region from the cloned SPA gene in pPA16 may explain why the protein A expressed in *E. coli* is smaller than that from the native *S. aureus*. This is corroborated by the results in Figure 19B which shows the differences in molecular weights produced by the full (pPA31 48 Kd) and tailess protein A (pPA34 43 Kd). The commercial products show a wide variation, although the protein A produced by Fermentech and Pharmacia are very similar with both being produced by *S. aureus*. The protein A produced by Sigma gave the largest number of molecular weights, resulting in a diffuse band ranging from 43-55 Kd. The reason for the multiple forms of protein A found in *E. coli* and *S. aureus* is not clear. Proteolysis may play a part or the unusual shape of the protein could give rise to differential binding of SDS, resulting in differing PAGE mobilities. These results may also explain the variations in molecular weight of protein A reported by different workers.

Due to the unique binding of protein A and protein G to immunoglobulins it has recently been found that protein G can also be measured using this ELISA system. There is however, a ten fold reduction in sensitivity although this is compensated for by the availability of the assay system.

4.2 BATCH CULTURE ANALYSIS OF GROWTH

Defining a synthetic medium for use in continuous culture is of great importance as this data forms the foundations for the experiments. The amino acid supplements required by most bacterial strains producing recombinant products may be especially important, as they may often be overlooked.

The chemically defined medium used in these experiments was based on that of Klemperer *et al.* (1979) with the addition of specific host requirements (i.e. proline and thiamine). Due to the effects of catabolite repression (see 1.5.1) glycerol has been used as an alternative carbon source to glucose, so that differences between these two substrates can be highlighted. The results clearly show that the concentration of each nutrient required to sustain logarithmic growth of JM83 both with and without pPA16 to an optical density of 1.0 was similar to that obtained by Klemperer *et al.* (1979; Table 5).

There were however, two major differences in the growth rate of the host organism as illustrated by the u^{MAX} in batch culture (Table 4). The first noticable difference was that the growth rate of the organism was reduced by 50% (depending on the nutrient being depleted) if a plasmid was present. This would indicate that the plasmid was increasing the metabolic load placed upon the host, resulting in a reduced growth rate, this effect has also been described by Da Silva and Bailey (1986) and Bassford *et al.* (1979). The second difference was that the specific growth rate was further reduced when the host either with or without the

plasmid was grown in glycerol based media (depleted or excess) as opposed to glucose. In fact the specific growth rate of the host is lowest when carrying a plasmid and grown on excess glycerol with depleted phosphate. Glycerol based media also had a longer lag phase, when compared to glucose depleted media. These results indicate that the glycerol is adding to the metabolic "stress" placed upon the host when compared to media containing glucose.

These conflicting results may be explained by the differences in the two carbon sources. Firstly glucose is taken into the cell by an active transport mechanism, while glycerol gains entry to the cell via facilitative diffusion. This mechanism depends on a concentration gradient across the cell wall being maintained by the metabolism of the substrate (Stowell *et al.* 1987). As the glycerol concentration is always restricted, even when in excess then the amount of glycerol crossing the membranes may be low. Secondly the number of molecules of ATP produced by glycerol are lower than that produced by an equivalent concentration of glucose. So although glycerol is often used as an alternative carbon source to glucose, to avoid the effects of catabolite repression, the host organism will be at a disadvantage due either to an increase in metabolic load by the plasmid or the fact the glycerol is effecting the growth rate of the host. Surprisingly the concentration of proline required to produce growth to an optical density of 1.0 was 1.5 mM which was higher than that used by other workers (Jones *et al.* 1980; Caulcott *et al.* 1985; Seo and Bailey, 1985). This may be crucial as the relatively small amounts

of amino acid supplements used by these workers may result in a medium which is not limited by a single nutrient. The medium defined here will allow experiments involving continuous culture to be carried out with the knowledge that the medium wil be depleted by a single nutrient.

Prior to using the chemically defined medium in the continuous culture experiments, it was thought appropriate to investigate the stability of pPA16 in JM83 by subculturing in various depleted media. The results indicated that the plasmid was stable when the host was grown in TSB or in glucose depleted medium (Table 6). While ammonium or proline depleted cultures only showed a small loss of ampicillin resistant cells from the population, magnesium and glycerol produced further reductions. Phosphate depletion produced the greatest instability which may be attributed to the fact that the substrate is required for DNA and RNA synthesis as well as protein synthesis, carbohydrate metabolism and cellular respiration.

4.3 STABILITY AND EXPRESSION OF PLASMIDS CONTAINING THE SPA GENE

In the expression of recombinant products under the control of the lac promoter glycerol is often used as an alternative to glucose, although little data has been published on this subject. The results obtained from the batch culture experiments (see 3.2) suggested that more work may be required in this area than was at first anticipated. It is for this reason that the main thrust of the work has involved comparing the effects of glucose and glycerol limiting conditions on the stability and expression of the SPA clones in *E. coli* JM83.

4.3.1 Stability and expression of pPA16 using glucose limited media

Initially the stability and expression of plasmid pPA16 in *E. coli* JM83 was investigated using glucose limited media at dilution rates (D) 0.1-0.4 hr^{-1}. At the lowest D, the percentage of ampicillin resistant cells in the population remained stable, although the copy number of the culture fell from 350 to 170 at the end of the experiment (Figure 28). SPA production reached a peak value of 11 $\mu g\ mg^{-1}$ of cell protein in the transitional phase of growth, which fell with a corresponding fall in copy number to 3 $\mu g\ mg^{-1}$, at the end of the experiment. The amount of SPA found in the culture supernatant only reached values of 1 $\mu g\ ml^{-1}$. Unfortunately the experiment at a D of 0.2 hr^{-1} was curtailed due to problems with the pH, although samples were obtained for electron microscopy analysis (Figure 32). A

repeat of this experiment showed that 100% ampicillin resistance was maintained for 60 generations, although there was not enough time to estimate the other parameters. Results at a D of 0.3 hr^{-1} show transient instabilty occurred at 30 generations, which may be accounted for by the fall in copy number from a peak value of 450 to a plateau level of 50 copies. SPA production paralleled this giving an initial peak value of 6 $\mu g\ mg^{-1}$ in the early continuous culture phase, which then fell (prior to the fall in copy number) to a plateau of 2 $\mu g\ mg^{-1}$ of cell protein. SPA found in the culture supernatant followed a similar pattern to that found in the previous experiment. Total equilibrium of the host/vector system was not attained until after 80 generations when the copy number, SPA production and the number of ampicillin resistant cells stabilised at a continuous level. At the highest D plasmid instability occurred as soon as the continuous culture phase was entered (Figure 34). The percentage of cells showing ampicillin resistance was reduced to 65% of the population after 120 generations. The copy number of the whole culture remained low at 100 copies throughout most of the experiment, while SPA production fell gradually from 4 to 2 $\mu g\ mg^{-1}$ of cell protein. This implies that the increase in D has increased the metabolic "stress" on the host organism, which is demonstrated by the increase in the loss of ampicillin resistant cells in the population at higher dilution rates. SPA production was also reduced from a peak value of 11 (D of 0.1 hr^{-1}) to 4 $\mu g\ mg^{-1}$ of cell protein (D of 0.4 hr^{-1}). These results were in agreement with Warnes and Stephenson

(1986) and Noak *et al.* (1981), who showed that the plasmids investigated became increasingly unstable with increases in dilution rate. This was however, contrary to that reported by Chew *et al.* (1988), who found plasmids to be stable at the higher dilution rates. In fact Brownlie (personal communications) has shown there are differences between complex and chemically defined media. The highest level of SPA production was 11 μg mg^{-1} cell protein or 1.1% of the total soluble protein. This was produced at a peak value in the transitional phase of growth at a D of 0.1 hr^{-1}. Due to the appearance of low levels of SPA in the culture supernatant (which followed that produced in the cells), this would indicate that protein export is not responsible but the appearance could be due to cell lysis. No structural instability of the plasmid was observed in these experiments according to the restriction analysis performed on the samples (e.g. Figure 29). According to western blot analysis the SPA produced was found to be in three major bands of 48, 45 and 43 Kd (e.g. Figure 31).

4.3.2 Stability and expression of pPA16 using glycerol limited media

The next set of experiments investigated the stability and expression of pPA16 in JM83 when grown under glycerol limiting conditions, at equivalent dilution rates to those investigated under glucose limiting conditions. In complete contrast to the results observed under glucose limitation, the plasmid was lost from the culture at all the dilution rates investigated. At a D of 0.1 hr^{-1} the onset of plasmid

instability was initiated after 25 generations in continuous culture (Figure 35). SPA production reached 8 μg mg^{-1} cell protein in the transitional phase, and then fell during the continuous culture phase, with the copy numbers following a similar pattern. This was followed by a loss of ampicillin resistance in the population, which resulted in the total loss of the plasmid from culture. Following the loss of the plasmid from the culture, SPA was not detected in either the cells or the culture supernatant. A similar pattern was observed at a D of 0.2 hr^{-1} where the copy number fell after 10 generations in continuous culture (Figure 39). There was a period however, where the copy number remained constant after the initial fall, this then fell from 400 to 80 copies per host chromosome. SPA production produced a peak value of 7 μg mg^{-1} cell protein in the early continuous culture phase, which then fell to trace amounts. This was again followed by a fall in the number of ampicillin resistance cells in the population. The SPA found in the culture supernatant paralleled that found in the cell extracts. At a D of 0.3 hr^{-1} (Figure 40), the onset of plasmid instability was in the transitional phase, occurring earlier than the two previous experiments. The loss of ampicillin resistance was preceded by a loss of copy number and SPA production, which fell from peak values of 270 copies and 8 μg mg^{-1} of cell protein to zero during the continuous culture phase. At the highest D of 0.4 hr^{-1} (Figure 41) the results obtained were similar to those obtained at a D of 0.3 hr^{-1}. Although the peak production of SPA reached only 3 μg mg^{-1} cell protein, substantially lower than the levels produced

in the other experiments. This would indicate that greater expression of SPA (under the control of the lac promoter) can be attained under glucose rather than glycerol limiting conditions. The plasmid was also found to be stable under glucose limiting conditions. Restriction analysis of the samples showed no major structural changes had occurred indicating that the instability of the plasmid was segregational.

Using glycerol limited media at a D of 0.2 hr^{-1} pUC8 in JM83 was used as a negative control and as expected no SPA was detected in either the cells or the culture supernatant. There was a slight loss of ampicillin resistance at the end of the experiment, although the copy numbers reached a level of 600 per host chromosome.

4.3.3 Stability and expression of pPA16 using ammonium limited media with either excess glucose or glycerol

As pPA16 was unstable at all the dilution rates investigated under glycerol limited media, it may be possible that glycerol itself was having an adverse effect on the host, resulting in the loss of the plasmid. To investigate this phenomena, the stability and expression of pPA16 was investigated using excess glucose and glycerol while limiting with ammonia. Ammonium limited medium was used as the results from the batch subculture experiments (Figure 21) indicated that ammonium limited media had the least effect on plasmid stability. A D of 0.3 hr^{-1} was used, to put the host/vector system under a reasonable amount of metabolic "stress" (Seo and Bailey, 1985).

Using excess glucose the results showed the plasmid to be stable throughout the experiment, as there was no loss of ampicillin resistance. The copy number, contrary to the results using limited glucose remained constant throughout the experiment at a level of 550 copies per host chromosome. Although the copy numbers were high throughout the experiment, the levels of SPA compared to that produced under glucose limiting conditions (Figures 28, 32, 33 and 34; 4 $\mu g\ mg^{-1}$ cell protein). This may be explained by the fact that the excess glucose is causing catabolite repression, by switching off expression of the SPA gene. The reduction in the ammonium concentration could also be affecting protein synthesis, with a reduction in SPA production.

When investigating ammonium limitation with glycerol in excess, the results showed the plasmid to be stable up to 20 generations in continuous culture. After this period there was a decline in the number of ampicillin resistant cells to 85% by the end of the experiment. This was preceded by a fall in copy number (from 700 to 200 copies), and SPA production (from 6 to plateau level of 2 $\mu g\ mg^{-1}$ cell protein). SPA production was maintained even though the copy number was falling throughout the experiment. The plasmid showed a small amount of instability which could have been due to the fall in copy number, with SPA production not affected. It appears that glycerol does not have an adverse effect on the host, as plasmid stability was enhanced under excess glycerol. The results obtained here indicate that plasmid instability under glycerol limiting conditions may be caused by an increased metabolic load

being placed upon the host. The fact that less energy is derived from glycerol as opposed to glucose, and glycerol is transported into the host via facilitative diffusion and not by an active transport mechanism only supports this conclusion.

4.3.4 Stability of pPA16 using phosphate limited media

Phosphate limitation was also investigated to see if the plasmid was unstable at lower rather than at the higher dilution rates as described by Wouters *et al.* (1980) and Chew *et al.* 1988). Two different dilution rates were used, 0.1 and 0.3 hr^{-1} (Figures 44A and 44B) with only ampicillin resistance being monitored in these experiments. Plasmid instability occurred simultaeneously at both diution rates, however the plasmid was rapidly lost at the lower D with an 80% loss of ampicillin resistance in 20 generations. At the higher D only 15% of the cells loss their resistance in the same time. These results confirm that under phosphate limiting conditions the plasmid was less stable at the lower D. Why this should occur under phosphate and not carbon limited conditions may be that phosphate is required for a number of esential purposes including; DNA replication, RNA and protein synthesis as well as cellular respiration. It is also known that at high dilution rates the available concentration of a limiting nutrient is greater than that of a lower value. It could be that a very small change in the nutrient concentration could result in a rapid loss of the plasmid from the culture.

4.3.5 Stability and expression of pPA31 and pPA34 using glycerol limited media

The stability and expression of the plasmids pPA31 and pPA34 (see 1.11.2), which are used in the large scale production of recombinant SPA were also investigated in continuous culture. As glycerol is used in the large scale fermenters (150 and 400 L) then the same carbon source was used in these experiments. A D of 0.2 hr^{-1} was used in these final experiments so that electron microscopy analysis could be performed, which would allow a comparison to be made with the other experimental results.

The results for the production clone pPA31 which expresses the full length SPA molecule (which includes the hydrophobic tail-piece), indicated that the plasmid was lost from the culture, with the total loss of ampicillin resistance from the population (Figure 46). The copy numbers did not correlate with this and although erratic did not fall below 200 copies per host chromosome. SPA production remained high throughout the whole of the experiment, with the peak value nearly reaching 100 $\mu g\ mg^{-1}$ of cell protein, or 10% of the total soluble protein. Minimal SPA was found in the culture supernatants. The explanation for the contradiction in results obtained for the stability of this plasmid are discussed in section 4.4.

In the experiment involving pPA34 (the production clone expressing the tail-less SPA), the plasmid was lost in the batch phase of growth. The plasmid was extremely unstable and no value could be estimated for the copy numbers and only a small quantity of SPA could be detected in the cell

samples. As the quantity of SPA was ten times greater in pPA31 when compared to that produced in pPA16, then it would be expected that similar values should have been produced for pPA34.

The results show that greater levels of SPA can be produced under glucose limiting media (11 $\mu g\ mg^{-1}$ cell protein) as opposed to the preferred carbon source glycerol (8 $\mu g\ mg^{-1}$ cell protein). The plasmid pPA16 was also stable under glucose limiting-conditions, while under glycerol limiting-conditions the plasmid suffered segregational instability. The use of excess glycerol with limited ammonium increased the stability of pPA16 indicating that the use of low levels of glycerol could well be instigating segregational instability. Expression of the production plasmid pPA31 reached levels of 10% the total soluble protein, while the other production plasmid pPA34 was extremely unstable (Table 8).

Table 8 Summary of the stability of the plasmids used throughout this study

Medium limited	Plasmid	Dilution rate (hr^{-1})	Stability	Peak SPA ($\mu g\ mg^{-1}$)
Glycerol	pUC8	0.2	Stable	0.0
Glycerol	pPA16	0.1	Unstable	8.1
Glycerol	pPA16	0.2	Unstable	7.0
Glycerol	pPA16	0.3	Unstable	7.8
Glycerol	pPA16	0.4	Unstable	3.0
Glucose	pPA16	0.1	Stable	11.0
Glucose	pPA16	0.2	Stable	9.5
Glucose	pPA16	0.3	Stable	7.0
Glucose	pPA16	0.4	Stable	4.0
Ammonium with excess glycerol	pPA16	0.3	Stable	6.2
Ammonium with excess glucose	pPA16	0.3	Stable	6.0
Phosphate	pPA16	0.1	Unstable	N.D
Phosphate	pPA16	0.3	Unstable	N.D
Glycerol	pPA31	0.2	Inconclusive	98.0
Glucose	pPA34	0.2	Unstable	0.8

Samples on which SPA estimation were not performed are designated N.D.

4.4 CELL STRUCTURE AND THE LOCALISATION OF RECOMBINANT PROTEIN A

The location of any recombinant protein is of great importance, as purification procedures require this knowledge to ensure efficient extraction takes place. It is therefore imperative that the recombinant protein reaches the desired destination within the cell. In the case of SPA there is a staphylococcal signal peptide which is thought to transfer the protein into the periplasmic space of *E. coli* (Lofdahl *et al.* 1983). Without a signal peptide the protein would almost certainly remain in the cytoplasm which could then lead to the formation of inclusion bodies (as described in 1.12). The formation of inclusion bodies or even defects in the hosts structure may also lead to the loss of the recombinant protein on purification (Carrier *et al.* 1983).

It is well documented that organisms expressing recombinant DNA may appear elongated (Carrier *et al.* 1983), Shuttleworth *et al.* (1987) also mentions that JM83 containing pPA31 is elongated. This would explain the problems associated with diluting out the bacteria containing pPA31 for the viable counts in section 3.4. Due to the problems surrounding the stability of pPA31 in JM83 (Figure 46), Gram-stains were performed on the host organism which revealed that the organisms were Gram-negative rods, 20-30 times the size of plasmid free *E. coli*. Because of the change in size of the host, batch experiments were performed on the various host vector systems and Gram-stains were

performed on samples taken in the log phase of growth. The results show JM83 as a "normal" Gram-negative rod (Figure 48), while JM83 with pPA16 was Gram-negative but more diplococci in shape (Figure 49). JM83 with pPA31 showed Gram-negative rods which could be up to 20-30 times longer than plasmid free *E. coli*, which confirmed the results obtained for the Gram-stains performed on the continuous culture samples. Finally the results obtained for JM83 with pPA34 showed the organisms to be Gram-negative and again more diplococci in shape. It must be remembered however, that the culture conditions were different to that used in the continuous culture experiments and although JM83 with pPA31 produced similar results it does not follow the other host/vector systems will also be comparable.

Electron microscopy analysis of cultures taken from the continuous culture experiments were performed to look at any structural changes and to localise the SPA produced using immunogold (see 2.12.2). The electron microscopy results first showed the structure of the hosts containing the various plasmids. Analysis of JM83 containing pPA16 when grown under glucose or glycerol limiting conditions produced similar results (see figures 52 and 53) showing the presence of electron lucent areas. However, the organisms were more elongated than those seen under the light microscope, which could be due to the different culture conditions. Electron micrographs showed a membrane running round these areas in the interior of the cell, which would indicate that they are in the periplasmic space. Similar results were also obtained for the negative control, JM83 containing pUC8, with the

bacteria again elongated and showing similar electron lucent areas (Figure 54). The structure of JM83 with pPA31 showed extremely long organisms which again contained electron lucent areas (Figure 55). Although the electron microscopy clearly shows elongated cells it must be remembered that these photographs are of cells embeded in resin and it is therefore dificult to show extemely large cells as they run out of focus. Another important feature clearly shown in Figure 56, is the formation of inclusion bodies. These major disruptions to the hosts cell structure could ultimately lead to cell lysis. This could be highlighted when the host is grown on media containing ampicillin (as for the antibiotic sensitivity patterns) then the accumulative production of the recombinant products could result in increasing cell lysis. As mentioned earlier, the plasmid pPA34 was lost from JM83 before samples were taken for electron microscopy analysis. Even so, examination of the fields of view showed the presence of electron lucent areas although many appeared as normal *E. coli* (Figure 57). In conclusion it seems that there is little or no difference in the structure of the host which can be attributed to the changes in the carbon source. It also appears that the electron lucent areas discovered in the hosts occur primarily in the periplasmic space. These areas have also been shown in JM83 containing pUC8. The reason for this may be due to the high expression of *B*-lactamase from pUC8 which is having a similar effect on the hosts cell structure (Table 7).

The final series of experiments was to try to localise

the recombinant SPA within the host. As an ELISA system had already been developed to detect SPA, the system was adapted for use in the relatively new immunogold probing techniques. Initially, whole *S. aureus* Cowan I was used and the cell probed as in section 2.12.2. The results gave a good positive response (Figure 58) indicating the presence of protein A on the cell surface. There was a ten fold increase in binding to the cell (120 gold paricles) to that seen in the background (11 gold particles in a mean of 5 similar areas). The results for JM83/pPA16 indicated there could be low levels of SPA on the cell surface (35 gold paricles) as opposed to the background (9 gold particles in a mean of 4 similar areas; Figure 59). The next series of experiments involved probing cell cross sections to see where the SPA was localised. Probing JM83 containing pUC8 (negative control) with immunogold showed little binding even though electron lucent areas were present in the host (Figure 62). Results for JM83 containing pPA16 when grown under either glucose or glycerol (Figures 60 and 61) showed immunogold binding in the electron lucent areas which would indicate that the location of the SPA is in the periplasmic space. These results correlated with the results obtained by Shuttleworth (personal communications) who had already tried traditional localisation studies. This involved purifying fractions of the host whose purity was ascertained by using marker enzymes. The fractions for the JM83/pPA16 system gave the following purity; periplasmic 94%, membrane 72% and cytoplasmic 80%. Results for the high expression systems (JM83 pPA31 and JM83/pPA34; production strains) showed that

the cell fractions could not be purified according to the enzymic assays; periplasmic 61%, membrane 71% and cytoplasmic 47%. This abnormality and the fact that the plasmid, and therefore expression was lost meant that the SPA could not be localised. However, using immunogold labelling on the high expression system JM83/pPA31 the SPA could be localised in the periplasmic space of the host. The results were different from that shown for pPA16 in that there was binding of immunogold not only in the electron-lucent areas but also around the outer-membrane of the host (Figure 63). The reason for the location of the SPA around the cell membrane may be due to one of two reasons the first being that pPA31 contains the hydrophobic so-called membrane binding region which may result in the full length SPA molecule binding to the membrane. This may also result in extremely long elongated forms of JM83 if this binding is interfering with septate formation within the host. The hydrophobic tailpiece may also be responsible for the beginning of inclusion body formation, so clearly seen in Figure 56. This could not be due to *B*-lactamase alone as inclusion bodies should have then been seen in JM83/pUC8 (Figure 62). The other alternative is that because this is a commercial production strain expressing 10% of the total soluble protein as SPA, then the overproduction may be responsible for the formation of inclusion bodies and the location of the SPA around the membranes of the cell. The final experiment involving JM83 containing pPA34 should have explained these results as the only difference between the two is the hydrophobic C- terminal sequence. However, due to

the loss of the plasmid (pPA34) from the host, the results from these two high expression systems could not be compared.

The results shown in Table 7 help to illustrate what is happening to the host at the time of samplimg. Clearly the largest amount of SPA was produced by the plasmid pPA31 as no SPA was produced by pPA34. Although the levels of SPA were reduced for pPA16 so was the level of *B*-lactamase. The removal of the *B*-lactamase gene may increase the expression of SPA as previously described with carboxypeptidase (Chambers *et al.* 1988). Other workers have also used minimal-antibiotic-resistance markers to increase the production of recombinant proteins (Panayotatos, 1988).

5. CONCLUSIONS

The work undertaken in this thesis describes how the effects of catabolite repression may be overcome. This was shown by growing an *E. coli* host containing a plasmid expressing recombinant SPA in continuous culture using either limited glucose or glycerol. As expression of the SPA gene is under the control of the lac promoter then differences between the two carbon sources could be evaluated. For pPA16, the highest amount of SPA (11 $\mu g\ mg^{-1}$ cell protein) was expressed under glucose limiting conditions at a D of 0.1 hr^{-1}. The plasmid remained stable at the lower dilution rates (0.1 and 0.2 hr^{-1}) while increases (0.3 and 0.4 hr^{-1}) lead to plasmid instability and the loss of SPA production (4 $\mu g\ mg^{-1}$ cell protein). Under glycerol limiting conditions the plasmid was unstable at all the dilution rates investigated, resulting in the total loss of the plasmid and consequently expression of SPA. The highest amount of SPA detected when using glycerol as the carbon source was 8 $\mu g\ mg^{-1}$ cell protein. Restriction analysis of samples taken from the above experiments, gave no indication of structural instability.

The stability of pPA16 was enhanced when excess glycerol and limited ammonium was used indicating that glycerol is not directly causing segregational instability. However, low levels of glycerol and the fact that it is transported into the host via facilitative diffusion may place too much "stress" on the host resulting in the loss of the plasmid. The results from the batch culture experiments in defining a synthetic medium also indicated that there was an increased

burden placed upon the host when glycerol was used as the carbon source. This was clearly shown, as the μ^{MAX} values were reduced when glycerol was used as the carbon source.

In the large scale production of recombinant SPA it may therefore be possible to use glucose as the carbon source, instead of using the current preferred substrate "glycerol". As can be seen from the results catabolite repression can be minimised when limited glucose is used. It must also be remembered that the level of excess glucose (25 mM) used in these experiments may well be thought of as limiting in an industrial environment (Caulcott *et al.* 1985). Due to the peak levels of SPA produced in the transitional phase of growth, fed-batch systems may be preferable if glucose is used as the carbon source as stability can be maintained. Although further work needs to be performed on the high expression host/vector systems (pPA31 and pPA34), it was encouraging to see that SPA production reached 10% of the total soluble protein in a chemically defined medium.

The work undertaken in this thesis also describes an ELISA system for the detection of staphylococcal protein A, which is sensitive (down to 1 ng), easy to perform, reliable and only uses commercially available substances. Although this assay has been superceded by Bloom *et al* (1988), the various antibodies have to be biotinylated "in house" rather than bought commercially. The assay described here is also used to quantitate all the recombinant SPA produced at CAMR using large scale processes, which is then made available for commercial sale. Furthermore the assay has been adapted for use in western blot analysis and the relatively new

immunogold labelling techniques. Western blot analysis has shown that the SPA produced in the continuous culture experiments exists in three bands at 45, 43 and 42 kilo daltons. Multiple forms of SPA were also shown to exist in other commercially available sources of SPA. This may explain the wide range in molecular weights quoted by various workers.

Electron microscopy analysis of the host containing pPA16 revealed the presence of electron lucent areas in the periplasmic space, which may be detremental to the cell. Using immunogold labelling these areas were found to contain SPA. However, similar electron lucent areas were shown in the negative control (JM83 containing pUC8), which was only expressing *B*-lactamase. The areas did not show the presence of SPA and may therefore contain *B*-lactamase (Georgiou, 1986). The host organism containing pPA31 was shown in many instances to be extremely elongated as seen under the light microscope. Numerous electron lucent areas were present not only in the periplasmic space, but a small number were also detected in the cytoplasm of the host. SPA was again localised in the electron lucent areas and also around the whole of the cell attached to the outer-membrane. As pPA31 contains a hydrophobic tail-piece at the C-terminal which is thought to bind the membrane of staphylococcus, then a similar binding could be occurring in *E. coli*. The binding of SPA to the outer-membrane could well be interfering with septate formation resulting in elongated hosts. As this is a high host/vector expression system then the binding of SPA to the membrane and the beginings of

inclusion body formation may well be an artifact of overproduction. Results from the other high host/vector expression system (JM83/pPA34) could have clarified this.

6. References

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