PLASMID VECTOR STABILITY IN *Streptomyces lividans* FERMENTATIONS

by

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ABSTRACT

PLASMID VECTOR STABILITY IN *Streptomyces lividans* FERMENTATIONS

Plasmid instability is frequently cited as a common problem in both laboratory and pilot-scale fermentations of recombinant micro-organisms. *Streptomyces* are increasingly being explored as hosts for the production of useful cloned gene products since they are already employed industrially for antibiotic production. A method was developed to facilitate the study of plasmid copy number and structural stability in *Streptomyces lividans* cultures. The protocol involves total deoxyribonucleic acid (DNA) extraction followed by gel electrophoresis and scanning densitometry.

The protocol has been successfully applied to follow transient changes in plasmid copy number of the multi-copy *Streptomyces* vectors pIJ303 and pIJ702. The stability of the pIJ702-derived recombinant plasmids pMT605 and pMT608 was also studied to a lesser extent. Experiments were conducted using shake-flask cultures and 5L working volume stirred vessels.

Plasmid vector stability was tested by varying medium composition, growth temperature and levels of dissloved oxygen tension. The effects, upon plasmid copy number of the drug novobiocin and simulated inoculum development protocols were also examined. Also the effect of different enzyme induction strategies upon tyrosinase expression encoded by pIJ702 was investigated.

In general, comparable plasmid copy numbers were obtained from both shaken cultures and 5L fermentations. A dramatic increase in plasmid copy number was measured during initial rapid growth followed by a decline during stationary phase. Maximum plasmid copy number was always obtained in early stationary phase. Under low dissolved oxygen tension or high temperature conditions, recombinant cultures retained plasmid molecules. The yield of DNA under such conditions was low, but plasmid copy number remained stable.

The relative quantities of plasmid forms (topoisomers) was found to change at various stages during growth. Supercoiled forms were often seen to increase as the culture ages. No structural instability was detected. These host-vector systems were generally stable but copious acid production by the host was found to be undesirable.

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To my husband, Steve and my parents

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1 INTRODUCTION

The theme of this project is plasmid stability in recombinant *Streptomyces* cultures since there is much scope for the generation of useful products *via* gene cloning in this genus.

There has been considerable interest in studying plasmid stability in unicellular micro-organisms since many early multi-copy vectors showed various degrees of instability and some were rapidly lost from host cells (see later Sections). For this reason a fair amount of quantitative and qualitative data on plasmid loss (and structural stability) has been gathered. However, it should be emphasized that there is little evidence for a systematic approach to the investigation of this problem. Many workers studied only a single host-vector system (or a limited number of closely-related vectors). Despite this lack of continuity some common properties were noted.

As a background to this project I have therefore reviewed some of the more pertinent studies of plasmid stability (with emphasis on plasmid copy number evaluation) in unicellular (and a few filamentous) micro-organisms. I have also attempted to draw some conclusions from the diverse collection of literature available, but have discovered many contradictions and can therefore make few generalisations. The relevance of plasmid stability studies in unicellular organisms to those in *Streptomyces* hosts remains to be discovered since virtually nothing is known about plasmid stability (and copy number) in the latter group. A comparison of findings from this project with published reports will be made in the Discussion Section.

1.1 Plasmid Replication and Mechanisms of Inheritance.

This sub-section is an introduction and summary of the current information available on the stability and inheritance of bacterial plasmids.

Plasmids are autonomously replicating molecules of deoxyribonucleic acid (DNA) which nearly always carry genes which are non-essential to their host. They allow temporarily useful functions to be expressed which permit a bacterium to occupy a particular ecological niche (Sherratt, 1982). Plasmids are also recombinogenic and can promote genetic diversity. Because of these attributes plasmids were thought to exert a

biosynthetic burden on their hosts and a non plasmid-bearing cell may have a growth advantage over its plasmid-bearing neighbour (Sherratt, 1982). Since a plasmid disadvantages an individual cell, but is needed within the population as a whole, there are encoded determinants which ensure efficient plasmid maintenance from generation to generation.

The mechanisms of plasmid partitioning into daughter cells at cell division have been reviewed by Austin (1988) and Nordstrom and Austin (1989). It has been found that natural bacterial plasmids are inherited with a high degree of stability with a loss rate as low as 10^{-7} per cell division. All plasmids have a region called the minimal replicon which consists of an origin of replication and usually a negative-feedback control system which limits the propagation of the plasmid to a characteristic copy number (defined as the number of plasmid molecules per host chromosome or cell). Since all copies of a plasmid are homologous with each other then they may recombine to form multimers. The existence of plasmid multimers makes partitioning very difficult, therefore natural plasmids probably have an active site-specific recombination system.

Some plasmids *e.g.* the F plasmid, possess a function which kills new segregants which do not bear a plasmid copy (Jaffe *et al*, 1985). The plasmids produce a killer substance and an agent that blocks its activity. When the plasmid is lost the blocking agent decays but the killer substance persists in the cytoplasm and kills the cell.

Another effective system is an active partitioning function which selectively moves DNA molecules to ensure that each daughter cell receives a copy. It is likely that all low copy number plasmids have a function which may be analogous to the segregation of chromosomes in mitosis. One example of such a system is the *par* function of plasmid pSC101 (Meacock & Cohen, 1980) which has a moderate copy number of 15. The enzyme which promotes DNA supercoiling, DNA gyrase, was found to bind specifically to this locus (Wahle & Kornberg, 1988).

The only mechanism which ensures adequate partitioning of the multi-copy ColE1 plasmid is the *cer* recombination site locus (Summers & Sherratt, 1984). This locus is thought to resolve multimeric forms. The widely used vector pBR322 (a ColE1 derivative) does not possess the *cer* locus, therefore is prone to the accumulation of multimeric forms and is less stably inherited than the parent plasmid. A considerable increase in

stability was obtained when the *cer* locus was cloned into previously unstable plasmids (Summers and Sherratt, 1984).

Plasmids may be regarded as selfish DNA (Nordstrom & Austin, 1989) and it is crucial that the plasmid:

a) does not affect its host seriously;

b) is stably maintained;

c) competes favourably with cells that do not carry the plasmid; and

d) is able to spread to plasmid-free cells.

In general small plasmids have few maintenance functions and rely on high copy numbers to achieve stability. Large plasmids have multiple functions that, in concert, can achieve great stability at low copy number. Also the plasmid content typically amounts to 2-5% of the total DNA irrespective of the size of the plasmid.

1.2 Studies of plasmid stability in Gram negative bacteria.

The following sub-section will deal with some specific studies of plasmid stability in G-ve bacterial cultures and the effect of hosting the plasmid on the culture's characteristics.

Segregational instability is defined as the failure of plasmid molecules to partition correctly at cell division. The other source of plasmid instability is structural instability which is due to structural re-arrangement of the molecules. If either or both of these events occur the productivity of a recombinant culture is impaired resulting in higher costs. The plasmid stability of various low and high copy number vectors in batch and chemostat culture has been reviewed by Primrose *et al* (1983) and Zabriskie and Arcuri (1986). An overview of the substantial amount of data currently available on the stability of various vectors used in bacterial fermentations is given.

1.2.1 Batch fermentation studies.

1.2.1.1. Stability and copy number of plasmid vectors in relation to growth rate.

One of the earliest studies of plasmid stability in recombinant *Escherichia coli* was conducted by Enberg and Nordstrom (1975). These workers cultivated a R1 plasmid *E. coli* K12 strain in shake-flask culture using a variety of carbon sources to achieve different specific growth rates. It was found that the initiation mass of R1 plasmid replication was proportional to the growth rate and that plasmid replication was under a negative control mechanism.

The nutritional requirements of a RP1-bearing *E. coli* culture was investigated by Klemperer *et al* (1979). The maximum growth rate of P^+ (plasmid bearing) and P^- (non-plasmid bearing) cells were found to be similar in a chemically-defined medium. However, P^+ cells showed a greater nutrient requirement for Mg^{2+}, Fe^{2+}, K^+ and PO_4^{3-} ions.

Stueber and Bujard (1982) studied the effect of efficient promoters on plasmid replication (and hence stability) in a ColE1-derived vector in *E. coli*. They found that the copy number (the ratio of chromosome to plasmid DNA) of pBR322 increased 4-fold from early exponential phase to stationary phase. However, inactivation of the *rop* gene (repressor of primer protein) further raised the copy number of pBR322 by a factor of 2.

Frey and Timmis (1985) also showed that ColD-CA23 vectors

autoamplified (increased in plasmid copy number) in stationary phase. A major consequence of the increase in copy number is a substantial increase in cloned gene expression. ColD-type vectors were remarkably stable in *E. coli* and were found to be compatible with ColE1 type vectors. The copy number during exponential growth in minimal media was only 15-20 copies per chromosome while in stationary phase it increased to 150-200 copies per chromosome.

Seo and Bailey (1985b) also described work in which the effect of recombinant culture growth rate upon plasmid copy number was investigated. They employed the vector RSF1050 and four other closely related copy number mutant plasmids. Growth was investigated in complex medium and minimal medium containing either casamino acids or various concentrations of α-methylglucoside which is a competitive inhibitor of glucose transport. The maximum specific growth rate (μ_{max}) was found to decrease with increased plasmid content in both types of media. However, plasmid copy number increased when μ was reduced by altering the medium composition. It was also found that the ratio of the cloned β-lactamase specific activity to plasmid content decreased significantly with high plasmid content in complex media. Also, even at lower plasmid copy numbers, a non-linear relationship with enzyme activity was found. All the vectors in this study were highly stable with no detectable loss of plasmids at cell division. Interestingly, the authors also noticed that a maximum copy number was obtained at a relatively low specific growth rate but that copy number decreased at even slower growth rates.

Despite early assumptions that plasmids enforced a metabolic burden upon their host cell (Sherratt, 1982), it has been found that the copy number of the plasmid alone had little effect on the host's metabolic stoichiometry (Da Silva and Bailey, 1986) but Mason and Bailey (1989) found that plasmid presence could cause a slowdown of intracellular reactions. Other workers have also found that plasmid replication alone does not bring about a reduction in growth rate (Betenbaugh *et al*, 1989 and Bentley *et al*, 1990).

The low copy number (4-7 copies), broad host-range plasmid pTJS26 was studied by Bernardez and Dhurjati (1987). The plasmid was transformed into *E. coli* and *Pseudomonas putida* and was cultured at 30°C and 37°C. At 30°C (sub-optimal temperature) the growth kinetics were not affected by the

presence of the plasmid but instability was observed. Growth rates and yields at 37°C were lower than those of the host bacterium but no instability occurred. pTLS26 was found to be segregationally unstable in both hosts but its stability could be improved with temperature due to an increase in plasmid copy number. The effect described above is not universal since work done by Lancaster *et al* (1989) suggests otherwise. They studied the optimum fermentation conditions for a cloned carboxypeptidase gene in *E. coli*. Several different strains were tested at different growth temperatures. Maximum production was obtained using recombinant RV308 strain grown at 28°C. In this case plasmid copy number was found to decrease during exponential growth and it was not observed to correlate with enzyme production levels.

Betenbaugh *et al* (1989) also studied the effects of plasmid amplification and recombinant gene expression (separately and together) on recombinant *E. coli* growth kinetics. Gene expression of the low copy number (6-10) plasmid pVH106/172 could be obtained by adding the inducers IPTG (isopropyl-β-D-thiogalactoside) and cAMP (cyclic adenosine monophosphate) whereas plasmid amplification could be controlled by temperature. The growth rates of the recombinant organisms were found to be considerably reduced by the expression of high levels of product and also by plasmid amplification.

1.2.1.2 Stability of pBR322-derived plasmid vectors.

This class of vector is very popular since pBR322 was the first general purpose plasmid vector to be developed (Bolivar *et al*, 1977). Consequently, there is a fair amount of data available regarding the stability of this plasmid in batch culture.

Lin-Chao and Bremer (1986) investigated the copy number of pBR322 and the concentration of its replication inhibitor RNAI and the RNAII pre-primer under various culture growth rates. These workers again found that the number of plasmids decreased with increasing growth rates. The maximum plasmid copy number (*i.e.* plasmids per chromosome) was 23 at $0.6h^{-1}$ growth rate. A subsequent paper from the same laboratory (Chiang and Bremer, 1988) described the deletion of the *rom* (=*rop*) gene which resulted in a noticable increase in plasmid copy number. However, rapid pBR322 plasmid loss was seen in cultures without antibiotic selection. Another plasmid pCL43, which was an unstable derivative, was found to be highly stable after large

deletions resulted from consecutive overnight cultures. Cells containing the *tet* gene product died sooner in stationary phase than those which lacked this gene. This plasmid-encoded protein is thought to be located in the inner membrane and acts as an energy-dependent 'antiport' which prevents the accumulation of tetracycline in the cell by secreting it. The *tet* gene may reduce the availability of energy by altering the cell membrane and this effect could be exacerbated in stationary growth when nutrients are scarce.

Mason and Bailey (1989) reported an investigation into the effect which plasmids (at different copy numbers) had upon the activities of key enzymes involved in carbon metabolism and biosynthesis of macromolecules in the host. The plasmids used were the same as those of Seo and Bailey (1985). Although these plasmids were derived from pBR322 no segregational instability was observed. However, a decrease in μ_{max} using defined media was accompanied by increasing copy numbers in the plasmid series. Unlike their previous work (Seo and Bailey, 1985) it was found that for *E. coli* DH5α the level of cloned gene product was proportional to the plasmid copy number. The authors found that the β-lactamase specific activity to gene ratio (at one point) in late exponential growth was similar for 4 different plasmids. They also found that recombinant *E. coli* exhibited a diversion of carbon metabolism away from the Embden-Meyerhoff pathway (energy-generating) to the pentose phosphate pathway (biosynthetic).

A study of the effect of various fermentation conditions upon recombinant *E. coli* plasmid stability was studied by Reinikainen *et al* (1989). The plasmid selected was the pBR322 derivative, pKTH1220 and the fermentation temperature, pH and turbidity at amplification were investigated. Plasmid amplification was achieved using chloramphenicol. The results showed that recombinant plasmid copy numbers could be influenced by controlling temperature and pH. Also the maximum copy number during growth did not co-incide with optimal conditions of cell growth and division. Final plasmid concentrations after amplification varied from 0.2 to 4.5 mgL^{-1} (it was not possible to convert these values to copy number since no values for chromosomal DNA concentration were given). The best yields of plasmid DNA were obtained at low temperature (34°C) at either low or high pH and when amplification was initiated at high cell density. The growth of the host was optimal at pH 6.5-7.0 and 40-42°C. Another study of the

effects of amplification, but without chloramphenicol, was described by Angelov and Ivanov (1989). These workers found that by adding both the amino acids methionine (Met) and threonine (Thr), a copy number of 90-100 could be obtained for pBR322. An even greater level of plasmid DNA could be obtained by adding Fe^{3+} with certain amino acids. When the same treatment was tried with expression vectors an increase of 5-10 fold of cloned gene product was achieved. The reasons for these observations were not stated.

1.2.2 Continuous fermentation studies.

Although much information may be obtained from batch cultures a better understanding of the physiological effects of the plasmid upon the host can be obtained from chemostat studies.

Noack *et al* (1981) studied the stability of pBR322 and pBR325 in chemostat fermentations under nutrient limitation at two dilution rates. pBR322 was found to be stable under conditions of either glucose or ammonium chloride limitation at high and low dilution rates. However pBR325 was only retained under conditions of low dilution and ammonium limitation. Subsequent studies using *E. coli* K12 *redB redC sbcB* hosting pBR322 revealed that the plasmid was rapidly lost from the culture. These results suggest that plasmid instability is influenced by host genotype, cultivation conditions and plasmid size. Considerable differences may be encountered even between closely related plasmids.

The stability of TOL catabolic plasmid of *Pseudomonas putida* was studied by Keshavarz *et al* (1985). Benzoate is catabolized via the *ortho* cleavage pathway in TOL^- strains. Greater stability was observed when the plasmid encoded genes were not expressed *i.e.* when the culture was grown on succinate as carbon source. Benzoate-limited chemostat experiments however resulted in rapid plasmid loss in one case but this was not observed in parallel experiments. No significant differences in the growth rates of TOL^+ and TOL^- strains were seen, this was therefore unlikely to be the cause of the instability. Despite the rapid plasmid loss a small proportion of cells still retained the plasmid.

Warnes and Stephenson (1986) investigated the effect of vector size upon the stability of recombinant plasmids in chemostat culture of *E. coli* HB101. A single vector was used but carried different sized inserts of cytomegalovirus DNA. None of the vectors yielded foreign protein since it was not expressed. The plasmid with the smallest insert (2kb insert) was

found to be the most stable. The intermediate-sized plasmid (8kb insert) remained stable only under conditions of slow growth. The largest plasmid (21kb insert) was rapidly lost from the culture and a similar decrease in its plasmid copy number was also observed. Some oscillations in the plasmid copy number of the medium-sized vector was seen over a period of 80 generations. There was no evidence of structural instability. The copy number of the smallest plasmid remained virtually constant throughout each experiment. It is interesting to note that the host phenotype changed (reproducibly) from *leu*$^-$ to *leu*$^+$, a change which was accompanied by the recovery of the medium-sized plasmid. The authors concluded that large plasmids place a metabolic burden on the cells which leads to their loss at cell division.

Using *E. coli* HB101 transformed with plasmid pDM246, Seo and Bailey (1986) found no segregational or structural instability. The plasmid copy number was maximal at fairly low dilution rates and decreased at faster growth rates. Batch studies by the same workers also showed this to be true. It was also found that the cell mass yield per mass glucose consumed declined with increasing dilution rate.

Another study of recombinant *E. coli* HB101 by Chew *et al* (1986) employing the plasmid pAT153 (derived from pBR322), revealed that plasmid loss was most acute at low dilution rates. The rate of plasmid loss was thought to be due to the increased growth rate of the plasmid-bearing cells compared to the host cells alone. Under carbon and phosphate limitation faster growth brought about the delay of plasmid loss when $\%P^+$ cells were plotted as a function of number of generations. Plasmid copy number remained constant at 160 with no detectable increase with increased growth rate.

Reinikainen and Virkajarvi (1989) also employed recombinant *E. coli* HB101. The strain was transformed with the vector pBR322 and a 14kb derivative, pKTH1220. The kinetics of plasmid loss of both plasmids and growth kinetics of the host strain were compared in continuous culture. The growth rate of the recombinant vector was slighlty slower than either the vector strain or the host. Plasmid copy number (determined as plasmid DNA molecules per chromosome) and plasmid DNA per dry cell mass increased 2-3 fold with increased dilution rate. The authors employed ampicillin selection in both plasmid-bearing strains which makes the data less useful

than if no selection pressure had been applied.

The same host strain was also used by Peretti *et al* (1989) to host a range of related plasmids with different copy numbers. The level of the cloned gene product, β-lactamase, was found to increase dramatically in those strains which harboured higher plasmid copies. Also for higher copy number plasmids, the level of mRNA was linearly proportional to copy number. Despite getting good yields the authors suggest that increasing the promoter strength would increase polymerase usage more efficiently than increasing copy number (since adding plasmid to the cell also augments the concentration of non-promoter sites available for non-specific polymerase binding). Their results also indicate that it is the stability of cloned gene mRNA and protein which primarily determine the productivity of recombinant organisms.

1.2.3 Fed-batch fermentation studies.

Horn *et al* (1990) recently addressed the problem of plasmid stability in high density cultivation systems. This data is the first relating to plasmid copy number in recombinant *E. coli* cells grown as a fed-batch culture. *E. coli* K12 BMH71/18 was transformed with the vector pUR290 and a recombinant derivative pFB99. Semi-synthetic and synthetic media were used without antibiotic selection. The recombinant plasmid was not induced since cloned gene expression was not desired. Both segregational stability and plasmid copy number were measured. It was found that as phosphate concentration decreased to nearly $0gL^{-1}$ (after 7h) the pUR290 plasmid copy number increased from 50 to 200 copies per chromosome. At such a high copy number it is not surprising that no segregational instability was detected. Plasmid pFB99 also increased in copy number from 50 to about 400 copies per chromosome. The specific growth rate and the growth yield were both slightly lower for the recombinant culture compared to the vector-hosting culture. The specific growth rate of the plasmidless host cells was found to be even lower.

In summary therefore there exists a large amount of information available on plasmid stability in *E. coli* and a few other Gram -ve species. On the other hand it is very difficult to make any general conclusions since there are vast differences in behaviour between the various host-

vector systems which have been studied. The method of cultivation is also important since the same plasmid may well behave differently under varied growth conditions. However, it can be stated that many plasmids showed increased copy number during stationary phase and under conditions of slow growth in chemostat culture. Also, at levels of high plasmid copy cloned gene product was rarely proportional to the level of plasmid present.

1.3 Plasmid stability in *Bacillus* species.

Bacillus species have also been investigated as potential hosts for recombinant gene expression in addition to *E. coli*. Unfortunately plasmid instability problems have also been encountered using this genus. The characteristics of *Bacillus* plasmids and their instability are discussed below.

1.3.1 *Bacillus* cloning vectors.

Although *Bacillus* species harbour indigenous plasmids which are cryptic they do not bear any easily selectable markers (Saunders and Saunders, 1987). However, small multicopy resistance plasmids from *Staphylococcus aureus* can replicate in *B. subtilis*. Old and Primrose (1989) have summarized some of the *S. aureus* plasmids which are used as cloning vectors in *B. subtilis*, they include pC194 and pUB110. Unfortunately both segregational and structural instability have been major problems in the application of gene cloning techniques in *Bacillus* species. Some examples of plasmid instability are discussed in the following sub-sections.

1.3.2 Structural stability of *Bacillus* plasmids.

Hahn and Dubnau (1985) have investigated the phenomenon of structural stability in bacilli. They discovered that two re-arranged plasmids were reproducibly formed in recombinant *B. subtilis* transformed with a co-integrate of pUB110 and pC194. The new molecules were the results of an illegitimate recombination event which took place reciprocally between short regions of homology. The regions of homology appeared as direct repeats and were bounded by sequences showing dyad symmetry. All the evidence suggested that the cross-over could only have occurred within the molecule. It was also found that the same recombination event also occurred in six *rec*$^-$ (recombination deficient) mutants. The authors proposed that since palindromic sequences may potentially form cruciform structures then recombination enzymes could recognise these and cleave at such regions.

1.3.3 Segregational stability and copy number of *Bacillus* plasmids.

Bron and Luxen (1985) have studied the stability of a pUB110-derived plasmid pL13 (3.6kb) and a shuttle vector pLB5 in *Bacillus*. DNA inserts of both homologous and heterologous origins were cloned into both vectors to create a series of closely-related plasmids differing only in size. It was found that the larger plasmids were very unstable in *B. subtilis* regardless of the origin of the DNA inserts. Interestingly, no

instability was detected in any of the plasmids when hosted in *E. coli*. It was found that the increased size of the pLB plasmids resulted in strongly reduced copy numbers. However, a main criticism of this observation is the fact that the authors took the plasmid's size into consideration when calculating the copy number. Consequently, similar levels of plasmid DNA from each vector would automatically lead to very different copy number values merely because of the way it is calculated. The authors also discovered that, unlike *B. subtilis*, good plasmid copy numbers (40) were obtained when all the vectors were hosted in *E. coli*. It should be noted that these measurements were made by a different technique in *E. coli*. This study is one of the very few where the relative amounts of the different extracted plasmid forms are mentioned. It was found that only monomeric cccDNA and open circle DNA molecules were extracted from *B. subtilis* while only dimeric or oligomeric forms could be extracted from *E. coli*. The presence of multimers of these plasmids evidently did not lead to inefficient segregation in *E. coli*. Also, since the predominant plasmid forms differed in the two host strains then this may explain the large discrepancy in their relative copy numbers.

B. subtilis is not the only species used since *B. stearothermophilus* CU21, transformed with plasmid pLP11 carrying a penicillinase gene, was studied by Aiba and Koizumi (1984) in continuous culture. It was found that productivity at 50°C increased remarkably with increased dilution rate and plasmid stability was also enhanced. These observations suggested that copy number in chemostat culture may depend on operational parameters *e.g.* dilution rate and temperature.

A subsequent paper by the same research group (Koizumi *et al*, 1985) also supported the conclusion that the copy number of pLP11 increased with higher dilution rates. Plasmid content, plasmid copy number and cloned gene expression were measured at high and low dilution rates at growth temperatures of 44°C, 47.5°C and 50°C. It was found that high plasmid copy number did not correspond with optimum cloned gene expression. Different growth conditions were needed to (independently) optimise these parameters. It is unfortunate that these workers did not measure plasmid copy number for all their data but rather preferred to measure plasmid content (as mg plasmid per g biomass). Unfortunately, the data presented in the paper shows that these measurements of plasmid levels are not analogous to

plasmid copy number (perhaps because the mass of an individual cell changes under different growth conditions).

Watson *et al* (1986) also used continuous culture to investigate plasmid stability in *Bacillus subtilis*. These workers had previously observed that plasmid loss during batch fermentations was negligible in exponential growth but increased dramatically during stationary phase. When plasmid loss was plotted as a function of the number of cell doublings, maximum loss appeared to occur at low dilution rates. However, plasmid loss plotted as a function of time revealed no differences in loss between different dilution rates.

McLoughlin *et al* (1987) studied the stability of pPGV2 and pPGV138 in *B. subtilis*. They employed three fermentation systems; batch, exponential rate fed-batch and continuous culture. The effects of specific nutrient limitation and temperature were also investigated. Plasmid stability was different depending on growth rate or physiological state. Instability was most severe at elevated temperatures, perhaps due to higher maintenance energy requirements but improved at normal temperatures.

Kadam *et al* (1987) studied an amylase producing strain of *B. subtilis* using batch and continuous culture. In batch (shake-flask) fermentations, segregational instability was observed since the chloramphenicol resistance marker was lost. No plasmid DNA could be isolated from cells in stationary phase and those which were chloramphenicol sensitive did not produce amylase . The authors thought the instability arose from the host rather than the inherent characteristics of the plasmid. Chemostat studies of the same host-vector system revealed that the plasmid instability witnessed was not an artefact of batch cultivation. Plasmid DNA analysed from a chemostat experiment revealed a molecule of smaller size which was maintained more stably.

In a recent paper Shoham and Demain (1990) investigated the effect of medium composition on the stability of plasmid pUB110 and a recombinant derivative pCED3 in *B. subtilis* BR151. They were particularly concerned about the problem of structural re-arrangements in recombinant *Bacillus* plasmids and proposed to apply selective pressure in order to alleviate these problems. Kanamycin was included in the medium so that no segregational instability occurred and the number of colonies showing another plasmid-borne phenotype ($LacZ^+$) was calculated. It was found that

pCED3 was both segregationally and structurally unstable despite the application of a selective pressure. The medium composition was found to be important since the plasmid was more stable at lower growth rates in defined medium than in a complex medium which supported faster growth. A major criticism of this type of approach is that observing any plasmid-borne phenotype in order to follow structural instability is misleading. Ideally, the plasmid should be purified and examined by restriction analysis.

Segregational and structural plasmid instability is therefore also a major problem in unicellular Gram positive micro-organisms and may hinder the scale-up of recombinant *Bacillus* cultures due to poor productivity.

1.4 Plasmid stability in eukaryotic micro-organisms.

Yeast can be used as an alternative host to prokaryotic micro-organisms, particularly where post-translational modifications of the cloned gene product is required. However, similar problems regarding vector stability have also been encountered using eukaryotic microbial hosts.

1.4.1 Yeast cloning vectors.

Old and Primrose (1989) have divided yeast cloning vectors into six main groups. They are YIp (yeast chromosome integrated plasmids), YEp (episomal plasmids), YRp (replicating integrated plasmids), YCp (centromere plasmids), YAC (yeast artificial chromosomes) and Ty (retrovirus-like vectors). The properties of the non-integrated plasmids are discussed briefly in the following section since they exhibit most instability.

Most yeast episomal plasmids are based on the naturally-occurring 2μm circle plasmid. The properties of the 2μm plasmid have been reviewed by Murray (1987). It is a cryptic plasmid of 6.3kb in size and exists at copies of 50-100 per haploid cell. Recombinant plasmids based on YEp vectors tend to exhibit structural instability. This may result from recombination between either vectors and resident 2μm sequences or the inverted repeat sequences present in the 2μm plasmid molecule (Hinnen and Meyhack, 1982). 2μm circle-based plasmids are also easily lost through segregational instability since the most stable hybrid plasmids are lost at a frequency of 1-5% per cell division (Hinnen and Meyhack, 1982). However the authors also report that segregational instability can be substantially reduced by using 2μm-free mutants as host cells.

Another class of yeast multi-copy vectors is the yeast replicating plasmid (YRp) group. Such vectors carry autonomously replicating sequences (ARS) usually derived from yeast *trp* or *arg* regions (Saunders and Saunders, 1987). YRp vectors are highly unstable with very few copies segregated into daughter cells. Murray and Szostak (1983) discovered that due to the budding method of yeast cell division, the recombinant plasmids tended to remain in the mother cell despite high plasmid copy number. Yeast centromere plasmids (YCp) carry regions from close to the chromosome centromere but exist as cccDNA molecules. However, YCp vectors showed greater stability than YRp vectors probably due to the presence of a centromere which eliminates bias at cell budding.

1.4.2 Yeast plasmid stability.

Several studies of plasmid stability in recombinant yeast fermentations have been conducted. In general, it is the stability of YEp (2μm-based) vectors which have been examined. This section briefly reviews the data available on segregational plasmid stability in recombinant yeast cultures.

Futcher and Cox (1984) reported a study of the stability of 2μm circle-based plasmid vectors in *Saccharomyces cerevisiae*. Previous work had revealed considerable instability in such vectors and the authors decided to investigate by measuring plasmid copy number and loss. A novel method of copy number assessment was developed; densitometric peaks of *Eco*R1 digested ribosomal DNA fragments, of an assumed concentration, were compared with plasmid DNA fragments. The results showed that P^+ cells and P^- cells grew at virtually the same rate in non-selective medium. Also it was found that high instability correlated with low copy number and that the efficiency of partitioning was less than that of random segregation. Despite common ancestry, copy numbers varied widely between plasmids and there was also evidence that the distribution of copies was variable. In general the partitioning of 2μm-based plasmids was more efficient than those derived from ARS (autonomously replicating sequence) of yeast.

Kleinman *et al* (1986) also studied the stability of 2μ-based plasmids in yeast but used chemostat culture. Earlier results (by Caulcott, 1984) had shown that plasmid replication rate, segregation, copy number, growth rate and the inherent effect of plasmid on the host were all important factors in determining stability. Kleinman and co-workers investigated the importance of growth rate on plasmid stability. Their plasmid was found to be more stable at higher growth rates, (a similar observation was made by Parker and DiBiasio (1987) using a 2μ plasmid grown in selective, continuous culture). The same laboratory later reported studies of the 2μ plasmid pJDB248 in chemostat culture (Bugeja *et al*, 1989). It was found that at "fast" growth rates the plasmid level (copy number) remained constant but at "slow" growth rates a considerable increase in plasmid level was observed. This increase coincided with a rapid decrease in the number of plasmid-containing cells in the population. However, if the copy number was averaged over plasmid-containing and non-containing cells then levels remained virtually constant throughout both culture conditions. The authors concluded that this suggested a breakdown

of plasmid segregation leading to an accumulation of plasmid molecules in a minority of the population under conditions of slow growth.

Attempts to stabilise yeast plasmids in non-selective continuous culture by using double mutants have been tried (Marquet *et al*, 1986 and Marquet *et al*, 1987). Such approaches were successful since no plasmid loss was observed. Another tactic has been to use diploid yeast (Mead *et al*, 1986) in carbon limited chemostat culture. The improved stability was thought to be the result of increased plasmid copy number and the reduced selective burden of the plasmid.

Other yeasts have also been studied *e.g. Hansenula polymorpha* (Tikhomirova *et al*, 1986). However, the hosted plasmid was very unstable and disappeared after a few generations when grown in non-selective conditions. Growth in selective media yielded polymeric (multimeric) forms of the plasmid which were more stable. This observation contradicts those made in recombinant bacteria where multimer formation is thought to reduce plasmid stability.

Caunt *et al* (1989) studied plasmid stability in recombinant *S. cerevisiae* in continuous culture under conditions of environmental stress. These authors were interested in the effect of step changes in dissolved oxygen tension (DOT) on plasmids at pseudo-steady state at different dilution rates. Since their plasmid contained the *E. coli lacZ* gene then it was possible to follow β-galactosidase expression as a measurement of the recombinant cell fraction. No instability was detected under normal growth but when the DOT was reduced to below 10% of saturation, the plasmid-containing fraction fell sharply in an exponential decay pattern. The authors suggested the drop in DOT had caused either a fall in the growth rate of P^+ cells or an increase in the formation of P^- cells. Since there was little change in the total cell number after DOT change the major reason for the increased loss of P^+ cells was due to segregational loss. Overall the data which these authors presented confirms that oxygen limitation has a detrimental effect on plasmid stability in yeast . It was also emphasized that during process scale-up careful reactor design is needed to reduce the undesired effect of culture exposure to oxygen limitation.

There are also reports of advances in the use of recombinant filamentous fungi (Barnes and MacDonald, 1986). The fungus *Aspergillus*

niger was transformed with a pBR322-derived plasmid which also incorporated an ARS from yeast. However the recombinant plasmid exhibited severe structural instability in *A. niger*.

In summary therefore, problems of plasmid loss are also evident in recombinant eukaryotic micro-organisms and vectors rarely showed the same degree of stability as their parental wild-type plasmids.

1.5 Large-scale recombinant fermentations.

1.5.1 General considerations.

The development and application of recombinant cultures on a large scale has been reviewed by Bok (1983), Bull (1983), Atkinson *et al* (1984) and Zabriskie and Arcuri (1986). The major factors involved in obtaining high levels of cloned gene expression was summarized by Atkinson *et al* (1984) as the following points:

1. Nature of the bacterial host;
2. Stability of the recombinant plasmid;
3. Construction of the recombinant system;
4. Codon utilisation within the mRNA;
5. Fidelity of the translation and post-translational modification; and
6. Applicability of the whole system to scale-up.

Bull (1983) also considered the problems of process scale-up. He points out that often cultures are grown as rapidly as possible by optimizing the fermenter conditions to this end. However, recombinant plasmids may well be less stable under such conditions and will eventually get diluted out by plasmidless host cells. The use of auxotrophs was suggested as a means of overcoming this problem but nearly all industrial fermentations employ complex media. The beneficial properties of amino acid auxotrophs can be exploited but their growth requires the supply of a defined medium which generally results in slower growth rates, higher costs and lower cell densities.

Zabriskie and Arcuri (1986) stated that with the recent (unreported) advancement of many recombinant products to pilot plant development a greater number of publications would ensue. However there is still a considerable lack of information available on the physiology and production of large-scale recombinant cultures perhaps due to company secrecy. With respect to the use of *Bacillus* and *Streptomyces* species as hosts the authors considered them good candidates since they can secrete much protein. A main disadvantage is that they may also produce proteases which could degrade recombinant proteins. Both genera are routinely grown in large-scale industrial fermenters and a great deal is known of their fermentation characteristics. The choice of plasmid copy number is also important since high copy number vectors are sometimes more stable than low copy number systems (unless a *par* function is present). The difference in

growth rate between plasmid-bearing and plasmid-less cells should also be minimised, perhaps by avoiding the use of the *tet* resistance gene as a selectable marker. It can therefore be assumed that recombinant micro-organisms are widely used but few reports appear in the literature relating to large-scale culture.

1.5.2 Recombinant microbial products of commercial importance.

Recombinant proteins of pharmaceutical value include somatostatin, insulin, human growth hormone, foot and mouth disease vaccine and human interferon (Bok, 1983). Itakura *et al* (1977) were the first to achieve expression of the somatostatin gene which had been chemically synthesized and fused with a β-galactosidase gene on the vector pBR322. A large number of plasmid molecules per cell were obtained resulting in the production of a substantial level of β-galactosidase-somatostatin hybrid protein (at least 3% of the total cellular protein). Recombinant cultures were initially grown in 10L working volume fermenters. Despite the good level of product formed, exogenously added somatostatin was rapidly degraded. Considerable segregational instability was observed since after 15 generations only 50% of the cell population contained plasmids. There was also much variation in the product level obtained but even the best fermentations yielded 10-fold less than the maximum predicted concentration.

Human insulin has also been produced commercially using recombinant bacteria. Early shake-flask fermentations produced β-galactosidase-insulin hybrid protein which accounted for 20% of the total cellular protein (Johnson, 1983). By 1983 the company was routinely using 10,000 gallon fermenters for recombinant cultures. Some production strains of recombinant *E. coli* produced up to 20% cellular volume occupied by insulin inclusion bodies (Williams *et al*, 1982). Such structures consisted entirely of insoluble chimeric protein which could be viewed by electron microscopy.

Goeddel *et al* (1979) reported the production of human growth hormone (hGH) using recombinant cells. Maximum protein levels ($2.4 \mu g mL^{-1}$) were obtained from bacteria in mid-exponential growth. Yields from cells in stationary phase were much less possibly due to proteolytic activity (or plasmid instability). In the case of Martial *et al* (1979) the hGH fusion protein accounted for 3% of the cellular protein (only 17% of the expected

level).

Kleid *et al* (1981) cloned the foot and mouth picnovirus capsid protein into plasmid pFM1. Remarkably, an 800 mL shake-flask fermentation yielded sufficient chimeric protein (17% of total) to permit preliminary animal trials. Human leucocyte interferon cDNA has also been cloned into pBR322 (by Yelverton *et al*, 1981) and the recombinant *E. coli* culture produced 2.5×10^{8} units of interferon per litre of culture in shake-flasks.

Bok (1983) concludes his review by stating that it is important to optimize environmental factors for the over-production of desired products *e.g.* medium design, pH control, temperature control, oxygen transfer, growth rate, growth phase and culture stability. However, as we have seen from work reported earlier, the conditions for optimum growth are not necessarily those which support good cloned gene expression.

Yeast is also widely used as a host for recombinant plasmids and has certain advantages over a prokaryotic host *e.g.* glycosylation of proteins. Hintzeman *et al* (1981) described the expression of human interferon by recombinant yeast cells. However, the production level was rather low (only 1-2% of total protein) probably due to segregational instability. These authors suggested chromosomal integration of the cloned gene as a solution to segregational instability.

One of the few published works on large-scale recombinant cultures was that performed by the DuPont de Nemours Company in the US (as reported by Pierce and Gutteridge, 1985). The cloned gene was a plant carboxylase enzyme carried on plasmid pBR322 hosted in *E. coli*. It was found that under production fermentation conditions, recombinant cells were rapidly outgrown by plasmid-free cells, resulting in a very poor yield of product. A 1.7-fold difference in growth rate was measured. The workers then applied a selection pressure by adding ampicillin to the medium, but this was rapidly inactivated by the overproduced β-lactamase from the recombinant plasmid thereby removing the selection pressure. The problem was partly overcome by inoculating the 300L fermenter with only 0.3μL of freshly-grown recombinant cells. After 20h growth in rich medium with antibiotic selection a yield of 74g of RuBisCo protein was obtained.

In summary therefore, most early recombinant bacterial fermentations have been conducted using pBR322-derived vectors. The plasmids were also found to be segregationally unstable and were frequently

lost after prolonged growth. Some proteins were optimally produced during exponential growth *e.g.* human growth hormone (Goeddel *et al*, 1979). This observation is probably related to plasmid instability which was, unfortunately, rarely measured. In order to compensate for segregational plasmid loss, antibiotic selection pressure was often applied, but overproduction of resistance enzyme sometimes resulted in its removal. Heterologous proteins were also occasionally degraded by the host's protease enzymes. Some of the strategies developed to stabilize plasmids are discussed in Section 1.8.

1.6 Plasmid stability under environmental stress.

The maintenance of adequate levels of dissolved oxygen (DO) available to a microbial culture in a large fermenter or bioreactor can sometimes be a problem through inefficient agitation. Transient pockets of culture broth with DO tensions below the critical level could result in increased plasmid instability by imposing environmental stress upon the host organism. A few workers have investigated the effect of reduced DO concentrations on plasmid stability in *E. coli* and yeast.

Hopkins *et al* (1987) used the temperature-sensitive *E. coli* plasmid pKN401 to study the effect of induced DO limitation upon the stability of the recombinant population. Although a temperature-sensitive plasmid was employed no plasmid amplification was desired therefore all experiments were conducted at 32°C. During a batch fermentation experiment under normal conditions (with ampicillin selection) the plasmid was found to be segregationally very stable since no P^- segregants were observed. Another batch experiment without selection pressure, revealed that only 80% of cells contained plasmid after 6h growth. The DO limitation experiments were conducted (with ampicillin selection) by switching off the sterile air supply at the very early exponential growth stage. The DO tension gradually fell to 5% of saturation, after which the air supply was resumed. As a result of oxygen starvation the number of plasmidless segregants increased by two to three orders of magnitude greater than the number of recombinant cells. In a further experiment the DO shock was delayed until early exponential phase and this time the DOT dropped more rapidly and plasmid segregational instability was found to be even more pronounced. In both DO shock experiments the final percentage of P^+ cells was <1%, but this level was achieved more rapidly in the second experiment. The rate of plasmid loss after environmental shock may be related to the culture's metabolic status at the point it was stressed. Stress imposed during high metabolic activity seemed to lead to a higher plasmid loss rate.

The same plasmid was also investigated by Tolentino and San (1988) but they used *E. coli* C600 as host. These workers applied the DO shock by alternating between the sterile air supply and a pure nitrogen source in order to achieve anaerobic conditions. Enough time was allowed between switching to enable at least one generation of growth to occur. In contrast to the previous report of Tolentino and San, these workers found

no plasmid instability under any of the anaerobic or aerobic conditions employed, including the switching-between-environment experiments. The authors concluded that in their host-vector system the plasmid was stably maintained under both aerobic and anaerobic conditions but that cloned gene expression was affected by the availability of oxygen.

In comparing the work of Hopkins *et al* (1987) and Tolentino and San (1988) it must be concluded that the host's physiology may well have a considerable impact on the stability of the same plasmid even when the hosts are different strains of the same species. It is therefore virtually impossible to draw conclusions on the effect of DO limitation when different host-vector systems are employed. However, under certain circumstances plasmid segregation in *E. coli* is affected by lack of oxygen.

In addition the effect of oxygen tension upon *Saccharomyces cerevisiae* recombinant plasmid stability has also been investigated (Lee and Hassan, 1987). Montrachet 522 wine yeast was transformed with the chimeric plasmid pADH10A which encodes killer toxin. The expression and stability of the plasmid was investigated in glucose-limited chemostat culture. The vessel was supplied with either air, nitrogen or pure oxygen. It was found that growth under anaerobic conditions yielded the best killer toxin concentrations, but highest plasmid stability (100% P^+ cells) was observed with air sparging. Plasmid stability also increased with increasing dilution rate when air was supplied. It is therefore evident that changes in oxygen tension can affect not only metabolism but also the expression and stability of recombinant plasmids in yeast cultures. Another study of the effect of DO limitation on yeast plasmids was conducted by Caunt *et al* (1989) and was discussed in Section 1.4.

1.7 Mathematical modelling of plasmid stability in micro-organisms.

1.7.1 General models.

Due to the problems of plasmid instability (mentioned earlier) there has been much interest in the development of mathematical models to predict the fate of unstable plasmids during recombinant fermentations. An early model of unstable recombinant fermentations was proposed by Ollis (1982). It is stated that growth models are generally classified as either structured, where various molecular mechanisms are included, or unstructured where modelling details are limited to the cellular level. Models may also be classified as segregated (single cell), where all cells are assumed to be homogenous or unsegregated (population) models where cell-to-cell variations are taken into account. The ultimate aim of developing models is their subsequent application in order to optimize, design and control recombinant fermentation processes. At the moment models are being used to formulate hypotheses concerning the dominant rate-limiting mechanisms and are tested by comparison with simulated experimental results (Zabriskie and Arcuri, 1986).

Seo and Bailey (1985a) have studied instability in binary fission organisms using a segregated, population balance model. Such models are said to have the advantage of direct incorporation of basic data on the mechanisms and kinetics of plasmid replication and segregation at the cellular level. It was stated that it is necessary to assess the distribution of cell states, particularly cellular plasmid content and product synthesis activities in individual cells. It was found that plasmid replication kinetics during cell growth significantly influenced the plasmid content distribution.

The same workers (Lee *et al*, 1985) have also developed a kinetic model for product formation by unstable recombinant micro-organisms in both batch and continuous bioreactors. In their model the product formation kinetics were based on quasi steady-state, transcription-translation expression models. Simulation results based on these models indicated:

1) Overall plasmid stability depended on product expression and reactor operating conditions as well as on intrinsic segregation and mutation rate parameters;

2) There existed an optimum combination of plasmid copy number and cloned gene transcription and translation efficiencies to maximize reactor

productivity; and

3) Continuous reactor dilution rate influenced the fraction of productive cells.

This model was subsequently applied by the same group in a later paper (Seressiotis and Bailey, 1987). Results revealed that the more deleterious the influence of full gene expression on cell growth, then it is necessary to incorporate an origin of replication and/or cloned gene promoter which permits the environmental switching from low to high levels of expression in a controlled fashion.

Host-plasmid interactions in recombinant *E. coli* fermentations were also modelled by Peretti and Bailey (1987). The model was of the single-cell (segregated) type and considers the competition for RNA polymerase between chromosome and plasmid DNA. The distribution of active ribosomes among chromosome- and plasmid-derived mRNA was also included. It was found that simulations of recombinant cell growth rate and cloned gene productivity as a function of relative plasmid number per cell agreed closely with experimental results. Also the effects of changing cloned-gene promoter and ribosome binding affinities and the effects of augmenting cell transcription or translation capacity were studied using this single-cell model. In conclusion, it was found that cloned-gene expression was limited by cellular transcription capacity.

Other workers who have presented models include Koizumi and Aiba (1986). Theirs describes the dymamics of the amount of plasmid copies per cell in relation to the specific growth rate of the host cell. However it was stated that a major drawback of this model was that it could only be applied to one particular host-vector system.

A more general model was proposed by Satyagal and Agrawal (1989) which permits the determination of plasmid content for a large number of host-vector systems. It incorporates host, vector and environmental influences on plasmid replication through the use of empirical expressions. It can be applied to continuous or batch cultures. Another model was developed later (Satyagal and Agrawal, 1990) which can predict cellular plasmid content and cloned gene production levels. Their equations were applied to several published sets of data which agreed well with experimental results. The authors advocate the use of the (assumed) linear relation between plasmid content and product yield to assess plasmid

content indirectly. It should be emphasized that a linear relationship is only valid at low plasmid copy numbers.

1.7.2 Modelling of cycling strategies in chemostat culture.

Stephens and Lyberatos (1988) have studied the possible differences in the periods required for plasmid-bearing and non-bearing organisms to adjust their metabolic activity to step changes in their environment. The possibility of maintaining an engineered strain in continuous culture by transient operation was also addressed. It was found that when the recombinant culture growth rate was similar to that of the host strain, a cycling strategy was feasible.

Weber and San (1989) also examined the effects of dilution rate cycling upon a pBR322-containing *E. coli* strain in chemostat culture. During constant dilution rate control experiments the $\%P^+$ cells decreased after a long lag period until eventually no plasmid-containing cells were present. Under forced perturbations in dilution rate the culture maintained a mixed population for a longer period of time. There was also an increase in average plasmid copy number during cycling. An unstructured model was developed from the results and it suggested that P^+ cells responded quicker to environmental changes than did P^- cells.

1.7.3 Modelling of recombinant yeast fermentations.

A mathematical model of recombinant yeast culture stability was developed by Srienc *et al* (1986). The influence of genetic factors was explored, particularly the effect of plasmid replication origin on segregational instability. A mutant host was employed which was deficient in *ura*3 gene, a functional copy of which was carried on the plasmid. Consequently, only P^+ cells grew in a uracil-free medium.

A 'distributed' model has been proposed by Sardonini and DiBiasio (1987) which allows for the effect of growth and production of a metabolite by the recombinant strain on the resulting metabolite concentration. The host strain was a trp^- mutant hosting a pBR322-2μ hybrid plasmid with a functional *trp*1 gene. Despite this strategy a large proportion of the population lost the plasmid, approximately 33% of which had arisen from segregational loss and the remainder from plasmid-free cell growth.

Another model was developed by Greenhalf *et al* (1989) which could predict the behaviour of recombinant yeast. A significant advantage was its ability to determine the distribution of plasmid copy numbers in the

individual cells of the population, a point frequently overlooked in earlier models.

Despite the efforts currently being made to develop useful, widely-applicable models, there is still a lack of basic metabolic information which is needed for their formulation. Fermentation simulations frequently differ considerably from actual experimental results even when a well-characterised bacterium such as *E. coli* is used as host. More work is therefore needed before models can be successfully applied in order to optimise industrial fermentations. No models have been developed to predict the behaviour of recombinant filamentous cultures.

1.8 Examples of plasmid stabilization strategies.

As we have seen from earlier sub-sections of this introduction, plasmid instability is a widespread problem both from the point of view of segregational and structural instability. This section will review some of the various methods which have been devised to improve the performance of recombinant cultures where plasmid instability has occurred.

Many early attempts to stabilize plasmids relied on the application of antibiotic selection pressure. This approach was studied by Dennis *et al* (1985) in order to observe the effect of increasing ampicillin concentration on the plasmid copy number and morphology of a recombinant *E. coli* HB101 strain in shake-flasks. It was found that as the selection pressure increased, cells with lower plasmid copy numbers became increasingly disadvantaged. It was also found that the production of a cloned gene product is not necessarily proportional to the number of plasmid copies, especially at high levels.

The use of antibiotics to maintain plasmid selection should be avoided since it considerably increases production costs and leads to problems of waste culture disposal. The remainder of this sub-section will deal with alternative methods which have been designed or discovered that will stabilize recombinant plasmids.

Chew *et al* (1986) found that the transposition of an insertion sequence (IS1) from the chromosome of *E. coli* HB101 brought about the stabilization of the plasmid pAT153. Normally this vector was rapidly lost from carbon-limited chemostat cultures, but during one experiment this did not happen. The altered plasmid was isolated and analyzed. It was found that IS1 DNA had spontaneously inserted into the aminoterminal region of the *tet* gene of pAT153 thereby stabilizing the vector which was now 700-800 bp larger. Similar plasmids were isolated from phosphate-, ammonium- and sulphate-limited chemostat experiments. Previous work had suggested that the tetracycline resistance gene was particularly prone to structural re-arrangements. The use of IS1 sequence for stabilization is not ideal since <80% of cells retained the plasmid after 30 generations under carbon-limited chemostat culture.

Another approach which stabilizes plasmids is the immobilization of recombinant *E. coli* cells in carrageenan gel beads (Poet *et al*, 1986). The pBR322-derived plasmid pTG201 was stably maintained for 80 generations

by immobilized cells in chemostat culture (60% P^+ after 60 generations). Biomass concentrations 20 times greater were found in carrageenan beads compared to free-cell systems. Immobilized cells grew within compartments in the gel but this physical confinement alone could not explain the increased segregational stability. The authors speculated that mechanical constraints could influence the morphology of the immobilized cells but growth rate was not affected.

The same workers (Nasri *et al*, 1987a) went on to compare the stability of their plasmid (pTG201) in three different *E. coli* strains, again using free and immobilized cultures. In free culture the plasmid was maintained with various degrees of stability in all host organisms but was highly segregationally stable in the immobilized cells. The instability (loss) rate in free continuous culture was found to be much higher than that predicted by random distribution of pTG201 between daughter cells, the reason for this was not clear. Plasmid copy numbers were similar in all strains therefore this could not explain the more rapid loss of plasmid from HB101 cells. Also the plasmid copy number of free and immobilized cells compared favourably.

A subsequent paper, also from the same group (Sayadi *et al*, 1988), examined the stabilizing effect of immobilization upon the plasmids pBR322, pBR325 and pBR328 hosted in *E. coli* W3101 cells. In free-cell culture pBR325 and pBR328 were lost after a short lag period, a decrease in plasmid copy number preceded the loss but pBR322 was found to be stably inherited. In the immobilized cell system, plasmid-free segregants were not detected even after 250 generations. The plasmid copy number was also found to remain constant at approximately 90.

Sayadi *et al* (1989) reported an increase in plasmid copy number of pTG201 from 100 to 350 as the chemostat dilution rate decreased from $0.47h^{-1}$ to $0.06h^{-1}$ in 40 generations of free cell growth. The effect of various limiting nutrients was examined in batch culture. In minimal medium the copy number was 90, under ammonium limitation 145, glucose limitation 220, phosphate limitation 71 and magnesium limitation 60. In conclusion the authors state that pTG201 could be stably maintained for over two weeks in immobilized *E. coli* W3101 in minimal media and that gel beads can act as a reservoir of recombinant cells with high plasmid copy number. Such a system could be of considerable industrial importance.

Another plasmid stabilization approach was proposed by Porter *et al* (1990) where an essential gene was deleted from the *E. coli* genome and was replaced, intact, on a recombinant plasmid. The gene chosen was *ssb* which encodes the SSB (single strand binding) protein and is required for DNA replication and cell viability. The protein is not secreted therefore crossfeeding between plasmid-free and plasmid-containing cells was not possible. Any plasmid-free segregants would be incapable of sustained growth. Recombinant vectors were designed and included the 'helper' plasmid pACYC*ssb* and mini-F-*ssb* both of which carry the wild type *ssb* gene. All recombinant DNA studies must be conducted using *E. coli* strain AB1157Δ(*ssb*) transformed with an *ssb* helper plasmid since plasmid-free segregants are not viable. This strain transformed with a suitable helper plasmid yielded no plasmid-free cells after prolonged chemostat culture. The main drawback of this system is the fact that different plasmids have to be introduced into the culture by a plasmid displacement technique. The antibiotic resistance marker must be different on the plasmid being introduced compared with the 'resident' plasmid and both should have a functional *ssb* gene. A pUC19*ssb* plasmid yielded 50 units/10^9 cells of β-lactamase activity which is very good expression under chemostat conditions. The authors point out that their host-vector system allows rapid growth of the recombinant culture using inexpensive complex media without the need for antibiotic selection.

Sakoda and Imanaka (1990) also reported a novel method of stabilizing recombinant plasmids. These authors developed a strain of *E. coli* W3110 which lacked the ability to actively transport tryptophan in addition to its previous *Tna*$^-$ phenotype. Strain FA14 could not grow normally even in complete medium. When transformed with a pSC101-derived plasmid carrying the *trp* operon the growth rate was restored to the original level. The host strain was deficient in both tryptophan biosynthesis and uptake hence plasmid-free segregants grow slowly and are rapidly diluted out of the culture. 100% segregational plasmid stability was achieved after 100 generations of growth in a complete medium, compared with only 25% P^+ in the original host-vector system.

The *par*B locus of the low copy number plasmid R1 has also been used as a general plasmid stabilization mechanism (Gerdes, 1988). The locus consists of 580 bp of DNA and mediates efficient segregation by post-

segregational killing of plasmid-free cells. The locus encodes two genes: the *hok* gene product is a lethal protein the expression of which is regulated by the *sok* product which is a complementary antisense RNA molecule. The *hok* mRNA is very stable while *sok* RNA is rapidly degraded. If the plasmid fails to segregate the prolonged presence of the *hok* mRNA results in production of the Hok protein thereby rapidly killing the cell. Recombinant plasmids carrying the *par*B locus can then be efficiently maintained in the culture. A variety of unstable vectors showed remarkable stability after incorporation of the *par*B locus. The *hok/sok* system also stabilized plasmids in the Gram -ve bacteria *Pseudomonas putida* and *Serratia marsescens* in addition to *E. coli*. No special medium is required and the system is equally efficient in both low and high copy number vectors.

Wood *et al* (1990) also exploited the *par*B locus to stabilize a recombinant vector in addition to introducing a *recA* mutation in the *E. coli* AMA1004 host chromosome and changing the selectable marker from ampicillin to kanamycin. The stability of the new vector, pTKW106, was increased to 100% P^+ cells in chemostat culture under antibiotic selection. Also at full induction, the level of β-galactosidase protein reached 10% of total intracellular protein. Structural instability was minimized by employing a *recA* host which reduces the frequency of homologous recombination events between the chromosome and plasmid. By using kanamycin resistance as a marker the problem of β-lactamase leakage from the periplasmic space into the medium, thereby degrading exogenous ampicillin, was avoided.

It is therefore evident that other methods of plasmid stabilization besides selection are currently being explored since the use of antibiotics has proved expensive and sometimes ineffective. Unfortunately, knowledge of basic plasmid biology has lagged behind the enormous advances made in gene cloning technology. It is only relatively recently that mechanisms such as the *par*B locus were discovered and exploited. Whether natural plasmids from other micro-organisms possess such characteristics which ensure efficient inheritance remains to be seen.

1.9 Gene cloning in *Streptomyces*.

Since the theme of this project is plasmid stability in *Streptomyces* the following sub-section gives an introduction to the properties of members of the genus and their plasmids.

Streptomycetes are prokaryotic micro-organisms which are mainly found in the soil. They are Gram +ve bacteria although the name 'streptomyces' means 'chain fungus' due to their characteristic filamentous appearance (Chater, 1984). These micro-organisms differentiate by forming spores on aerial hyphae which branch away from the substrate mycelium. The aerial hyphae usually bear long chains of spores (>50) but certain species have fewer (Cross and Goodfellow, 1973). It is the presence of these organisms in soil that give it that 'earthy' smell due to the presence of the compound geosmin. The genus can produce a range of extracellular enzymes which help to solubilize substrates in the soil. Their main attribute is the production of a wide range of secondary metabolites (molecules not essential for growth) many of which have antibiotic and other biological activities. Amongst antibiotics produced by bacteria over 84% were derived from Actinomycetes (Berdy, 1974). This property makes the genus very important to the industrial microbiologist and gene cloning can be applied to improve or diversify the secondary metabolite yield. *Streptomyces* are also good producers of extracellular enzymes and hence may be suitable candidates for heterologous gene expression.

1.9.1 *Streptomyces* plasmids.

The first covalently closed circular DNA (ccc DNA) molecule isolated from a *Streptomyces* species was initially named plasmid SCP1 (Schrempf *et al*, 1975) from *S. coelicolor*. It was discovered that this plasmid existed at low copy number (1-5 per chromosome) by measuring the molecule's contour length under electron microscopy and comparing the relative yields of plasmid and chromosomal DNA in extracts. More recent studies however, suggest that SCP1 is in fact a linear plasmid (Kinashi *et al*, 1987) and that the plasmid isolated by Schrempf *et al* was SCP2. A closely related plasmid, SCP2* (31.5kb), which is a sex plasmid has been developed to provide useful cloning vectors (Lydiate *et al*, 1985). Such large, low copy number vectors can accept long fragments of DNA and are fairly stable but have a less broad host range.

Kieser *et al* (1982) set out to discover high copy number vectors

from the '*Streptomyces violaceoruber*' group. These authors felt that high copy number vectors were preferable for uses such as gene amplification or the transfer of DNA between a variety of different Streptomycete hosts. A very useful plasmid, pIJ101 was discovered in this way. It was isolated from *S. lividans* ISP5434. The plasmid was reported to have a copy number of between 40-300 with a broad host range within the genus. The plasmid copy number was found to vary with culture age and physiological status. Maximum copy number was found in 48h old shaken cultures.

The genus *Streptomyces* has proved a fruitful source of plasmids especially when electrophoresis techniques improved thereby leading to the discovery of new molecules. There is a bias towards the discovery of cccDNA molecules but SLA2 of *S. rochei* is linear (like SCP1) and pUC1 of *S. fradiae* is a prophage (Hopwood *et al*, 1986). A low copy number plasmid, pMG200 was isolated from the bacteriocin-releasing strain *S. chrysomallus* by Krugel and Fiedler (1986). Another high copy number plasmid, pVE1 (11.0kb) was isolated from *S. venezuelae* by MacNeil and Gibbons (1986). pVE1, like pIJ101, is a conjugative plasmid which forms 'pocks' *i.e.* zones of apparent lethal zygosis upon transfer onto a confluent lawn of recipient strain.

Pock formation is a property unique to the self-transmissible plasmids of *Streptomyces* and is brought about by the spread of plasmids along the mycelium after primary intermycelial transfer by mating (Kieser *et al*, 1982). The plasmid migrates away from the pock centre and along mycelium containing recipient genotype. According to Bibb and Hopwood (1981), much experimentation and discussion has been devoted to discovering the process of stable plasmid inheritance in *Streptomyces*. It is the regulation of the initiation of plasmid replication at the origin which determines its copy number. The essential replication region of a plasmid is usually 1.5-2.5kb in length and contains not only the site of initiation, *oriV*, but usually also the genes which regulate it. A tentative model for the transfer and spread of *Streptomyces* plasmids proposes that inter-hyphal migration occurs via a process (perhaps involving simple hyphal fusion) which may depend on host genes but requiring *cis*-acting plasmid functions. The *tra* region of pIJ101 has been putitively suggested to function by facilitating plasmid spread in this way (Kendall and Cohen, 1987). Because of such self-coded specific functions the plasmid would then

spread within the hyphae. Perhaps a specific function might also allow the plasmid to cross the transverse septa that it would encounter in the mycelium.

An extensive review of the conjugative sex plasmids of *Streptomyces* has been written by Hopwood *et al* (1985a) and Hopwood *et al* (1986). In summary, *Streptomyces* plasmids range in size from <4kb to >200kb and can occur at copy numbers of between one and several hundred per chromosome. Most plasmids discovered so far exist as cccDNA molecules and the base composition (of the few that have been sequenced) is the same as that of the host chromosome *i.e.* 72-74% G+C.

1.9.2 Choice of *Streptomyces* host strains.

According to Martin and Gil (1984) most cloning studies have been carried out in *S. lividans* 66 (Lomovskaya *et al*, 1972) and *S. coelicolor* A3(2). There is little information available on the use of other *Streptomyces* as hosts for cloning vectors but Epp *et al* (1987) successfully used *S. griseofuscus* to host pIJ702 and pOJ159. Also Matsushima *et al* (1987) developed a mutant of *S. fradiae* which was deficient in restriction enzyme activity. The organism was successfully used to host both plasmid and phage based vectors.

Problems of structural instability in *E. coli* plasmids have been partly overcome by the use of recombination-deficient (*rec*$^-$) host organisms. Tsai and Chen (1987) reported the isolation of a *S. lividans* TK64 mutant blocked in a recombination pathway. The mutant, JT46, was able to host a plasmid (pWCL1) specifically constructed to be highly susceptible to re-arrangements through homologous recombination, without any deletions occurring. The parent host yielded a range of deleted forms of pWCL1.

Kieser *et al* (1989) attempted to further characterize the *S. lividans* JT46 mutant, but they could not place it with certainty into any of the *E. coli rec*$^-$ mutant categories. The availability of JT46 will partly overcome some of the problems associated with structural re-arrangements which occur in *Streptomyces* recombinant plasmids.

Transformation of protoplasts with plasmid DNA and transfection with phage are based on polyethyleneglycol-assisted DNA uptake, followed by regeneration of transformed or transfected protoplasts. A series of isogenic strains is only available for *S. lividans* (Kieser *et al*, 1982). It may be difficult to obtain totally plasmid-free strains of other species

within the genus due to the abundance of wild-type plasmids sometimes existing at copy numbers of <1 per chromosome.

Martin and Gil (1984) listed the desirable characteristics of an ideal *Streptomyces* host in the following points:

1) Good growth and formation of conidia in a variety of fermentation media;
2) Protoplasts easy to obtain and regenerate;
3) Lack of restriction enzyme activity;
4) Sensitivity to antibiotics used as resistance markers in vectors;
5) Lack of extracellular DNAase activity;
6) Preferably has *rec*$^-$ (recombination deficient) mutants; and
7) Lack of pathogenicity or side effects in animals or plants.

From the above list it is noticable that little or no consideration is given to the suitability of the *Streptomyces* host strain to large-scale fermentation culture. The work presented in this project will partly address this issue.

1.9.3 Development of plasmid vectors in *Streptomyces*.

Reviews on the development of cloning vectors have been written by Hopwood (1981), Chater and Hopwood (1982), Chater *et al* (1982), Hopwood *et al* (1983), Martin and Gil (1984), Fayerman (1986), Hopwood *et al* (1986) and Tomich (1988a and 1988b).

Many of the widely-used mutli-copy plasmid vectors currently available are derived from the 8.9kb plasmid pIJ101 (Kieser *et al*, 1982). Vectors were derived carrying antibiotic resistance genes for thiostrepton (from *S. azureus*), viomycin (from *S. vinaceus*) or neomycin (from *S. fradiae*). Such pIJ101 derivatives exist at copy numbers of between 40-300 which, at the time, were thought to be the highest described in any bacterium (recent developments in plasmid copy number estimations allow more accurate determinations which show such numbers are not uncommon amongst bacteria especially in stationary phase, refer to Section 1.2). The development of the pIJ101 series of vectors represented a new class of plasmids which has facilitated the genetic manipulation of *Streptomyces*. All the multi-copy vectors studied in this project were derived from pIJ101.

Another *Streptomyces* plasmid which has been used to develop cloning vectors is pVE1 (MacNeil and Gibbons, 1986). This plasmid was isolated from *S. venezuelae* and has a broad host range and is 11.0kb in

length. This plasmid is also a fertility factor which mediates chromosomal recombination. Genes for thiostrepton and neomycin resistance were cloned into the plasmid as well as regions of pBR322 which yielded *E. coli-Streptomyces* shuttle vectors. Many other plasmids have been used in a similar way to construct vectors both in industrial and academic laboratories.

Most low copy number vectors are derived from the *S. coelicolor* A3(2) plasmid SCP2* (Lydiate *et al*, 1985). Hygromycin, thiostrepton and viomycin resistance genes were all cloned into a deletion derivative, pSCP103. An *E. coli-Streptomyces* shuttle vector, pIJ903 (25.8kb) confers ampicillin resistance in *E. coli* and thiostrepton resistance when cultured in *Streptomyces*. Many derivatives retained the 'pock'-forming phenotype of the parent and were able to maintain large DNA inserts (>30kb) at a copy number of 1-2 per chromosome. Plasmid pMG200 of *S. chrysomallus* has also been used to develop useful low copy number cloning vectors (Krugel and Fiedler, 1986).

1.10 Stability of cloning vectors in *Streptomyces*.

1.10.1 Structural stability.

Streptomyces plasmids are known to exhibit structural instability since Kieser *et al* (1982) observed that pIJ101 derivatives sometimes gave rise to deletion variants when hosted in *S. lividans* 66 and *S. rimosus*. The smaller plasmids pIJ102. pIJ103 and pIJ104 are probably all *in vivo* deletion derivatives of pIJ101 which co-exist in *S. lividans* ISP5434.

Low copy number vectors derived from SCP2* are not thought to be structurally unstable since large inserts (>30kb) can be maintained without DNA re-arrangements (Lydiate *et al*, 1985). However, an earlier report (Bibb and Hopwood, 1981) described an example where SCP2*, upon transfer from *S. coelicolor* A3(2) to *S. parvulus*, underwent a structural change. A 9kb segment of the plasmid was duplicated to give molecules of between two and four tandemly repeated copies of the segment. There may exist a very heterogenous population of large or small molecules, some with copy numbers of <1 per chromosome suggesting considerable heterogeneity between hyphae in the same mycelium.

Lee *et al* (1986) constructed a recombinant plasmid carrying the hepatitis B viral surface antigen (HBsAg) on a pIJ702-pUC12 (*Streptomyces-E. coli)* shuttle vector. However the recombinant plasmid pWCL1 was stably maintained in *E. coli* but was structurally unstable in *S. lividans* 1326. Deletions had occurred which ranged in size from 2.75 to 5.65kb and were most often located around the *mel* gene, the reason for this is not known. This led to the loss of the insert and hence no expression of the recombinant product was achieved. Their results indicated that the structural instability of the pIJ702-derived plasmid depended on the interaction between the *mel* sequence and the inserted sequence. It should be noted that the HBsAg gene could be stably maintained in pIJ702 but insertion of the pUC12 plasmid induced deletion.

A subsequent paper by the same workers, Chen *et al* (1987) investigated this phenomenon further. Unpublished communications from several laboratories had shown structural instability in pIJ702 derivatives. The authors therefore set out to investigate the cause of the instability using sequence repetitions. Direct repeated sequences were constructed on a shuttle vector (pIF132) which were stably maintained in *E. coli* (in both *recA*$^-$ and Rec$^+$ strains) but it was unstable in *S. lividans*

where a deletion derivative was also isolated, pIF138. It was also noted that oligomeric (multimeric) plasmids were found in *E. coli* but not in *S. lividans*. The authors calculated that the time needed to convert half of the pIF132 to pIF138 (*i.e.* its half life) was about 14 days in *S. lividans* TK64.

Pigac *et al* (1988) also studied the phenomenon of *Streptomyces* plasmid structural instability. These authors constructed a *Streptomyces-E. coli* shuttle vector by ligating pIJ350 (a pIJ702 predecessor) and pBR322 resulting in the construction of plasmid pZG1(8.6kb). When introduced into *S. lividans* and *S. rimosus*, pZG1 exhibited structural instability. The authors also reported the intracellular accumulation of single-stranded plasmid DNA (ssDNA) molecules in *Streptomyces* strains. After analysing the plasmid sizes at different colony ages it was found that the younger colonies contained larger plasmids and the older colonies smaller plasmids. Up to 4 days old, intact pZG1 could be detected in addition to plasmids with different deletions all co-existing in the mycelium of a single transformant colony. After 2 or more weeks of growth colonies harbouring only the plasmids with deletions (up to 4.5kb) could be found. Plasmids derived from pIJ101 are known to replicate via ssDNA intermediates (Deng *et al*, 1988) and such structural instability may arise through illegitimate recombination during replication. Other G+ve bacterial plasmids are also known to replicate via ssDNA intermediates, te Riele *et al* (1986) have studied this phenomenon in plasmid-bearing *Bacillus subtilis* and *Staphylococcus aureus* . Generation of ssDNA can indicate an asymmetric replication mechanism where leading and lagging strand synthesis is uncoupled: a complete ssDNA copy may be displaced if lagging strand synthesis is not initiated (at a secondary site) before the termination of leading strand synthesis. Absence of the lagging strand initiation site resulted in considerable accumulation of ssDNA. Double stranded DNA copies are, however, maintained probably through lagging strand synthesis initiation at other, less efficient, sites.

The site of lagging strand synthesis on the *Streptomyces* pIJ101 plasmid has been identified as the *Sti* locus which is a *cis*-acting function (Deng *et al*, 1988). The *Sti* (abbreviation for strong incompatibility) phenotype initially defined plasmid compatibility categories since only plasmids of similar phenotype could co-exist in the

same mycelium *i.e.* *Sti*$^{+}$, *Sti*$^{+}$ or *Sti*$^{-}$, *Sti*$^{-}$. Plasmids of dis-similar phenotype cannot co-exist since the *Sti*$^{-}$ plasmid is always lost. It was observed that *Sti*$^{-}$ derivatives *e.g.* pIJ702, accumulate more ssDNA than *Sti*$^{+}$ derivatives *e.g.* pIJ303. Plasmids which lacked the *sti* sequence were observed to have copy numbers of between 50 and 100 on average (Deng *et al*, 1988). Also when the *sti* region was cloned from pIJ101 into a *Sti*$^{-}$ phenotype plasmid the copy number increased dramatically to 1000. Sequencing data of the *sti* region showed palindromic sequences which could form secondary structures that may be required for the initiation of lagging strand synthesis.

1.10.2 Segregational stability.

1.10.2.1 Solid media cultures.

The stability of many *Streptomyces* plasmids has been assessed on the basis of inheritance during spore-to-spore cycles. Essentially the percentage of plasmid-bearing spores is determined by plating and counting the number of colonies which exhibit a plasmid-borne phenotype *e.g.* pock formation or antibiotic resistance. Kieser *et al* (1982) used this method to assess the segregational stability (into spores) of pIJ101 derivatives. Plasmid pIJ303 segregated efficiently without antibiotic selection since all spores of *S. coelicolor* and *S. lividans* contained the plasmid. Other species *e.g.* *S. albus* and *S. griseus* did not retain the vector as efficiently. It was also possible to identify regions of the plasmid which confer stability by deletion and subsequent assessment of segregational stability. Plasmid pIJ350 (a predecessor of pIJ702) was found to be stably inherited in *S. lividans* spores.

Kendall and Cullum (1984) reported the cloning of a *S. coelicolor* agarase gene into *S. lividans* using the plasmid vector pIJ702. The agarase-bearing pIJ702 derivatives pMT605 and pMT608 were found to be segregationally stable with 70.4% and 99.6%, respectively, of *S. lividans* TK64 spores bearing the plasmid. It was initially found that pMT605 was rapidly lost from cells growing on solid media without thiostrepton selection.

The low-copy number *Streptomyces-E. coli* shuttle vector pIJ903 was also found to be stably inherited into spores (Lydiate *et al* , 1985). More than 99.7% of spores retained the thio^{R} plasmid-borne phenotype.

One vector has been designed to test the segregational stability

(into spores) of *Streptomyces* plasmids (Kendall and Cullum, 1988). The vector pMT603 is a derivative of SCP2* plasmid but lacks its stability region. The vector also carries the thio^R selectable marker and the *mel* gene of pIJ702. The number of both melanin and thiostrepton resistant colonies yields an estimate of the number of plasmid-bearing spores. The vector pMT603 is inherently unstable (only 20% retention), therefore it is possible to clone putitive stability regions from elsewhere and look for an increase in stability.

1.10.2.2 Fermentation cultures.

The only report to date which specifically studied the segregational stability of *Streptomyces* plasmids during fermentations is that of Roth *et al* (1985). These workers examined the maintenance of the low copy number recombinant plasmid pIJ2 in chemostat cultures of *S. lividans* 66. Plasmid pIJ2 is derived from SLP1.2 and carries the gene for neomycin resistance. The host organism was seen to yield large pellets except when high agitation rates were employed under nitrogen limitation whereby "small" pellets were obtained. Segregational stability was monitored by replica plating onto selective media.

It was found that when the growth was limited by glucose or phosphate the plasmid was lost faster than under nitrogen limitation. Also at low dilution rates in all media plasmid-free clones appeared after a shorter period of time compared with higher growth rates. Plasmid loss was higher at 36°C than at 28°C. The authors quite rightly point out that the interpretation of segregational plasmid loss from *Streptomyces* mycelium is more difficult than from unicellular organisms. Each mycelial pellet contains many hundreds of nucleoids and plasmid copies. It is possible that branches in the mycelium may arise without any plasmid copies present. If such a branch were to fragment from its 'parent' mycelium this would result in a plasmid-free organism. Successive decreases in plasmid copy number caused by the reduction in plasmid replication initiation due to nutrient limitation, could lead to inefficient 'partitioning' and hence plasmid loss.

The authors realised that the plating of mycelial pellets would not give an accurate representation of the actual percentage of plasmid-bearing cells. In order to overcome this problem they "fragmented" the mycelium into protoplasts by treatment with lysozyme. Protoplasts obtained

from mycelium grown in glucose-limited culture revealed that only 47% possessed the plasmid while platings of mycelium from the same culture revealed 100% plasmid-bearing colonies. The basic flaw of this method is that when plating mycelial pellets, probably as few as only one functional plasmid copy per pellet is needed to yield a colony on selective agar. The results obtained are consequently an overestimate of the true level of plasmid molecules in the culture as a whole.

1.10.3 Copy number profiles of *Streptomyces* plasmids.

There are very few reports of the fluctuation in *Streptomyces* plasmid copy numbers. When developing the pIJ101-derived vectors Kieser *et al* (1982) measured the copy numbers of pIJ101, pIJ208, pIJ303, pIJ312, pIJ350, pIJ355 and pIJ366 in *S. lividans* 66. It was found that plasmid copy numbers ranged from 40 to 300 with an average of 170. The vector pIJ350 was studied in more detail and it was found that the copy number after 24h incubation in liquid culture was 70 and increased to 250 after 48h. It was theorised that the observed wide range in copy numbers might reflect different physiological ages of the cultures rather than inherent differences in the plasmids studied.

MacNeil and Gibbons (1986) when characterizing and developing plasmid vectors from the *S. venezuelae* plasmid pVE1 (11kb) also examined its copy number. The method used was densitometric scanning of separated total DNA. The copy number when hosted in *S. lividans* TK64 was measured at several points in the growth curve in order to study the effect of physiological age of the cells upon copy number. At the start of exponential growth (Klett 15) the copy number was 50. But in exponential growth (Klett 25 to 100) the copy number was measured as 1000. As the culture entered stationary phase the copy number increased from 200 to 650 after 120h growth.

There are currently no reports of systematic studies of plasmid copy number profiles in *Streptomyces* organisms grown under laboratory-scale fermentation conditions. It is also not known whether such vectors are structurally stable under these conditions.

1.11 Applications of recombinant *Streptomyces* cultures.

Streptomyces strains are very important industrially because of their ability to produce a diverse range of useful secondary metabolites. It is therefore understandable that the major thrust of gene cloning applications within this genus have been directed towards manipulating this important feature. This section will review the developments to date in the application of gene cloning in *Streptomyces*.

1.11.1 Production of hybrid antibiotics.

It was widely anticipated that gene cloning would provide a means of generating new and possibly more efficacious antibiotics through the generation of so called 'hybrid' molecules. The first review of this method was by Eckhardt (1983). It was postulated that since some enzymes involved in antibiotic biosynthesis have wide substrate specificity then this could be exploited in the generation of hybrid molecules. Existing pathways of one strain or a combination of pathways of two different species could be amalgamated into a newly-created strain, either through protoplast fusion or gene cloning. The ultimate host would be the primary producing strain. A recent review of hybrid antibiotic production was written by Tomich (1988).

So far there are relatively few examples of the production of hybrid antibiotics through genetic engineering. The first example was described by Hopwood *et al* (1985b). Gene transfer between *Streptomyces* strains producing actinorhodin, granaticin and medermycin yielded the hybrid structures named dihydrogranatirhodin and mederrhodins A and B. It was not stated whether these hybrid molecules were better antibiotics than the 'parent' metabolites. A subsequent paper by Omura *et al* (1986) reported that mederrhodin A, although active against G+ve bacteria, was less active than medermycin. Comparable activities against G-ve bacteria were obtained. Mederrhodin B was inactive against all bacteria.

A more recent example of the generation of a hybrid molecule is described by Epp *et al* (1989). A gene from *S. thermotolerans* which is involved in carbomycin biosynthesis was cloned and introduced into the spiramycin-producing strain *S. ambofaciens*. This resulted in spiramycin being converted to the hybrid antibiotic isovaleryl spiramycin. All these antibiotics are macrolide molecules. The same molecule was also produced when the *carE* gene, cloned on pIJ702, was introduced into *S. lividans* and

spiramycin added exogenously. There is no information presented on the antimicrobial activity of isovaleryl spiramycin.

1.11.2 Improvement of antibiotic yield.

To date there are very few examples of where improved antibiotic yields have been obtained using gene cloning techniques, however its potential application was recently reviewed by Chater (1990). It has been assumed that most sets of antibiotic pathway genes contain linked regulatory genes that could be used to increase yield. Also some of these genes may act on heterologous sets of pathway genes. In *S. coelicolor* there are at least nine genes involved in the general positive control of secondary metabolism, some of which have potential for generating a yield improvement. If a large segment of DNA encoding a biosynthetic pathway is isolated then it is very likely that it will contain a pathway-specific positive regulatory gene. The insertion of extra copies of this gene into the wild-type strain may well yield higher antibiotic titres.

Horinouchi *et al* (1989) have shown that the pleiotropic regulatory gene *afs*B of *S. coelicolor* stimulates the production of actinorhodin and undecylprodigiosin in *S. lividans* and in a non-producing *S. coelicolor* mutant. It was found that cloned *afs*B gene in *S. lividans* stimulated transcription of the *act* genes under conditions in which they are normally silent in that strain. The nucleotide sequence of *afs*B suggested that its gene product was a DNA-binding protein. The *afs*B gene also stimulated the production of A-factor (an autoregulatory molecule) in *S. lividans*.

Another example of where increased antibiotic yield was obtained through gene cloning is that of improved cephalosporin production by the fungus *Cephalosporium acremonium* (Skatrud *et al*, 1989). An extra copy of the *cef*EF gene which encodes the rate-limiting enzyme deacetoxycephalosporin C synthetase/deacetocephalosporin C synthetase brought about a yield improvement of 20-39% in a production strain.

1.11.3 Cloning of heterologous genes.

Many genes have been cloned and expressed in various *Streptomyces* species, examples to date have been summarized by Tomich (1988a). Murooka *et al* (1986) cloned a *Streptomyces* cholesterol oxidase gene and obtained expression in *S. lividans*. They used the vector pIJ702 but found one recombinant plasmid to be unstable during cultivation. Cholesterol oxidase

was produced for 8 days by *S. lividans* 1326 pCO-3 and yielded intracellular and extracellular levels of 4.8 and 3.1 Umg^{-1} cells, respectively. The level of extracellular yield was 2-fold greater than the wild-type producer and intracellular yield 13-fold higher. Such values are not high considering the multicopy nature of the plasmid. Antibiotic selection was used for all the recombinant strains.

Dehottay *et al* (1986) also used the vector pIJ702. They cloned an extracellular β-lactamase gene from *S. albus* G and obtained good yields from the recombinant *S. lividans* TK24 strain. A 10-fold higher yield of β-lactamase was obtained from recombinant *S. lividans* than from *S. albus* G. The pIJ702-derived plasmid pDML6 was segregationally very stable since 100% of the spores examined contained the plasmid.

Eukaryotic genes have also been cloned and expressed in *S. lividans*. Gray *et al* (1984) cloned the bovine growth hormone and the human interferon α2 gene was cloned by Pulido *et al* (1986). The pIJ702-derived plasmids pNIS19(7kb) and pNIS91(7kb) produced $1x10^{5}$ IUL^{-1} of interferon antiviral activity when recombinant *S. lividans* 1326 was cultured until stationary phase. The yield was not affected by the use of rich or minimal liquid medium and all the activity remained within the mycelium. No evidence for proteolysis or degradation was found.

It is not always convenient for a desirable cloned gene to remain within the cells since there have been attempts to design vectors which allow the secretion of proteins from the *Streptomyces* host organism. Koller *et al* (1989) reported the expression of a fusion protein which was effectively secreted from *S. lividans* TK24. The fusion protein consisted of the α-amylase inhibitor (tendamistat) of *S. tendae* and monkey pro-insulin. The fusion protein exhibited α-amylase inhibiting activity and biologically active insulin was obtained after proteolytic digestion with trypsin. The tendamistat protein was a good candidate for such studies since it is secreted via a signal peptide-mediated mechanism, is produced in high yields and is correctly processed in the heterologous host, *S. lividans*. The fusion protein was produced at levels up to $100mgL^{-1}$ of culture. The fusion protein was encoded from a pIJ702-derived vector. The authors also noted that strains carrying the construct showed a reduced growth rate compared to the host without the plasmid. Workers from the same laboratory (Bender *et al*, 1990) also attempted to obtain good yields of human

interferon from a recombinant *S. lividans* using a pIJ680 derivative.

Another *Streptomyces* secretion vector system is described by Taguchi *et al* (1989). These workers used the gene for the *Streptomyces albogriseus* subtilisin inhibitor (SSI) cloned onto the vector pIJ702 . It was possible to exploit the secretory and protease inhibitory characteristics of SSI to obtain efficient production of a sex pheromone peptide (cAD1) heterologous protein. Approximately 28mg of SSI-cAD1 fusion protein was obtained from 1L of culture filterate of *S. lividans* 66. The original strain *Enterococcus faecalis*, produced only 600ngL^{-1} of cAD1.

The expression of the T-cell CD4 surface receptor in *S. lividans* was recently reported by Brawner (of Smith Kline Beecham at the UCLA Colloquium on the Molecular Biology of *Streptomyces* held at Colorado, USA, January, 1990). Correctly folded, active, soluble protein was produced at a yield of 100mgmL^{-1} during optimised fermentations.

There are very few examples in the literature where the culture conditions were examined in relation to cloned gene expression in a *Streptomyces* host. Erpicum *et al* (1990) have studied the production of various cloned extracellular enzymes by *S. lividans* TK24 under different fermentation conditions in shaken culture. Large variability in culture yields led the workers to attempt optimization of their fermentations in a reproducible manner. The plasmids used were all pIJ702-derived and encoded various β-lactamases and DD-peptidases. Production reached maximum after 36 to 48h but thereafter enzyme activity decreased drastically. The increase in pH (to pH9) at the onset of stationary growth was thought to affect enzyme yield but this alone could not explain the sudden decrease in cloned gene expression. Protease activity from the host organism was assayed but was found to be insignificant. The authors therefore postulated that *S. lividans* was producing an inactivator. These effects were minimized by buffering the medium with the zwitterionic buffer BES (N,N bis(2-hydroxyethyl)-2-aminoethane sulfonic acid) and by decreasing the water activity to 0.98 or 0.96 with added sucrose at 270gL^{-1} and 380gL^{-1}. The organism did not utilize sucrose as a carbon source. The normal carbon source was glucose with NH_4Cl as the nitrogen source. The replacement of Bactotryptone with undigested protein ensured a slow progressive supply of amino acids which also improved the fermentation characteristics. By employing these improved cultivation conditions a maximum enzyme level of

0.39gL^{-1} in the culture supernatant (compared with 0.2mgL^{-1} in the wild strain) was obtained. This was achieved after 216h of cultivation yielding 7.7gL^{-1} dry cell mass. The workers did not investigate the stability of their pIJ702-derived vectors nor did they extend their work to stirred vessel fermentations.

Of the many heterologous genes now cloned and expressed in *Streptomyces* the vector most widely used is pIJ702. Also the most popular host for these recombinant plasmids is *S. lividans* since very few other species have been explored as suitable hosts.

1.12 Assessment of plasmid copy number.

Plasmid copy number is widely defined as the ratio of the number of moles of plasmid DNA to the number of mole equivalents of chromosomal DNA. Early methods of assessing plasmid copy number involved approximations of molecule contour lengths obtained from electron micrographs and subsequent estimation of the plasmid molecular weight. By comparing the chromosomal and plasmid DNA yield ratios an estimate of plasmid copy number could be made. This method was used by Schrempf *et al* (1975) to estimate the copy number of the first *Streptomyces* plasmid to be isolated at the time named SCP2.

Reviews of methods of plasmid copy number estimations have been written by Shepard and Polisky (1979), Caro *et al* (1984) and Thomas (1987) amongst others. Currently there are several methods available to assess copy number *e.g.* DNA-DNA hybridisation, dye-bouyant density gradients, comparison with an internal standard, high pressure liquid chromatography separation and electrophoresis separation (followed by scanning densitometry).

Projan *et al* (1983) developed a method for the determination of plasmid copy number in recombinant *E. coli* by direct fluorescence densitometry scanning of ethidium bromide (EtBr) stained DNA gels. Essentially, they recognised that the existing methods were based on one of three analytical principles:

1) Physical separation of plasmid from chromosomal DNA;

2) DNA hybridization; and

3) Measurement of gene dosage.

All these methods were regarded as being unsatisfactory. The authors obtained whole cell lysates on a mini-preparative scale which were then subjected to electrophoresis through a 1% agarose gel. After staining with EtBr, individual lanes were scanned with a densitometer and the resulting peak areas were integrated. Integral values of chromosome and plasmid DNA bands could then be used to calculate plasmid copy number. It was found that different DNA bands did not fluoresce with equal intensity. Linear or open circle (OC) plasmid DNA fluoresced approximately 1.36 times greater than supercoiled (SC) plasmid DNA. Lin-Chao and Bremer (1986) also found a difference in intensity but it was only 10%. Seo and Bailey (1985b) however found no difference in intensity between linearized and supercoiled DNA.

Because of Projan *et al*'s observation a correction factor (to compensate for the fluorescence difference) was introduced into their equation in addition to a chromosome: plasmid size ratio multiplication factor. They postulated that the discrepancy is due to the observation (by Bauer and Vinograd, 1968) that the level of bound EtBr is related to the topological configuration of the double stranded DNA helix and this is the reason for the discrepancies between SC and OC fluorescences.

A similar method for rapid copy number assessment was proposed by Lewington and Day (1986). It was designed to be fast and ideal for processing large numbers of samples from bacterial cultures while also being reproducible. These workers scanned photographic negatives rather than measuring the fluorescence of gel bands directly.

Another rapid and straightforward method of copy number estimation was proposed by Coppella *et al* (1987). *E. coli* cultures were sonicated, centrifuged and after filtering the extracts were loaded onto a Nucleogen 400 DEAE HPLC column. The resulting peaks of chromosomal and plasmid DNA were used to calculate copy number.

Recently another method of plasmid copy number assessment was proposed by Labes *et al* (1990) which is specifically designed for *Streptomyces* cultures grown on agar. Again, whole cell lysates are obtained and separated by electrophoresis followed by scanning densitometry of photographic negatives. The main drawback of this protocol is that it requires all the biomass from one colony leaving no material left for duplicate estimates. Also, the extracts shown are not very 'clean' this results in densitometric traces which are difficult to interpret. Such a method would be suitable for the screening of several colonies but would not be suitable for accurately analyzing transient changes in the plasmid copy number of a single culture.

In order to study plasmid copy number fluctuations during liquid cultures of recombinant *Streptomyces*, existing protocols need to be extensively modified. During this project a *Streptomyces* total DNA extraction method has been adapted and in conjunction with the Projan *et al* (1983) method of scanning densitometry, the plasmid copy number of *Streptomyces* vectors can be measured.

1.13 Project aims.

The aims of this project were to develop and apply a method of plasmid copy number estimation during *Streptomyces* shake-flask and stirred vessel fermentations. This would permit the stability of several widely used vectors to be assessed and to check upon any structural re-arrangements that may have occurred.

The host organism chosen was *S. lividans* TK24 since its use is very widespread but little is currently known about its fermentation characteristics. The plasmid vectors pIJ303(*sti*$^{+}$) and pIJ702(*sti*$^{-}$) were chosen since they represent the two main classes of pIJ101-derived multi-copy vectors. There are also many reports of the use of pIJ702 as a cloning vector and it was of interest to assess its suitability for large-scale recombinant culture.

The effect of environmental stresses such as high incubation temperature and low dissolved oxygen tension upon plasmid copy number were also investigated. The effect of different induction strategies upon the expression of the pIJ702 *mel* gene were also studied as an example of a *Streptomyces* expression system.

Data from this project will expand our currently limited understanding of *Streptomyces* plasmid stability and assess the suitability of these host-vector systems for use in shake-flask and industrial fermentations.

2 MATERIALS and METHODS.

2.1 MATERIALS.

2.1.1 Organism.

Streptomyces lividans 66 strain TK24 (Kieser *et al*, 1982) SLP2$^-$, SLP3$^-$, *str-6* phenotype was used throughout this study.

2.1.2 Plasmids.

Plasmid pIJ303, 10.69kb (Kieser *et al*, 1982) and pIJ702, 5.6kb (Katz *et al*, 1983) were obtained from Professor D.A. Hopwood, John Innes Institute, Norwich, UK. The pIJ702 derivatives pMT605 (12.6kb) and pMT608 (6.35kb) (Kendall and Cullum, 1984) were a gift from Professor J. Cullum, UMIST, Manchester, UK.

2.1.3 Media.

All media were autoclaved for 20 min, 121°C and 15 pisg after making up.

2.1.3.1 Solidified media.

The following media were used for growing *S. lividans* TK24 on agar.

a) R2YE agar (Hopwood *et al*, 1985).

$MgCl_2.6H_2O$	(AnalaR; Fisons, Loughborough, UK)	10.12 gL^{-1}
Glucose	(AnalaR; BDH, Poole, UK)	10 gL^{-1}
Bacteriological agar	(Gibco, Paisley, UK)	22 gL^{-1}
K_2SO_4	(AnalaR; BDH, Poole, UK)	0.25 gL^{-1}
Sucrose	(AnalaR; BDH, Poole, UK)	103 gL^{-1}

Made up to 800mL in distilled water and autoclaved. After autoclaving the following sterile solutions were added;

0.5 %w/v KH_2PO_4	(AnalaR; BDH, Poole, UK)	10mLL^{-1}
14.7 %w/v $CaCl_2$	(AnalaR; BDH, Poole, UK)	20mLL^{-1}
20.0 %w/v L-proline	(Sigma, UK)	15mLL^{-1}
5.73%w/v TES	(Sigma, UK)	100mLL^{-1}
1 M NaOH	AnalaR; BDH, Poole, UK)	5mLL^{-1}
10.0%w/v Yeast extract	(β-Lab, East Molesley, UK)	50mLL^{-1}

b) Soft nutrient agar (Hopwood *et al*, 1985).

Nutrient broth (Oxoid, Basingstoke, UK) 8gL^{-1}

Bacteriological agar (Gibco, Paisley, UK) 3gL^{-1}

Made up to 1L in distilled water and autoclaved.

c) Modified R2YE agar (modified from Hopwood *et al*, 1985).

$MgCl_2.6H_2O$	(AnalaR; Fisons, Loughborough, UK)	10	gL^{-1}
Glucose	(AnalaR; BDH, Poole, UK)	10	gL^{-1}
Bacteriological agar	(Gibco, Paisley, UK)	22	gL^{-1}
Yeast extract	(β-Lab, East Moseley, UK)	5	gL^{-1}

Made up to 1L in 100mM TRIS HCl (Sigma, UK) pH7.5 and autoclaved. After autoclaving the following sterile solutions were added;

5%w/v KH_2PO_4	(AnalaR; BDH, Poole, UK)	1	mLL^{-1}
1M $CaCl_2$	(AnalaR; BDH, Poole, UK)	20	mLL^{-1}

d) Nutrient agar.

Nutrient agar	(Oxoid, UK)	28	gL^{-1}

Made up to 1L with distilled water and autoclaved.

e) Yeast-starch agar (modified from Grafe *et al*, 1981).

Yeast extract	(β-Lab, East Molesley, UK)	5	gL^{-1}
Soluble starch	(Connaught Labs, Canada)	10	gL^{-1}
NaCl	(AnalaR; BDH, Poole, UK)	5	gL^{-1}
Bacteriological peptone	(Oxoid, UK)	1	gL^{-1}
Bacteriological agar	(Gibco, Paisley, UK)	20	gL^{-1}

Made up to 1L with distilled water, pH7.0 and autoclaved.

2.1.3.2 Liquid media.

a) Malt extract-peptone (MEP) medium (modified from Reading and Cole, 1977).

Glycerol	(AnalaR; Fisons, Loughborough, UK)	20	gL^{-1}
Malt extract broth	(Oxoid, Basingstoke, UK)	10	gL^{-1}
Bacteriological peptone	(Oxoid, Basingstoke, UK)	10	gL^{-1}

Made up to 1L in distilled water, pH7.0 and autoclaved. For MEP+10mMPO_4^{3-} media, K_2HPO_4 (AnalaR; BDH, Poole, UK) was added at 1.74 gL^{-1}.

b) Modified YEME medium (modified from Hopwood *et al*, 1985).

Yeast extract	(β-Lab, East Molesley, UK)	3	gL^{-1}
Bacteriological peptone	(Oxoid, UK)	5	gL^{-1}
Malt extract	(Oxoid, UK)	3	gL^{-1}
Glucose	(AnalaR; BDH, Poole, UK)	10	gL^{-1}

Made up to 1L with distilled water and autoclaved. After sterilization 2.5M $MgCl_2$ (AnalaR, Fisons, UK) was added at 2mLL^{-1}.

c) Tryptone soya broth medium.

Tryptone soya broth	(Oxoid, UK)	30 gL^{-1}

Made up to 1L with distilled water, natural pH and autoclaved.

d) Glucose yeast tryptone medium.

Tryptone	(Difco, Detroit, USA)	5 gL^{-1}
Yeast extract	(β-Lab, East Molesley, UK)	4 gL^{-1}
KH_2PO_4	(AnalaR; BDH, Poole, UK)	2.72gL^{-1}
K_2HPO_4	(AnalaR; BDH, Poole, UK)	3.48gL^{-1}
$CaCl_2$	(AnalaR; BDH, Poole, UK)	0.1 gL^{-1}

Made up to 800mL with distilled water and autoclaved. After autoclaving 200mL of 15%w/v glucose (AnalaR; BDH, Poole, UK) sterile solution was added.

e) MOPS medium.

3-(N-morpholino)-propanesulphonic acid	(Sigma, UK)	20.8 gL^{-1}
Tryptone	(Difco, Detroit, USA)	5 gL^{-1}
Yeast extract	(β-Lab, East Molesley, UK)	4 gL^{-1}
$CaCl_2$	(AnalaR; BDH, Poole, UK)	0.1 gL^{-1}

Made up to 800mL with distilled water and autoclaved. After autoclaving 200mL of 15%w/v glucose (AnalaR; BDH, Poole, UK) sterile solution was added.

f) Glucose yeast malt medium (Lerch and Ettlinger, 1972).

Yeast extract	(β-Lab, East Molesley, UK)	10 gL^{-1}
Malt extract broth	(Oxoid, UK)	10 gL^{-1}
Glucose	(AnalaR; BDH, Poole, UK)	5 gL^{-1}
$CuSO_4.5H_2O$	(AnalaR; BDH, Poole, UK)	2.5mgL^{-1}

Made up to 1L with distilled water and adjusted pH to pH7.3 with NaOH and autoclaved. For tyrosinase induction the following sterile medium was added to achieve final concentrations;

Bactotryptone	(Difco, Detroit, USA)	10 gL^{-1}
$CuSO_4.5H_2O$	(AnalaR; BDH, Poole, UK)	5 mgL^{-1}

g) Modified minimal defined medium (Hobbs *et al*, 1989).

$NaNO_3$	(AnalaR; BDH, Poole, UK)	4.5 gL^{-1}
Na_2SO_4	(AnalaR; BDH, Poole, UK)	5.0 gL^{-1}
NaCl	(AnalaR; BDH, Poole, UK)	5.0 gL^{-1}
K_2HPO_4	(AnalaR; BDH, Poole, UK)	2.0 gL^{-1}
CuSO4	(AnalaR; BDH, Poole, UK)	2.5mgL^{-1}

Made up to 1L with distilled water and autoclaved. After autoclaving the following sterile solutions were added to achieve final concentration;

Glucose	(AnalaR; BDH, Poole, UK)	10 gL^{-1}
$MgSO_4$	(AnalaR; BDH, Poole, UK)	1.0 gL^{-1}

2.1.4 Buffers.

a) P-buffer (Hopwood *et al* 1985).

Sucrose	(AnalaR; BDH, Poole, UK)	103 gL^{-1}
K_2SO_4	(AnalaR; BDH, Poole, UK)	0.25gL^{-1}
$MgCl_2$	(AnalaR; BDH, Poole, UK)	2.02gL^{-1}

Made up to 800mL with distilled water and divided into 80mL aliquots and autoclaved. After autoclaving the following sterile solutions were added to each 80mL aliquot;

0.5%w/v KH_2PO_4	(AnalaR; BDH, Poole, UK)	1mL
3.68%w/v $CaCl_2.5H_2O$	(AnalaR; BDH, Poole, UK)	10mL
5.73%w/v TES pH7.2	(Sigma, UK)	10mL

b) L-buffer (Hopwood *et al* 1985).

The following sterile solutions were added together;

10.3%w/v Sucrose	(AnalaR; BDH, Poole, UK)	100 mL
5.73%w/v TES pH7.2	(Sigma, UK)	10 mL
2.5%w/v K_2SO_4	(AnalaR; BDH, Poole, UK)	1 mL
0.5%w/v KH_2PO_4	(AnalaR; BDH, Poole, UK)	1 mL
2.5M $MgCl_2.6H_2O$	(AnalaR; BDH, Poole, UK)	0.1 mL
1M $CaCl_2$	(AnalaR; BDH, Poole, UK)	0.25mL

Just before use lysozyme was dissolved in the buffer at a concentration of 1mgmL^{-1} and filter sterilized.

c) Lysozyme solution (Hopwood *et al*, 1985).

2 mgmL^{-1}	Lysozyme	(Sigma, UK)
0.3M	Sucrose	(AnalaR; BDH, Poole, UK)
25 mM	TRIS buffer pH8.0	(Sigma, UK)
25 mM	Sodium EDTA pH8.0	(Sigma, UK)
50 µgmL^{-1}	RNAaseI	(Sigma, UK)

d) Buffered phenol/chloroform (Hopwood *et al* 1985).

500 g	Phenol	(AnalaR; Fisons, Loughborough, UK)
30 mL	1M TRIS HCl pH7.4	(Sigma, UK)
60 mL	0.2M Sodium EDTA pH8.0	(Sigma, UK)
15 mL	2M NaOH	(AnalaR; BDH, Poole, UK)
200 mL	Distilled water	

All the ingredients were mixed together, left with occasional mixing until dissolved. The aqueous layer was discarded and the remainder mixed with equal volume chloroform (AnalaR; Fisons, UK).

e) TRIS-EDTA buffer.

10 mM	TRIS-HCl pH7.5	(Sigma, UK)
1 mM	Sodium EDTA pH8.0	(Sigma, UK)

f) TRIS-Borate electrophoresis buffer (x10 concentrate).

108 gL^{-1}	TRIS-Base	(Sigma, UK)
55 gL^{-1}	Boric acid	(AnalaR; Fisons, UK)

After dilution ethidium bromide (Sigma, UK) was added at 50µLL^{-1}.

g) Restriction digest buffer (x10 concentrate).

10 mM	TRIS-HCl pH7.5	(Sigma, UK)
10 mM	$MgCl_2$	(AnalaR; BDH, Poole, UK)
1 M	NaCl	(AnalaR; BDH, Poole, UK)

h) Gran's iodine (reported by Hodgson and Chater, 1981).

50 mM	Iodine	(AnalaR; BDH, Poole, UK)
120 mM	KI	(AnalaR; BDH, Poole, UK)

i) DNaseI storage buffer (modified from Maniatis *et al* (1982)).

50 mM	NaCl	(AnalaR; BDH, Poole, UK)
20 mM	Hepes buffer pH7.9	(Sigma, UK)
5 mM	$MgCl_2$	(AnalaR; BDH, Poole, UK)
5 mM	$CaCl_2$	(AnalaR; BDH, Poole, UK)
20%	Glycerol	(AnalaR, Fisons, Loughborough, UK)

j) Stop mix buffer.

0.1	M	Sodium EDTA pH8.0	(Sigma, UK)
40%		Sucrose	(AnalaR, BDH, Poole, UK)
0.15	$mgmL^{-1}$	Bromophenol blue	(Sigma, UK)

2.1.5 Enzymes.

All restriction enzymes were obtained from Anglian Biotechnology plc, UK. Other enzymes were obtained from Sigma Chemicals Ltd. (UK).

2.1.6 Antibiotics.

Thiostrepton was a gift from E.R. Squibb & Sons Ltd. USA. Novobiocin was obtained from Sigma (Poole, UK).

2.2 Equipment.

2.2.1 Incubator.

A New Brunswick Scientific (New Brunswick Scientific Ltd., New Jersey, USA) orbital shaking incubator was used throughout. It was set at 28°C and 200rpm shaking with a 2 inch throw.

2.2.2 Fermentation vessel.

All stirred vessel fermentations were conducted in a 7L capacity (5L working volume) LH 2000 series fermenter (LH Fermentation, Stoke Poges, UK). The fermenter was fitted with two Rushton turbines and was used with LH 3000i series instrumentation. The pH and dissolved oxygen tension were measured with steam sterilizable Ingold electrodes (Ingold, Switzerland). Data logging from the fermenter was carried out using BIOi software package (Biotechnology Computer Systems Ltd, UK) run on a Micro Vax computer (Digital Equipment Corporation, Maynard, MA, USA).

2.2.3 Gas analysis.

Fermenter inlet and outlet gases were analysed by mass spectrometry (VG Gas Analysis Ltd, UK, model MMG-80). Data from the mass spectrometer was also logged using BIOi software.

2.2.4 Ultraviolet/visible spectrophotometry.

A Perkin-Elmer (Perkin-Elmer, UK) 124 double beam spectrophotometer was used for all tyrosinase enzyme assays, deoxyribose assays, protein assays and DNA quantitation. Where necessary temperature control was achieved by circulating water from a water bath through the chamber.

2.2.5 Gel electrophoresis.

Agarose gels were cast in glass plates (150mm x 150mm) and were run in home-made electrophoresis tanks.

2.2.6 Photography.

Ethidium bromide stained agarose gels were illuminated under short wavelength (λ 302nm) ultraviolet light using an Ultraviolet Products Inc. (California, USA) transilluminator. Gels were photographed with a Polaroid MP4 Land camera and Ilford (Ilford, UK) HP5 film. The film was developed with Ilford Microphen developer and Ilford Microphen fixer.

2.2.7 Scanning densitometry.

All photographic negatives were scanned with a Joyce-Loebl Chromoscan 3 (Joyce-Loebl, UK) fitted with a 0.1mm diameter aperture and 530nm filter.

2.3 METHODS.

2.3.1 Preparation of spores.

Spores of *S. lividans* strains were prepared by inoculating liquid medium-grown cultures onto modified R2YE agar (Section 2.1.3.1) with added thiostrepton at 50μgmL^{-1} (stock solution 50mgmL^{-1} thiostrepton in dimethylsulphoxide) for recombinant strains. Agar cultures were incubated at 28°C until good sporulation had occurred (3-5 days). Release of spores from aerial hyphae was achieved by gentle scraping with a sterile loop and were suspended in 20% glycerol (Fisons, UK) and 0.1% Tween 80 (Polyoxyethylene sorbitan mono-oleate, Sigma, UK) solution. Spores from cultures of each strain were pooled and aseptically aliquotted into 1mL amounts in sterile Eppendorf tubes. Spore suspensions were kept at -20°C until required.

2.3.2 Shake-flask cultures.

Liquid cultures were either grown in 250mL Erlenmeyer flasks containing 50mL of medium or 2L Erlenmeyer flasks containing 250 or 500mL of medium. All flasks were plugged with a foam bung to allow aeration. To initiate growth 0.2 mL (into 50mL medium) or 1mL thawed spore suspension was used to inoculate sterile medium in flasks. Thiostrepton was added at 5μgmL^{-1} to recombinant srains when used as seed cultures. Flasks were then placed in an orbital shaker (Section 2.2.1) set at 28°C temperature and 200rpm shaking.

2.3.3 Stirred vessel fermentations.

The 5L vessel described in Section 2.2.2 was filled with 5L of

MEP medium (Section 2.1.3.2) with added 10mM K_2HPO_4 where necessary and 0.1mLL^{-1} of antifoam (Polypropylene glycol, Type 2025, BDH, UK). The medium was sterilised *in situ* for 20 min at 121°C, 15psig. After cooling the instruments were allowed to equilibrate. A sterile air supply was connected and air flow rate set at 2Lmin^{-1} (0.4 vvm^{-1}) except where low dissolved oxygen tension was needed and a nitrogen cylinder (O_2-free nitrogen, BOC, UK) was connected to the air supply. The agitation rate was set at 1000rpm and culture temperature controlled at 28°C. An alkali reservoir of 4M NaOH (BDH, Poole, UK) permitted pH control during the first phase of growth while the pH was allowed to rise towards the end of the fermentation. All fermentation parameters and exhaust gas compositions were logged using BIOi software.

Fermentation experiments were initiated by aseptically inoculating the vessel with 6%v/v culture grown for 3 days in 2x250mL MEP medium (with thiostrepton at 5μgmL^{-1} where necessary) as described in Section 2.3.2.

2.3.4 Measurement of dry cell weight.

Aliquots of 5mL or 10mL mycelium were suction filtered through pre-weighed and dried Millipore AP5 pre-filters (Millipore, UK) using a Buchner funnel. The mycelium was then dried in a domestic microwave oven (Toshiba, Japan) at maximum setting for 15 min (*i.e.* until constant weight). The filters were then re-weighed and the dry cell mass determined as gL^{-1}. The accuracy of the method was investigated and is described in APPENDIX 1.

2.3.5.1 Harvesting of biomass.

Aliquots of up to 50mL culture were harvested by centrifugation in a bench centrifuge (MSE Minor, UK) using a swing-out rotor. The speed was set at maximum (1000g) for 10min. The supernantant was discarded and the mycelia were stored at -20°C until required for extractions.

2.3.5.2 Preparation of protoplasts.

Streptomyces protoplasts were prepared as described by Hopwood *et al* (1985c) and stored at -70°C.

2.3.6 DNA purification methods and plasmid transformation.

2.3.6.1 Total DNA mini-preparation method.

Approximately 100mg wet weight thawed mycelium was used as material for the Hopwood *et al* (1985c) procedure 4 protocol. The method was

applied as described in the reference but with the addition of more phenol/chloroform extraction steps to remove all protein. Also the propan-2-ol and spermine precipitation steps were conducted on ice rather than at room temperture.

This method was also scaled-up 10 times and DNA preparations were further purified to separate supercoiled covalently closed circular DNA (cccDNA) molecules on a caesium chloride (CsCl)-ethidium bromide (EtBr) density gradient. 5.5g of caesium chloride (AnalaR; Poole, UK) was dissolved in the resuspended DNA yielding a total volume of 5mL suspension and 200µL of 10mgmL^{-1} ethidium bromide (Sigma, Poole, UK) was also added. The suspension was placed in a Beckman quickseal tube and balanced. A Beckman Ti70.1 rotor was used for ultracentrifugation at 50000rpm at 20°C for 18h. After illumination with UV light the lower (SC plasmid) fluorescent band was removed with a syringe. The EtBr was extracted by adding equal volume propan-2-ol saturated with CsCl (10g CsCl added to 10mL water and 100mL propan-2-ol). This was repeated until no further EtBr was extracted. The DNA was salt precipitated after making up to 1mL and adding 1/10 volume 4M Na acetate and 2 volumes ethanol. Precipitation was conducted at -20°C for 20min followed by centrifugation in a bench centrifuge for 10min at maximum setting. Final resuspension was made in 200µL.

2.3.6.2 Plasmid DNA mini-preparation method.

Approximately 100mg wet weight thawed mycelium was used as material for the Kieser (1984) protocol. Propan-2-ol precipitation and spermine precipitation steps were conducted on ice. For larger quantities of plasmid DNA the method was scaled up 10 times and further purified by bouyant density centrifugation as described above.

2.3.6.3 Transformation of protoplasts.

Plasmid DNA was introduced into protoplasts and cultures re-generated as described by Hopwood *et al* (1985c) using the rapid small scale procedure.

2.3.7 Restriction enzyme digests.

Digests were conducted at 50°C for *Bcl*I enzyme and at 37°C for *Bgl*II and *Pst*I enzyme. Restriction buffer (Section 2.1.4) was added at 1/10 volume to all digests. 10 units of restriction enzyme were used for small-scale digests while 50 units were used in bulk digests. Reactions were

stopped by adding 1/2 volume stop mix (Section 2.1.4 (j)).

2.3.8 Assessment of DNA concentration.

DNA concentration in preparations were determined by measuring the absorbance at 260nm in a spectrophotometer. It was assumed that 50μgmL^{-1} double-stranded DNA gives an absorbance reading of 1 at 260nm (Maniatis *et al*, 1982). The purity of DNA preparations was determined by measuring the absorbance at 260nm and 280nm. The ratio gave an indication of the DNA preparation's purity. Typically ratios >1.1 were obtained which suggests the presence of some contaminating material since pure DNA yields a ratio of 1.8 (Maniatis *et al*, 1982).

2.3.9 Agarose gel electrophoresis.

Gel electrophoresis was performed using 0.7%w/v (some early gels were run using 1%w/v) agarose (Park Scientific Ltd., Northampton, UK) dissolved by boiling in 0.09M Tris-Base (Sigms, UK) 0.09M boric acid (FSA, Loughborough, UK) buffer with EtBr at $0.5mgL^{-1}$. DNA samples were loaded using 1/2 volume stop mix buffer (Section 2.1.4 (j)). The electrophoresis running buffer was the same. Early gels were run at $1.7Vcm^{-1}$ ("slow") for 16h (see Section 3.1.2) subsequent electrophoresis, for plasmid copy number determination, was conducted at $10Vcm^{-1}$ ("fast") for 2-3h.

Note: No EDTA (ethylenediaminetetra-acetic acid) was used due to smearing effects of chromosomal DNA upon electrophoresis experienced during this work and also reported by Zhou *et al* (1988).

2.3.10 Gel photography.

After electrophoresis the gels were visualised by illumination with UV light at 302nm using a transilluminator (Ultraviolet Products Inc., California, USA) and photographed. A Polaroid MP4 land camera was used for photography with Ilford HP5 film (Section 2.2.6). The aperture was set at 4.5 and exposure time was 15s using a red filter to eliminate background fluorecsence.

2.3.11 Densitometric scanning.

Photographic negatives were scanned with a scanning densitometer (Chromoscan 3) yielding a trace revealing peaks corresponding to the darkness of individual DNA bands. A built-in integrator also calculated the peak areas which were re-calculated as necessary to eliminate background fluorescence. Re-calculation was done by assuming 12 arbitrary Chromoscan

area units=1mm^2. The densitometric scanning method is essentially that of Projan *et al* (1983). In order to convert DNA length to mass it was assumed that 1kb=0.64mega daltons and *S. lividans* chromosomal size was assumed to be 6.9x10^6 daltons (Benigni *et al*, 1975). The plasmid size correction factors for copy number calculations were as follows;
pIJ303=968, pIJ702=1783, pMT605=856 and pMT608=1700.

2.3.12 Deoxyribose assessment method.

Deoxyribose was extracted using perchloric acid as described by Hanson and Phillips (1981) which is a modification of the original Burton (1956) method. The assay is colorimetric and depends on the reaction of deoxyribose and diphenylamine reagent. Stored mycelium was thawed and filtered before weighing a 0.2g amount of biomass which was resuspended in buffer and acidified with 2.5M perchloric acid (60%v/v BDH, UK). The protocol was followed as described in the reference. Pure deoxyribose (Sigma, Poole, UK) was used to construct a standard curve as shown in Figure 1 and DNA concentration was determined as deoxyribose equivalents. The accuracy of the deoxyribose assay was investigated and is described in APPENDIX 2.

2.3.13 Tyrosinase enzyme assays.

Intracellular extracts were obtained by ultrasonicating 1g wet weight mycelium suspended in 10mL 0.05M sodium phosphate buffer (made up according to Dawson *et al* (1986). Sonication was conducted for 2min with 10s on 10s off cycles at 14μm amplitude (Soniprep 150, MSE). Sonicates were centrifuged at 20000g, 4°C in a Sorvall centrifuge using a SS34 rotor.

Tyrosinase enzyme activity was measured in the supernatant using the dopachrome method of Lerch and Ettlinger (1972) with L-dihydroxyphenylalanine (L-DOPA, Sigma, UK) as substrate. 7mM L-DOPA was dissolved in 0.1M sodium phosphate buffer pH6.0 (made up according to Dawson *et al*, 1986). Dopachrome formation was followed at 30°C in a Perkin-Elmer 124 spectrophotometer fitted with a Perkin-Elmer 56 chart recorder. All absorbance readings were taken at 475nm wavelength. Disposable cuvettes (Sarstedt, W. Germany) were used with a total reaction mixture volume of 2mL. One unit of tyrosinase enzyme activity is defined as the amount of enzyme that catalyses the oxidation of 1μmol L-DOPA min^{-1} (Katz *et al*, 1983). The molar extinction co-efficient for the reaction at 475nm is 3600 (Katz *et al*, 1983). Tyrosinase enzyme activity was calculated thus;

$$\frac{\Delta \text{ Absorbance } 475\text{nm}}{3600} = x \text{ moles}$$

$$\frac{x \text{ moles}}{\text{time (min)}} = x \text{ moles formed } \text{min}^{-1}$$

1 unit enzyme activity = μ mol min^{-1}

Specific enzyme activity is defined as units of activity per mg protein.

2.3.14 Total protein assays.

Protein concentration was determined in intracellular extracts using the Bio-Rad protein assay dye reagent concentrate (Bio-Rad Lab, USA) according to the manufacturers' instructions. The method is based on that of Bradford (1976). Bovine serum albumin (Sigma, UK) was used to construct a standard curve (refer to Figure 2) and all readings were taken at 600nm wavelength.

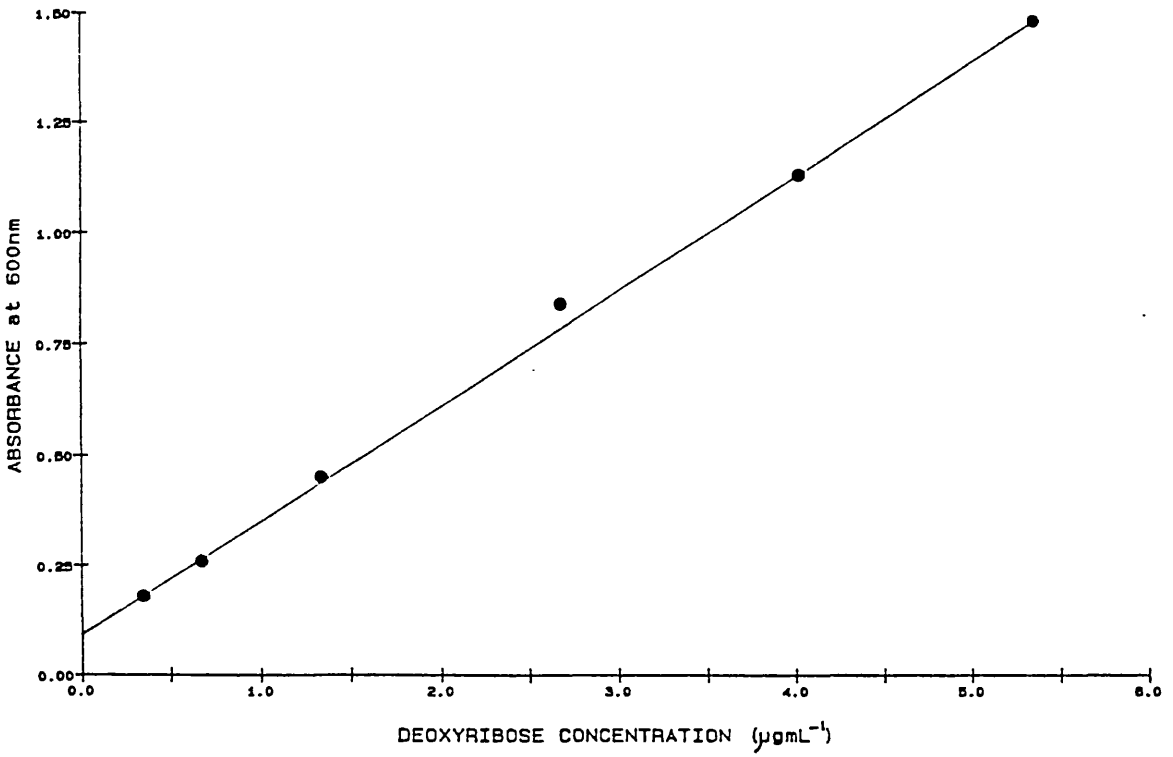

Figure 1 An example of a deoxyribose standard curve used for deoxyribose concentration determination according to Burton (1956) method as reported by Hanson and Phillips (1981).

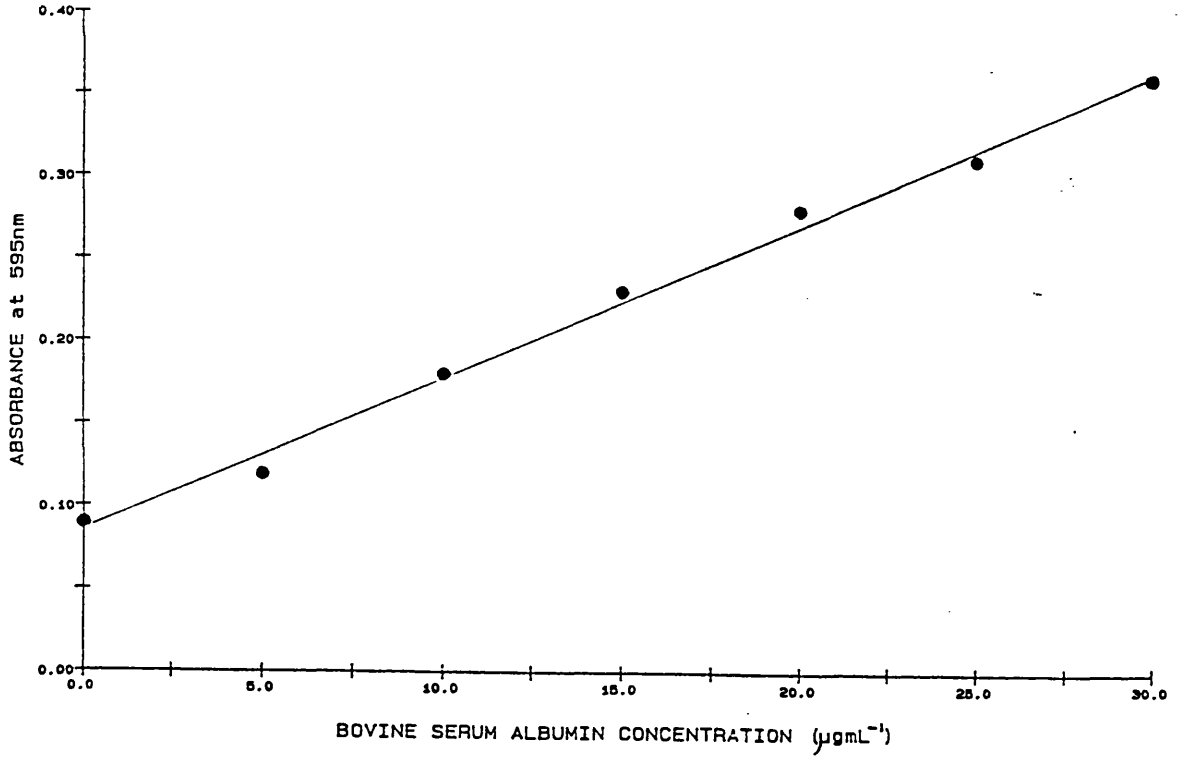

Figure 2 An example of a bovine serum albumin standard curve used for total protein concentration determination according to Bradford (1976) method.

3 Results.

3.1 Development of the plasmid copy number assessment and structural stability methods.

3.1.1 Linearity of fluorescence and film response to increasing DNA level per band.

3.1.1.1 Aim.

The aim of this experiment was to establish the range of DNA levels per band which yielded a linear relationship when plotted against Chromoscan peak integral. It is assumed that the plasmid DNA used in these experiments is representative of plasmid molecules of similar size.

3.1.1.2 Protocol.

Plasmid DNA was obtained as described in Section 2.3.6.2 using the large-scale method. The concentration of DNA in the suspension was assessed as described in Section 2.3.8. Bulk restriction digests were performed using 50 units of *Bgl*II , 200-300µL (containing 16µg to 25µg) of plasmid DNA and 1/10 volume of 10x restriction buffer. Incubation was performed at 37°C for 3h to ensure complete digestion of plasmid DNA.

Chromosomal DNA was obtained as described in Section 2.3.6.1 using plasmidless *S. lividans* TK24 mycelium as the source. The concentration of DNA was assessed as described in Section 2.3.8.

Aliquots containing increasing amounts of linearized plasmid DNA or uncut chromosomal DNA were loaded onto agarose gels and subjected to electrophoresis as described in Section 2.3.9. The gels were subsequently photographed (Section 2.3.10) and scanned (Section 2.3.11).

3.1.1.3 Results and discussion.

The dilution series of linearized pMT605 plasmid DNA is shown in gel photograph Figure 3 and the dilution series of TK24 chromosomal DNA is shown in gel photograph Figure 4. The curves obtained by plotting the peak integral obtained from the Chromoscan (similar to that shown in Figure 8) versus amount of DNA (µg) per band are shown in Figure 5 and Figure 6.

The results of a series of gels with DNA at various levels revealed that a linear relationship exists between the peak integral and the level of plasmid or chromosomal DNA in the approximate range 0.15µg to 3µg DNA per band. Results from gels where higher concentrations were loaded showed that the linear relationship breaks down at levels of DNA (regardless of source) higher than 3.5µg per band. It was interesting to

Figure 3 Photograph of gel where pMT605 plasmid was digested with *BglII* and different aliquots of DNA loaded into each lane. Amount of DNA in each lane were as follows;
Ln1=0.17μg, Ln2=0.33μg, Ln3=0.50μg, Ln4=0.66μg, Ln5=0.83μg, Ln6=1.0μg

Figure 4 Photograph of gel where different aliquots of TK24 chromosomal DNA was loaded into each lane as follows;
Ln1=2.85μg, Ln2=2.45μg, Ln3=2.04μg, Ln4=1.63μg, Ln5=1.22μg,
Ln6=0.82μg, Ln7=0.41μg

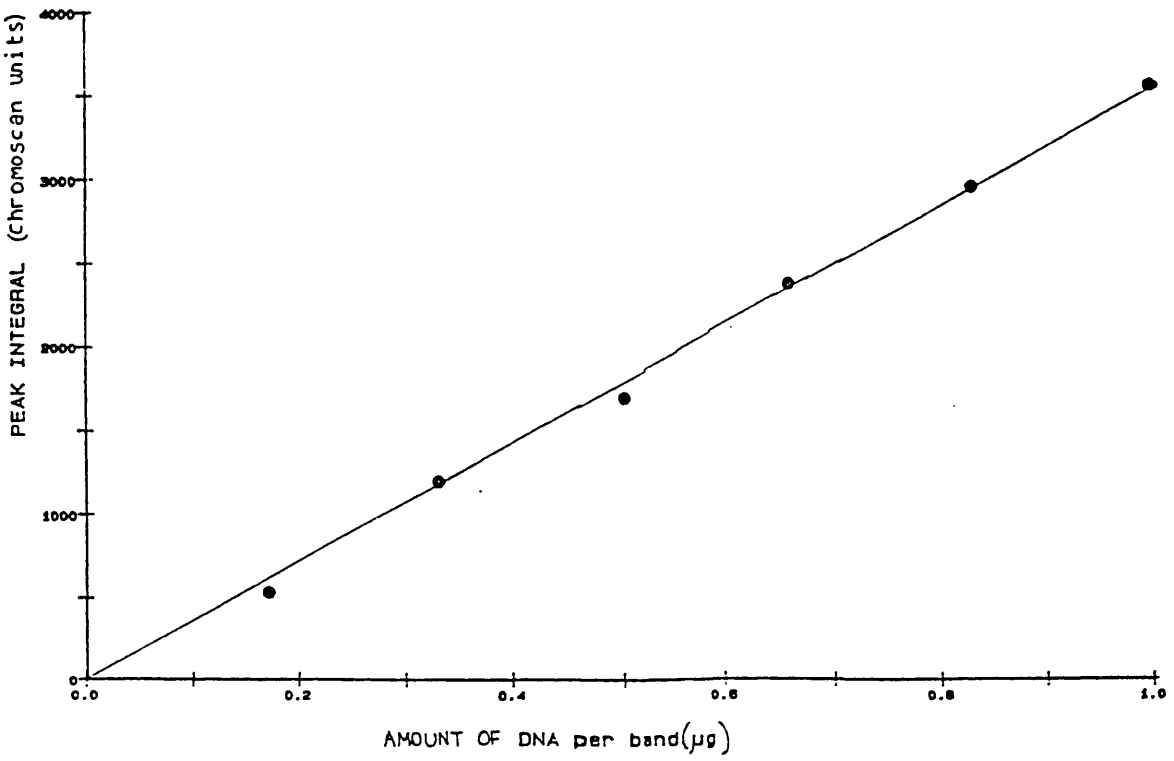

Figure 5 Fluorescence intensity (peak integral) results of Figure 3 plotted as a function of amount of linearized pMT605 DNA in each band.

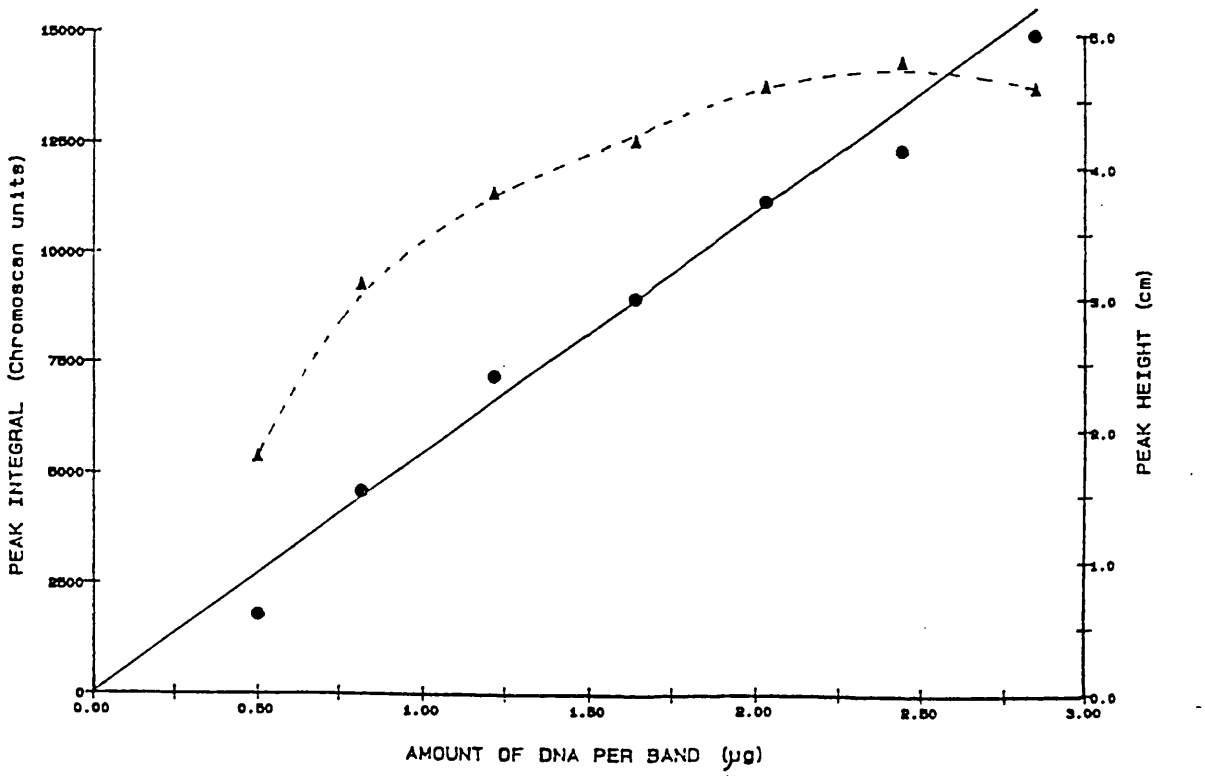

Figure 6 Fluorescence intensity (peak integral, ●) and peak height (▲) results of Figure 4 plotted as a function of amount of *S. lividans* TK24 chromosomal DNA in each band.

note that when peak height was plotted against DNA a plateau is reached at levels above approximately 2-2.5μg DNA (refer to Figure 6). At such levels, therefore, an individual band cannot continue to fluoresce with greater and greater intensity but a broadening of the band is observed. This may be due to DNA saturating the agarose at that particular point in the gel. However, by plotting the integral of the band a linear relationship is maintained.

3.1.2 Accuracy of plasmid copy number assessment by testing with DNA of known concentrations.

3.1.2.1 Aim.

The aim of this experiment was to use separately prepared plasmid DNA and chromosomal DNA of known concentrations to determine the accuracy of plasmid copy number determinations. A range of plasmid copy numbers could be artificially achieved through reconstitution of the two sources of DNA, thereby defining the sensitivity of the method.

3.1.2.2 Protocol.

The procedure for plasmid DNA was the same as described in Section 3.1.1.2.

Chromosomal DNA was obtained as described in Section 2.3.6.1. Different amounts of linearized plasmid DNA were added to a constant amount of TK24 chromosomal DNA, both of known quantity. Electrophoresis, photography and scanning were as described in Sections 2.3.9, 2.3.10 and 2.3.11.

Certain plasmid topoisomeric forms co-migrate with chromosomal DNA under slow electrophoresis conditions (refer to Section 3.1.4). Since it is not always possible to obtain good separation (from chromosomal DNA) of all the plasmid forms which may be present, a novel solution to this problem was investigated. This approach explored the possibility of digesting total DNA extracts in order to linearize plasmids (rather than measure two or more plasmid topoisomers). The contribution of the resulting chromosomal DNA background smear upon copy number calculations was assessed by adding together known amounts of chromosomal and plasmid DNA and then measuring the artificial "copy number".

3.1.2.3 Results.

Gels with different aliquots of bulk-digested linear pMT605 mixed with constant amounts of (non-digested) chromosomal DNA are shown in Figure

7. The Chromoscan traces of lanes from Figure 7 are shown in Figure 8. The calculated "copy number" of lane 1=337, lane 2=306, lane 3=205, lane 4=95. Since the amount of DNA loaded into each lane is known then an expected "copy number" can also be calculated by substituting the precise quantity of DNA, rather than peak integrals, into the plasmid copy number equation (Section 2.3.11). The expected "copy numbers" for lanes 1-4 were 1050, 788, 525 and 263 respectively. The possible reasons for the discrepancy between observed and expected "copy numbers" are discussed in Section 3.1.2.4.

The results of "copy numbers" obtained by mixing linearized pMT608 with known amounts of chromosomal DNA are shown in Table 1. For comparison the expected values for copy number are also shown and the observed value has been expressed as a percentage of the expected "copy number".

Figure 9 shows the results of a reconstitution (digest) gel where plasmid pIJ303 was digested using *Bgl*II, with and without the addition of chromosomal DNA. Various levels of plasmid DNA were added to a fixed amount of chromosomal DNA. "Conventional" reconstituted copy numbers were also obtained by adding non-digested chromosomal DNA in alternate lanes at fixed levels. Digested reconstituted copy numbers were calculated by adding the total sum of chromosome smear peaks to represent the amount of chromosomal DNA in the lane and substitution into the copy number equation.

The Chromoscan tracings of some of the lanes from Figure 9 are shown in Figure 10 where comparable peaks from neighbouring lanes were superimposed. The contribution of the chromosomal background smear at each linear plasmid band position is shown by the height of the double headed arrows. The plasmid "copy numbers" calculated from the results of the gel shown in Figure 9 are listed in Table 2 as is the expected plasmid copy number.

As seen in Table 2 the calculated plasmid copy number using the digested chromosomal DNA yields a value approximately half that obtained from non-digested chromosomal DNA. However, despite the discrepancy the expected downward trend in plasmid copy number was observed as the level of added pIJ303 decreased in relation to both digested and non-digested chromosomal DNA. The results shown in Table 2 are discussed in the following sub-section.

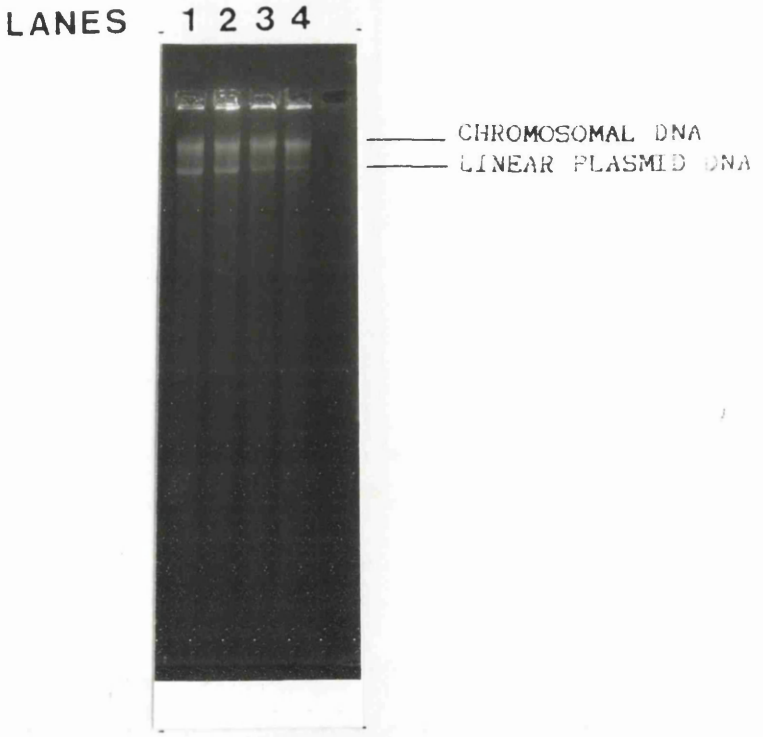

Figure 7 Photograph of gel where aliquots of linearized (*BglII* digestion) pMT605 plasmid were added to constant aliquots of *S. lividans* TK24 chromosomal DNA prior to loading.

Each lane contained the followimg amounts of DNA;

Ln1=1.38µg TK24 DNA + 1.66µg pMT605, Ln2=1.38µg TK24 DNA + 1.23µg pMT605 , Ln3=1.38µg TK24 DNA + 0.83µg pMT605, Ln4=1.38µg TK24 DNA + 0.42µg pMT605

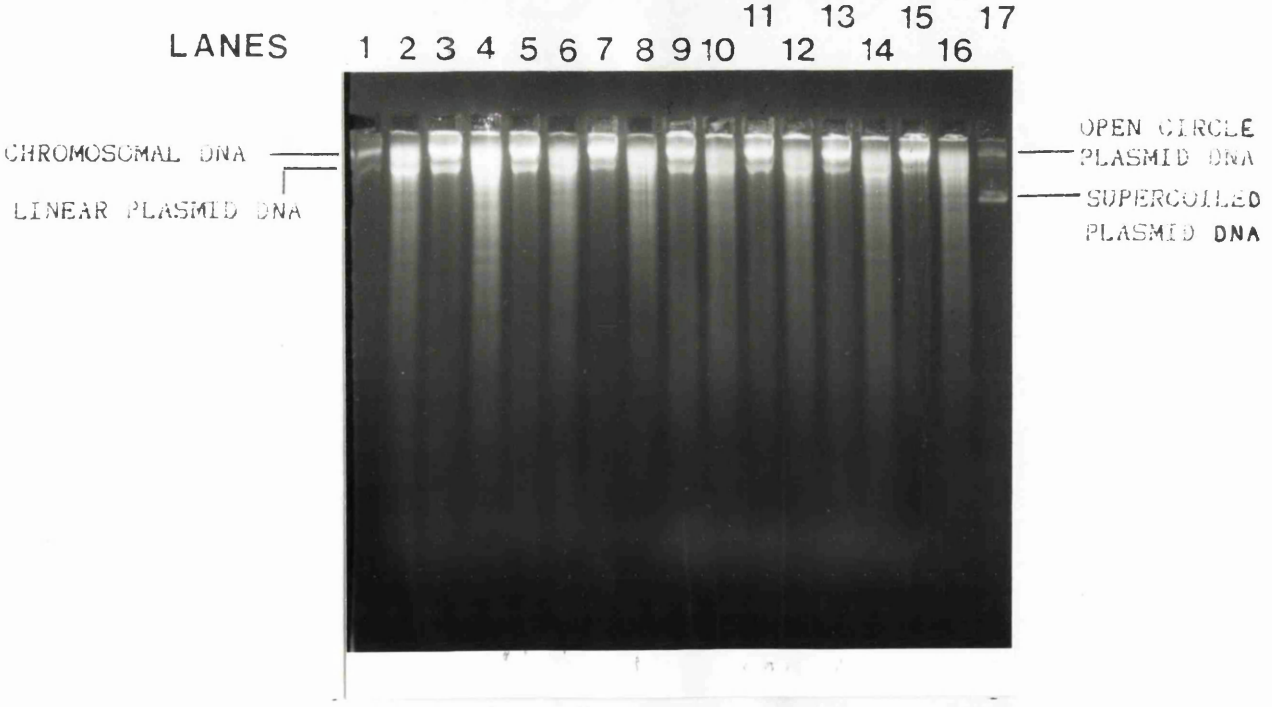

Figure 9 Photograph of gel where pIJ303 plasmid was digested (using *BglII*) with and without chromosomal DNA. Complete chromosomal DNA was added to linearized plasmid prior to loading in alternate lanes. Refer to TABLE 3 for amounts of DNA's in each lane. Lane 17 contains pure, non-digested pIJ303 plasmid.

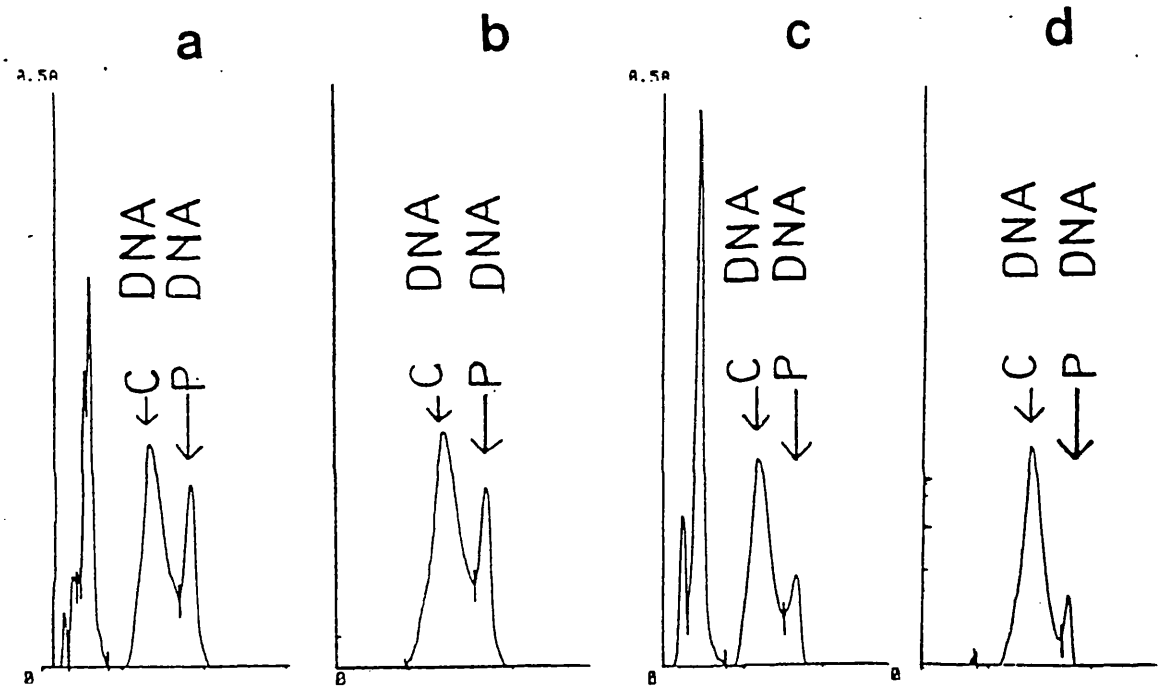

Figure 8 Chromoscan traces of lanes 1-4 of gel in Figure (corresponding to a, b, c, and d here) where different aliquots of linearized pMT605 DNA were added to constant aliquots of *S. lividans* TK24 chromosomal DNA.

TABLE 1 Results of reconstitution gel where varying quantities of linearized pMT608 DNA were added to fixed amounts of chromosomal DNA.

pMT608 DNA per lane(μg)	Chromosomal DNA per lane (μg)	Observed copy no.	Expected copy no.	Observed % Expected
0.44	1.58	124	478	26%
0.56	1.58	160	597	27%
0.67	1.58	188	717	26%
0.78	1.58	211	836	25%
0.89	1.58	239	955	25%
1.11	1.58	247	1194	21%
1.33	1.58	287	1433	20%
1.67	1.58	427	1791	24%
2.22	1.58	494	2389	21%

TABLE 2 Results of reconstitution (digest) gel where varying quantities of pIJ303 DNA were digested with or without fixed amounts of chromosomal DNA.

Lane no. of Figure 9	pIJ303 DNA per lane (μg)	Chromosomal DNA per lane (μg)	Observed copy number	Expected copy number
3	0.6	1.2 (undigested=u)	481	484
4	0.6	1.2 (digested=d)	156	484
5	0.48	1.2 (u)	418	387
6	0.48	1.2 (d)	201	387
7	0.24	1.2 (u)	313	194
8	0.24	1.2 (d)	162	194
9	0.72	1.2 (u)	566	581
10	0.72	1.2 (d)	283	581
11	0.6	1.2 (u)	391	484
12	0.6	1.2 (d)	216	484
13	0.48	1.2 (u)	332	387
14	0.48	1.2 (d)	156	387

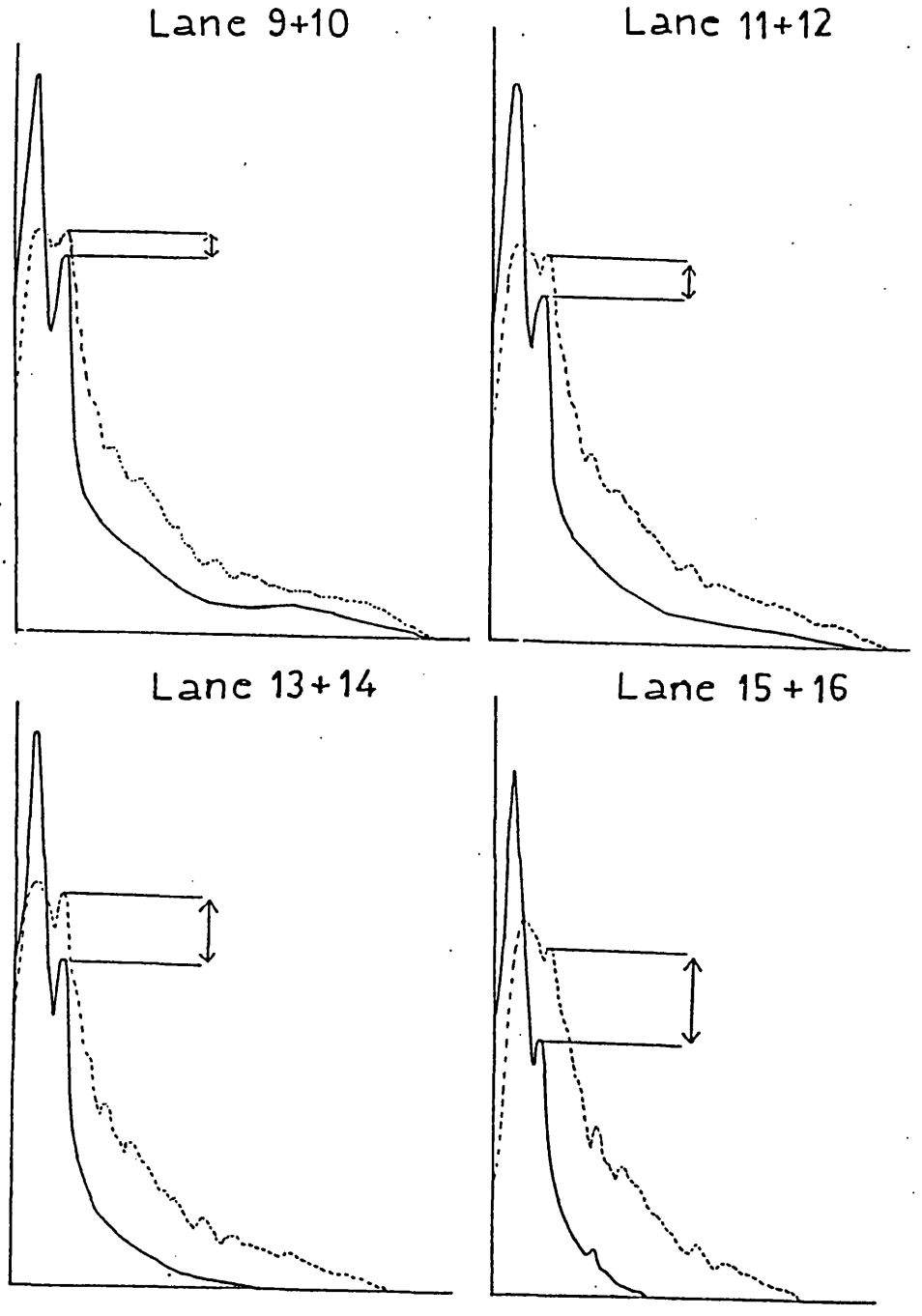

Figure 10 Superimposed chromoscan traces of lanes 9+10, 11+12, 13+14 and 15+16 of gel in Figure 9

KEY

Lanes 9, 11, 13 and 15 =

Lanes 10, 12, 14 and 16 =

3.1.2.4 Discussion of reconstitution gel results.

The results described in the previous sections show that an expected fall in plasmid copy number was obtained when decreasing levels of linearized plasmid DNA were mixed with chromosomal DNA. The difference between observed and expected plasmid copy number using certain plasmid DNA extracts (*i.e.* pMT605 or pMT608) is considerable (approximately 4-fold). When expressed as a ratio of observed/expected the discrepancy was fairly constant at 20-27% (see Table 1). The reason for this could be that the use of absorbance readings to quantitate DNA is unreliable. The OD_{260} to OD_{280} ratios of the DNA preparations used in this experiment were approximately 1.2. According to Maniatis *et al* (1982) a pure DNA extract gives a ratio of about 1.8, therefore the preparations used here contained a proportion of contaminating material. A large amount of small molecular weight DNA, nucleotides or other contaminants may have led to an overestimate of the concentration. The actual concentration of DNA particularly in the plasmid preparation, was therefore probably less than that suggested by absorbance readings. Attempts to obtain more pure DNA by gel filtration through liquid chromatography columns failed to give good yields.

There was less discrepancy between the observed and expected plasmid copy number results of undigested lanes shown in Table 2. However, there were considerable differences between the copy number values measured from parallel digested and undigested lanes. Digested lanes yielded values which were consistently less than their neighbouring undigested lane. The reason for this is probably that the sum of the chromosomal DNA peaks resulting from the digested DNA smear is greater than the area of a single (undigested) chromosomal DNA peak. This consequently results in an artificially low copy number value. Since the standard (undigested) method of plasmid copy number assessment yielded consistently more accurate values (as shown in Table 2), the novel (digested) method was abandoned.

The assessment of DNA concentration in the pIJ303 plasmid extract used to generate the data shown in Table 2 was probably more accurate than that of either pMT605 or pMT608 extracts used for the results shown in Figure 7, Figure 8 and Table 1. The reason for this may have been that less contaminating material was present in this DNA extract. Another reason for pMT605 and pMT608 reconstitution results being widely different between expected and observed copy number values was that fluorescent material

between bands was assessed as part of the chromosomal DNA peak since the scanner "perceived" it to belong to that band (refer to Figure 8). Some manual re-calculation is needed where fluorescent material of unknown origin is present so that it contributes neither to chromosomal nor plasmid DNA peak integrals. For future calculations therefore, the extent of the bands visible on photographs was correlated to the corresponding Chromoscan peak traces and some peak integrals were re-calculated as necessary (refer to Section 2.3.11) in order to eliminate contributions by fluorescent material of unkown source.

3.1.3 Statistical analysis of within total DNA extract and between total DNA extracts variation in plasmid copy number determination.

3.1.3.1 Aim

The aim of this experiment was to define the accuracy of the plasmid copy number assessment method by calculating the errors between gel lanes of the same extract and between parallel extracts of the same culture at a single point during growth.

3.1.3.2 Protocol

Total DNA was prepared as described in Section 2.3.6.1, electrophoresis and photography were performed as described in Sections 2.3.9, 2.3.10 and 2.3.11. Aliquots of the same total DNA extract were run in parallel lanes in order to assess the errors in the running of gels *i.e.* within the extract. Mycelium samples from one culture at one time point were processed in parallel to extract total DNA in order to assess the errors inherent in the sampling and preparation of DNA. Both the undigested total DNA and digested total DNA methods of plasmid copy number estimation were assessed for relative accuracy. Statistical analysis was conducted as described by Parker (1979), method for small samples.

3.1.3.3 Results and discussion.

The results of plasmid copy number estimations of 9 parallel lanes of a single total DNA extract using *S. lividans* TK24 pIJ303 mycelium were as follows;

218, 220, 236, 227, 164, 221, 180, 232 and 174.

The sample mean was 208 and the standard deviation 27.4. The confidence limits of the mean was calculated to be 21 (95% confidence limits with 8 degrees of freedom, calculated according to Parker, 1979).

The accuracy of plasmid copy number values from a single extract was therefore within 10% of the mean.

The results of plasmid copy number estimations from samples of *S. lividans* Tk24 pIJ303 extracted in parallel are shown in Table 3. The mean of the undigested plasmid copy numbers was 497 and 403 for the digested lanes. The confidence limits of the mean were 50.2 (or 10%) for the undigested lane values and 75.7 (or 19%) for the digested lane values.

The standard method of assessing the plasmid copy number by densitometric scanning of total DNA extracts was found to be accurate to within approximately 20% (since within-extract and between-extracts variation is cumulative). However, the novel proposal of linearizing plasmid DNA molecules in total DNA so as to obtain a single plasmid band, was not as accurate as the standard (non restriction digest) method. For this reason the plasmid copy number data presented in this work were calculated via the standard method. It should be borne in mind that open circle plasmid molecules of a certain size (*e.g.* pIJ303 and pMT605) often co-migrate with chromosomal DNA during "slow" electrophoresis and hence remain hidden and were excluded from some early calculations. Therefore for this reason the "fast" method of total DNA electrophoresis was adopted since this helps separate certain plasmid topoisomers away from chromosomal DNA bands (refer to Section 3.1.4). Altering electrophoresis conditions partly overcame these problems but the novel approach of plasmid linearization was not only less accurate but would not reveal relative changes in the proportion of plasmid topoisomers which have since been noted (refer to Section 3.6.3).

3.1.4 Identification of different plasmid topoisomeric forms in DNA extracts.

3.1.4.1 Aim.

The aim of this series of experiments was to identify, with a fair degree of certainty, which plasmid bands were the open circle and supercoiled plasmid topoisomeric forms. Also it was intended to discern whether dimers, as well as monomers, were present and to identify different dimer topoisomers. The experiments were designed to utilize the DNA nicking activity of bovine pancreatic DNaseI enzyme resulting in a high proportion of nicked (open circle) plasmid DNA. The relative increase or decrease in

TABLE 3 Plasmid copy number estimations of different extracts of the same mycelial culture using undigested and digested total DNA methods.

Extract number	Plasmid copy number (from undigested lanes)	Plasmid copy number (from digested lanes)
1	505	281
2	503	-
3	528	232
4	591	353
5	537	345
6	492	341
7	614	347
8	532	291
9	557	348
10	413	326
11	462	589
12	515	635
13	584	-
14	-	677
15	-	517
16	-	220
17	308	518
18	316	422

intensity of the various plasmid bands will help to identify them. Supercoiled plasmid bands are expected to disappear as progressively more molecules are nicked and open circle plasmid bands will increase in intensity.

3.1.4.2 Protocol.

pIJ303 and pIJ702 plasmid DNA was prepared as described in Section 2.3.6.2 using the large-scale method. Timecourse nicking experiments were intiated by adding 0.5pg of DNaseI (bovine pancreatic, Sigma, Poole, UK), kept as a $1mgmL^{-1}$ stock solution in storage buffer (Section 2.1.4(i)) before use. DNaseI was added to approximately 25µg of plasmid DNA. The reaction was allowed to proceed at 37°C and aliquots were removed from time zero onwards at one minute intervals. The reaction was stopped by adding stop mix (Section 2.1.4 (j)) to each aliquot which was then loaded into 0.7%w/w agarose gel and electrophoresis conducted as described in Section 2.3.9. Electrophoresis was conducted slowly for a period of 16h since this resulted in gels that were easier to interpret because of better band separation. Photography was conducted as described in Section 2.3.10 and restriction enzyme digests were conducted as described in Section 2.3.7 using *Bgl*II.

3.1.4.3 Results and discussion.

The results of the DNaseI nicking timecourse experiment of pIJ303 plasmid are shown in Figure 11 (refer to Figures 14 and 15 for DNA marker sizes). As seen from the gel the fastest migrating band is seen to disappear as does the second fastest migrating band. This suggests that they are both supercoiled plasmid molecules with the monomeric form migrating furthest. The two slowest migrating bands are seen to increase in intensity and are probably the open circle monomeric and dimeric forms. A faint band was seen between the open circle and supercoiled forms and was most likely to be linear plasmid DNA since it migrates just behind the 9.416kb λ *Hind*III fragment (pIJ303 is 10.69kb). The linear plasmid was probably generated by DNaseI nicks occurring very close on opposite DNA strands.

The results of the DNaseI nicking timecourse experiment of pIJ702 plasmid DNA are shown in Figure 12. Again, the fastest migrating band was seen to diminish (indicating that it is supercoiled DNA) while the slower migrating band was seen to increase in intensity (indicating that it is

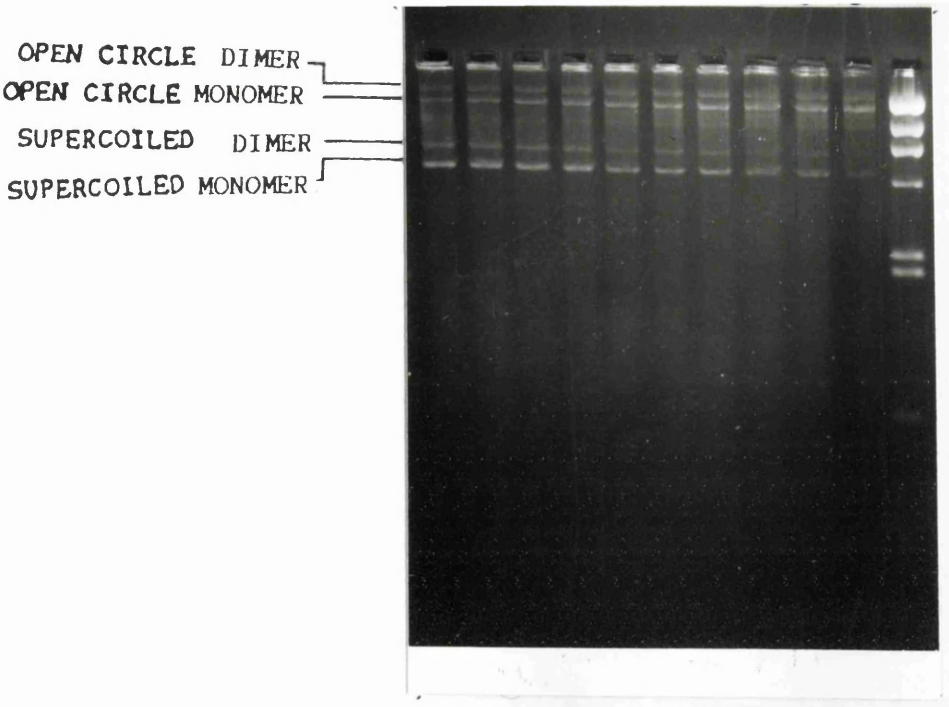

Figure 11 Photograph of gel showing the progress of pIJ303 plasmid DNA nicking by DNaseI enzyme after different periods of time. All aliquots 25µL volume. Ln1= 0 min, Ln2= 1 min, Ln3= 2 min, Ln4= 3 min, Ln5= 4 min, Ln6= 5 min, Ln7= 6 min, Ln8= 7 min, Ln9= 8 min, Ln10= 20 min, Ln11= λ*Hind*III size marker.

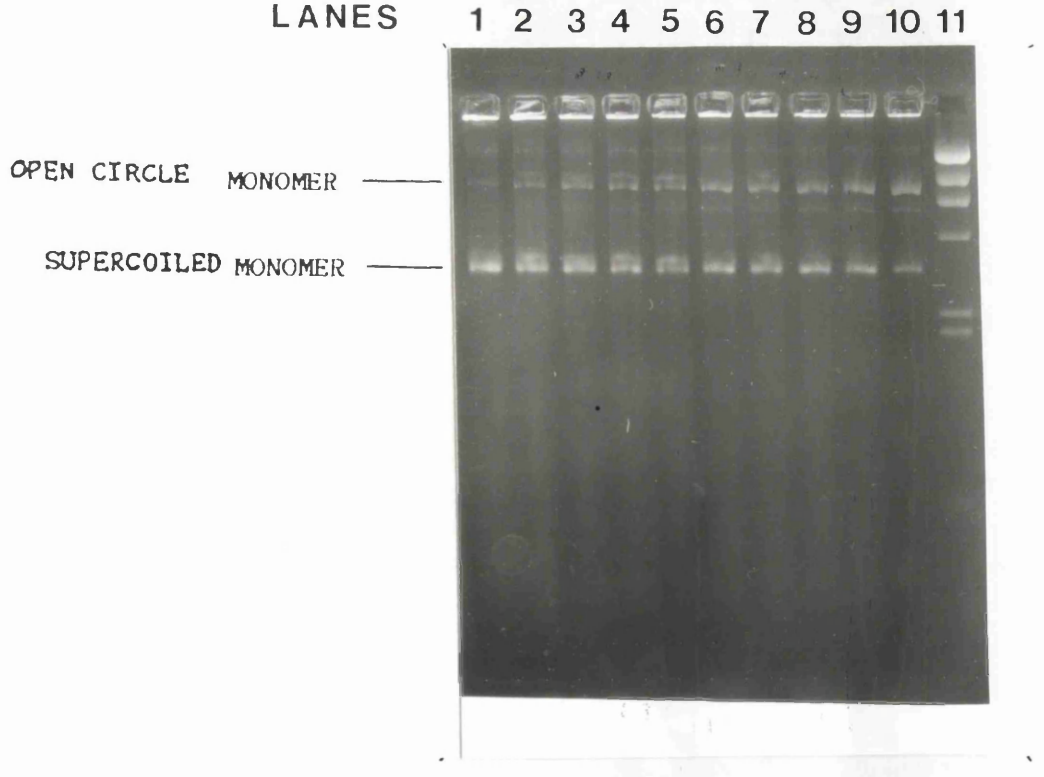

Figure 12 Photograph of gel showing the progress of pIJ702 plasmid DNA nicking by DNaseI enzyme after different periods of time. All aliquots 25µL volume. Ln1= 0 min, Ln2= 1 min, Ln3= 2 min, Ln4= 3 min, Ln5= 4 min, Ln6= 5 min, Ln7= 6 min, Ln8= 7 min, Ln9= 8 min, Ln10= 20 min, Ln11= λ*Hind*III size marker.

probably open circle DNA). Only faint pIJ702 open circle dimeric forms were visible. Again a faint pIJ702 linear plasmid band is seen migrating just ahead of the 6.56kb λ *Hind*III fragment (pIJ702 is 5.6kb).

In order to compare the relative band positions of undigested and restriction enzyme digested plasmid DNA with those of total DNA such extracts are shown in Figure 13. As seen in the photograph, open circle plasmid DNA migrates slower than linear plasmid DNA while supercoiled DNA migrates faster. The additional band in Lane 2 is a partially digested plasmid of unknown conformation. When comparing total *S. lividans* TK24 pIJ303 DNA extracts with pure pIJ303 DNA it may be noted that under these electrophoresis conditions the open circle plasmid migrates ahead of chromosomal DNA. For convenience it was decided to employ fast 0.7%w/v gel electrophoresis conditions in further calculations (refer to Section 2.3.9).

When total TK24 pIJ702 DNA extracts were compared with pure pIJ702 DNA it was seen that under slow electrophoresis conditions open circle plasmid migrates ahead of chromosomal DNA. The open circle band of pIJ702 was very faint and hence fast gel electrophoresis conditions were also used for plasmid copy number determinations of pIJ702 since this protocol improved the speed of the method.

All future plasmid band identifications were based on the assumptions made here *i.e.* under "fast" gel conditions open circle forms were seen to migrate behind chromosomal DNA while supercoiled plasmid DNA migrates ahead.

3.1.5 Assessment of plasmid structural stability by restriction fragment analysis.

3.1.5.1 Aim.

Structural re-arrangements during the cultivation of recombinant cultures can be a problem as discussed in Section 1.10.1. In order to check upon the structural integrity of the plasmid vectors used in this project a method of restriction fragment analysis was employed. The size and number of restriction fragments derived from plasmid DNA isolated at the beginning of an experiment were compared with those from a sample taken near the end. It was anticipated that any change in size and number of fragments could thus be detected thereby indicating structural re-arrangements.

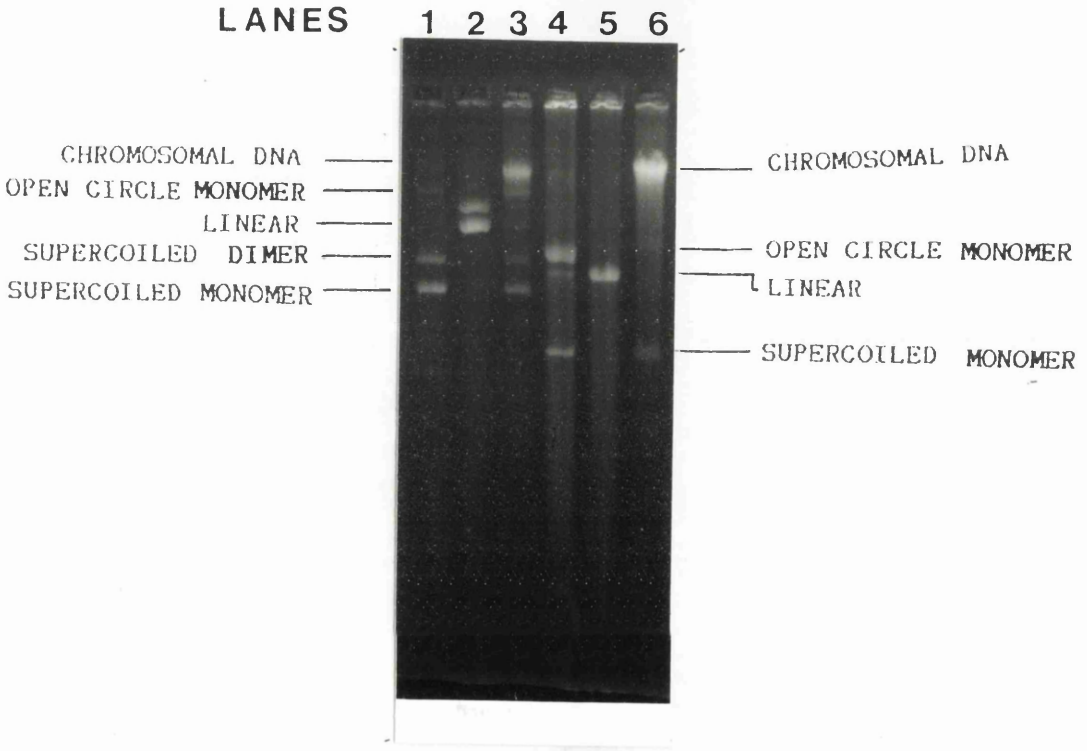

Figure 13 Photograph of gel comparing pure plasmid DNA (uncut), plasmid DNA (cut), total DNA. Slow electrophoresis conditions.
Ln1= uncut pIJ303, Ln2= *BglII* cut pIJ303, Ln3= TK24 pIJ303 total DNA,
Ln4= uncut pIJ702, Ln5= *BglII* cut pIJ702, Ln6= TK24 pIJ702 total DNA,

3.1.5.2 Protocol.

pIJ303 and pIJ702 plasmid DNA was prepared as described in Section 2.3.6.2 using the large-scale method. The starting material for DNA extracts was a 15h sample from a 5L stirred vessel fermentation and a 95h sample, respectively, of both recombinant fermentations (refer to fermentations described in Sections 3.6.3.2 and 3.7.3.1).

Restriction digests were performed as described in Section 2.3.7 using the enzymes *Bgl*II (single restriction site) and *Bcl*I (several restriction sites). Also *Pst*I restriction enzyme was also used (two restriction sites). Gel electrophoresis and photography was performed as described in Section 2.3.9 and Section 2.3.10, repectively.

3.1.5.3 Results and Discussion.

Restriction fragments of purified pIJ303 from a stirred vessel fermentation are shown in Figure 14. As seen in the photograph the linearized plasmid from both early and late samples was approximately 10.7kb in size. No gross structural re-arrangement had therefore occurred. The same number and size of *Bcl*I restriction fragments were also observed *i.e.* 3.02kb, 1.85kb, 1.76kb, 1.41kb, 1.2kb and 1.06kb (the 0.3kb fragment is small and was not visible). This result again confirmed that no large deletions, duplications or re-arrangments had occurred during these experiments.

The restriction fragments of purified pIJ702, again from a 5L fermentation are shown in Figure 15. As seen in the photograph the linearized plasmid from both early and late samples was observed to be approximately 5.6kb in size. Identical sizes and numbers of *Bcl*I restriction fragments were also observed *i.e.* 1.71kb, 1.34kb, 1.25kb and 1.05kb, therefore no gross structural plasmid re-arrangements were detected.

This procedure was conducted during all future stirred vessel fermentations and the results are discussed in Sections 3.6.4 and 3.7.4.

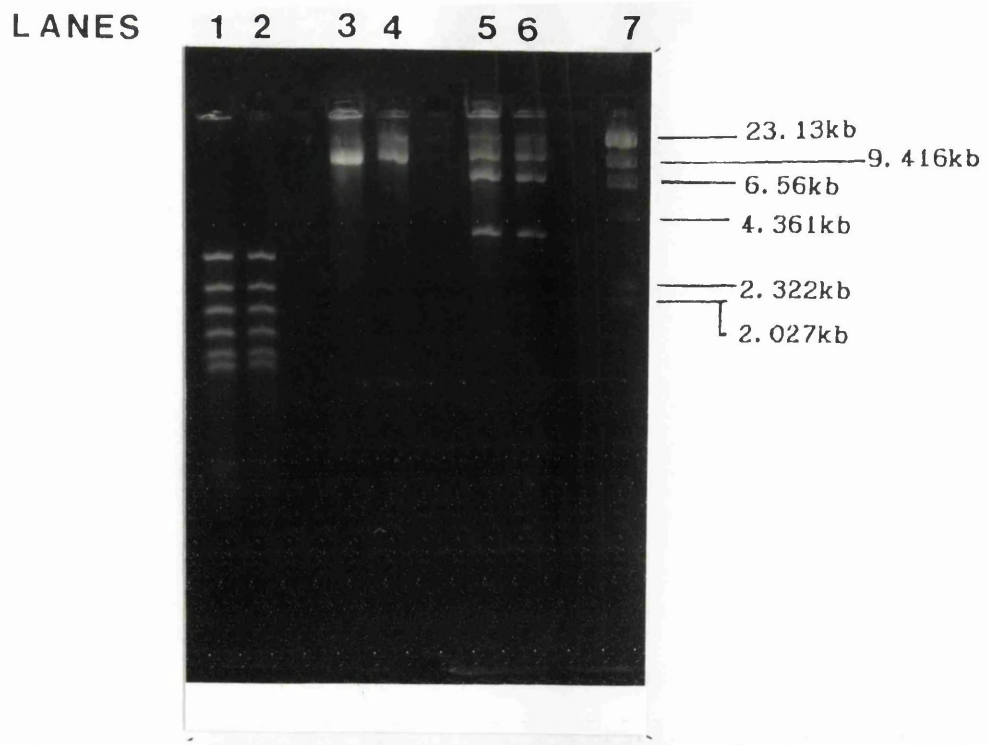

Figure 14 Photograph of gel showing restriction fragment analysis of pIJ303 extracted from 15h and 95h samples of a stirred vessel fermentation. Ln1= *BclI* digest (15h), Ln2= *BclI* digest (95h), Ln3= *BglII* digest (15h), Ln4= *BglII* digest (95h), Ln5= *PstI* digest (15h), Ln6= *PstI* digest (95h), Ln7= λ*Hind*III size marker.

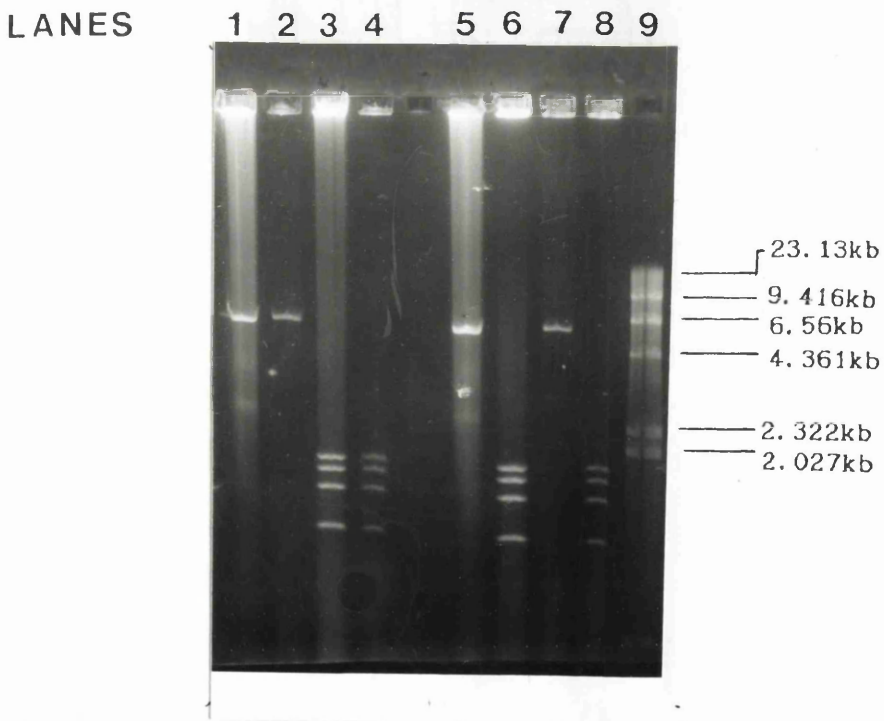

Figure 15 Photograph of gel showing restriction fragment analysis of pIJ702 extracted from 15h and 95h samples of two stirred vessel fermentations. Ln1= *BglII* digest (15h), Ln2= *BglII* digest (95h), Ln3= *BclI* digest (15h), Ln4 *BclI* digest (95h , Ln5= *BglII* digest (15h), Ln6= *BclI* digest (15h), Ln7= *BglII* digest (95h), Ln8= *BclI* digest (95h , Ln9= λ*Hind*III size marker.

3.2 Studies of pIJ303 plasmid vector stability in shake-flask fermentations.

3.2.1 Aim.

The *Streptomyces* plasmid vector pIJ303 was developed by Kieser *et al* (1982) and is derived from plasmid pIJ101 of *S. lividans* ISP5434. The only information currently available on the copy number of pIJ101 is that it varies from 40-300 depending on the age and physiological status of the culture (Kieser *et al*, 1982). The studies here are designed to increase our knowledge and understanding of the maintenance and stability of the pIJ101-derived plasmid vector pIJ303. The structural integrity of the plasmid was also checked.

3.2.2 Protocol.

Timecourse experiments conducted as one-stage cultures (from spores) were initiated by inoculating 1mL thawed spores into a large volume of medium as described in Section 2.3.2. Two-stage cultures were initiated by inoculating a small volume of spores, 0.2mL, into 250mL flasks containing 50mL medium which were incubated for three days. This "seed" culture was then used to inoculate a larger volume of medium in 2L flasks. All cultivation conditions, samplings, growth estimates, DNA extractions, plasmid copy number estimations *etc* were performed as described in Section 2 for both one- and two-stage experiments.

3.2.3 Results of one-stage shake-flask fermentations of *S. lividans* TK24 pIJ303.

3.2.3.1 MOPS medium.

The plasmid copy number (Cp) profile of this experiment is shown in Figure 16. As seen in the graph, the plasmid copy number of pIJ303 increased to approximately 450 after 63h incubation. It then remained at about this level until after 112h incubation when it decrease to approximately 180.

The biomass concentration of *S. lividans* in MOPS medium was relatively low since a large culture volume was needed for each sample. The culture morphology after 3d growth in MOPS is shown in Figure 17. Such mycelial fragments are not typical of *S. lividans* but are produced in MOPS and MEP media (but not MEP+10mMPO_4^{3-}, refer to photomicrograph Figure 18 showing 3d growth in phosphate-supplemented MEP medium). The organism continued growing slowly for a longer period of time and this probably

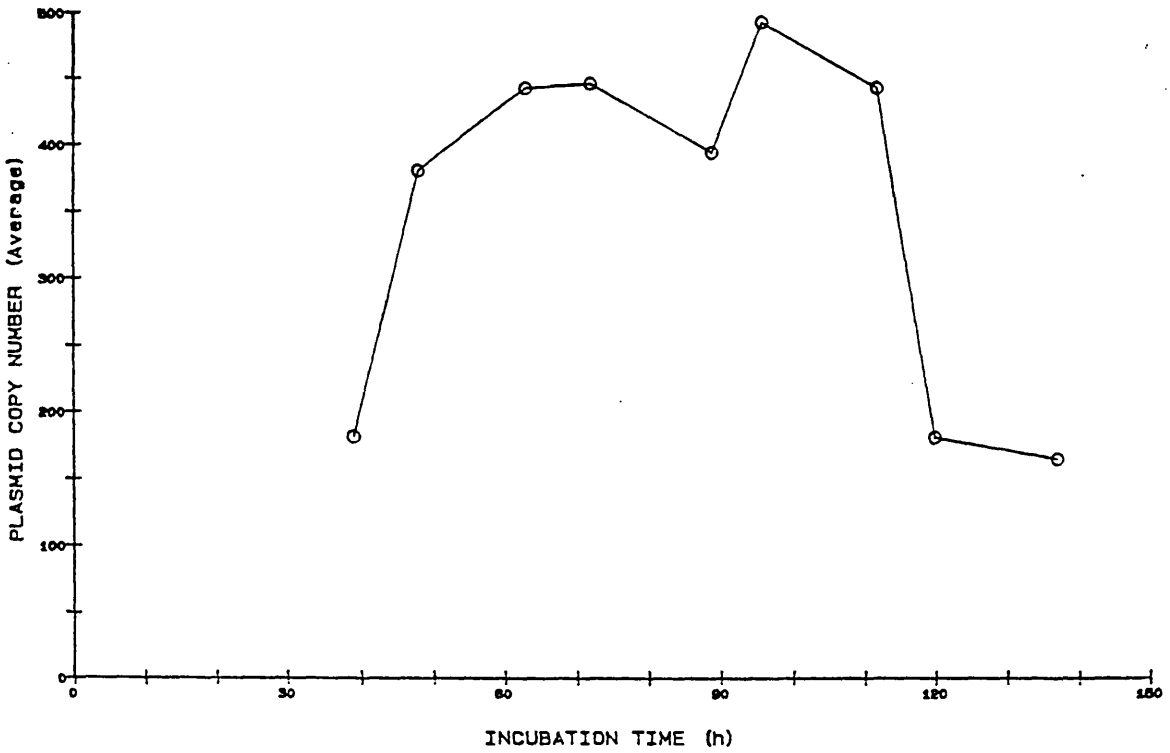

Figure 16 Plasmid copy number profile (O) of a one-stage MOPS shake-flask fermentation of *S. lividans* TK24 pIJ303.

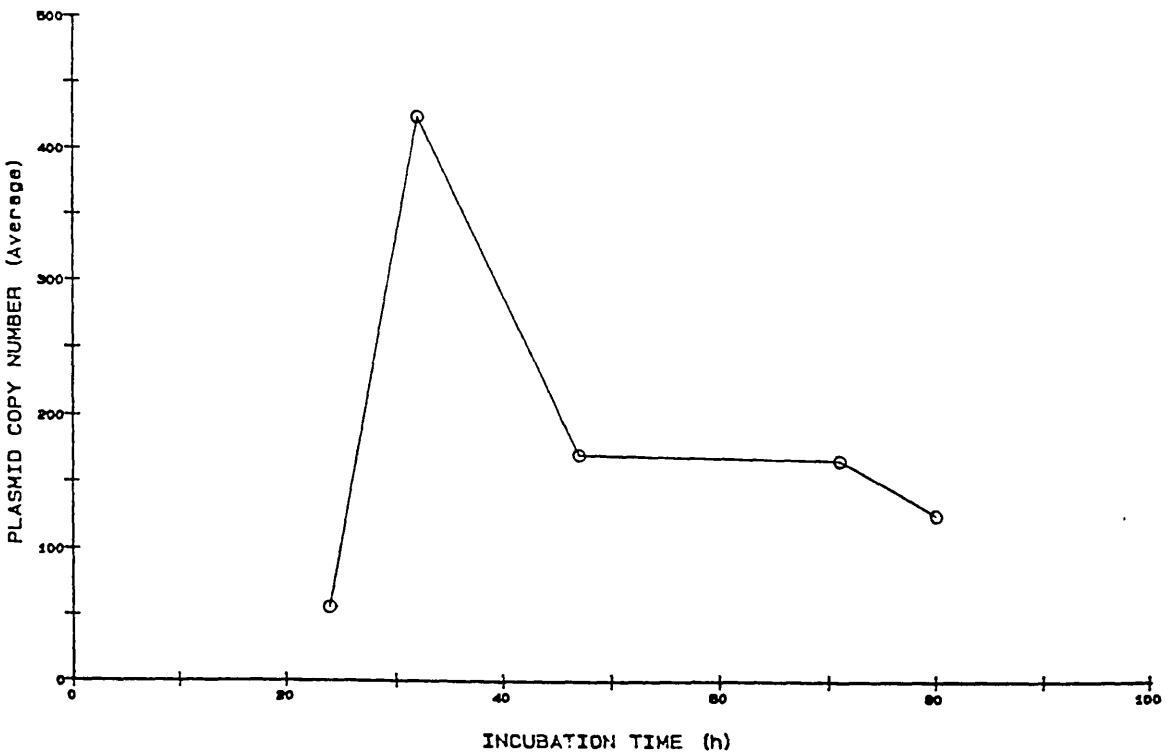

Figure 19 Plasmid copy number profile (O) of a one-stage TSB shake-flask fermentation of *S. lividans* TK24 pIJ303.

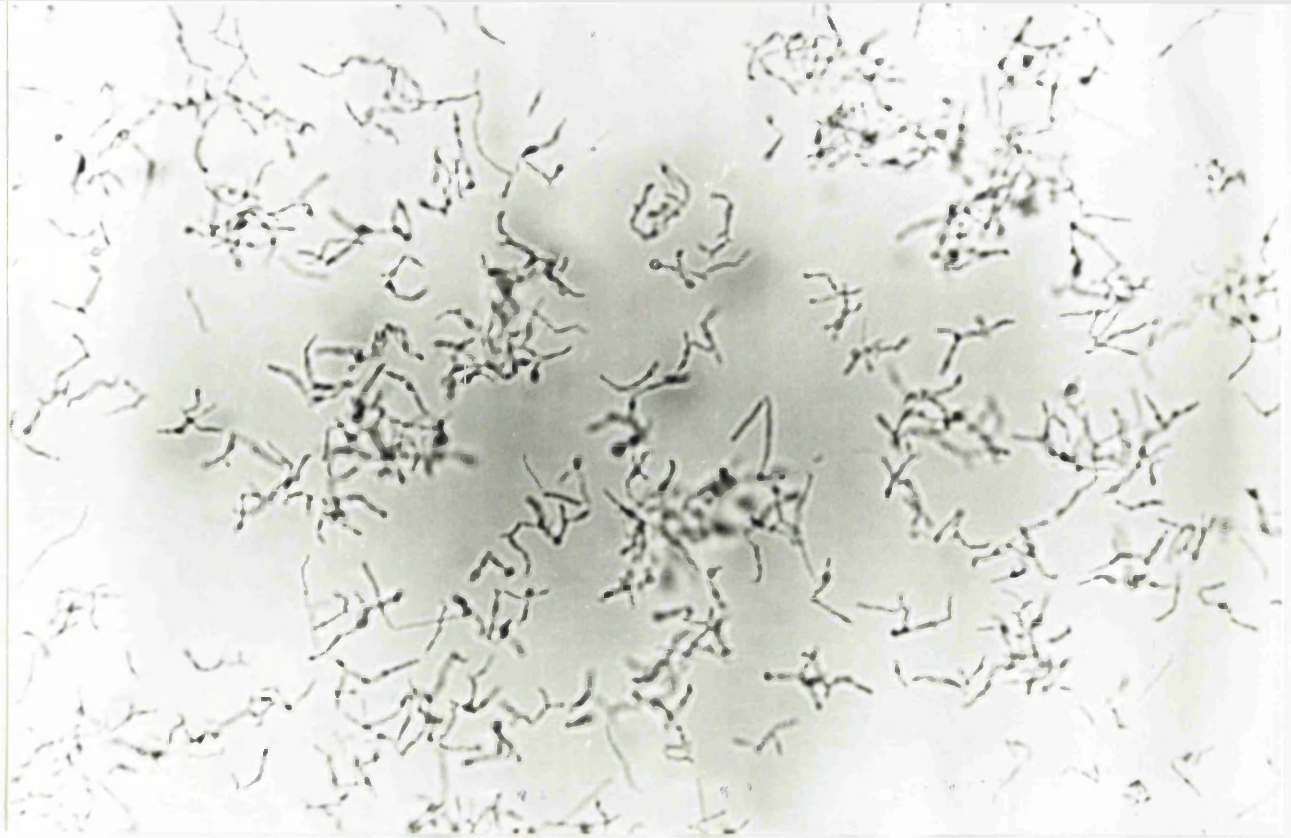

Figure 17 Photomicrograph of *S. lividans* mycelial fragments obtained when grown in MOPS medium under standard conditions. Phase contrast light microscopy at x400 magnification.

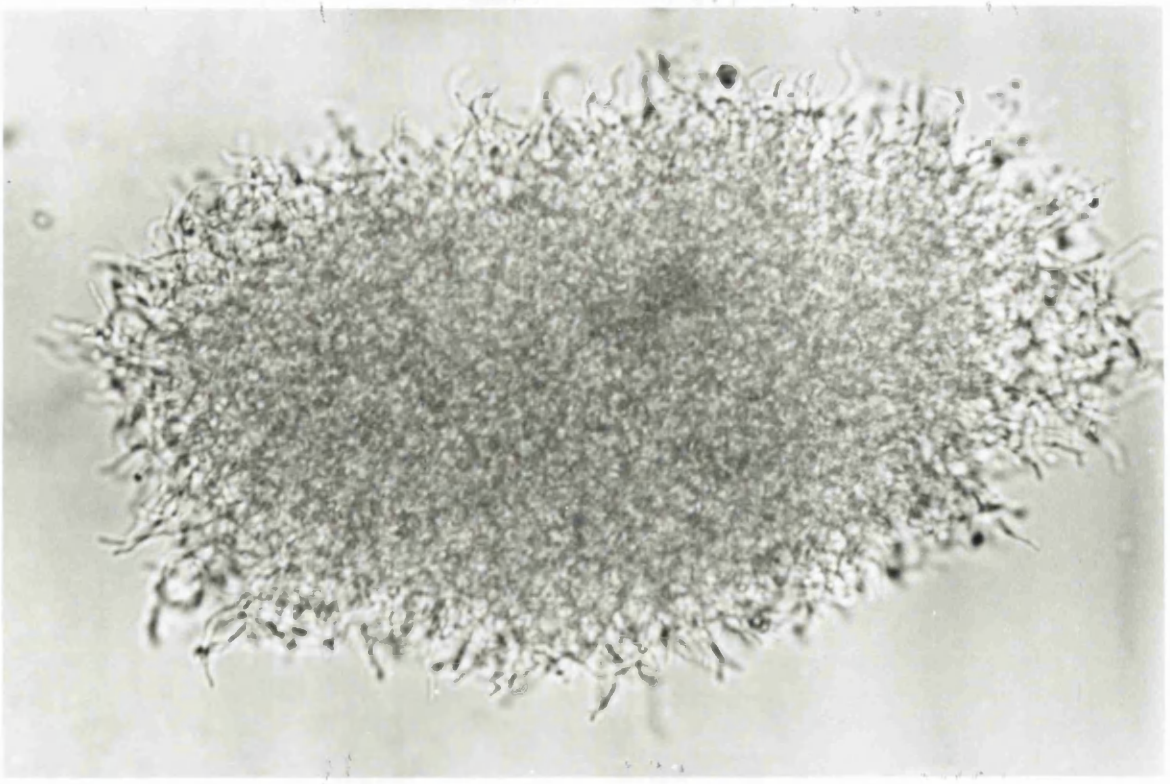

Figure 18 Photomicrograph of *S. lividans* mycelial pellet obtained when spores were inoculated into MEP+10mMPO_4^{3-} medium and grown under standard conditions. Phase contrast light microscopy at x400 magnification.

explains the extended period high plasmid copy number.

3.2.3.2 TSB medium.

Due to the low biomass concentration obtained with MOPS medium all further one-stage fermentations of recombinant *S. lividans* were conducted using TSB medium. The results of the plasmid copy number profile of this experiment are shown in Figure 19. The plasmid copy number rose quickly during the period of initial rapid growth to approximately 425 at 32h. Upon further incubation the plasmid copy number then decreased rapidly to about 150 during the latter part of the fermentation.

3.2.4 Results of two-stage shake-flask fermentations of *S. lividans* TK24 pIJ303.

3.2.4.1 MEP medium.

The seed culture for this experiment was grown for 3 days and transferred at a level of 6%v/v inoculum into a larger flask. The results of the plasmid copy number determinations during the second fermentation stage are shown in Figure 20 as are the biomass concentrations (dry cell weight measurements). The maximum plasmid copy number after transfer of the seed was approximately 340 after 24h incubation. The value then decreased to between 125 and 200 until after 80h incubation when it dropped to about 80. Interestingly, the plasmid copy number profile closely followed the biomass concentration. Dimeric forms of pIJ303 were visible in total DNA extracts from seed transfer to 48h incubation.

3.2.4.2 MEP + 10mMPO_4^{3-} medium.

The seed culture for this experiment was also grown for 3 days and was transferred at a 6%v/v inoculum level. The second fermentation stage plasmid copy number determinations are shown in Figure 21 with the biomass concentrations at each sampling point also plotted. From the graph it can be seen that maximum plasmid copy number was obtained after 24h growth which corresponds to late rapid growth phase. The plasmid copy number value then decreased rapidly to a level of about 250-350 until 89h incubation. Dimeric forms of pIJ303 were visible in total DNA extracts from seed transfer until after the first sampling. In comparison to the results obtained from growth in MEP medium, the biomass concentration was greatly improved by adding inorganic phosphate. Higher copy number values were also obtained when the phosphate-supplemented medium was used. This is probably

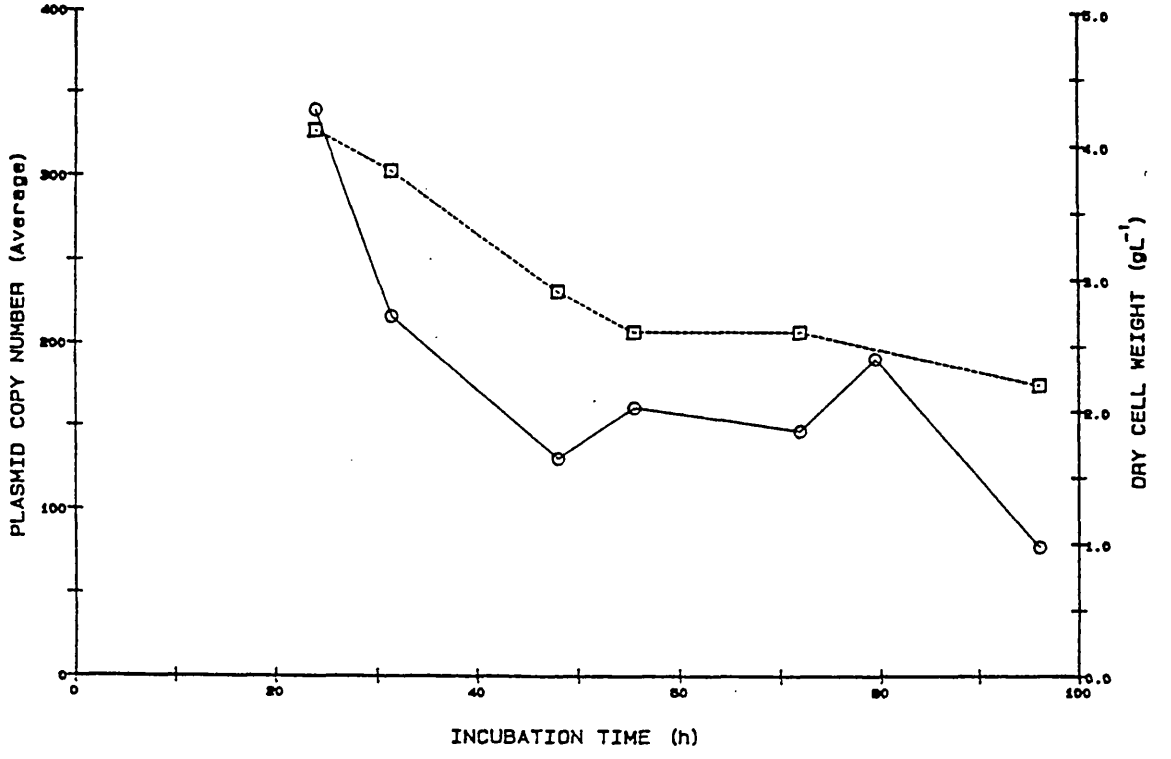

Figure 20 Plasmid copy number (○) and biomass concentration (□, gL^{-1}) data of a two-stage MEP shake-flask fermentation of *S. lividans* TK24 pIJ303.

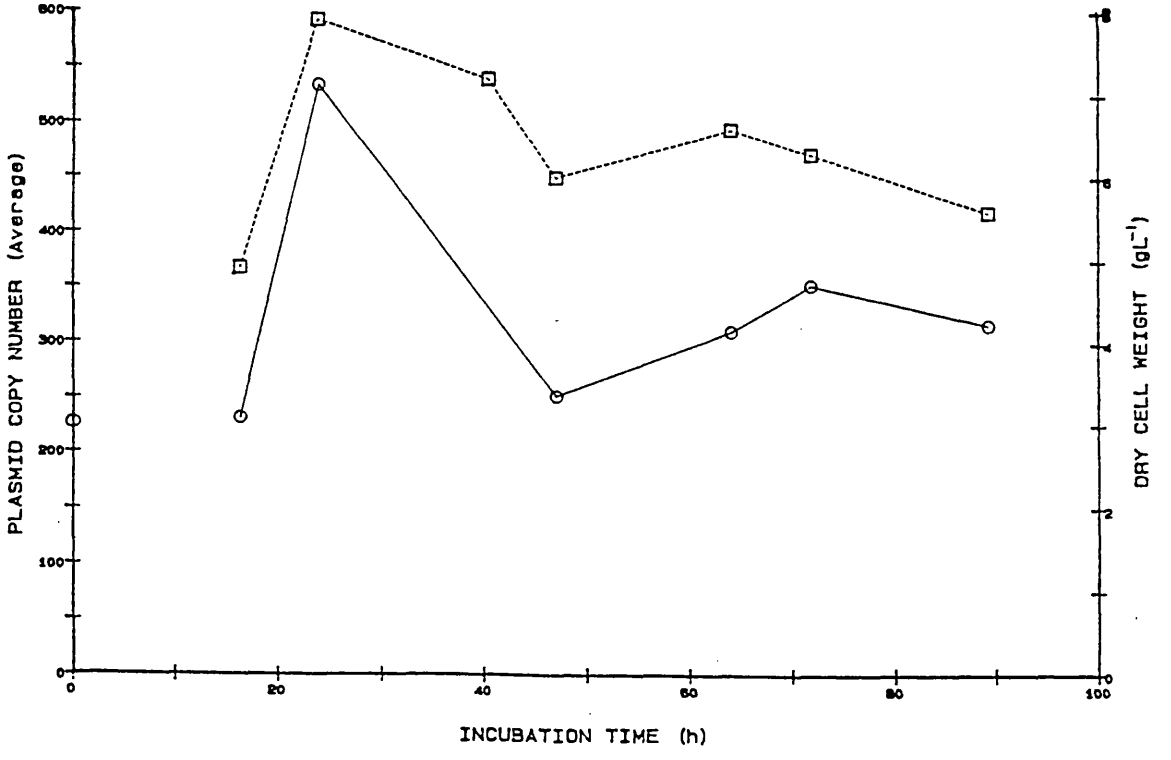

Figure 21 Plasmid copy number (○) and biomass concentration (□, gL^{-1}) data of a two-stage MEP+10mMPO_4^{3-} shake-flask fermentation of *S. lividans* TK24 pIJ303.

linked to the increased growth rate and biomass concentration rather than the increased availability of inorganic phosphate for DNA synthesis.

3.2.5 Results of two-stage shake-flask fermentations of *S. lividans* TK24 pIJ303 at elevated growth temperatures.

3.2.5.1 GYT medium.

A seed culture was grown for 3 days and was transferred at a level of 5%v/v into larger flasks containing GYT medium. One flask was incubated at the normal growth temperature of 28°C while a second flask was incubated at 37°C. Both cultures were sampled periodically, the plasmid copy number and biomass concentration data are shown in Figure 22. The growth rate of *S. lividans* pIJ303 is obviously reduced at 37°C since the biomass concentration reached only half of that at the optimum growth temperature of 28°C. The plasmid copy number profile however shows a different trend. At 28°C the maximum copy number value was obtained after 24-48h growth with the value decreasing rapidly upon further incubation. At 37°C the maximum copy number value was also obtained after 24h growth but it then dropped to between 20 and 40 without further decrease.

3.2.5.2 MEP + 10mMPO_4^{3-} medium.

A seed culture was again grown for 3 days and transferred at a level of 6%v/v into two larger flasks containing MEP + 10mMPO_4^{3-} medium. One flask was incubated at 37°C. The plasmid copy number and biomass concentration data for both growth temperatures are shown in Figure 23. The biomass concentration was again lower at 37°C compared with the culture grown at 28°C. There was a very noticable difference in the plasmid copy number data from the two cultures. When grown at 28°C, pIJ303 copy number increased quickly during initial rapid growth to about 450 after 17h incubation, it then fell steadily during stationary growth. When grown at 37°C, however, the maximum copy number was only approximately 280 but it then decreased to apparently zero since no plasmid bands could be detected thereafter. This is probably due to the arrest of plasmid replication initiation and perhaps its subsequent degradation under conditions of stress *e.g.* high temperatures. Total DNA extracts of 37°C-grown culture also revealed a decrease in intensity of chromosomal DNA bands. Dimeric forms of pIJ303 were visible from all samples at 28°C but only from early samples at 37°C.

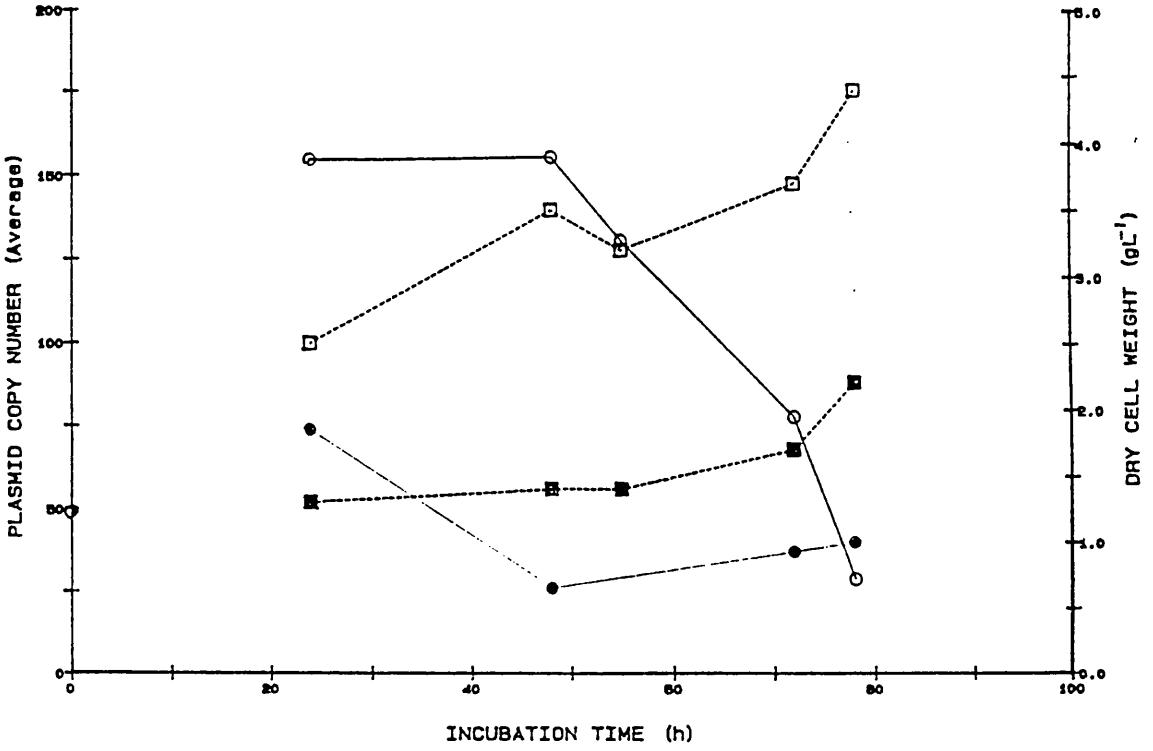

Figure 22 Plasmid copy number data at 28°C (○) and 37°C (●) and also biomass concentration data at 28°C (□, gL^{-1}) and 37°C (■, gL^{-1}) during a two-stage GYT shake-flask fermentation of *S. lividans* TK24 pIJ303.

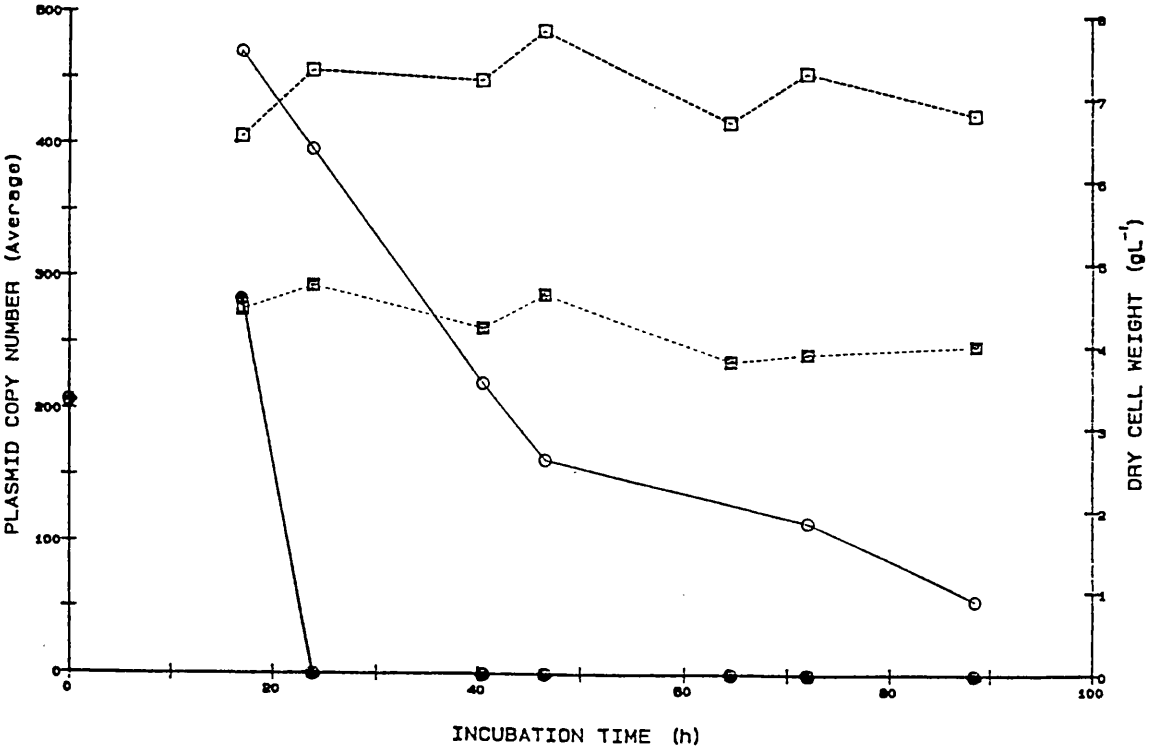

Figure 23 Plasmid copy number data at 28°C (○) and 37°C (●) and also biomass concentration data at 28°C (□, gL^{-1}) and 37°C (■, gL^{-1}) during a two-stage, MEP+10mMPO_4^{3-} shake-flask fermentation of *S. lividans* TK24 pIJ303.

3.2.6 Results of shake-flask fermentations of *S. lividans* TK24 pIJ303 with novobiocin antibiotic.

3.2.6.1 Aim.

Novobiocin is a DNA gyrase inhibitor which prevents the supercoiling of DNA by inhibiting the enzyme. Wolfson *et al* (1983) suggested that novobiocin interferes with the introduction of negative superhelical twists into plasmid DNA molecules and that negative superhelical twists are essential for initiation of a new round of DNA replication. Due to this action novobiocin has been used to effectively 'cure' plasmids from *E. coli* cells (McHugh and Swartz, 1977 and Wolfson *et al*, 1983). These experiments were designed to show if novobiocin could reduce the copy number of pIJ303 when hosted in *S. lividans*. The range of concentrations at which novobiocin is effective against *S. lividans* is shown in APPENDIX 1.

3.2.6.2 One-stage shake-flask fermentation in TSB medium with novobiocin.

A spore inoculum was used to initiate *S. lividans* cultures in TSB medium. The cultures were grown under normal conditions for 40h before adding novobiocin at 5μgmL^{-1} final concentration to one flask. The test and control flasks were then re-incubated and sampled periodically. The data from both cultures regarding the plasmid copy number is shown in Figure 24. There was no major difference in plasmid copy number value between the control and test cultures up to 50h incubation but the curves diverged thereafter. The expected reduction in plasmid copy number values did not occur since novobiocin may not be active at such a low concentration.

3.2.6.3 Two-stage fermentation in MEP + 10mM PO_4^{3-} medium with novobiocin.

A seed culture was grown for 3 days and transferred at a level of 6%v/v to initiate three secondary fermentations. After 17.5h growth novobiocin was added at 25μgmL^{-1}, 5μgmL^{-1} and 0μgmL final concentrations and the flasks were re-incubated and sampled periodically. The plasmid copy number data from all the cultures are shown in Figure 25. The plasmid copy number at transfer was low therefore the maximum value obtained even in the control culture was not very high. However, as seen in the graph, a shifting of the normal plasmid copy number trend occurred with increasing novobiocin concentration. This may be the result of novobiocin indirectly slowing down the growth rate rather than interfering with plasmid

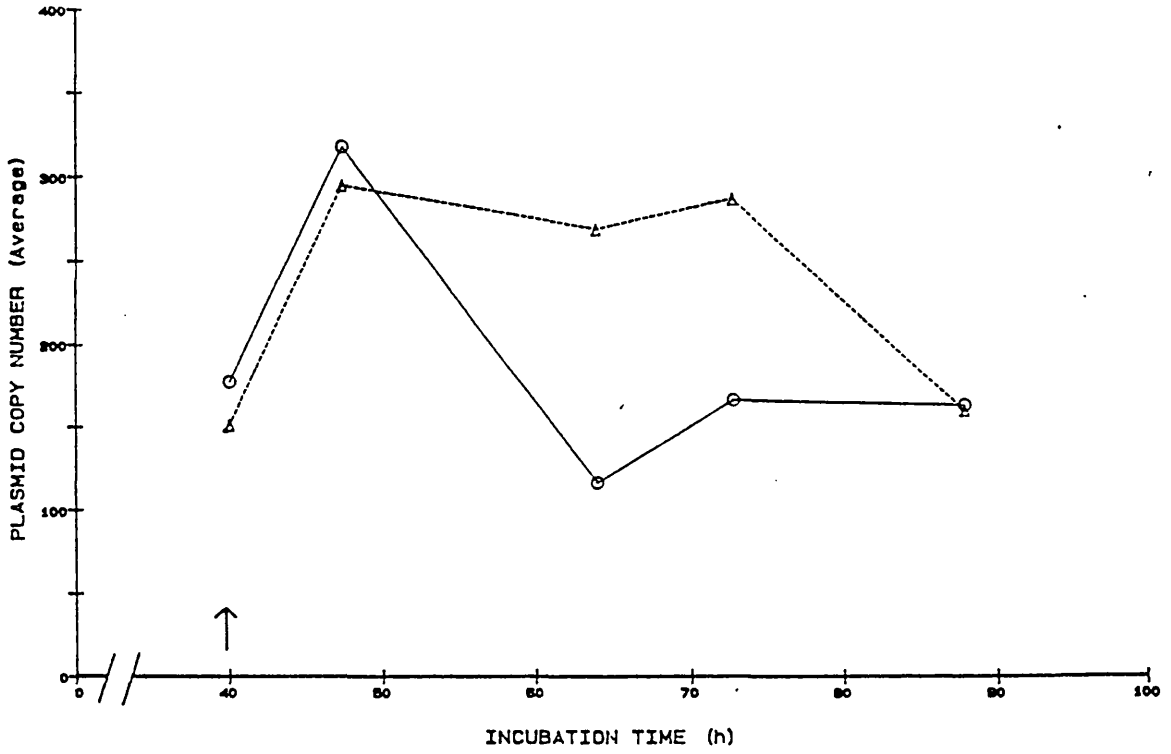

Figure 24 Plasmid copy number profile of a one-stage TSB shake-flask fermentation, with novobiocin at 0μgmL^{-1} (○) and 5μgmL^{-1} (△) of *S. lividans* TK24 pIJ303. Novobiocin added at point shown by arrow.

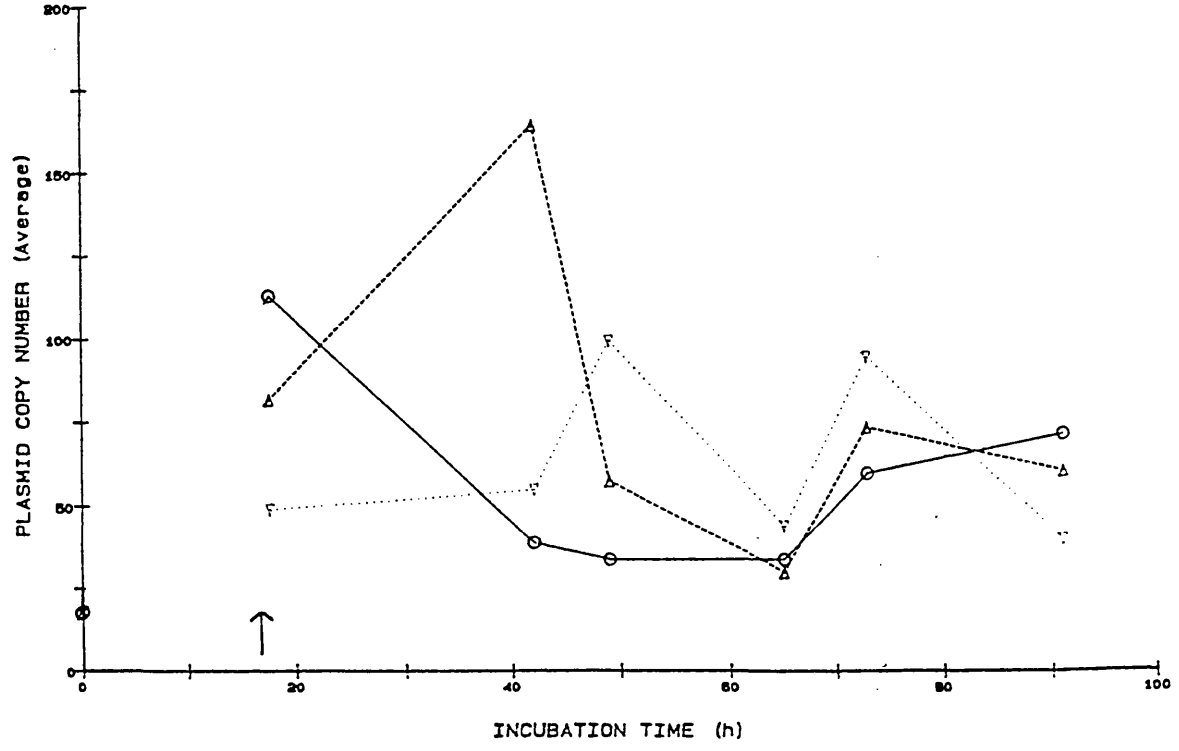

Figure 25 Plasmid copy number profile of a two-stage MEP+10mMPO_4^{3-} shake-flask fermentation, with novobiocin at 0μgmL^{-1} (○), 5μgmL^{-1} (△) and 25μgmL^{-1} (▽) of *S. lividans* TK24 pIJ303. Novobiocin added at point shown by arrow.

replication *via* DNA gyrase inhibition.

3.2.7 Results of seed age and multi-stage cultivation effects on *S. lividans* TK24 pIJ303 fermentations.

3.2.7.1 Aim.

There is evidence from two-stage fermentation results to suggest that the plasmid copy number at transfer affects the maximum copy number obtained in the second fermentation. Fermentation inoculum development on an industrial scale involves several transfer steps (not just one as in most of these fermentations). In order to investigate whether the seed age at transfer affects the subsequent maximum copy number value and whether repeated culture transfers affect plasmid copy number, the following experiments were conducted.

3.2.7.2 Effects of seed culture transfer at various stages of growth.

A seed culture was initiated in a large volume of MEP+thio medium and incubated under standard conditions. A proportion of the seed was transferred after 15h, 24h and 39h (at 6%v/v level) to initiate a second fermentation. All the second fermentations were sampled after 24h and 48h growth.

Because of a long lag in spore germination and subsequent low biomass concentration no plasmid copy number values were obtained from seed samples taken before 39h. The plasmid copy number at 39h was approximately 18, therefore the copy number before this point was probably less. The maximum plasmid copy number value of the seed culture was about 165 after 63h incubation (the culture would normally have been transferred after 72h). The 15h seed transfer culture resulted in an apparent plasmid copy number of zero at 24h and about 80 at 48h. The 24h seed transfer second fermentation resulted in a copy number of approximately 10 at 24h and 40 at 48h. The oldest seed transfer (39h) fermentation yielded a maximum copy number of about 100 after 24h and 30 at 48h.

The results of both younger seed transfers (at 15h and 24h) showed that there is long lag in the second stage fermentation since, even after 48h growth the culture is probably not at stationary phase therefore the plasmid copy number value is still rising. The seed transferred after 39h shows the reverse trend *i.e.* the maximum copy number value is seen at 24h with a decrease thereafter. The physiological status of the seed at transfer is therefore important and the normal protocol involving 3 days'

growth in MEP+thio was both convenient and no advantage could be gained by using a younger culture.

3.2.7.3 Effects of a multi-stage shake-flask fermentation.

A seed culture was grown for 3 days and transferred at a 6%v/v inoculum level to initiate the experiment. The second fermentation (in MEP+10mMPO_4^{3-}) was grown for 24h and at that point half the culture volume was removed and replaced with fresh medium to start a third fermentation stage. The procedure was repeated after 48h and 72h post-initiation to initiate fourth- and fifth-stage fermentations respectively. The results of the experiment are shown in Figure 26. Maximum biomass concentration was obtained after 24h but the same level was not subsequently achieved despite the addition of fresh medium at three points.

A dilution effect upon plasmid copy number values can be seen at each withdraw/refill point. The reason for this could be that the organism's growth rate remained high due to the availability of fresh nutrients. Plasmid copy number at higher growth rates is probably less than at slower growth rates (*e.g.* when grown in MOPS, refer to Figure 16). It is therefore possible to dilute out plasmid molecules by repeated transfers if the culture is not allowed to proceed to stationary phase where copy number is higher. This problem could be minimized by limitating the number of transfers made during inoculum development. Ideally a small volume should be transferred and the resulting culture allowed to grow until stationary phase.

3.2.8 Remarks on the observed stability of pIJ303 in shake-flask fermentations.

The plasmid copy number values estimated for pIJ303 during this study compare well with those quoted in the literature (Kieser *et al*, 1982), and are even higher in some circumstances. In general, the profile showed a rapid increase in plasmid copy number during initial fast growth up to a maximum value after 24-48h incubation in rich media. This occurs in both one- and two-stage fermentations. The use of poorer media *e.g.* MOPS yielded very high, sustained copy number suggesting that low growth rate improves plasmid copy number.

Novobiocin does not directly affect *Streptomyces* plasmid copy number although it affects the growth rate and may lead to a shifting of the characteristic profile. Growth at elevated temperatures influences

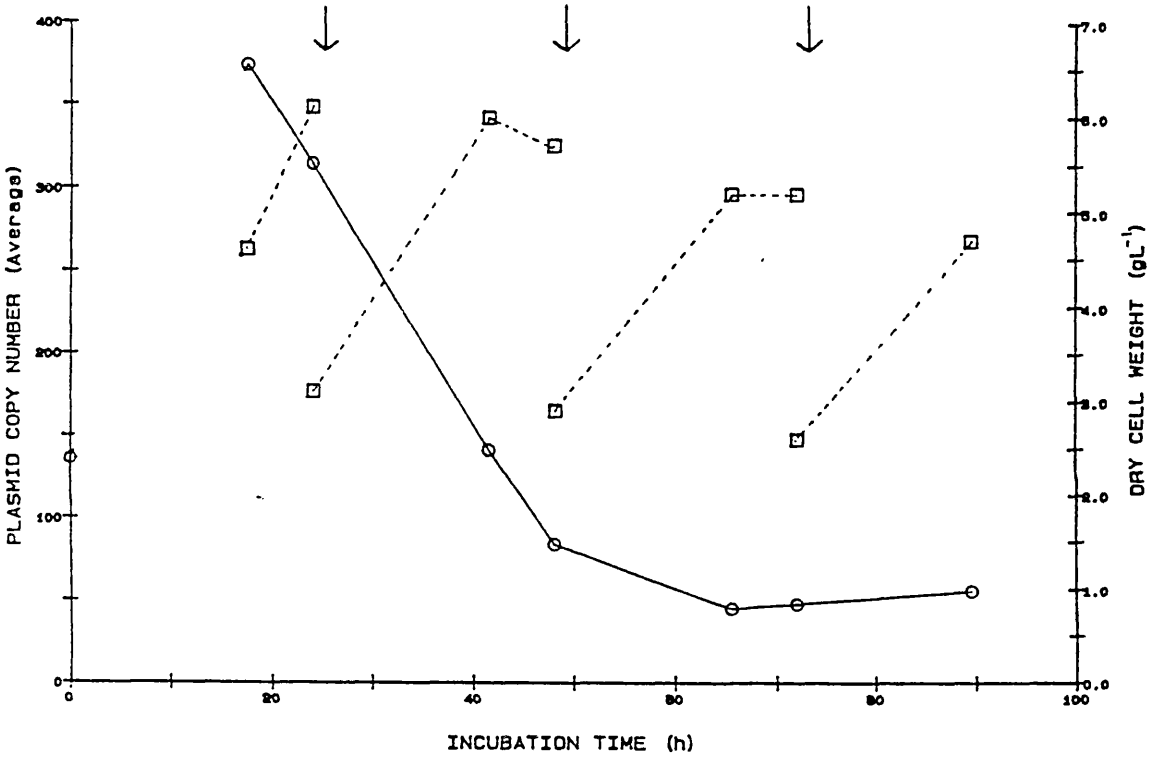

Figure 26 Plasmid copy number (○) and biomass concentration (□, gL^{-1}) data of a multi-stage MEP+10mMPO_4^{3-} shake-flask fermentation of *S. lividans* TK24 pIJ303. Culture transfers made at points shown by arrows.

plasmid copy number but its severity is medium-dependent and may lead to degradation of both chromosomal and plasmid DNA through environmental stress. It was found that employing multi-stage inoculum development strategies may lead to a "diluting out" effect upon plasmid copy number and should be avoided.

There was no evidence of structural re-arrangements of pIJ303 during these shake-flask experiments.

3.3 Studies of pIJ702 plasmid vector stability in shake-flask fermentations.

3.3.1 Aim.

The *Streptomyces* plasmid vector pIJ702 was developed by Katz *et al* (1983) and carries the *S. antibioticus* tyrosinase gene which catalyses the formation of the black pigment melanin. A spectrophotometric enzyme assay protocol is available for estimating the level of tyrosinase activity (refer to Section 2.3.13) using L-DOPA as substrate. It was therefore possible to follow both plasmid copy number and cloned enzyme activity in certain shake-flask cultures where the cloned gene expression had been obtained through induction.

Unlike pIJ303 (*Sti*$^+$), pIJ702 has a *Sti*$^-$ phenotype which results in the accumulation of ssDNA at each round of plasmid replication (Deng *et al*, 1988; also refer to Section 1.10.1). It is expected that the yield of double stranded DNA plasmid molecules will be decreased resulting in a lower plasmid copy number compared with pIJ303. Also the *Sti*$^-$ phenotype seems to lead to structural re-arrangements (Section 1.10.1.) therefore the structural integrity of the plasmid molecules was also checked.

3.3.2 Protocol.

Timecourse experiments which were conducted as one stage cultures *i.e.* from spores, were initiated by inoculating 1mL thawed spores into a large volume of medium as detailed in Section 2.3.2. Two-stage cultures were initiated by inoculating a small volume of spores, 0.2mL, into 250mL flasks which were incubated for three days. This "seed " culture was then used to inoculate a larger volume of medium in 2L flasks. All cultivation conditions, samplings, growth estimates *etc* were performed as described in Section 2 for both one- and two-stage timecourse experiments. Where tyrosinase induction was desired the culture was induced by the addition of sterile Bacto-tryptone and copper sulphate (refer to Section 2.1.3.2.(f)). Tyrosinase enzyme assays were performed as described in Section 2.3.13 and total protein concentration determined as described in Section 2.3.14.

3.3.3 Results of one-stage shake-flask fermentations of *S. lividans* TK24 pIJ702 using TSB medium.

The results of the plasmid copy number profile of this experiment are shown in Figure 27. The plasmid copy number of pIJ702 increased quickly during initial rapid growth to a maximum value of approximately 200 after

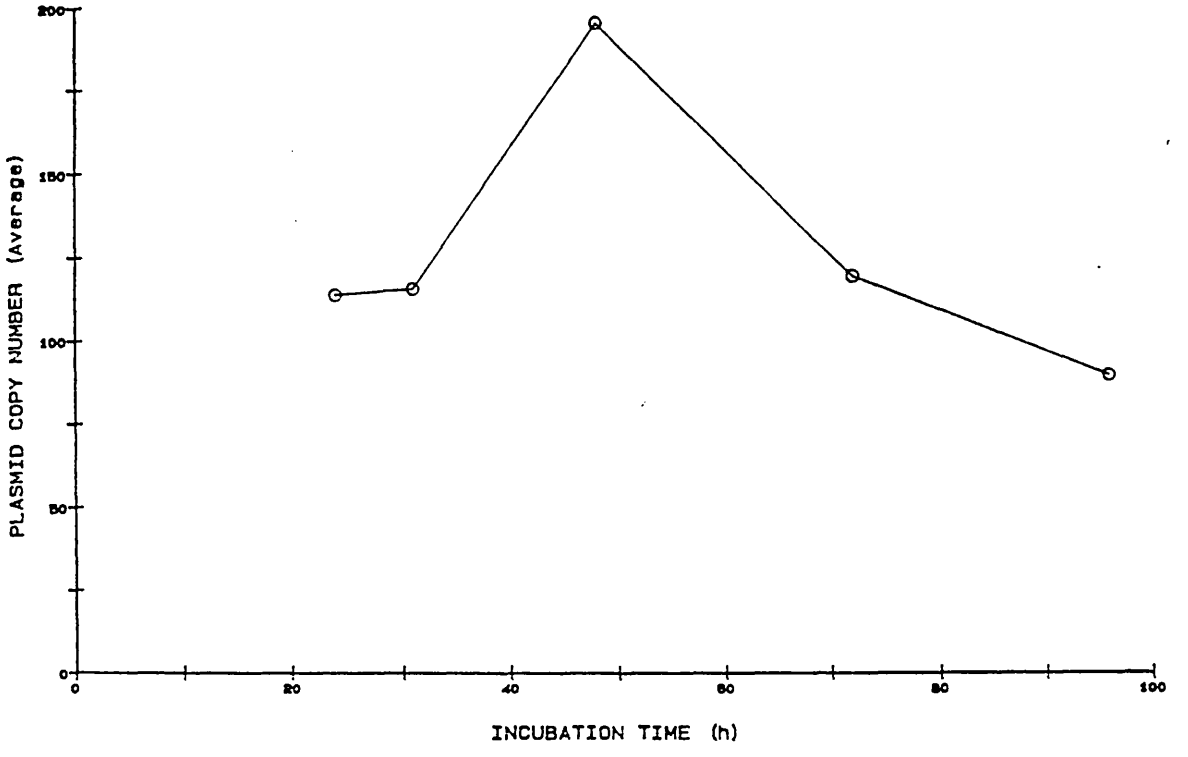

Figure 27 Plasmid copy number profile (○) of a one-stage TSB shake-flask fermentation of *S. lividans* TK24 pIJ702.

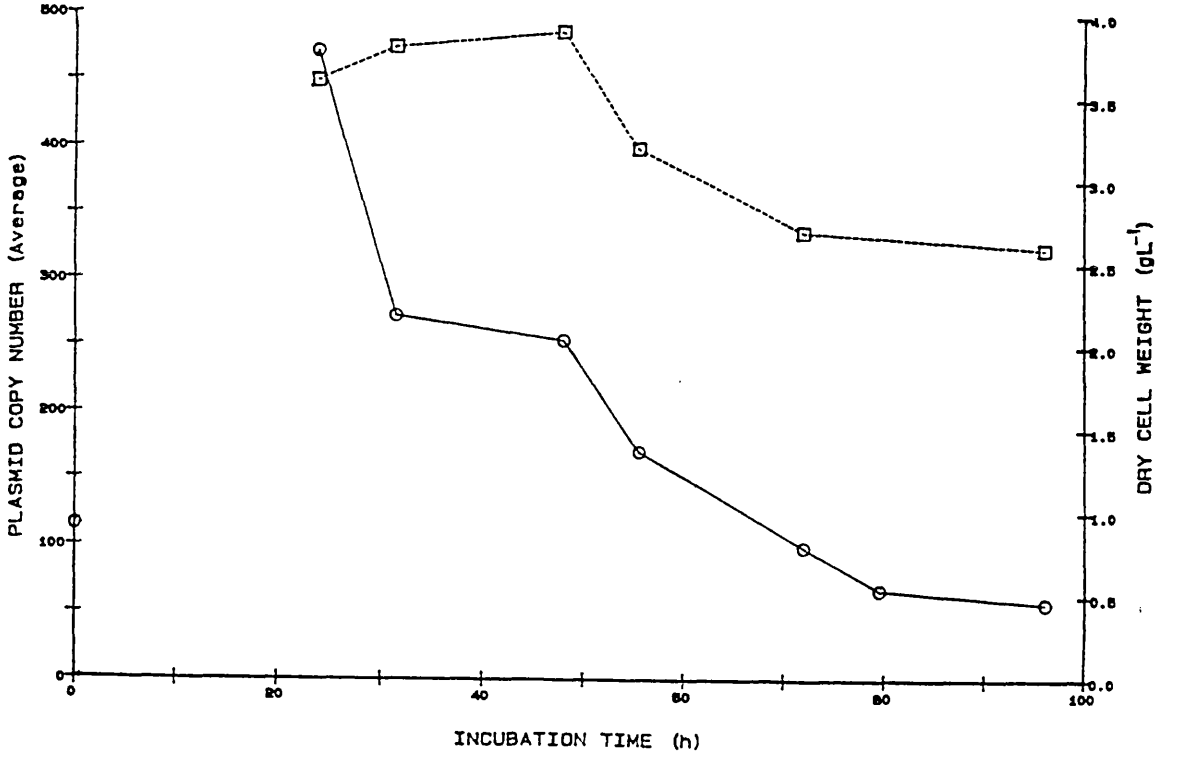

Figure 28 Plasmid copy number (○) and biomass concentration (□, gL^{-1}) data of a two-stage MEP shake-flask fermentation of *S. lividans* TK24 pIJ702.

48h incubation. The culture had probably reached the stationary growth phase at this point and a decline to 50% of the maximum value was observed after 96h.

3.3.4 Results of two-stage shake-flask fermentations of *S. lividans* TK24 pIJ702.

3.3.4.1 MEP medium.

The seed culture for this experiment was grown for 3 days at which point it was transferred at a level of 6%v/v into a larger flask. The results of the plasmid copy number during the second fermentation stage are shown in Figure 28 as are the biomass concentrations (determined as dry cell weight). The maximum plasmid copy number after transfer of the seed culture was approximately 470 after 24h incubation. The value then declined steadily until only faint plasmid bands were visible after 96h incubation. The culture was relatively old at this stage and a decrease in biomass concentration of $1.5gL^{-1}$ was observed between 50h and 96h.

3.3.4.2 MEP + $10mMPO_4^{3-}$ medium.

The seed culture for this experiment was grown for 3 days at which point it was transferred at a level of 6%v/v into a larger flask. The results of the plasmid copy number during the second fermentation stage are shown in Figure 29 as are the biomass concentrations. The maximum plasmid copy number measured was approximately 260 after 48h incubation. A decline in plasmid copy number was seen after 55.5h but good plasmid bands were obtained beyond this point. The biomass concentration of *S. lividans* pIJ702 when grown in phosphate-supplemented MEP medium was slightly better suggesting that the availability of phosphate may be limiting in non-supplemented MEP medium. A similar results was obtained for *S. lividans* TK24 pIJ303 (refer to Section 3.2.4).

3.3.5 Results of two-stage shake-flask fermentations of *S. lividans* TK24 pIJ702 at elevated growth temperatures.

A seed culture was grown for 3 days and then transferred at a level of 6%v/v into larger flasks. One flask was incubated under standard conditions *i.e.* 28°C and a second flask was incubated at 37°C. The medium used for both second stage fermentations was MEP + $10mMPO_4^{3-}$. The plasmid copy number and biomass concentration results are shown in Figure 30. As seen from the graph there was little difference in the biomass concentration at either temperature. However, plasmid copy number values

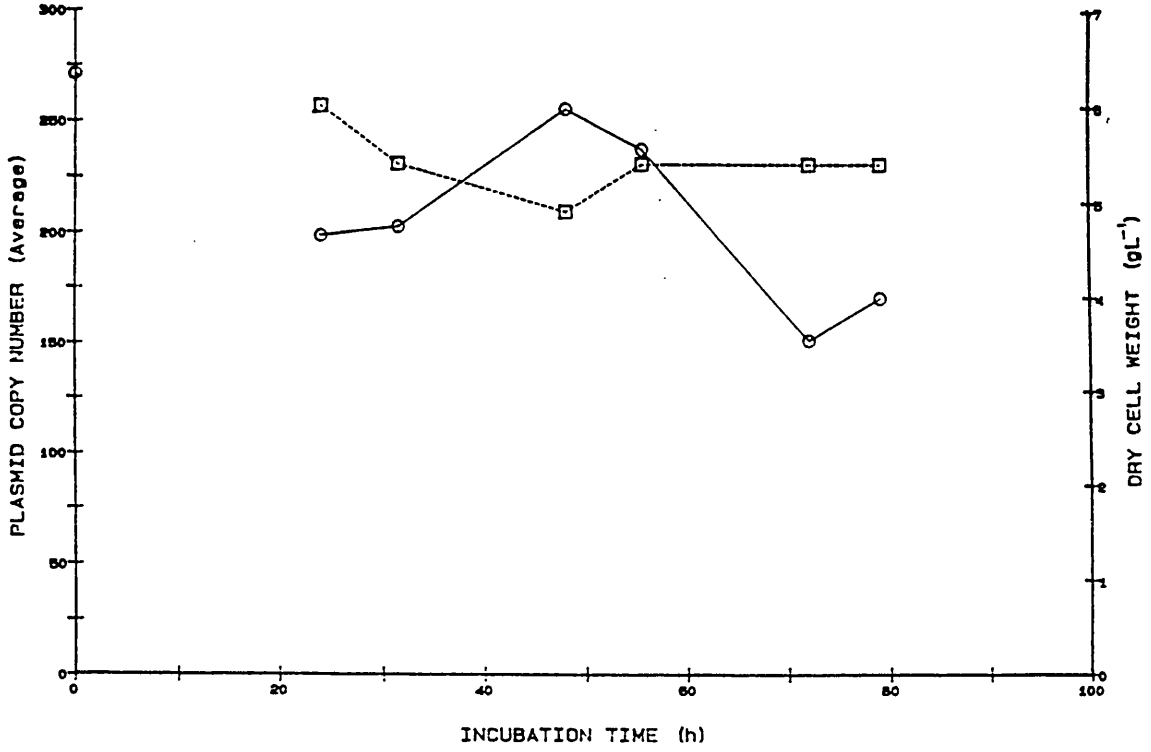

Figure 29 Plasmid copy number (○) and biomass concentration (□, gL^{-1}) data of a two-stage MEP+10mMPO_4^{3-} shake-flask fermentation of *S. lividans* TK24 pIJ702.

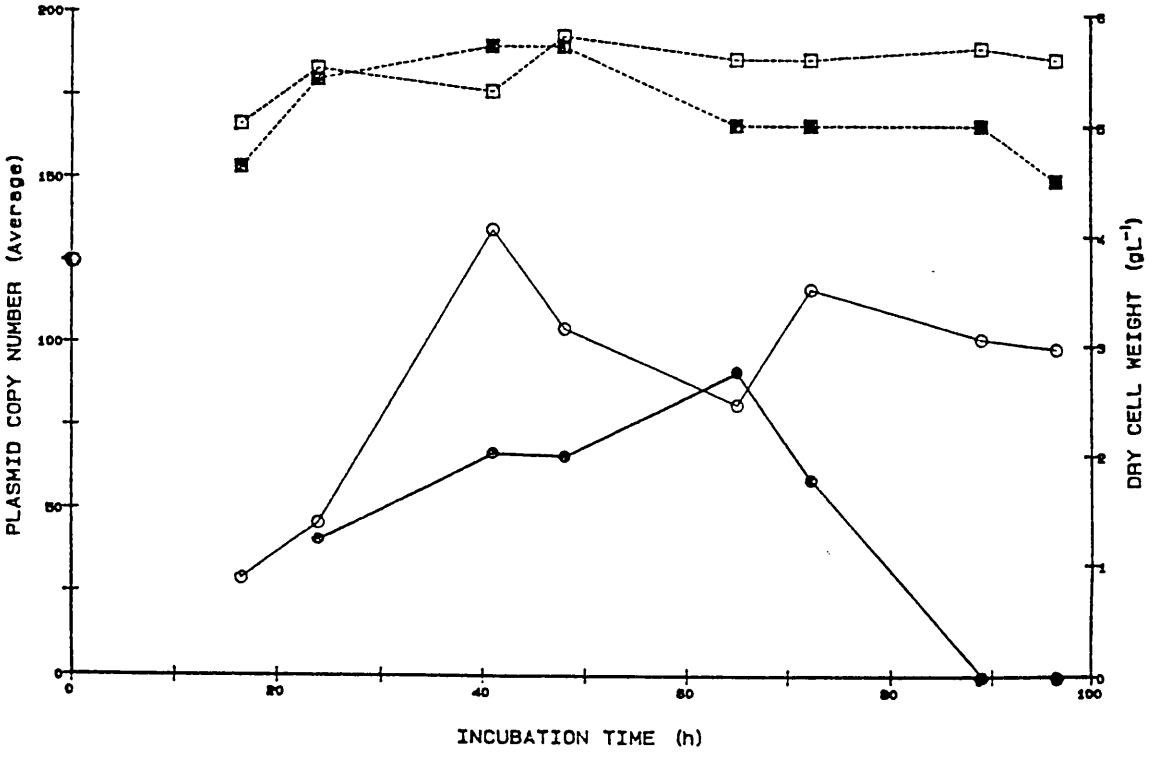

Figure 30 Plasmid copy number data at 28°C (○) and 37°C (●) and also biomass concentration data at 28°C (□, gL^{-1}) and 37°C (■, gL^{-1}) during a two-stage MEP+10mM$PO4^{3-}$ shake-flask fermentation of *S. lividans* TK24 pIJ702.

were greater in the culture grown under normal conditions compared to that grown at 37°C where the maximum value was approximately 90 after 65h incubation. The plasmid copy number decreased rapidly after 72h until no further plasmid bands could be seen. The stability of pIJ702 was observed to decrease at higher incubation temperatures compared to normal growth conditions.

3.3.6 Results of two-stage shake-flask fermentations of *S. lividans* TK24 pIJ702 where tyrosinase enzyme activity was measured.

3.3.6.1 Induction of tyrosinase activity using bactotryptone-copper.

A seed culture was grown for 3 days and then transferred at a 7%v/v inoculum level into larger flasks containing GYM medium. The larger flasks were subsequently re-incubated for a period of 48h when one culture was induced by adding bactotryptone-copper while the other remained as a control culture. The plasmid copy number and tyrosinase enzyme activity for the induced culture are shown in Figure 31. Some background activity in the non-induced culture was also obtained but the level was <10 units mg^{-1} total protein. The addition of the enzyme induction medium also stimulated growth since it contains a high concentration of nutrients. The stimulation of growth therefore led to a growth-related increase in plasmid copy number since under normal, non-induced conditions the plasmid copy number would be declining at this point (>48h). It not possible to directly compare the plasmid copy number in the induced and non-induced cultures due to the stimulation of growth observed in the former. The precise nature of the molecule which induces tyrosinase expression is not known but Hintermann *et al* (1985) reported that the amino acids L-tyrosine, L-methionine and L-leucine could induce the expression of wild-type *S. glauscens* tyrosinase. Consequently it was not possible to induce tyrosinase activity by adding non-growth stimulating effector molecules. However, the effect of copper on tyrosinase activity in defined and complex media is described in the following section.

3.3.6.2 Tyrosinase enzyme production in complex and minimal media with and without added copper sulphate.

This experiment was designed to show under which conditions tyrosinase enzyme activity could be detected. The following media were prepared; MEP with and without $2.5 mgL^{-1}$ $CuSO_4$, GYM with and without $2.5 mgL^{-1}$ $CuSO_4$, and modified Hobbs minimal media with and without $2.5 mgL^{-1}$

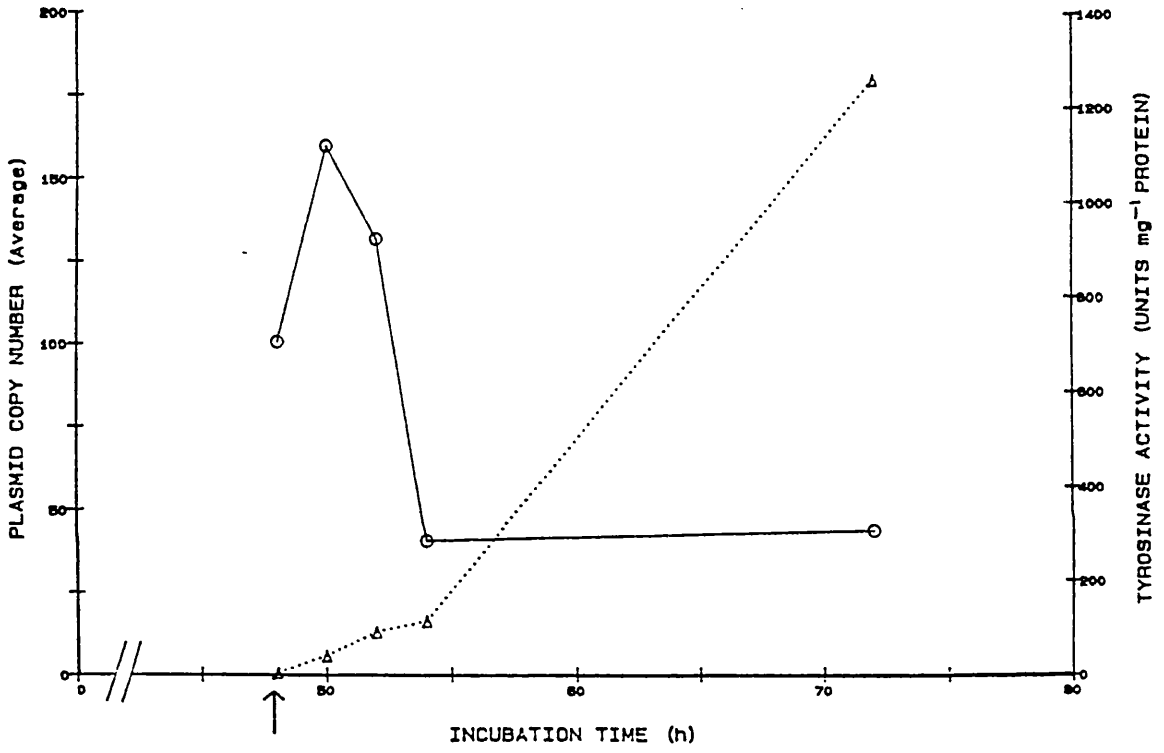

Figure 31 Plasmid copy number (○) and tyrosinase specific activity (△, units mg^{-1} protein) data of a two-stage GYM shake-flask fermentation of *S. lividans* TK24 pIJ702 (with "induction" at timepoint indicated by arrow).

$CuSO_4$. Each fermentation was conducted as a one-stage fermentation initiated from spores. 1mL volume of spore suspension was used to inoculate the media and the cultures were incubated under standard conditions for 3 days. No growth was obtained from the MM with Cu medium possibly due to the toxic effects of copper at the concentration used. The MM without Cu-grown culture was streaked onto YS agar with and without thiostrepton selection and incubated for several days at 30°C. The streaked agar plates are shown in Figure 32 and melanin pigmentation is clearly visible around all the colonies indicating no plasmid loss.

The plasmid copy number and tyrosinase enzyme activity of all the cultures are shown in TABLE 4. As seen in the table the best tyrosinase activity was obtained from GYM-grown cultures, although the highest level was obtained when copper was added to the medium. The poorest yields were obtained from the MEP-grown cultures. Surprisingly good levels of tyrosinase activity were obtained from the MM-grown culture but this may be because complete repression of the operon does not occur under these circumstances. The expression of this enzyme cannot therefore be completely controlled and some tyrosinase will be obtained regardless of culture conditions.

3.3.7 Remarks on the observed stability of pIJ702 in shake-flask fermentations.

On the whole, the plasmid copy number of pIJ702 was not as good as pIJ303. This is probably because of the accumulation of ssDNA molecules during plasmid replication (refer to Section 3.3.1). One-stage fermentations did not yield maximal plasmid copy number values as high as those from two-stage fermentations, where the maximum value occurred at the end of rapid growth phase.

Tyrosinase enzyme activity was obtained from cultures grown in a variety of media but this increased dramatically upon induction with the bactotryptone and $CuSO_4$ mixture. Where data are available on plasmid copy number and tyrosinase enzyme activity, enzyme levels did not follow the same trend as plasmid copy number. No structural re-arrangements of the plasmid were detected.

TABLE 4 Tyrosinase enzyme activity and plasmid copy number of *S. lividans* TK24 pIJ702 grown in various media with and without copper.

Medium	Tyrosinase activity units per mg protein	Plasmid Copy Number (Av)
GYM + Cu	16.4	162
GYM - Cu	10.2	117
MEP + Cu	1.4	207
MEP - Cu	0.6	197
MM - Cu	2.8	245

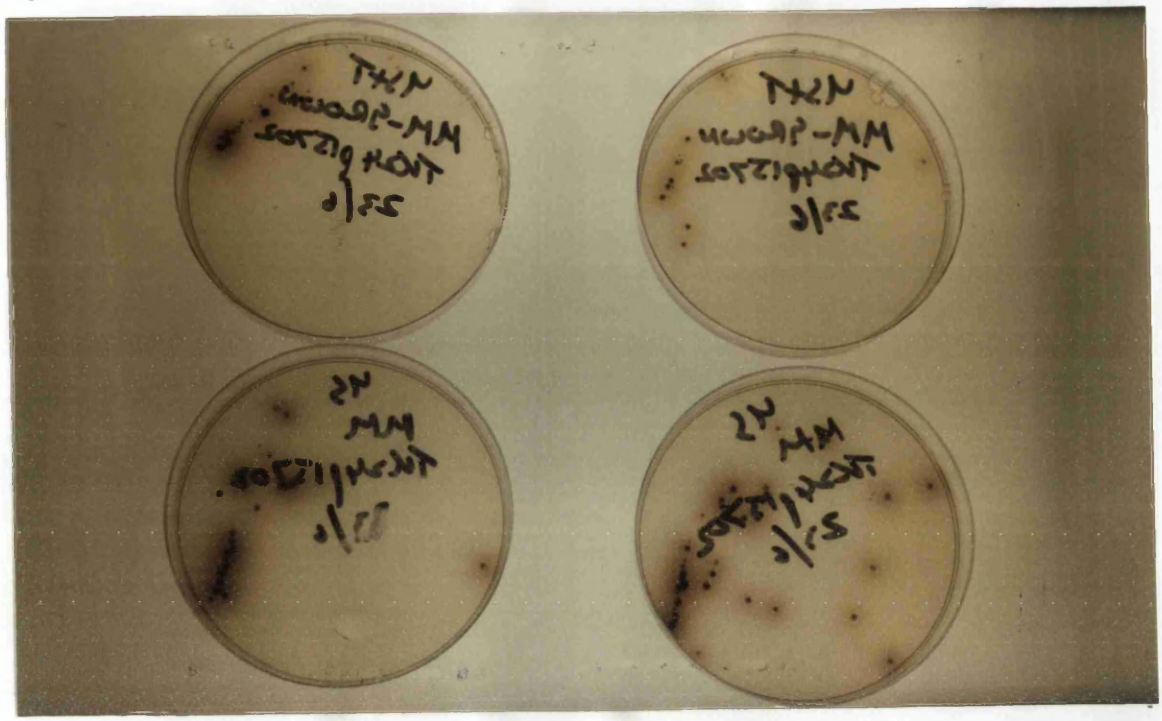

Figure 32 Photograph of *S. lividans* TK24 pIJ702 colonies producing melanin on YS agar with thiostrepton (top plates) and without thiostrepton (bottom plates). Colonies from mycelium originally grown in Hobbs' minimal medium.

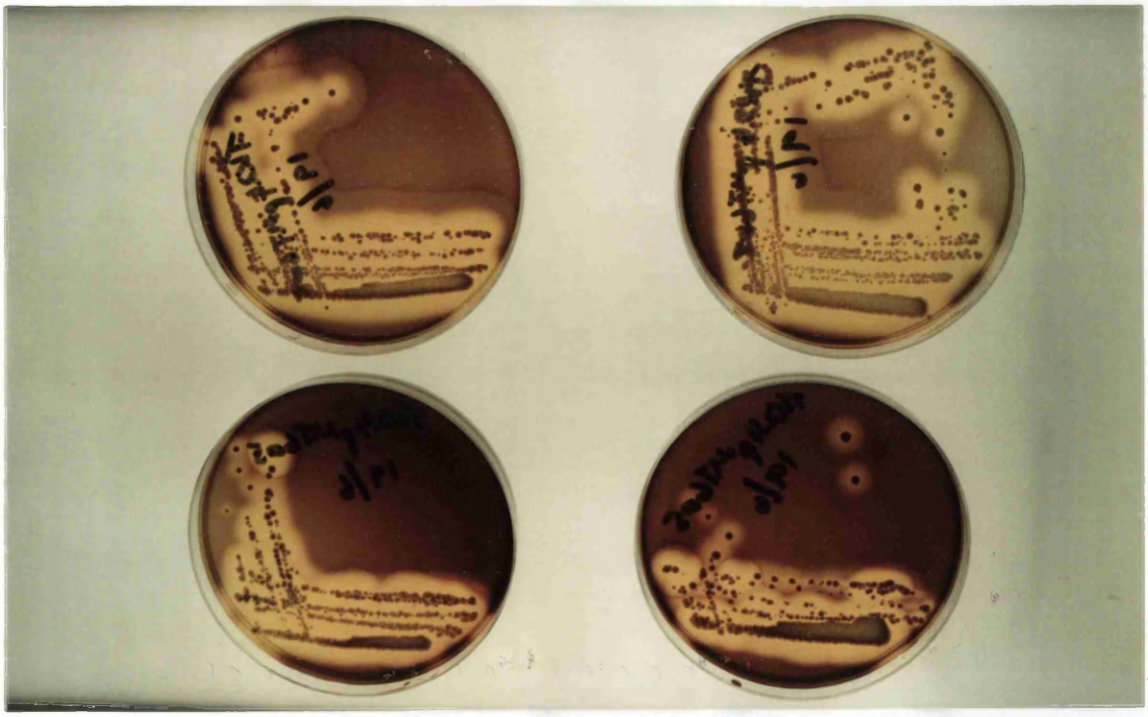

Figure 33 Photograph of *S. lividans* TK24 pMT608 (top plates) and *S. lividans* TK24 pMT605 (bottom plates) colonies producing agarase enzyme. Cultures were grown on nutrient agar for 7 days and then flooded with Gran's iodine solution.

3.4 Studies of pMT605 and pMT608 plasmid vector stability in shake-flask fermentations.

3.4.1 Aim.

The agarase gene of *S. coelicolor* A3(2) was cloned by Kendall and Cullum (1984) using the *Streptomyces* plasmid vector, pIJ702. Some instability was reported by the authors during spore-spore cycle tests (refer to Section 1.10.2.1). It was found that pMT605 was rapidly lost from cultures of recombinant *S. lividans* unless thiostrepton was included to provide a selective pressure. In order to examine these rather unstable plasmids further their plasmid copy numbers were followed during the course of shake-flask fermentations. It was also anticipated that cloned gene expression levels could be related to plasmid copy number.

3.4.2 Protocol.

Timecourse experiments which were conducted as one-stage cultures *i.e.* from spores, were initiated by inoculating 1mL thawed spores into a large volume of medium as detailed in Section 2.3.2. Two-stage cultures were initiated by inoculating a small volume of spores (0.2mL) into smaller flasks which were incubated for three days. This "seed" culture was then used to inoculate a larger volume of medium in 2L flasks. All cultivation conditions, samplings, growth estimates, DNA extracts and plasmid copy number estimations were performed as described in Section 2 for both one- and two-stage timecourse experiments.

Since the plasmids pMT605 and pMT608 encode an agarase enzyme it is possible to detect the level of enzyme production by flooding agar-grown cultures with an iodine solution. *S. lividans* pMT605 and *S. lividans* pMT608 were streaked onto nutrient agar plates (with and without added thiostrepton) which were incubated at 30°C for several days until good sporulation was obtained. The surface of the agar was then flooded with 5mL of Gran's iodine solution (Section 2.1.4 (h)) and the colour allowed to develop for a few minutes. Zones of clearing around the colonies could be seen where the agar had been broken down as shown in Figure 33.

The same technique was adapted to detect levels of extracellular agarase enzyme in culture supernatants. Wells of 7mm diameter were punched in nutrient agar plates using a flamed cork borer. A 50µL aliquot of culture supernatant was placed in the well and the plates were incubated at 30°C for 3 days to allow diffusion of the enzyme. The plates were again

flooded with Gran's iodine and the zones of clearing measured with a ruler and the enzyme activity could therefore be measured. *S. lividans* does not produce an agarase enzyme.

3.4.3 Results of one-stage shake-flask fermentations of *S. lividans* TK24 pMT605 using TSB medium.

The results of this experiment are shown in Figure 34. As seen in the graph the copy number of pMT605 increased quickly during initial rapid growth to a maximum value of about 250 after 48h incubation (first sampling). No samples were taken before this point due to insufficient biomass being available. It was not possible to measure the plasmid copy number in spores but the value was assumed to be very low. The plasmid copy number decreased rapidly after 70h incubation until no plasmid bands could be detected.

3.4.4 Results of one-stage shake-flask fermentations of *S. lividans* TK24 pMT608 using TSB medium.

The results of this experiment are also shown in Figure 34. The copy number of pMT608 also increased quickly during initial rapid growth to a maximum of approximately 350 at 48h. The profile of pMT608 was similar to pMT605 in that plasmid copy number declined rapidly during the stationary phase until only a faint plasmid band could be detected.

3.4.5 Results of two-stage shake-flask fermentations of *S. lividans* TK24 pMT605.

3.4.5.1 MEP medium.

The seed culture for this experiment was grown for 3 days at which point it was transferred, at a level of 15%v/v, into a larger flask. Plasmid copy number results during the second fermentation stage are shown in Figure 35. As seen from the graph the highest copy number value was measured after 55h incubation and declined thereafter. It was not possible to measure the plasmid copy number at transfer since the plasmid band was too faint.

3.4.5.2 MEP+10mM PO_4^{3-} medium.

The seed culture for this experiment was grown for 3 days and transferred at a level of 6%v/v. The average copy number at transfer was approximately 130 and the copy number profile of the fermentation is shown in Figure 36. The graph shows a maximal plasmid copy number of nearly 400 after 31.3h incubation post transfer. The plasmid copy number remained high

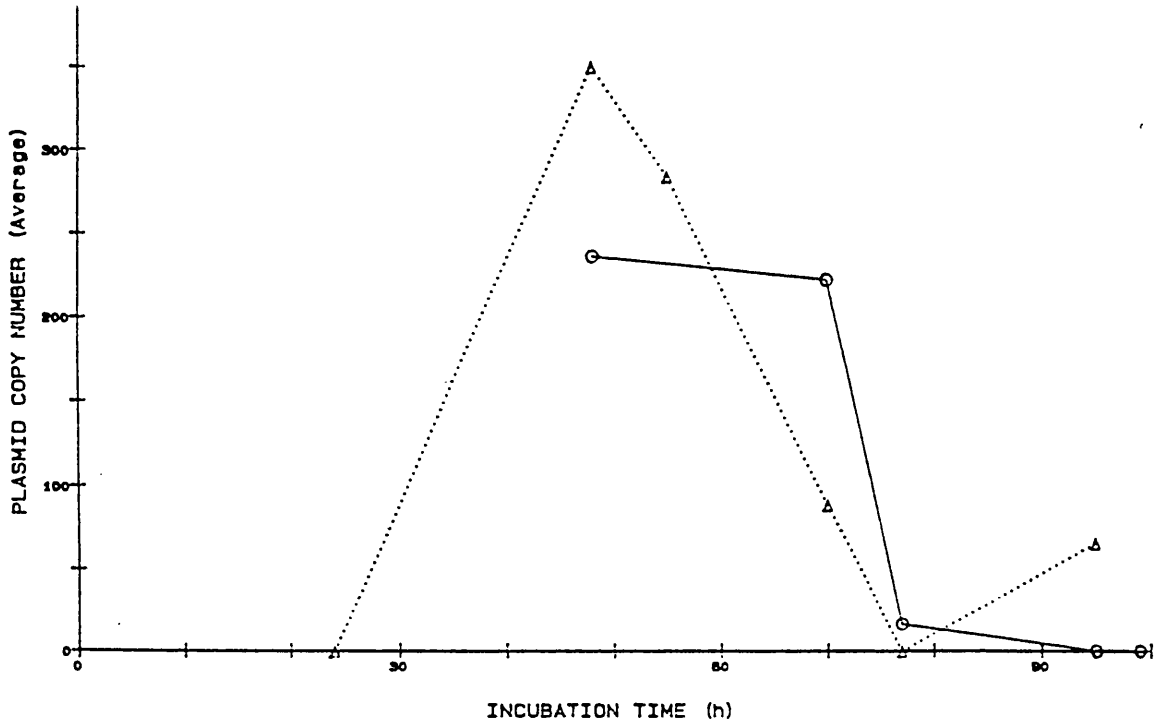

Figure 34 Plasmid copy number data of *S. lividans* TK24 pMT605 (○) and *S. lividans* TK24 pMT608 (△) during a one-stage TSB shake-flask fermentation.

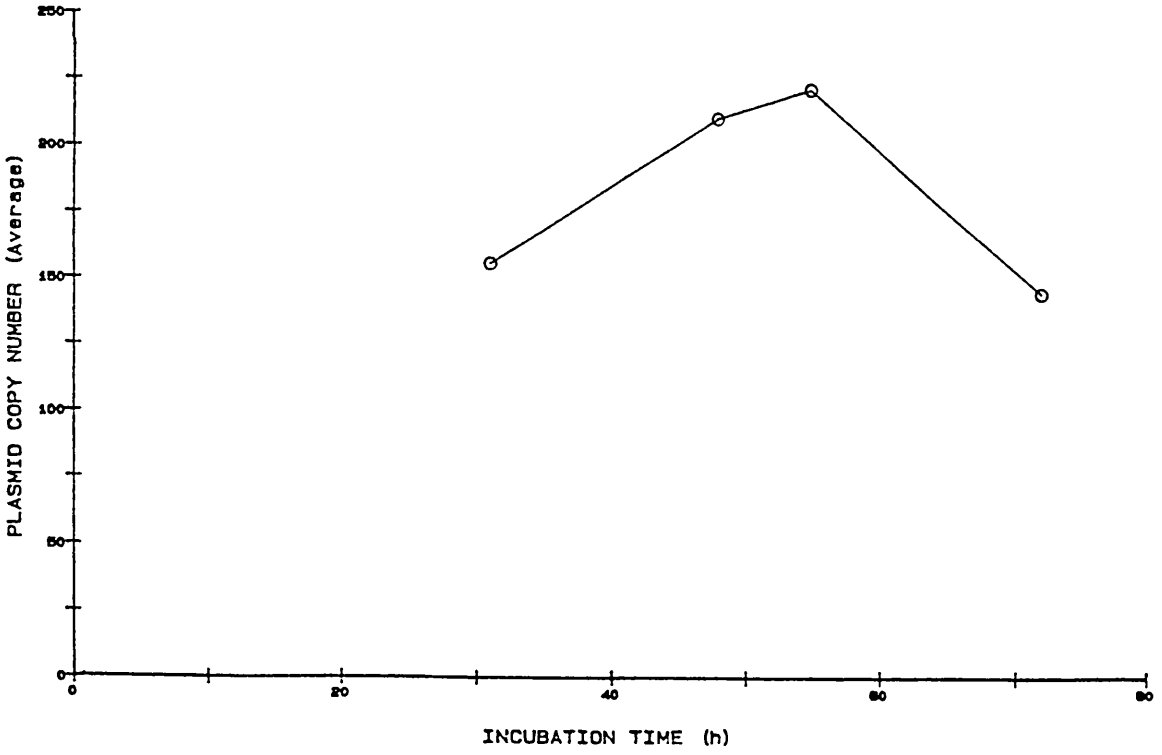

Figure 35 Plasmid copy number profile (○) of a two-stage MEP shake-flask fermentation of *S. lividans* TK24 pMT605.

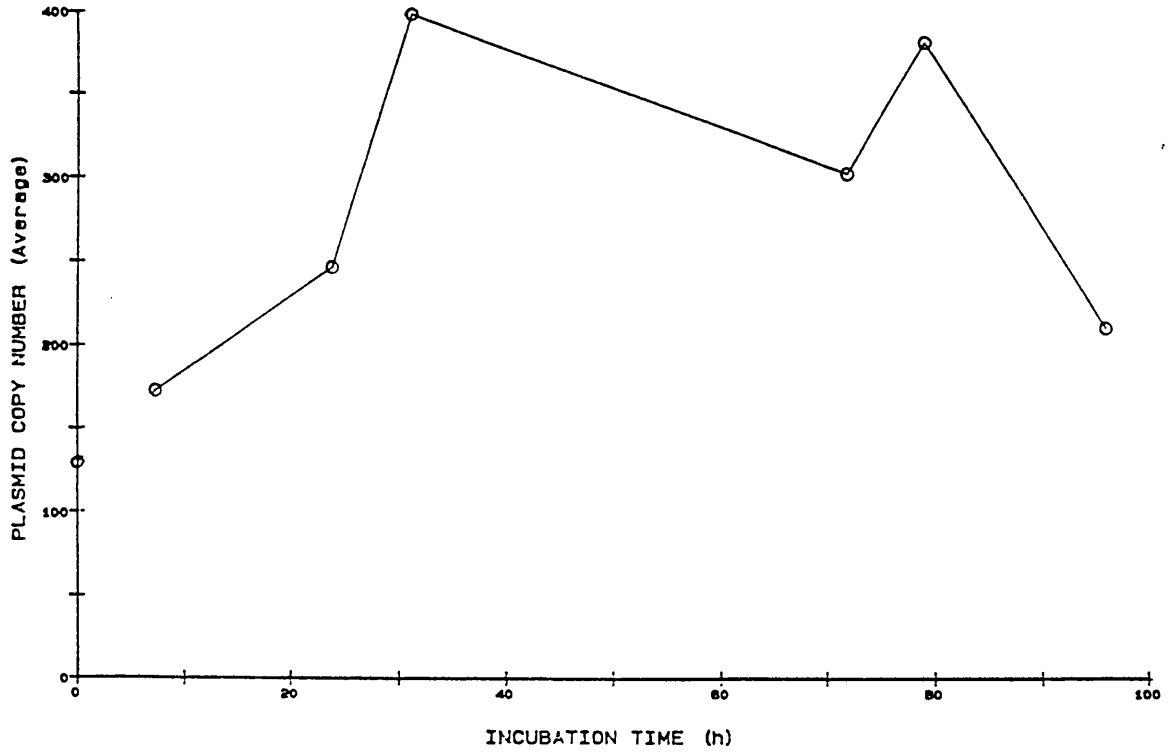

Figure 36 Plasmid copy number profile (○) of a two-stage MEP+10mMPO_4^{3-} shake-flask fermentation of *S. lividans* TK24 pMT605.

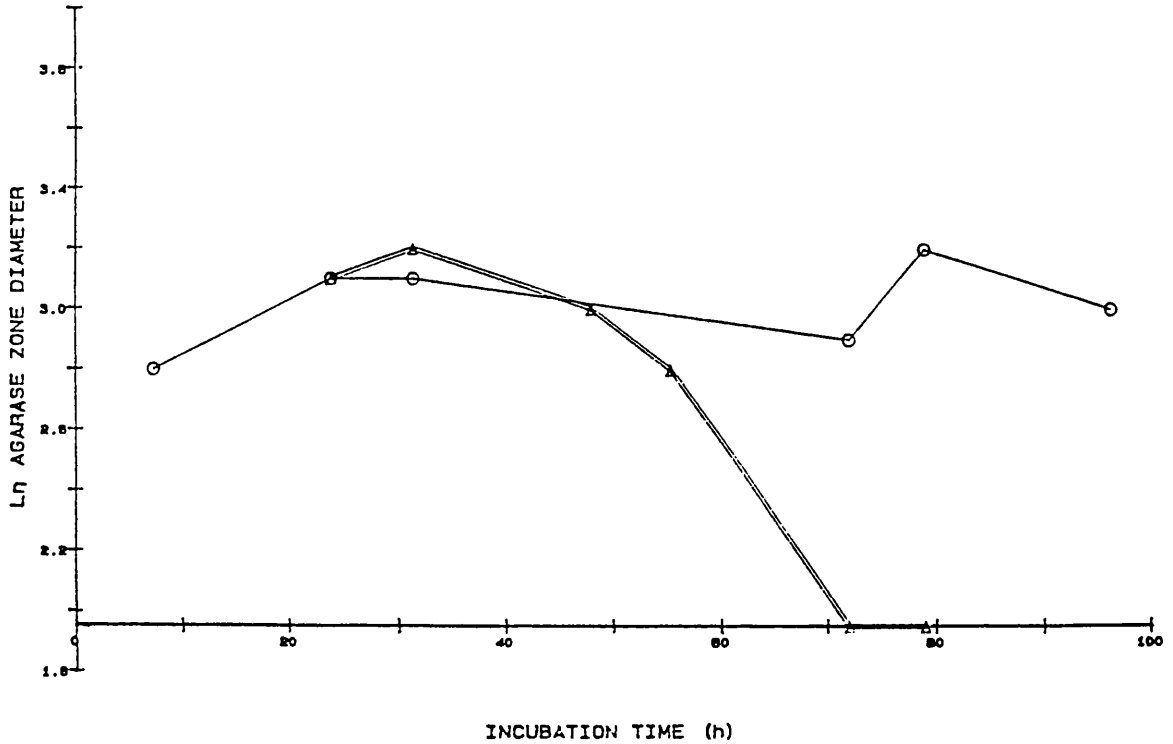

Figure 37 Agarase zone diameters (natural logarithm) of extracellular samples from two-stage MEP+PO_4^{3-} shake-flask fermentations of *S. lividans* TJ24 pMT608. First fermentation (○) and second fermentation (△).

even in late stationary phase, which contrasts with the results obtained from MEP medium culture (Section 3.4.5.1).

During this experiment the level of agarase enzyme production was also monitored in the culture supernatant. Plasmid pMT605 was found to be a poorer producer of agarase enzyme since only faint zones of clearing could be detected in all the samples. Kendall and Cullum (1984) also found that *S. lividans* TK24 pMT605 was a relatively poor producer of the cloned *S. coelicolor* agarase enzyme.

3.4.6 Results of two-stage shake-flask fermentations of *S. lividans* TK24 pMT608.

3.4.6.1 MEP medium.

Two experiments were conducted using MEP medium inoculated with a seed culture. Plasmid copy number data were unavailable from both experiments since the plasmid was found to be too unstable and yielded only extremely faint DNA bands. The seed medium was transferred at a level of 5%v/v and at 6%v/v. Since MEP medium promotes dispersed mycelial growth (refer to Section 3.2.3.1) it was possible to estimate the number of agarase-producing colony forming units on nutrient agar by flooding with iodine solution (refer to Section 3.4.2) and then counting the proportion of colonies with a zone of clearing around them. Up to the 31.5h sample all colonies were surrounded by a zone of clearing. However from 48h to 80h incubation an increasing number (>30%) of colonies could be seen which had lost the ability to produce agarase therefore they may have lost the pMT608 plasmid.

3.4.6.2 MEP+PO_4^{-3} medium.

Two experiments were also conducted using MEP+PO_4^{-3} medium inoculated at 6%v/v with seed culture. Again it was not possible to obtain a complete plasmid copy number profile of pMT608 during either timecourse experiment since only extremely faint plasmids bands could be seen. Where measurements were taken the values reached approximately 70 after 79.3h growth, however measurements are not accurate at such low levels of plasmid DNA.

Despite the lack of plasmid copy number measurements it was possible to estimate the agarase enzyme productivity. Unlike pMT605, pMT608 produced good yields of agarase as detected by agar well assays. The average zone diameter (including the well) were measured and the natural

logarithm calculated. Data from both experiments are shown in Figure 37. In the second experiment the zone size was observed to diminish more rapidly with no measurable zones appearing after 72h incubation.

3.4.7 Remarks on the observed stability of plasmids pMT605 and pMT608.

The data presented in Section 3.4 clearly shows that, on the whole, both of these plasmids are less stable than the parent pIJ702. Although the original workers (Kendall and Cullum, 1984) intended pMT605 and pMT608 to be used as cloning vectors, they are unsuitable because of instability. As reported here, the only experiments where the copy number of pMT608 could be measured were the one-stage (TSB medium) shake-flask timecourses. Such experiments can only be conducted on a small scale since a large number of spores would be required to inoculate even a small fermenter. In accordance with Kendall and Cullum's observations, pMT608 was found to be the best producer of agarase enzyme even when no plasmid bands could not be visualized. This observation supports the view that cloned gene expression alone is not a reliable indicator of plasmid stability since, in the case of pMT608, a very small number of plasmid molecules yielded measurable levels of enzyme activity.

3.5 Stirred-vessel fermentations of *S. lividans* TK24 host.

3.5.1 Aim.

The aim of these early fermentation experiments was to characterize the behaviour of *S. lividans* TK24 in a 5L fermentation vessel. There is little information available on the fermentation characteristics of this organism and these preliminary experiments were intended to highlight likely problems *e.g.* foaming.

3.5.2 Protocol.

All experiments were conducted as two-stage fermentations with vegetative "seed" inocula being used to initiate the 5L volume fermentations. Experiments were conducted as described in Section 2.3 with periodic samples taken for biomass and deoxyribose concentration estimations. No total DNA extractions were performed because of the plasmidless status of the host.

3.5.3 Results of *S. lividans* stirred vessel fermentations.

3.5.3.1 Results of a *S. lividans* 5L fermentation using MEP+PO_4^{3-} medium without pH control.

The results of this initial fermentation are shown in Figure 38. The fermentation was started by adding an inoculum at a 5%v/v level. As seen in the graph the maximum biomass concentration obtained was 4.8gL^{-1} after 24h, however a drop in biomass concentration is seen thereafter. The pH of the medium at the start of the fermentation was approximately pH7. The acidity of the culture increased rapidly during the early stages of growth decreasing to pH2-3. Such acidic conditions probably inhibited further growth after 24h and may have led to partial lysis of the culture. Foam production was successfully controlled with the automatic addition of polypropylene glycol antifoam and by adding a small volume of antifoam before vessel sterilization.

The results of this fermentation indicated that pH control was necessary in order to control the copious production of acids by *S. lividans* during the early stages of growth.

3.5.3.2 Results of *S. lividans* 5L fermentation using MEP+PO_4^{3-} medium with manual alkali additions.

The previous fermentation gave very poor biomass concentrations. After initiating a second fermentation by inoculating with a 4%v/v seed culture, the pH was adjusted at each sampling point by activating a pump

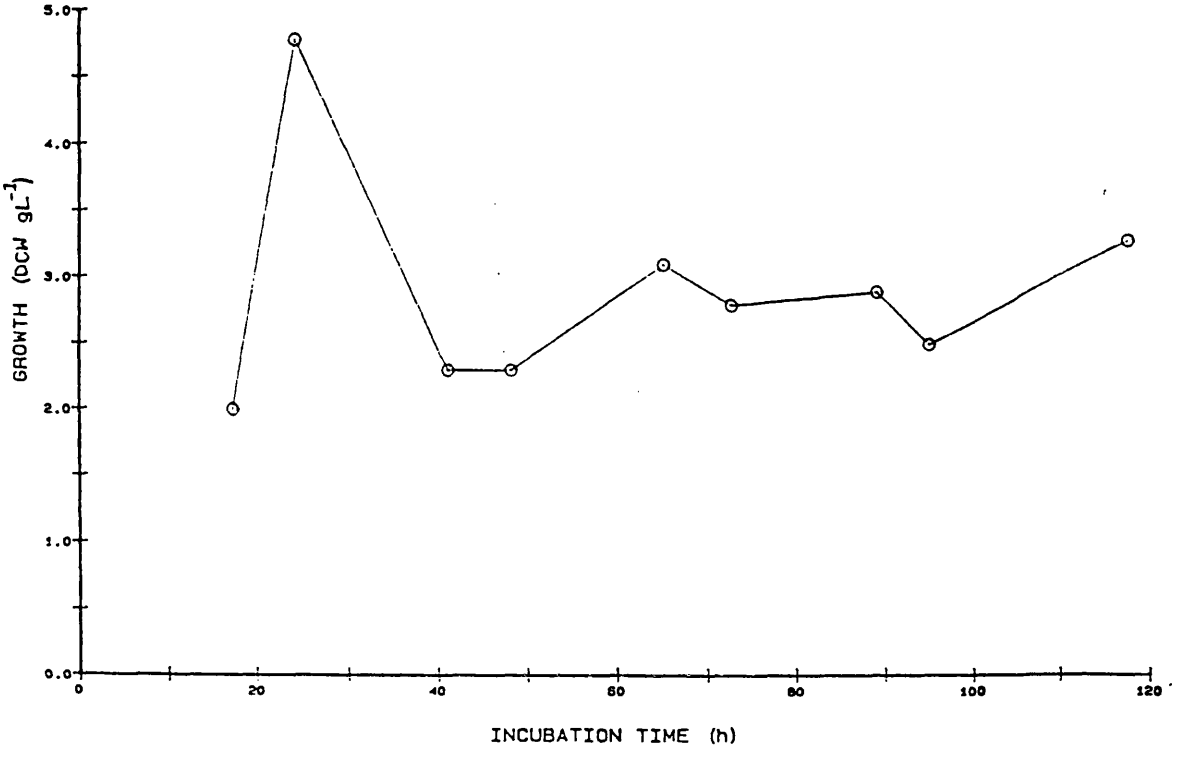

Figure 38 Biomass concentration data (○, gL^{-1}) of a MEP+10mMPO_4^{3-} 5L fermentation of *S. lividans* TK24 without pH control.

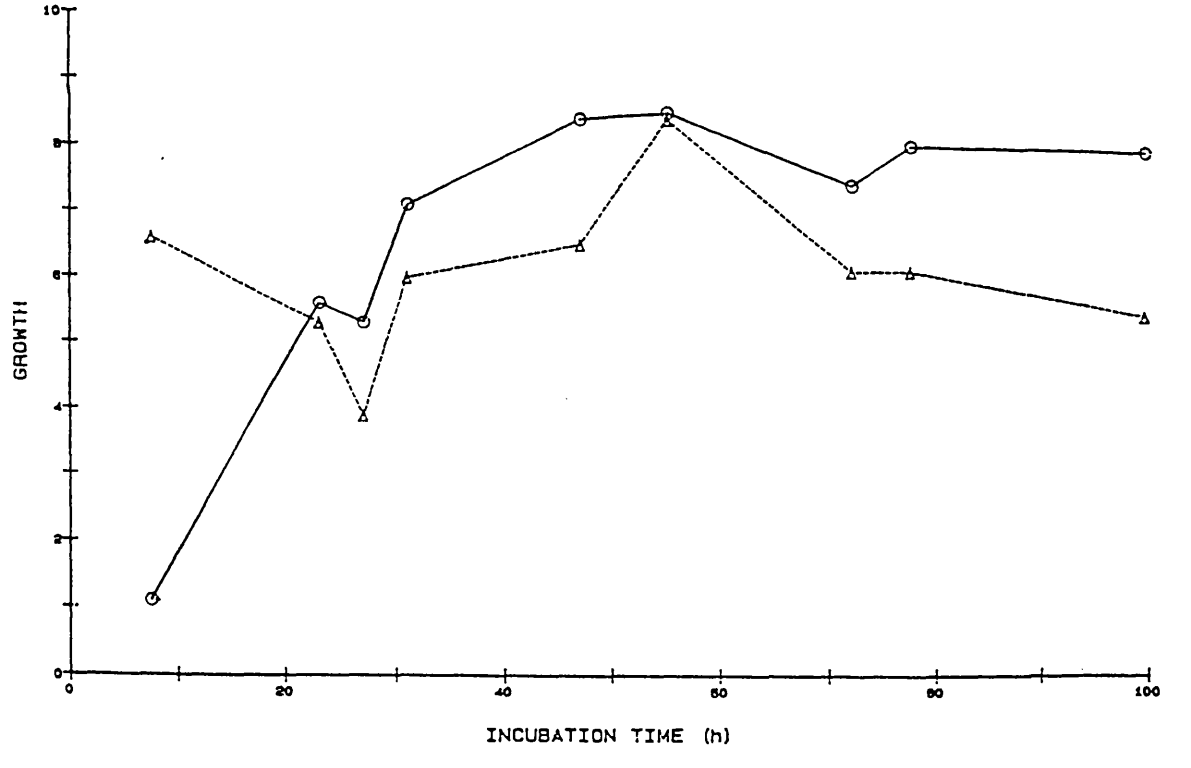

Figure 39 Biomass concentration (○, gL^{-1}) and deoxyribose concentration data (△, μgmL^{-1}) of a MEP+10mMPO_4^{3-} 5L fermentation of *S. lividans* TK24 with manual alkali additions.

connected to an alkali reservoir. Alkali (4M NaOH) was added until the culture pH reach 7.4. No manual additions of alkali were made after 55h since the culture pH had increased to >pH7.4 due to the organism's metabolism. The results of this fermentation are shown in Figure 39. The first phase of (rapid) growth occurred between 7.5h and 23h. A further increase in biomass concentration was seen between 23 and 47h which suggests a second slower phase of growth before the onset of true stationary phase. The deoxyribose concentration (determined from 0.2g of biomass) remained fairly constant at 5-7 μgmL^{-1}. However since the largest volume of broth needed to yield 0.2g of biomass was at the 7.5h sampling point, then deoxyribose concentration expressed as a function of broth volume was very low at this point *i.e.* $36\mu gmL^{-1}L^{-1}$ culture compared with $150\mu gmL^{-1}L^{-1}$ culture after 23h.

3.5.3.3 Results of *S. lividans* 5L fermentation using MEP+PO_4^{3-} with automatic pH control.

The fermentation was initiated by adding a seed inoculum at 6%v/v. Samples were taken from 15h onwards corresponding to the mid-rapid phase of growth. The results are shown in the graph Figure 40. Again evidence is seen from the slight increase in biomass concentration between 23.5h and 47h that a slower second phase of growth occurs before the onset of true stationary phase. The logged fermentation parameters are shown in Figure 41. The pH was controlled at pH7.0 from 10h to 48h, after that period the pH was allowed to increase naturally up to pH8.8 by 100h. The dissolved oxygen tension decreased rapidly during exponential growth to a minimum value of approximately 70% of saturation. The DOT level increased only gradually thereafter suggesting that the culture was still metabolically active and was taking up dissolved oxygen from the medium.

3.5.3.4 Results of *S. lividans* 5L fermentation using MEP medium with automatic pH control.

This fermentation was also initiated by adding a seed culture at 6%v/v level, but the fermentation medium used in the second stage was MEP medium without added inorganic phosphate. Evidence from shake-flask results (see Section 3.2.4) suggested that there was a difference in the maximum biomass concentration obtained from cultures grown in MEP medium and those from phosphate-supplemented MEP. In comparing this and the previous fermentation (Section 3.5.3.3) it will be possible to show whether there is

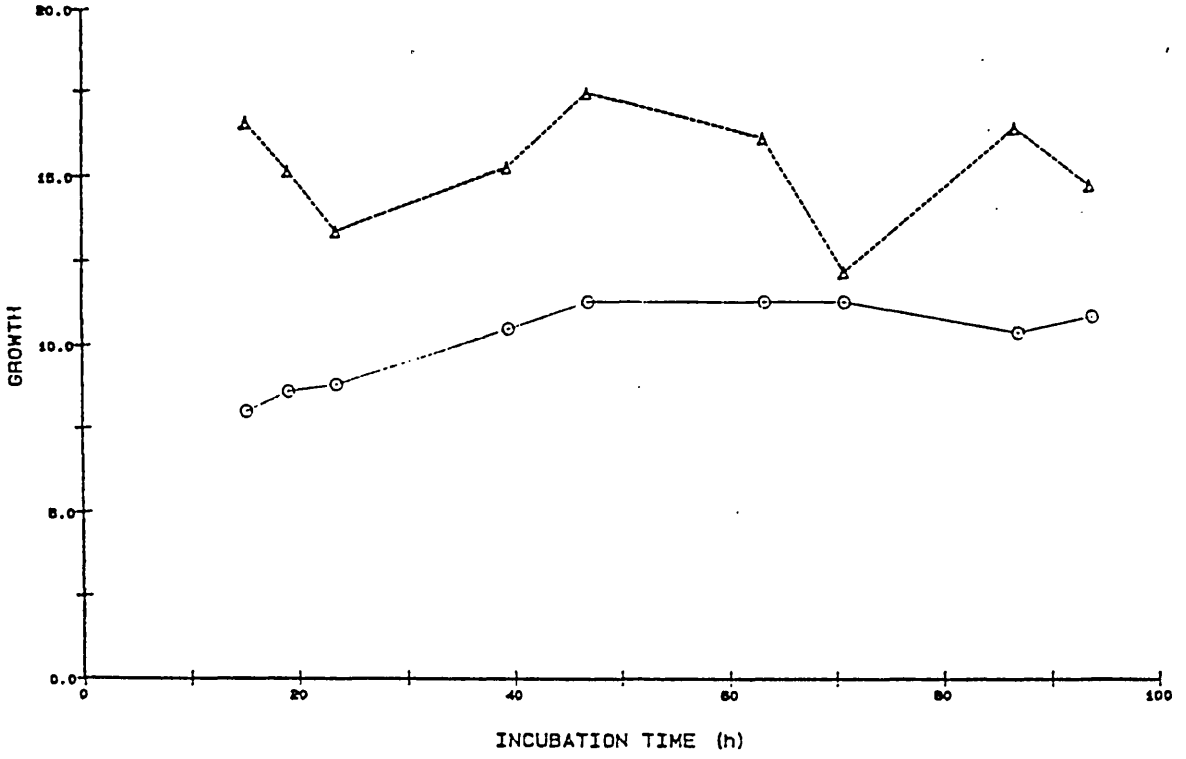

Figure 40 Biomass concentration (○, gL^{-1}) and deoxyribose concentration data (△, μgmL^{-1}) of a MEP+10mMPO_4^{3-} 5L fermentation of *S. lividans* TK24 with automatic pH control.

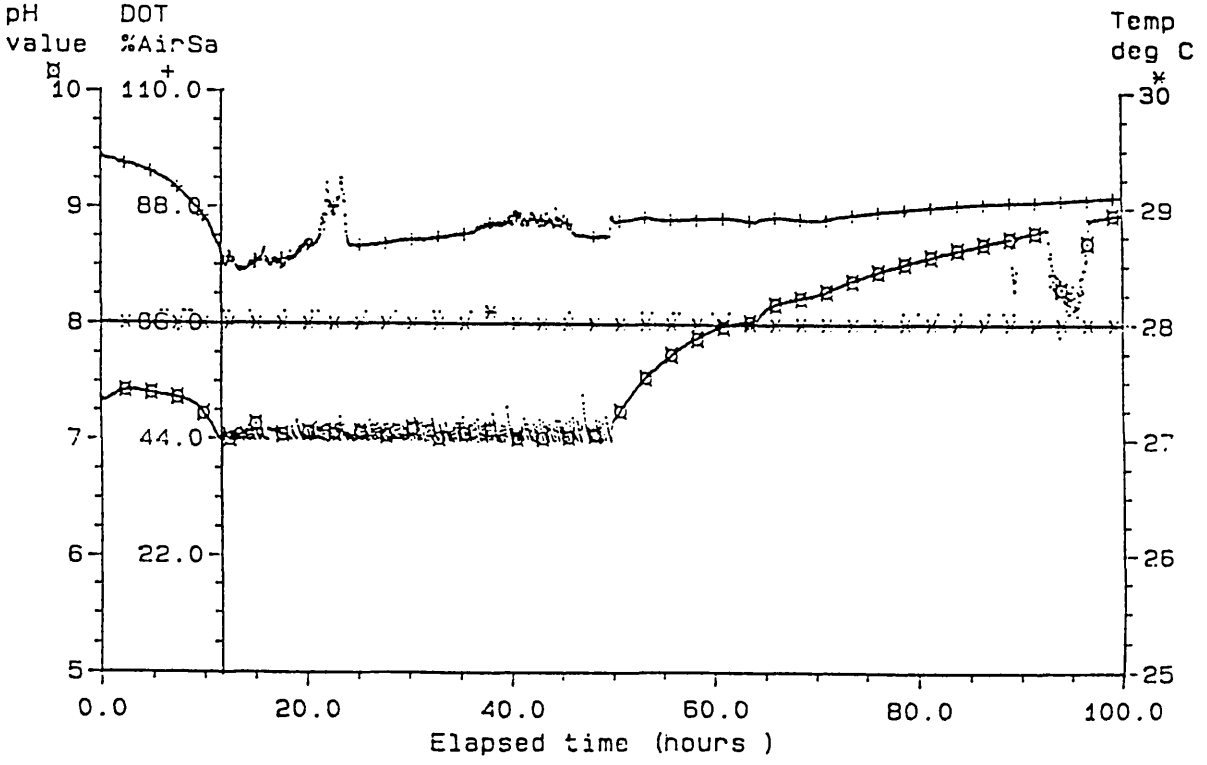

Figure 41 Fermentation parameter data of a MEP+10mMPO_4^{3-} 5L fermentation of *S. lividans* TK24 with automatic pH control. pH (⊡), temperature (✳) and DOT (+).

any a difference between stirred vessel fermentations employing the two media.

The growth data is shown in Figure 42 and the corresponding logged parameter data in Figure 43. When grown in MEP medium *S. lividans* showed a less pronounced rapid growth pattern since the increase in biomass concentration between 15h and 39.3h was linear. However, the maximum biomass concentration, 10.6gL^{-1}, was only slightly less than that achieved with MEP+PO_4^{3-} medium (see Figure 40). The pH profile of both fermentations were very similar. Also the minimum DOT level was slightly higher in the MEP fermentation with a very shallow rise throughout the fermentation suggesting extended metabolic activity.

3.5.4 Remarks on the stirred vessel fermentation characteristics of *S. lividans*.

The main features of the growth of *S. lividans* in MEP with and without added phosphate are that the first stage of rapid growth is accompanied by prolific acid production. The acid production, without neutralization with alkali, would lead to the inhibition of any further growth due to low pH. At the onset of stationary growth the pH was allowed to increase (without acid compensation) in a characteristic mode. The pattern of pH increase proved an useful tool in identifying the "typical" growth of *S. lividans* under these fermentation conditions. After rapid growth there occurs a period of slower (linear) growth immediately preceeding the pH increase. A typical biomass concentration of >10gL^{-1} dry cell weight can be obtained from cultures grown in either medium with automatic pH control. The average volume of 4M NaOH added during these fermentations was 125mL, when this is converted to the number of moles of alkali then it is equivalent to the production of 0.5 mole of organic acid.

DOT levels rarely fell below 70% of saturation which is well above the critical DOT level for *Streptomyces* (Scott *et al*, 1988 measured the critical DOT level of *S. clavuligerus* as 3μM).

In the following sections the features of recombinant *S. lividans* fermentations will be examined and compared to those of the host organism.

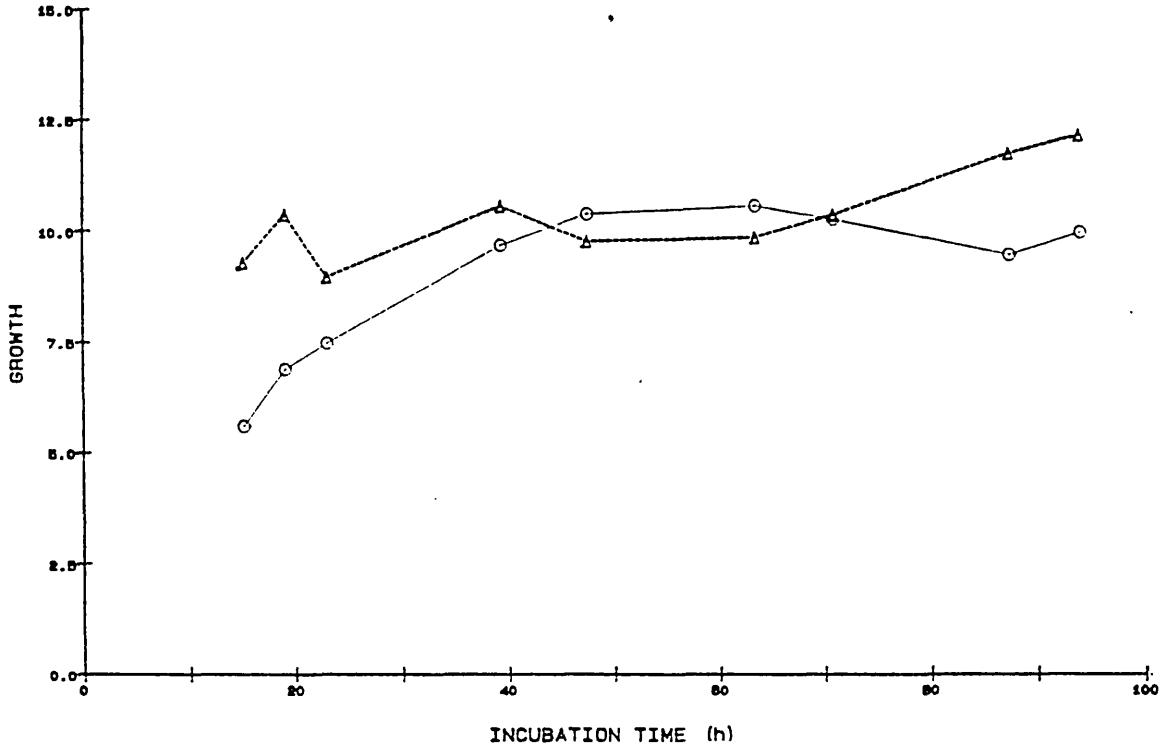

Figure 42 Biomass concentration (○, gL^{-1}) and deoxyribose concentration data (△, μgmL^{-1}) of a MEP 5L fermentation of *S. lividans* TK24 under standard conditions.

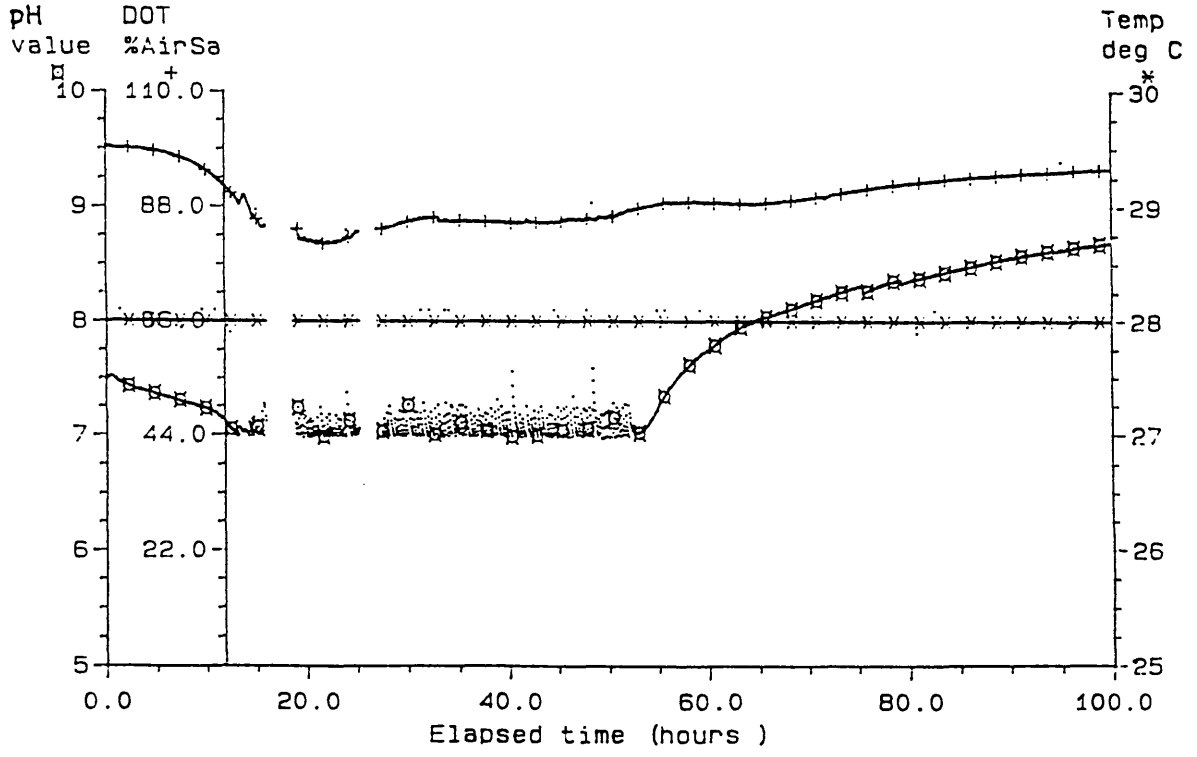

Figure 43 Fermentation parameter data of a MEP 5L fermentation of *S. lividans* TK24. pH (⌑), temperature (✳) and DOT (+).

3.6 Stirred vessel fermentations of *S. lividans* TK24 pIJ303.

3.6.1 Aims.

The aim of these experiments was to investigate the stability of the plasmid pIJ303 in 5L laboratory-scale fermentations. Results of pIJ303 stability in shake-flask experiments are given in Section 3.2. Plasmid copy number was shown to increase rapidly during early growth to a maximum value at early stationary phase. Values of plasmid copy numbers of >500 were achieved under certain growth conditions.

Greater control and monitoring of the fermentation conditions (*e.g.* pH control, good aeration) can be maintained in a stirred vessel fitted with a computer data logging facility. It is of interest, therefore, to study the plasmid stability under such conditions and to compare the profile obtained from shake-flasks with those derived from stirred vessel fermentations. It may then be possible to highlight potential problems of decreased performance upon increasing scale. Also the fermentation conditions can be altered such that decreased phosphate availability and environmental stress (*e.g.* low DOT) may also be studied.

3.6.2 Protocol.

All experiments were conducted as two-stage batch fermentations with a vegetative "seed" inoculum used to initiate the culture. Experiments were conducted as described in Section 2.3 with periodic samplings of biomass for plasmid copy number determinations, biomass concentration and deoxyribose concentration. In experiments where an environmental stress was imposed, a nitrogen gas supply was connected to the fermenter air inlet during the mid-rapid growth phase. The air flow rate was decreased and nitrogen flow rate adjusted to achieve a DOT of <5% saturation where possible. The total gas flow rate was kept approximate to that used during normal growth conditions (*i.e.* 2Lmin-1) in order to maintain similar mixing conditions.

Whenever possible the BIOi data logging package (Section 2.2.2) was used to collect and retrieve fermentation information and a mass spectrometer was used to analyse outlet gases (Section 2.2.3). However, due to frequent equipment and software malfunction, complete sets of fermentation data were difficult to obtain.

3.6.3 Results of *S. lividans* pIJ303 stirred vessel fermentations.

3.6.3.1 Results of *S. lividans* pIJ303 5L fermentations using MEP+PO_4^{3-} medium.

A series of four fermentations were conducted using this particular host-vector combination grown in phosphate-supplemented MEP medium. Due to the problems of excessive wall growth resulting in the unnecessary activation of the foam control loop, a large volume (*circa* 50mL) of antifoam was deposited into the culture. The antifoam used (polypropylene glycol) is not toxic to the organism, neither can it be utilised as a carbon source. However at this level it can seriously affect the dry cell weight determinations due to the broth failing to filter properly. Because of this problem two fermentations only have biomass concentration data up to 30h. During future fermentations where large amounts of wall growth was expected, only a small volume of antifoam was made available in the reservoir and the foam control loop was switched off after initial rapid growth.

The biomass and dexoyribose concentration data of the first fermentation are shown in Figure 44 and the plasmid copy number data is shown in Figure 45. Also the percentage of supercoiled plasmid DNA (as a fraction of total plasmid peak integral) is shown on the same graph. In previous published reports no distinction is made between the relative amounts of plasmid topoisomers (it is assumed that only SC plasmid bands were measured). This may have led to an underestimate of plasmid copy number, therefore in this work all visible plasmid topoisomers are in determinations. The plasmid copy number of pIJ303 increased quickly during initial rapid growth to a maximum value of about 250 at 24h, no significant change was seen during the remainder of the fermentation. The % supercoiled plasmid (%SC) at seed transfer was 22% but this proportion fell to zero during initial growth. The level of SC plasmid then rose steadily throughout the experiment until 61% of the plasmid was in a SC form after 79h. The biomass was over 50% greater in 5L fermentations compared to similar shake-flask results (refer to Section 3.2.6.3).

Comparable results of the three further fermentations are shown in Figures 44 to 50. In general the plasmid copy number pattern during initial rapid growth to 20h is similar in all the fermentations since a sharp increase was seen during this period. The reason for the decrease in

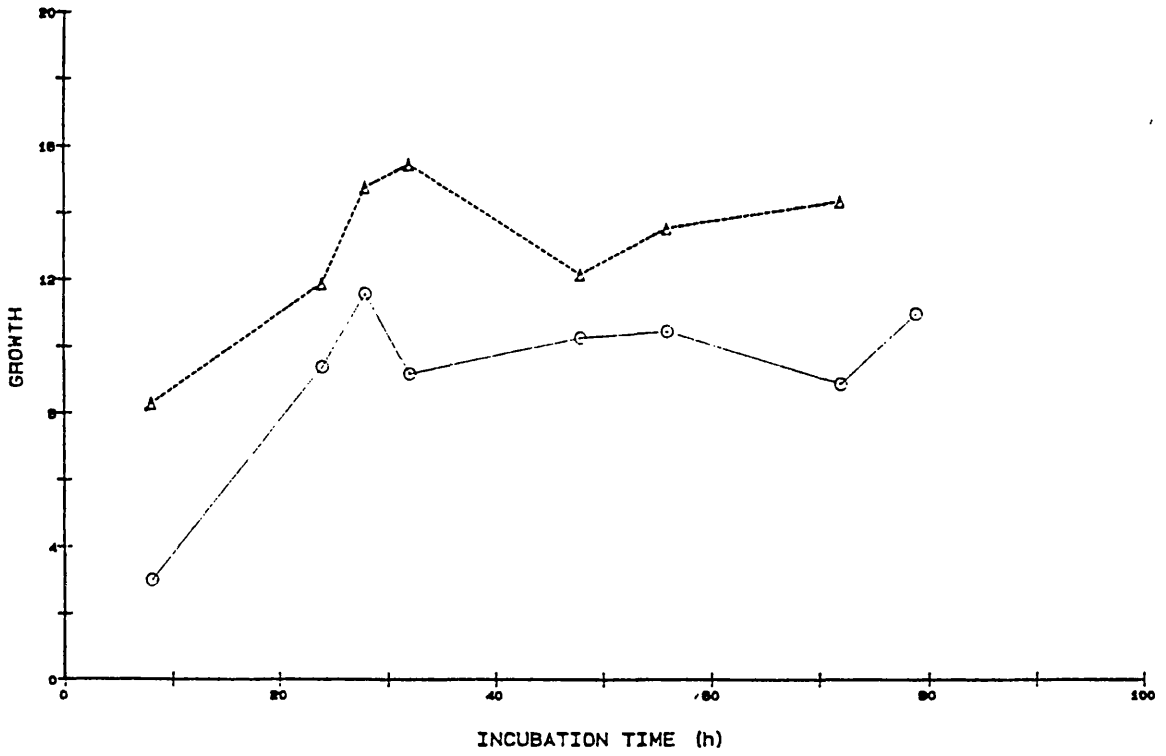

Figure 44 Biomass concentration (○, gL^{-1}) and deoxyribose concentration (△, μgmL^{-1}) data of a MEP+10mMPO_4^{3-} 5L fermentation of *S. lividans* TK24 pIJ303.

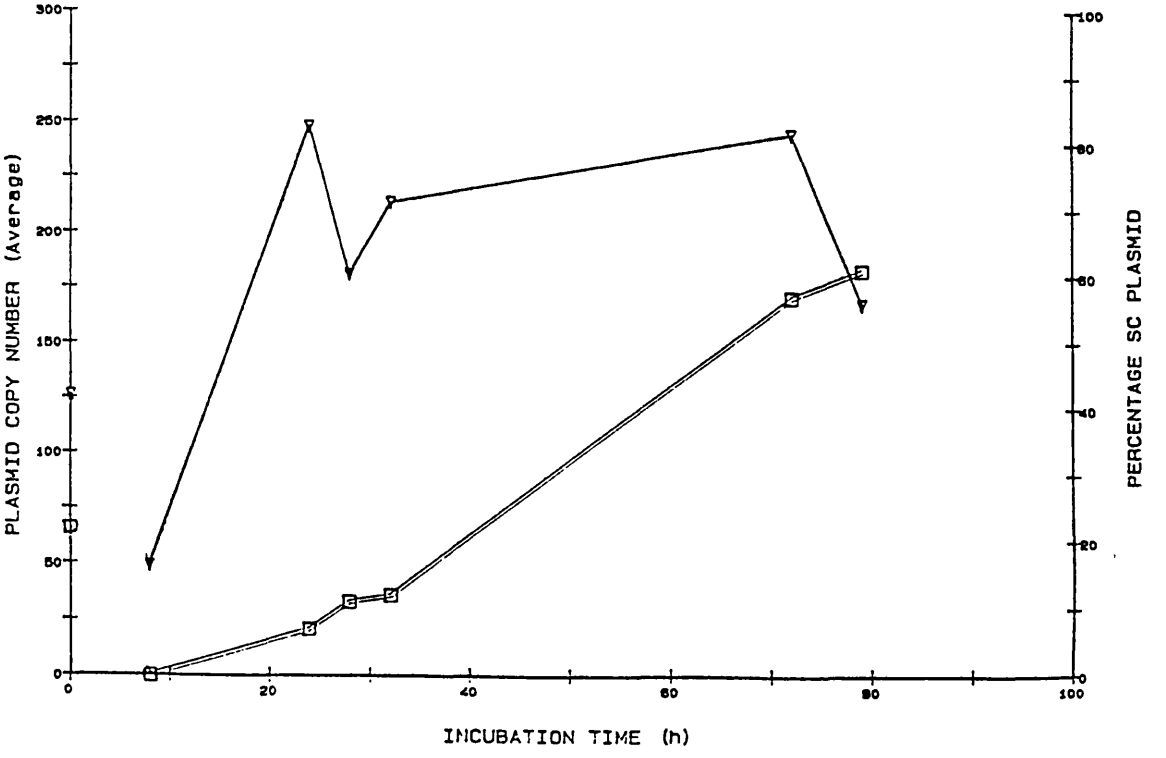

Figure 45 Plasmid copy number (▽) and percentage supercoiled plasmid (□) data of a MEP+10mMPO_4^{3-} 5L fermentation of *S. lividans* TK24 pIJ303.

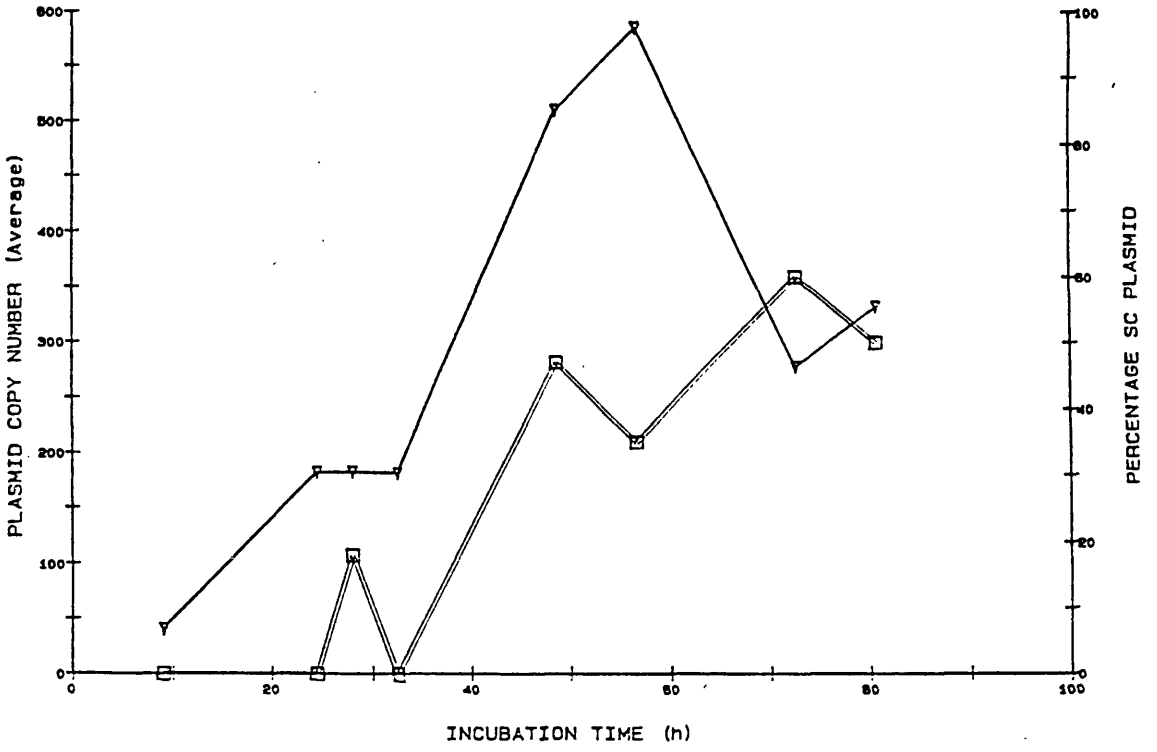

Figure 46 Plasmid copy number (▽) and percentage supercoiled plasmid (□) data of a MEP+10mMPO_4^{3-} 5L fermentation of *S. lividans* TK24 pIJ303.

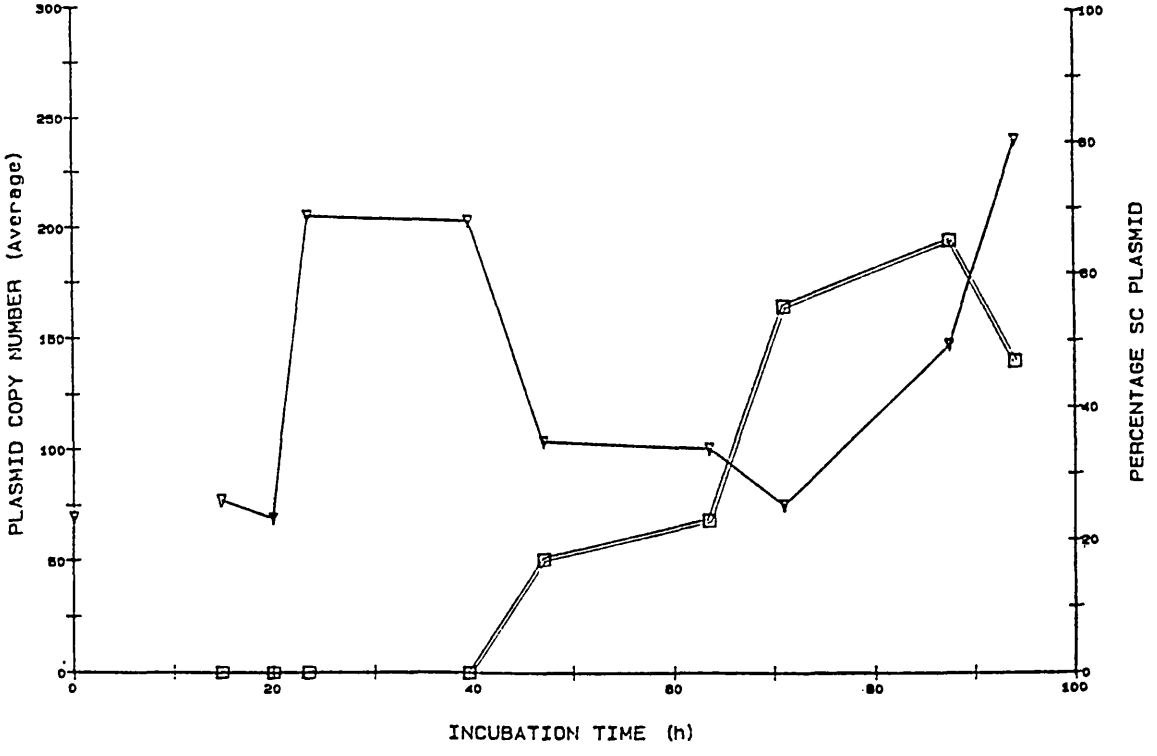

Figure 47 Plasmid copy number (▽) and percentage supercoiloed plasmid (□) data of a MEP+10mMPO_4^{3-} 5L fermentation of *S. lividans* TK24 pIJ303.

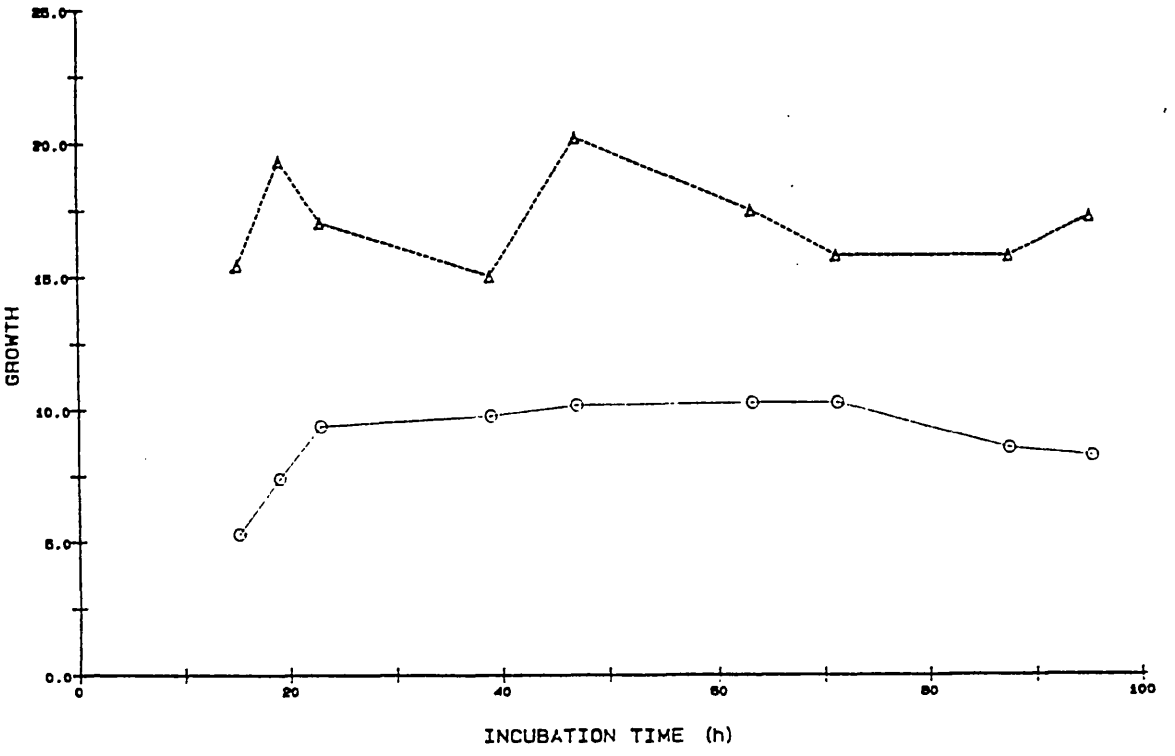

Figure 48 Biomass concentration (○, gL^{-1}) and deoxyribose concentration (△, μgmL^{-1}) data of a MEP+10mMPO_4^{3-} 5L fermentation of *S. lividans* TK24 pIJ303.

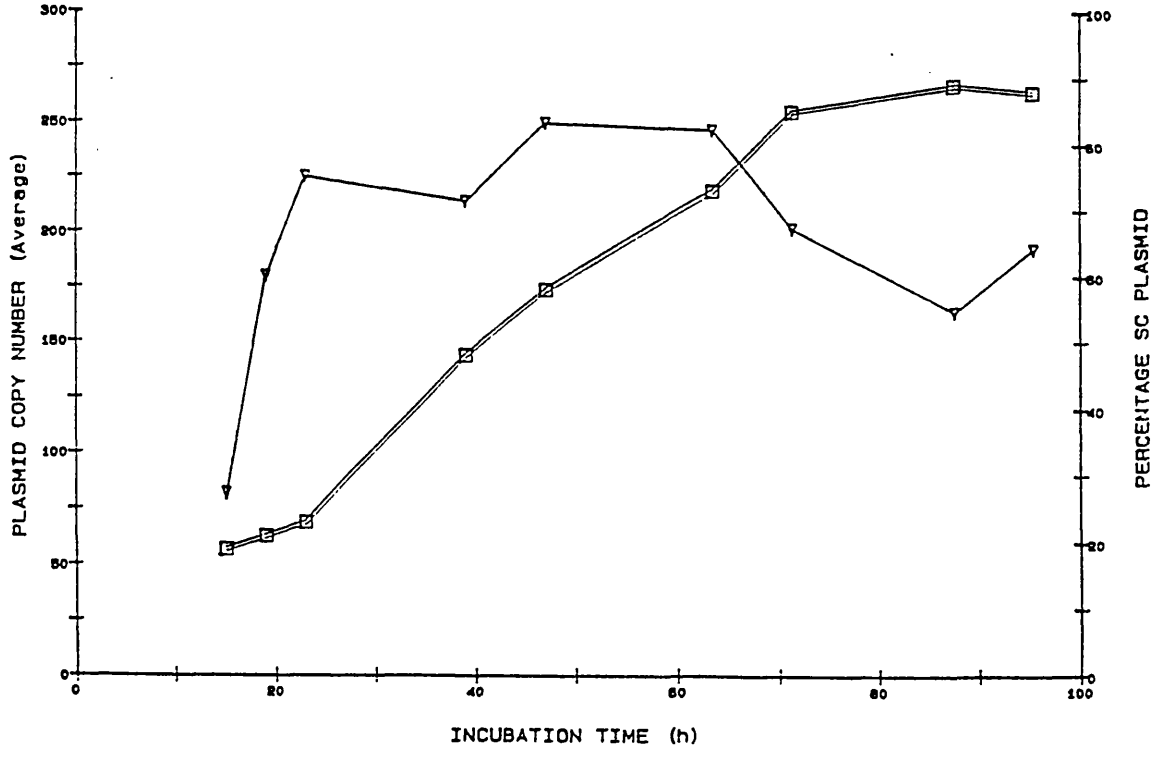

Figure 49 Plasmid copy number (▽) and percentage supercoiled plasmid (□) data of a MEP+10mMPO_4^{3-} 5L fermentation of *S. lividans* TK24 pIJ303.

copy number in Figure 47 between 47h and 71h is unknown but may be related to a period of rapid secondary growth. Since biomass concentration data from this experiment is not available then this assumption cannot be confirmed. The last fermentation in the series yielded the most complete set of data which are shown in Figures 48, 49 and 50. A gel used to calculate data presented in Figure 49 is shown in Figure 51, where the progressive accumulation of SC DNA can be observed.

Typically, a maximum biomass concentration of $>10gL^{-1}$ was achieved under these growth conditions. Also deoxyribose concentrations (derived from the extraction of 0.2 g of biomass) peaked in late rapid growth phase with up to $20\mu gmL^{-1}$ deoxyribose detected in the extract. A similar trend in values was seen throughout although the absolute concentrations were highly variable due to the variability of extraction. Such errors were minimized in any given fermentation by processing all the samples simultaneously.

The fermentation computer logged data of the last experiment are shown in Figure 50. As seen in the graph the DOT decreased during initial rapid growth to a minimum value of 75% of saturation. The DOT level then increased steadily during the remainder of the fermentation suggesting a second, less rapid phase of growth. The pH profile of the latter stages is typical of that obtained by growing *S. lividans* under these conditions. The pH was controlled at pH7.0 from approximately 15h at 50h. During this series of fermentations an average volume of 115mL of 4M NaOH (18g) was needed for pH control. This level of alkali was needed to neutralize 0.45 mole equivalents of organic acid produced. After this point the culture was allowed to become progressively more alkaline due to its own metabolic activity. The fermentation characteristics of *S. lividans* pIJ303 fermentations and *S. lividans* plasmidless host were found to be very similar (refer to Figure 41, Section 3.5.3.3 and Figure 50 this section).

3.6.3.2 Results of *S. lividans* pIJ303 5L fermentations using MEP medium.

Two fermentations were conducted using the MEP medium without added inorganic phosphate. Results of fermentations with the host organism suggested that there were few differences between growth characteristics for the two media (refer to Section 3.5.3.3 and 3.5.3.4) during stirred vessel culture. Experiments in shake-flasks suggested much better growth in phosphate-supplemented MEP but this seems not to be the case in 5L

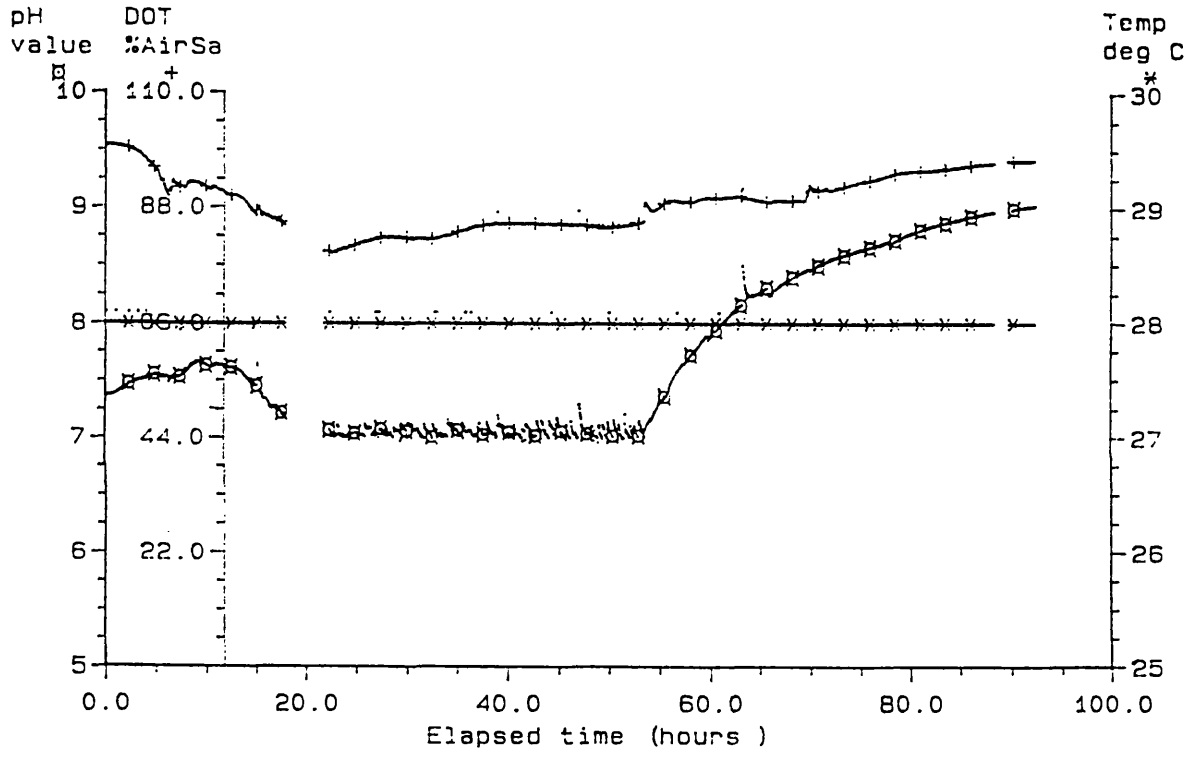

Figure 50 Fermentation parameter data of a MEP+10mMPO$_4^{3-}$ 5L fermentation of *S. lividans* TK24 pIJ303. pH (⊡), temperature (✳) and DOT (+).

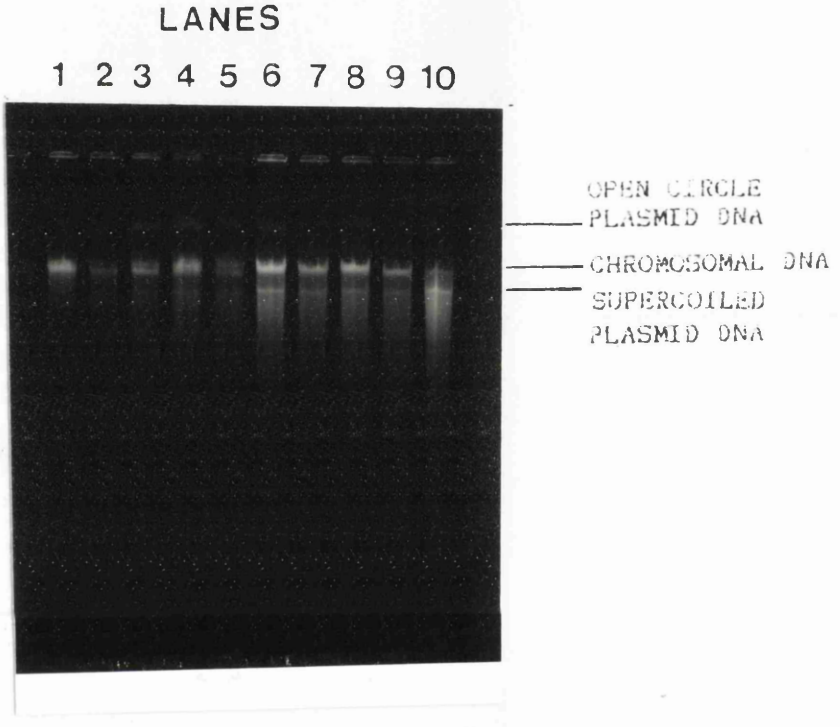

Figure 51 Total DNA extracts (at 20uL loadings) of samples taken during final MEP+10mMPO_4^{3-} 5L fermentation of *S. lividans* TK24 pIJ303. Change in relative proportions of plasmid topoisomers can be seen as incubation time increases.

Lanes 1-9 are timepoints 15h-97h, lane 10=160h.

fermentations. Despite these observations it was of interest to see whether the lack of available inorganic phosphate in MEP medium would affect plasmid copy number and/or the dominant topoisomers obtained.

The results of the first MEP fermentation are shown in Figure 52 where the biomass and deoxyribose concentrations are presented. The plasmid copy number and percentage SC plasmid data is shown in Figure 53 and the fermentation parameter data (and outlet gas analysis) are shown in Figures 54 and 55. The maximum biomass concentration obtained compared favourably with that from MEP+PO_4^{3-}-grown cultures, but was achieved after 70h growth. Plasmid copy number was maximal after 23h (approximately 380) followed by a decline to an average value of 250 for the remainder of the experiment. However, after 160h the copy number remained at a level of >100 (data not shown). The %SC plasmid profile was also very similar to those obtained from phosphate-supplemented fermentations as described in Section 3.6.3.1. Since outlet gas analysis data was available for this fermentation the respiratory quotient (RQ) could be calculated (Buckland *et al*, 1985). The maximum RQ was 1.06 at 14h.

The biomass and deoxyribose concentration data of the second MEP fermentation are shown in Figure 56 and the plasmid copy number and %SC data in Figure 57. As seen in the graphs a phase of rapid initial growth was accompanied by a higher copy number value. The rapid rise in plasmid copy number was not seen since it had already occurred before the first sampling point (14.8h). The average copy number at seed transfer was about 160 with 100% SC plasmid molecules. This observation may account for the subsequently high proportion of SC plasmid molecules seen throughout the fermentation (approximately 50%). The plasmid copy number after 160h growth was approximately 280 (63% SC molecules), data not shown. The logged fermentation and gas analysis data of this experiment are shown in Figures 58 and 59.

The maximum respiratory quotient for this fermentation was calculated to be 0.8 at 12h which was therefore less than that of the previous fermentation. The reduced growth rate of the second MEP fermentation may also explain the high copy number value sustained throughout this experiment.

A significant volume of 4M NaOH was required to control the pH in MEP fermentations as well as in phosphate-supplemented MEP fermentations.

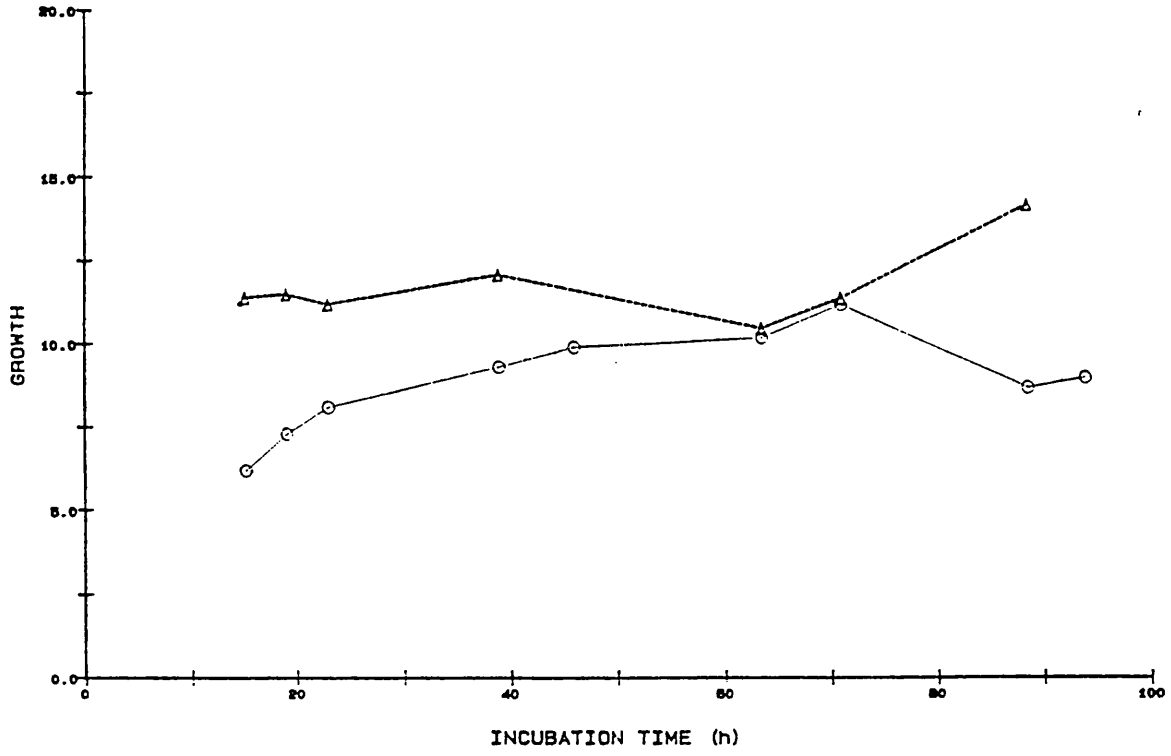

Figure 52 Biomass concentration (○, gL^{-1}) and deoxyribose concentration (△, μgmL^{-1}) data of a MEP 5L fermentation of *S. lividans* TK24 pIJ303.

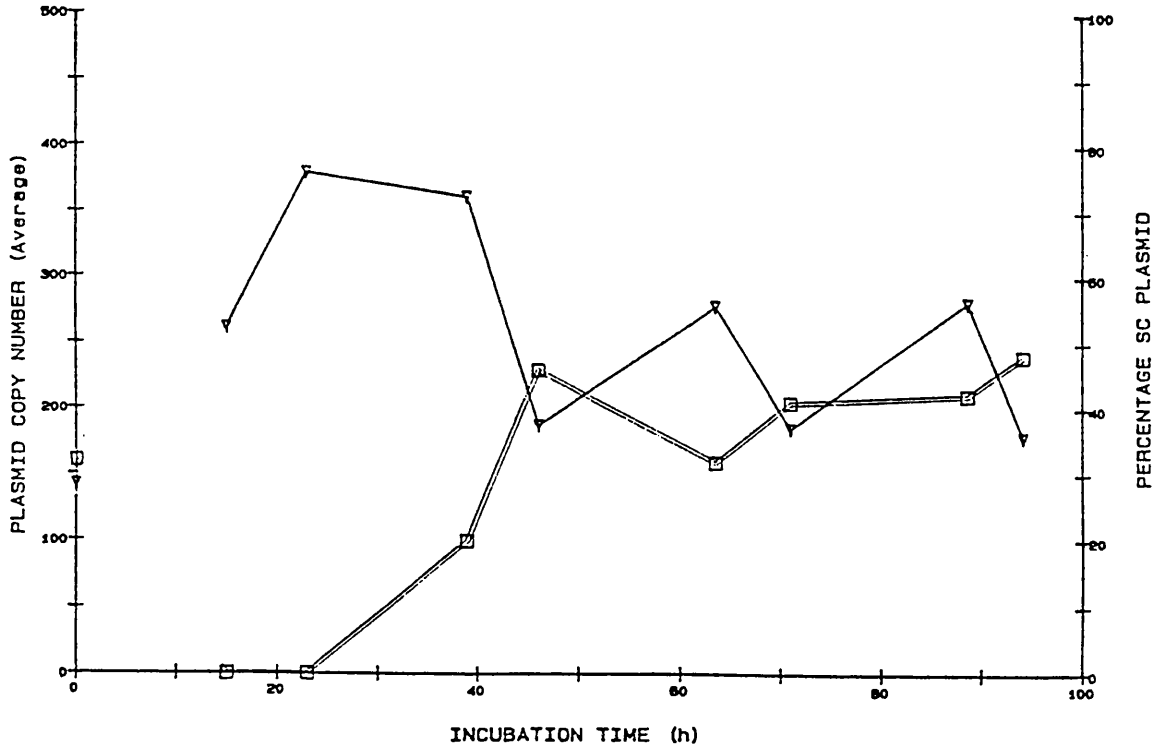

Figure 53 Plasmid copy number (▽) and percentage supercoiled plasmid (□) data of a MEP 5L fermentation of *S. lividans* TK24 pIJ303.

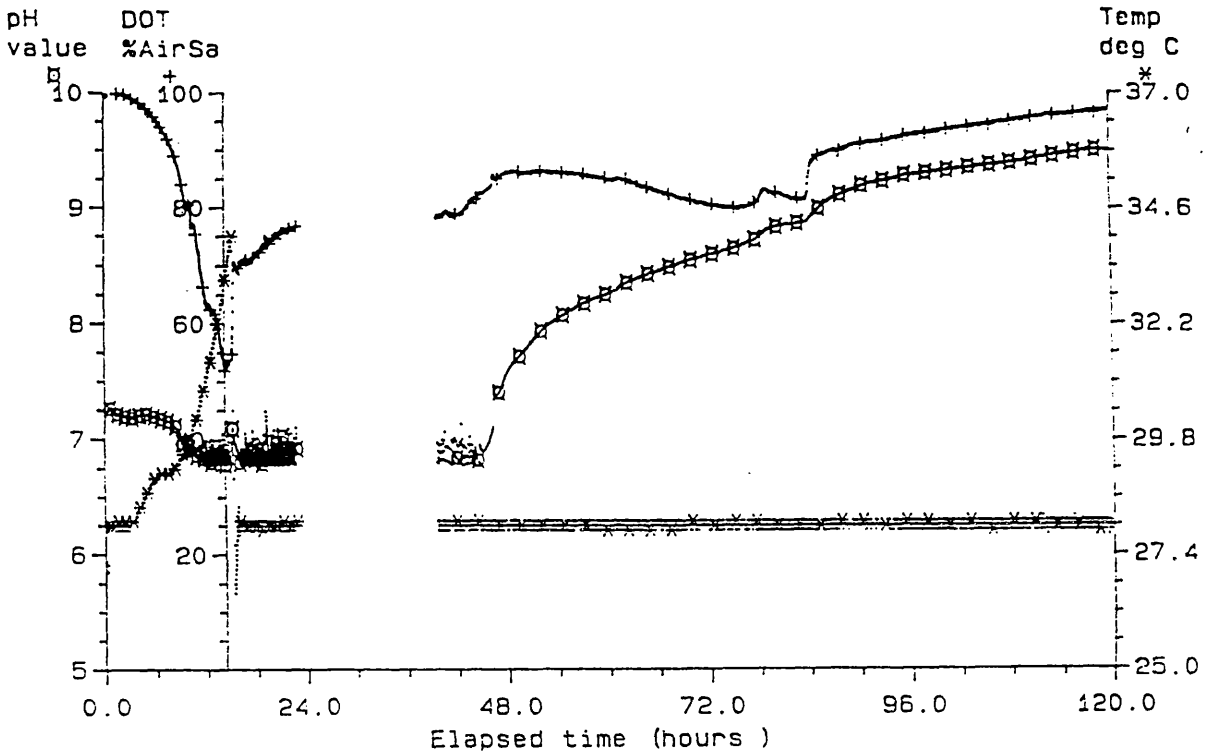

Figure 54 Fermentation parameter data of a MEP 5L fermentation of *S. lividans* TK24 pIJ303. pH (⊡), temperature (✳) and DOT (+).

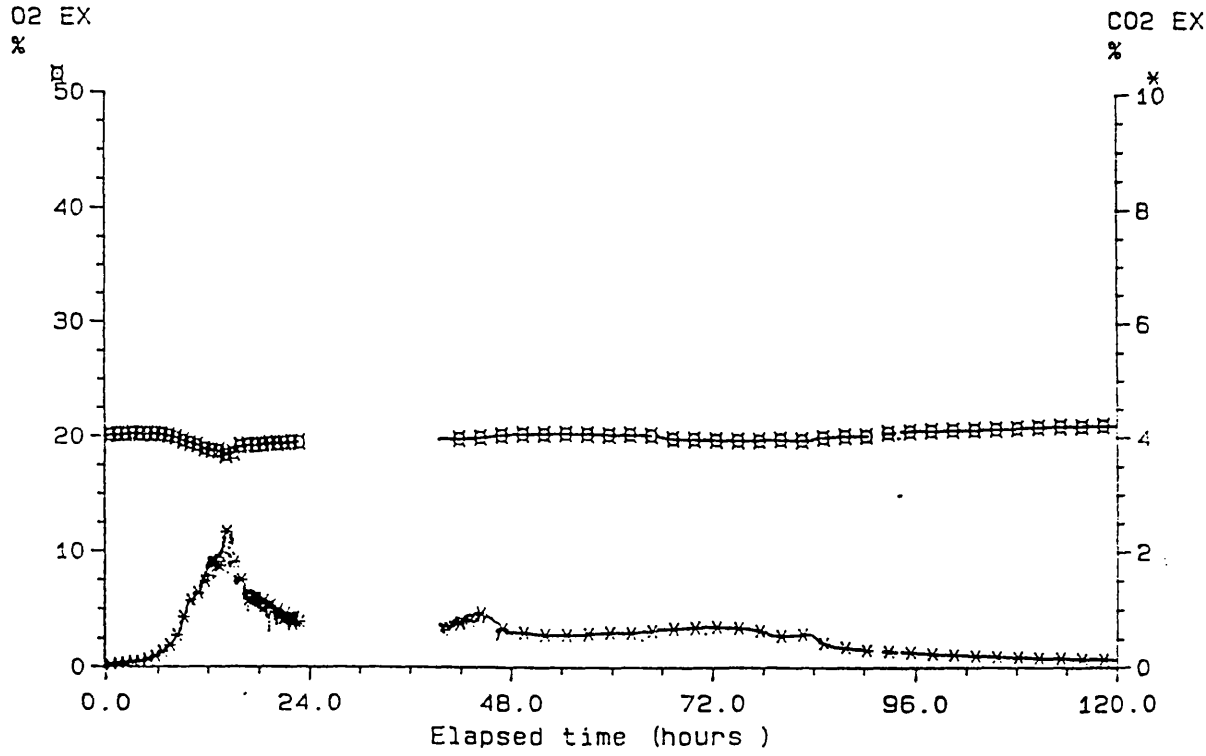

Figure 55 Gas analysis data of a MEP 5L fermentation of *S. lividans* TK24 pIJ303. $\%CO_2$ (✳) and $\%O_2$ (⊡).

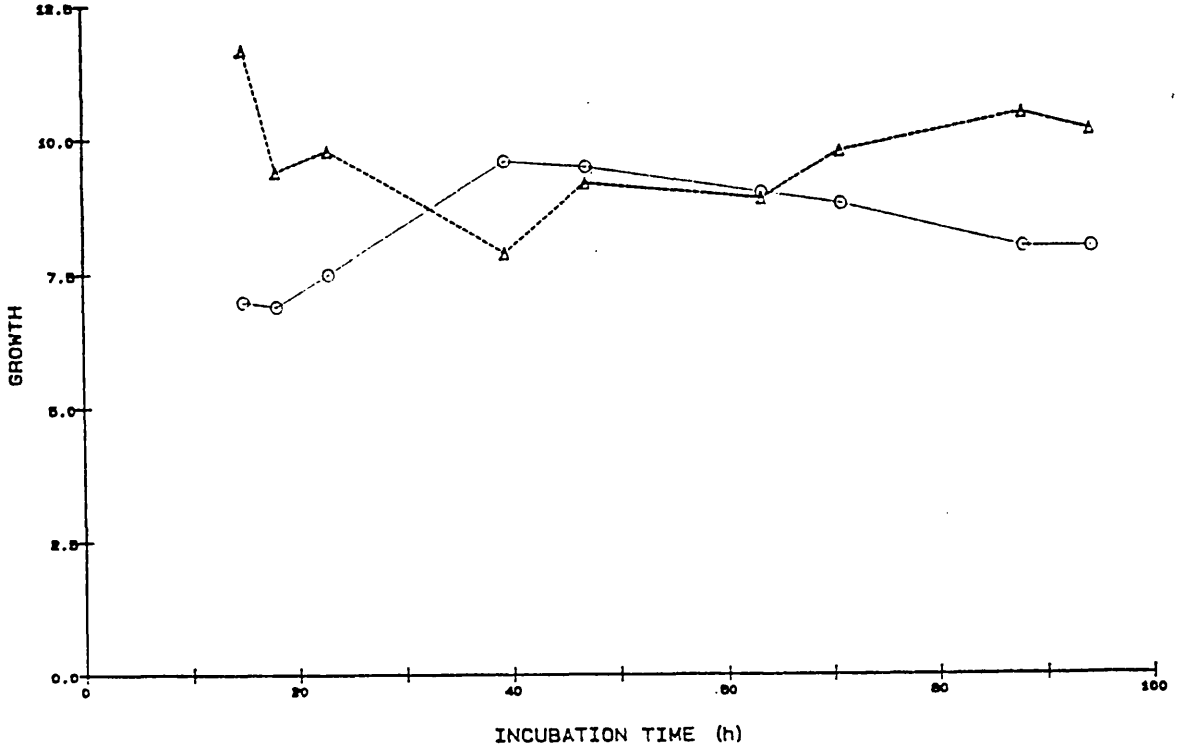

Figure 56 Biomass concentration (○, gL^{-1}) and deoxyribose concentration (△, μgmL^{-1}) data of a MEP 5L fermentation of *S. lividans* TK24 pIJ303.

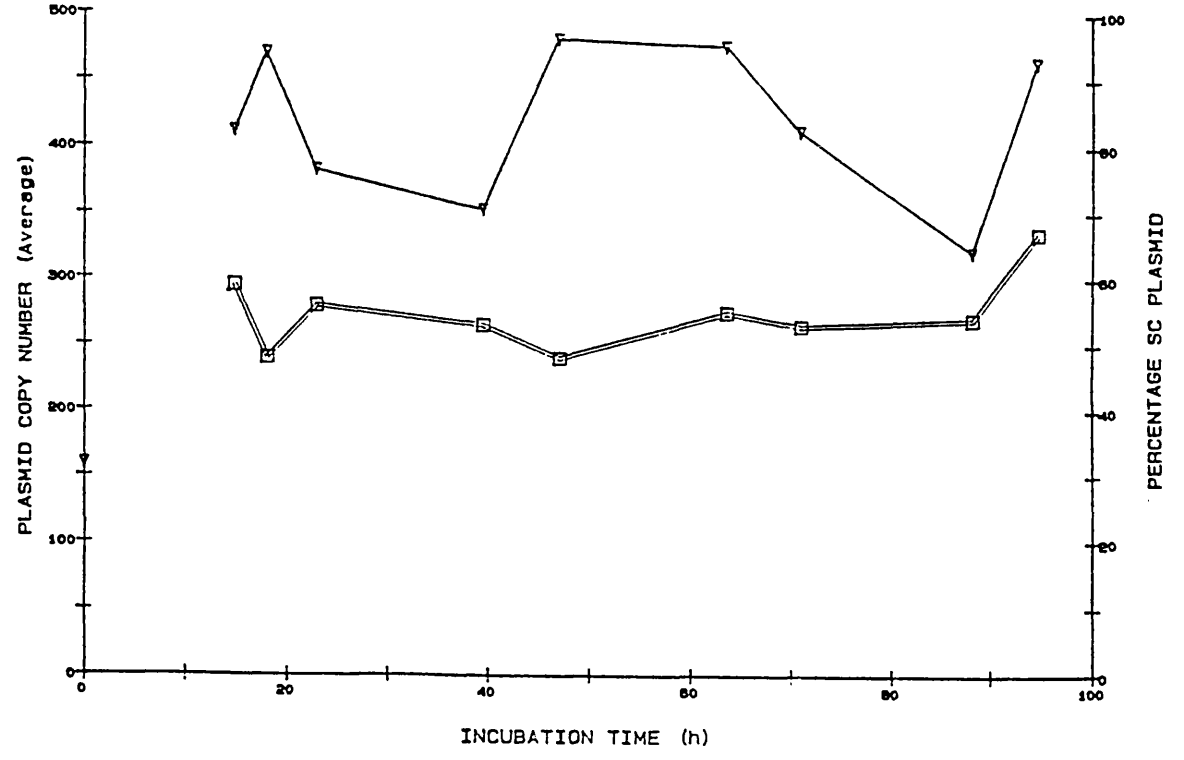

Figure 57 Plasmid copy number (▽) and percentage supercoiled plasmid (□) data of a 5L MEP fermentation of *S. lividans* pIJ303.

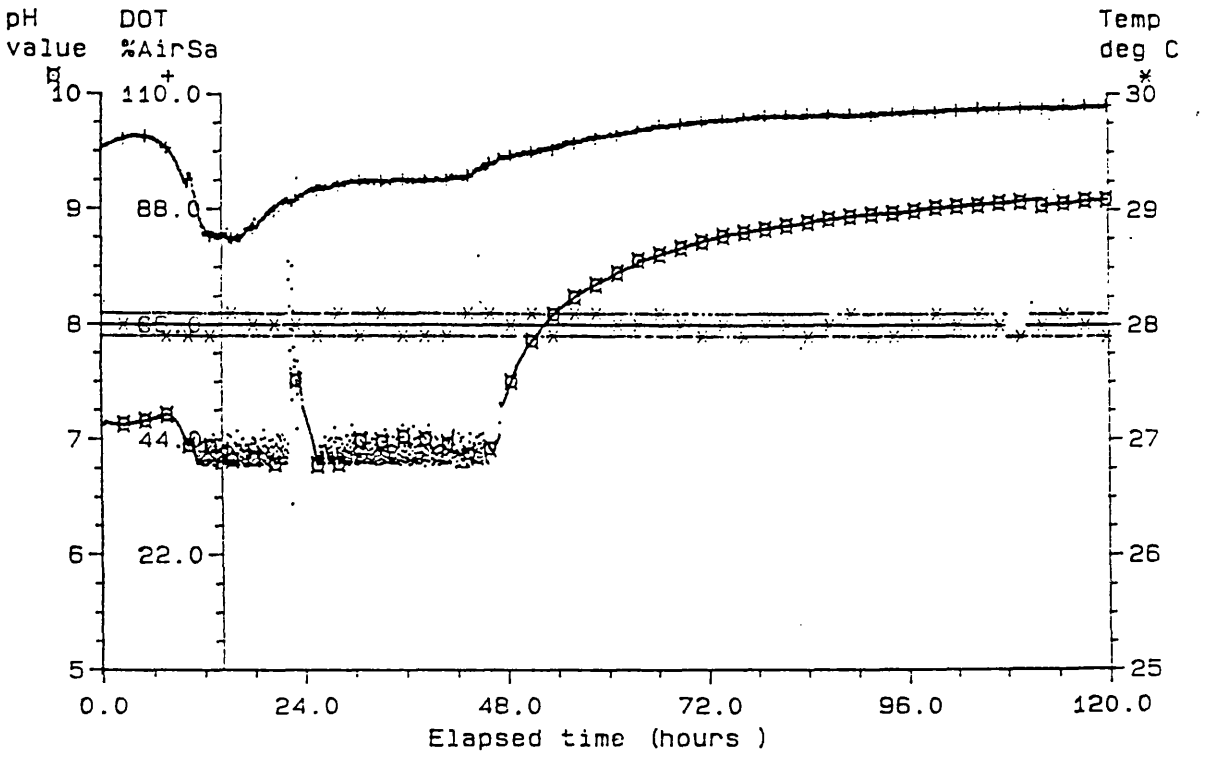

Figure 58 Fermentation parameter data of a MEP 5L fermentation of *S. lividans* TK24 pIJ303. pH (⊠), temperature (✳) and DOT (+).

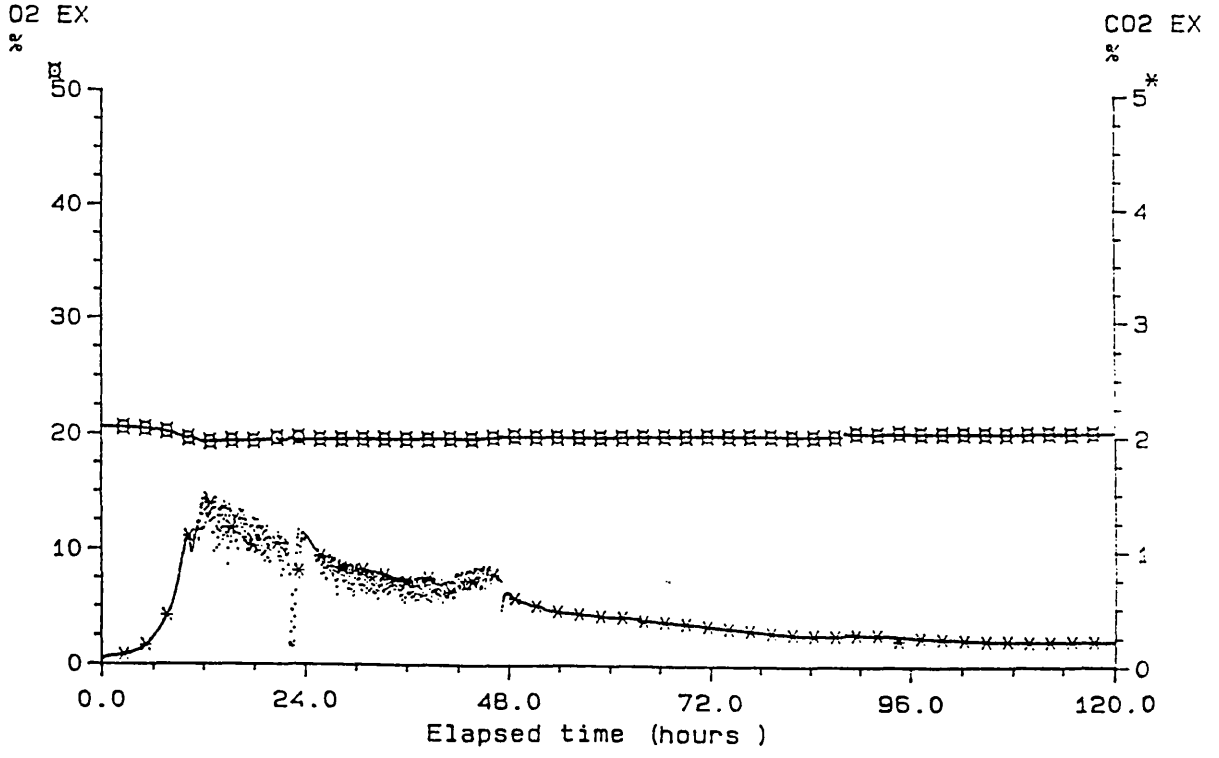

Figure 59 Gas analysis data of a MEP 5L fermentation of *S. lividans* TK24 pIJ303. $\%CO_2$ (✳) and $\%O_2$ (⊠).

During these two experiments an average volume of 140mL 4M NaOH (22g) was added to control the culture pH. Therefore 22g of NaOH was needed to neutralise 0.55 mole equivalents of organic acid produced.

The fermentation characteristics in MEP medium were therefore similar to those in phosphate-supplemented media. The effect upon plasmid copy number was not conclusive due to the noticable differences between the two MEP fermentations. The behaviour of the first was typical of *S. lividans* pIJ303 grown in MEP+PO_4^{3-} medium. The results of the second fermentation may be explained since the seed culture containing 100% SC plasmid (despite being grown under identical conditions). Another reason may have been the slightly reduced growth rate in MEP medium. The decreased availability of inorganic phosphate was not thought to directly result in the observed data. The logged fermentation data of the recombinant culture again compared favourably with those of the plasmidless host in MEP medium (see Section 3.5.3.4).

3.6.3.3 Results of *S. lividans* pIJ303 5L fermentations under DOT stress using MEP+PO_4^{3-} medium.

In order to explore the effect of low DOT levels on pIJ303 copy number, two fermentations were conducted using nitrogen as the inlet gas in order to displace dissolved oxygen from the culture at mid-exponential phase. The effect of high temperature on *S. lividans* pIJ303 cultures in shake-flasks has already been studied (refer to Section 3.2.5). From these experiments it was found that plasmid copy number was considerably reduced when grown at 37°C. A similar effect is expected under DOT stress, although DNA degradation and cell lysis may be more pronounced under anaerobic conditions.

The results of the first fermentation are shown in Figure 60 (biomass and deoxyribose concentration), 61 (plasmid copy number and %SC), 62 (fermentation parameter data) and 63 (outlet gas analysis). The biomass concentration shows a rapid phase of growth up to 15h. At this point the DOT was reduced to <5% (see Figure 62) and no further increase in biomass concentration is seen. The plasmid copy number however was still increasing until 21h. A rapid decline was seen thereafter until the plasmid copy number stabilized to 45-90 up to 135.5h incubation (data beyond 100h not plotted). Complete plasmid loss, therefore did not occur but degradation of both chromosomal and plasmid DNA was observed (refer to Figure 64 showing

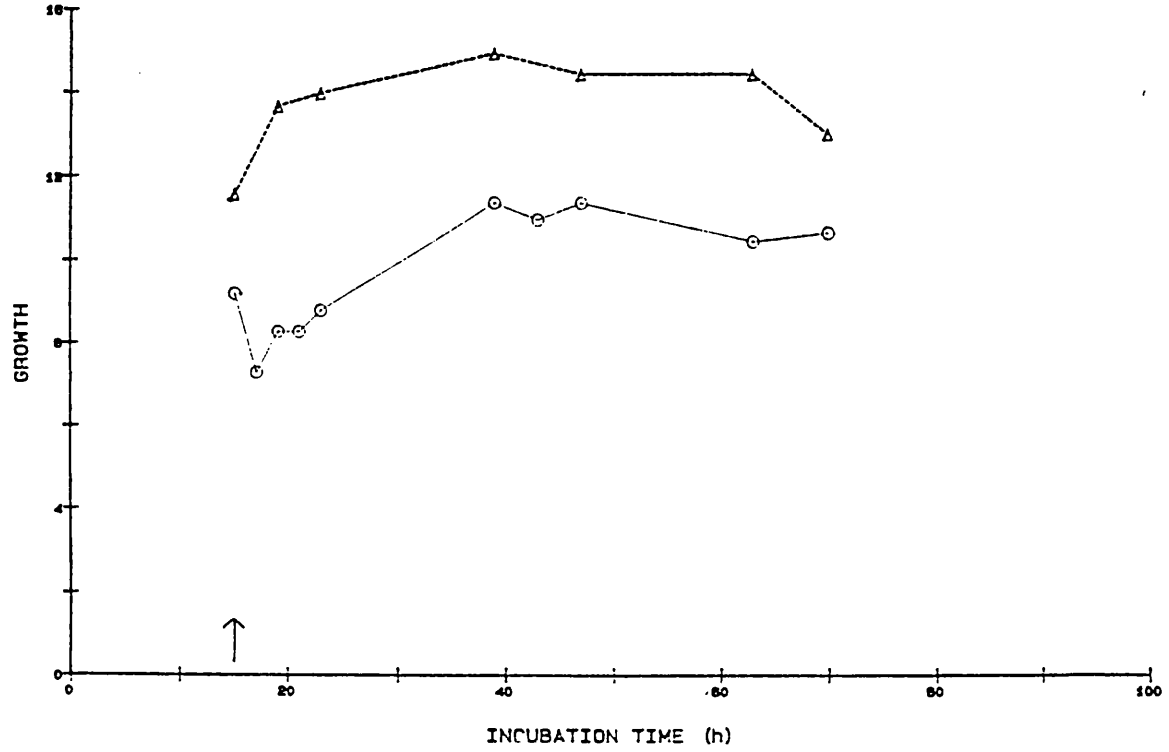

Figure 60 Biomass concentration (O, gL^{-1}) and deoxyribose concentration (Δ, μgmL^{-1}) data of a MEP+10mMPO_4^{3-} 5L fermentation of *S. lividans* TK24 pIJ303 under DOT stress after 15h (marked with arrow).

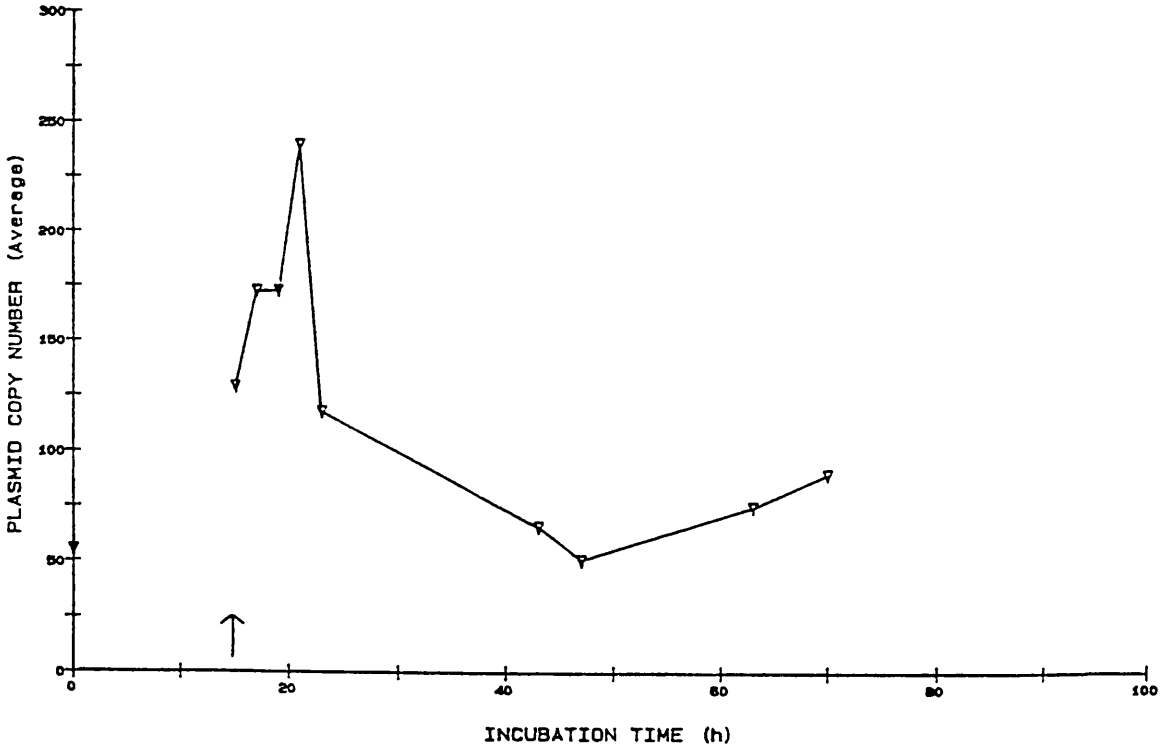

Figure 61 Plasmid copy number profile (∇) of a MEP+10mMPO_4^{3-} 5L fermentation of *S. lividans* TK24 pIJ303 under DOT stress after 15h (marked with arrow).

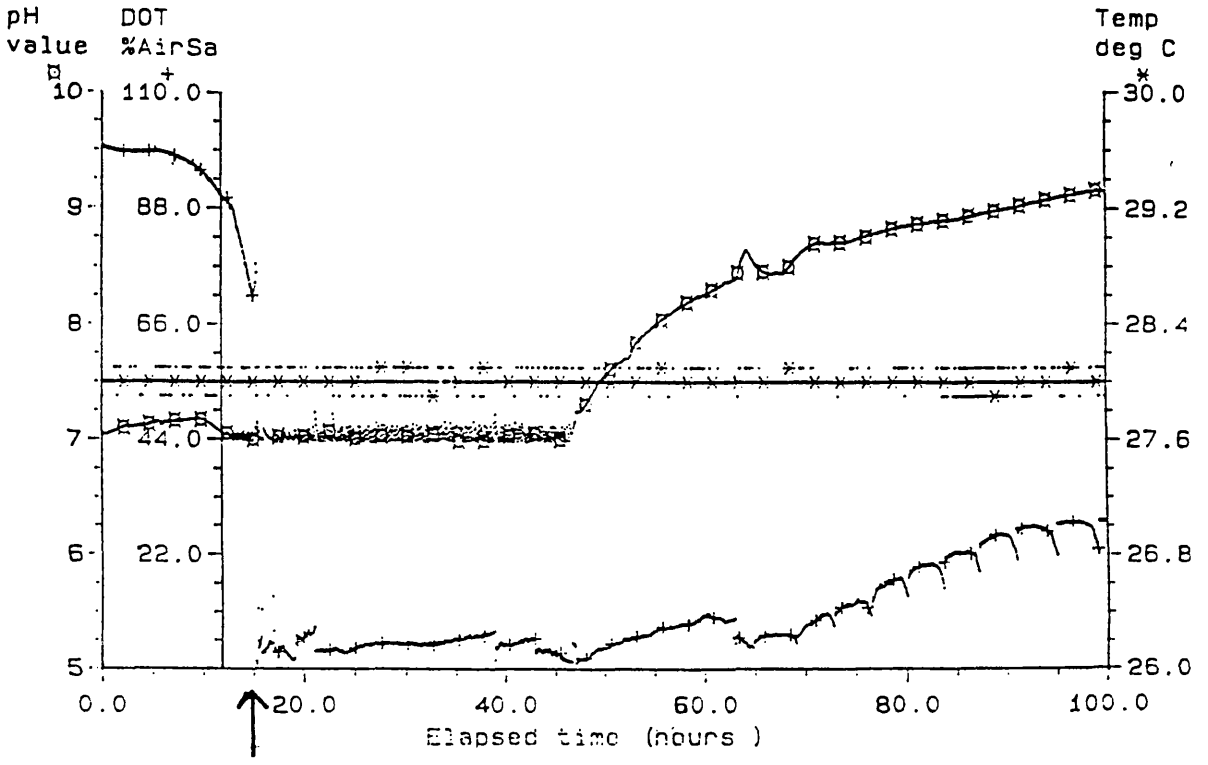

Figure 62 Fermentation parameter data of a MEP+10mMPO_4^{3-} 5L fermentation of *S. lividans* TK24 pIJ303 under DOT stress after 15h (marked with arrow). pH (⊠), temperature (✳) and DOT (+).

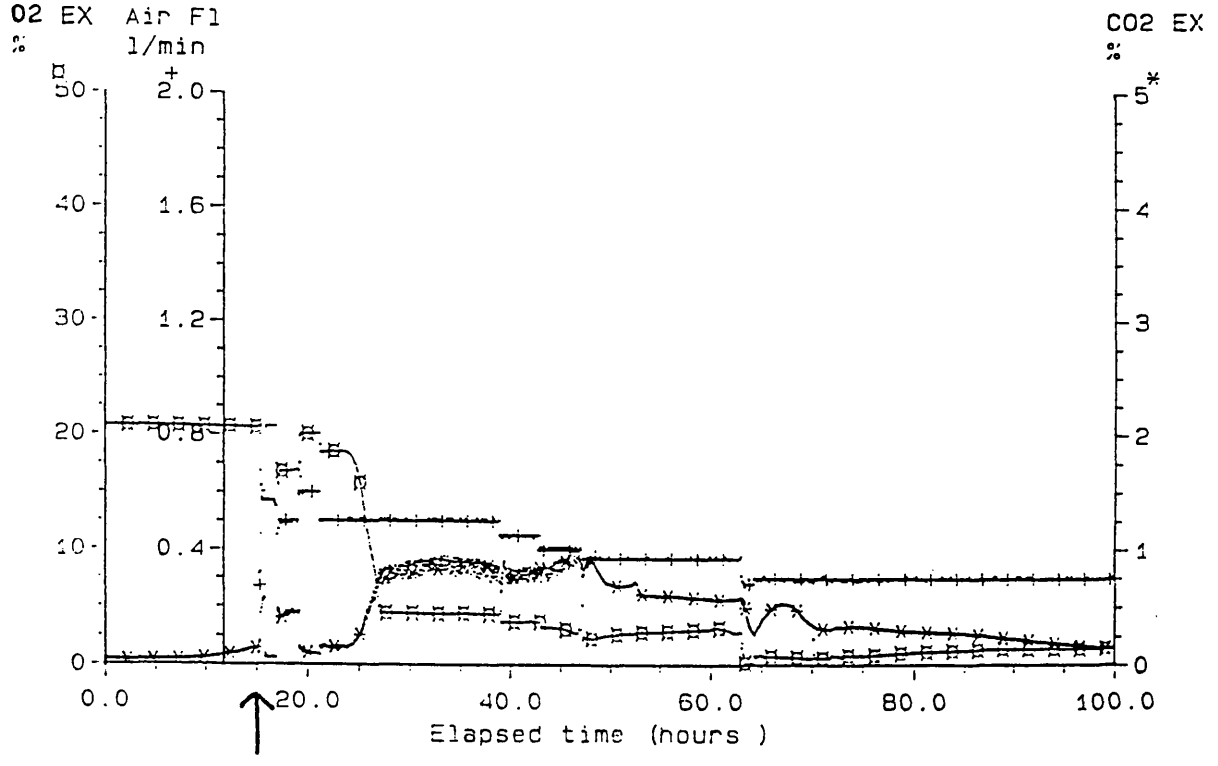

Figure 63 Gas analysis data of a MEP+10mMPO_4^{3-} 5L fermentation of *S. lividans* TK24 pIJ303 under DOT stress after 15h (marked with arrow). $\%CO_2$ (✳) and $\%O_2$ (⊠).

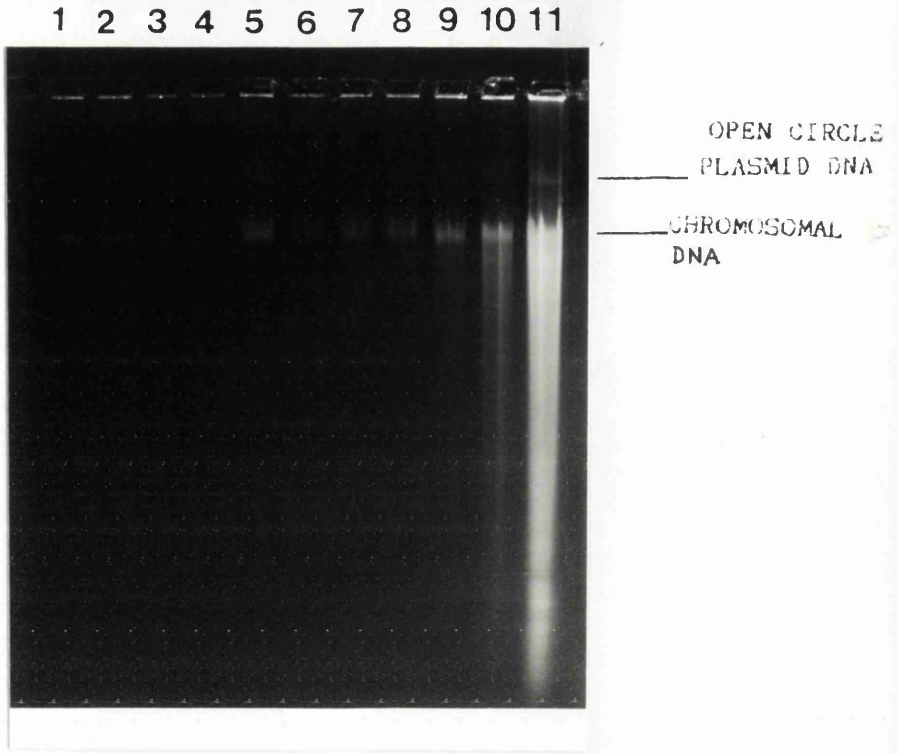

Figure 64a Total DNA extracts (at 35uL loadings) of samples taken during first DOT stress 5L fermentation of *S. lividans* TK24 pIJ303. Only OC bands observed and large amount of degraded DNA towards end of fermentation.
Lanes 1-10 are timepoints 0h-70h, respectively and lane 11=136h.

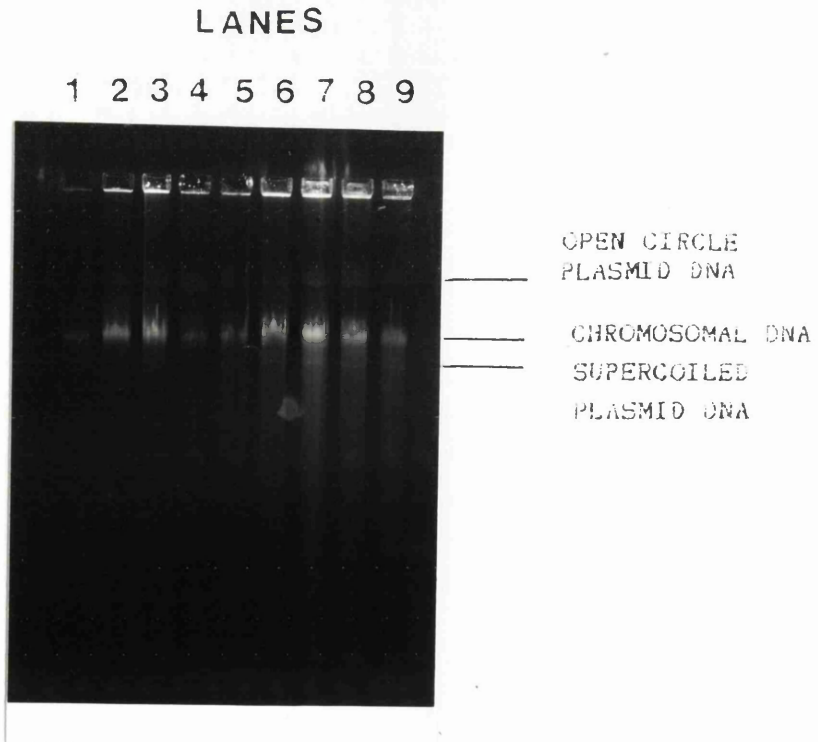

Figure 64b Total DNA extracts (at 35uL loadings) of samples taken during second DOT stress 5L fermentation of *S. lividans* TK24 pIJ303. OC plasmid bands observed throughout except lanes 7, 8 & 9.
Lanes 1-9 are timepoints 0h-88h.

total DNA extracts of culture under DOT stress). A low DOT level (<10% of saturation) was maintained for up to 50h, however despite further reducing the air flow rate (and increasing N_2 flow) a "rise" in DOT was seen towards the end of the fermentation. The logged fermentation data was also very similar to that obtained under normal growth conditions. These results suggested that the critical DOT level for *S. lividans* was >5% of saturation.

A second fermentation was conducted where the DOT was reduced to <2% of saturation. The data of the second fermentation are shown in Figures 65, 66, 67 and 68. The biomass concentration stopped increasing after 15.5h but a slight increase was seen from 23h to 39.5h. The pIJ303 plasmid copy number did not exceed about 125 which was less than half the expected maximum plasmid copy number. The apparent fluctuation between 15h and 24h was probably not real since measurements are less accurate in this range. As with the first fermentation no complete plasmid loss was observed (refer to Figure 69). A "rise" in DOT was again seen (after 36h) but according to the mass spectrometer data, the culture was completely anaerobic at this stage. Despite the anaerobic conditions, significant cell lysis did not occur. Additional evidence of sustained growth under these conditions was that a large volume of alkali was needed for pH control, 19g of NaOH was required to neutralize 0.48 mole equivalents of organic acid produced.

The growth of recombinant *S. lividans* was reduced under conditions of low DOT and the maximum copy number of pIJ303 was significantly less. However, complete plasmid loss did not occur and plasmid DNA bands were still visible (refer to Figure 64a and 64b). Only OC plasmid bands were visible. The results of these experiments suggest that pIJ303 is very stable even under conditions of environmental stress.

3.6.4 Remarks on the stability of plasmid pIJ303 in stirred vessel fermentations.

Plasmid pIJ303 showed very similar behaviour in 5L fermentations and in shake-flask cultures. Better biomass concentrations could be obtained in 5L fermentations but plasmid copy number profiles were very similar. Maximum copy number values were slightly less when recombinant *S. lividans* was grown in 5L fermentations, however this was dependent on growth conditions (*e.g.* medium).

Plasmid stability under conditions of low DOT resulted in the

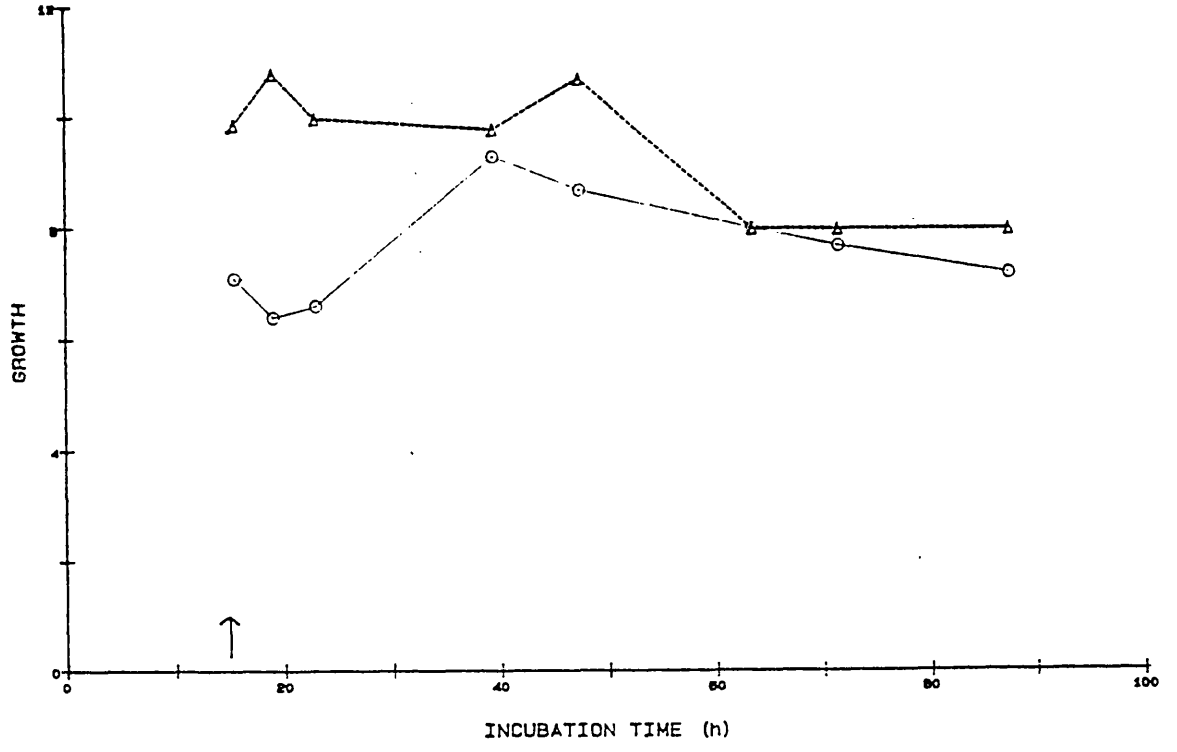

Figure 65 Biomass concentration (○, gL^{-1}) and deoxyribose concentration (□, μgmL^{-1}) data of a MEP+10mMPO_4^{3-} 5L fermentation of *S. lividans* TK24 pIJ303 under DOT stress after 15h (marked with arrow).

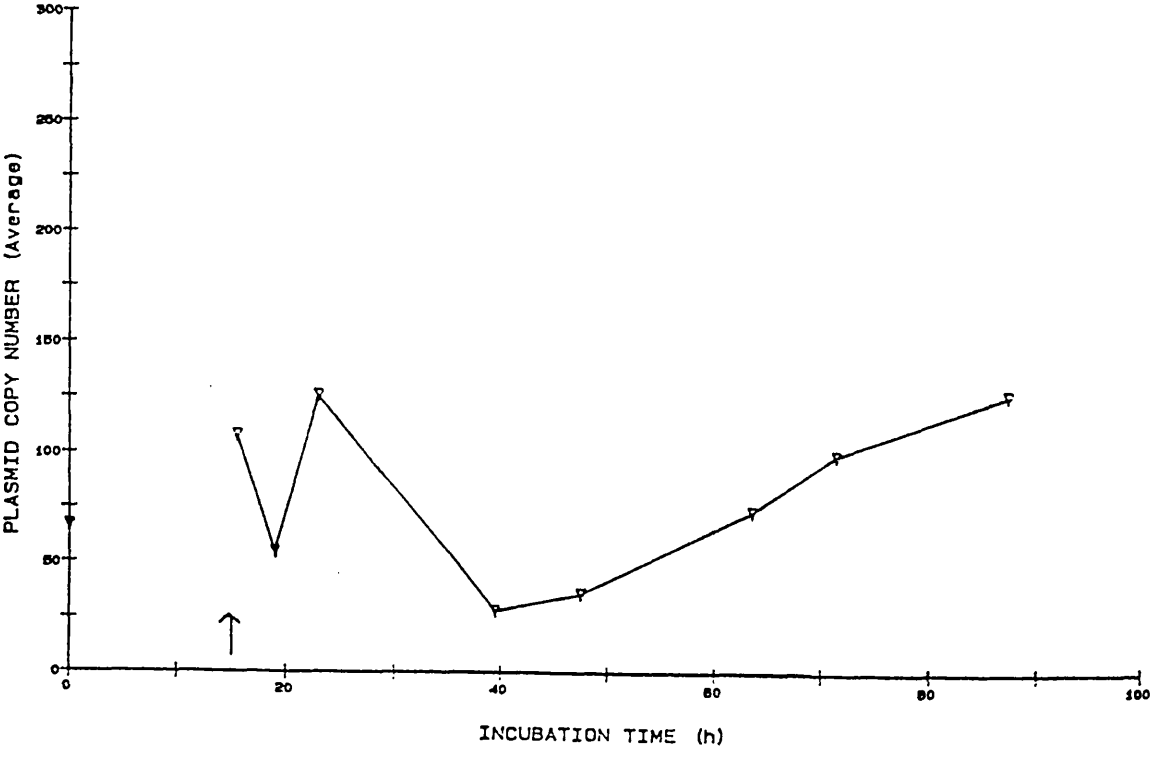

Figure 66 Plasmid copy number profile (▽) of a MEP+10mMPO_4^{3-} 5L fermentation of *S. lividans* TK24 pIJ303 under DOT stress after 15h (marked with arrow).

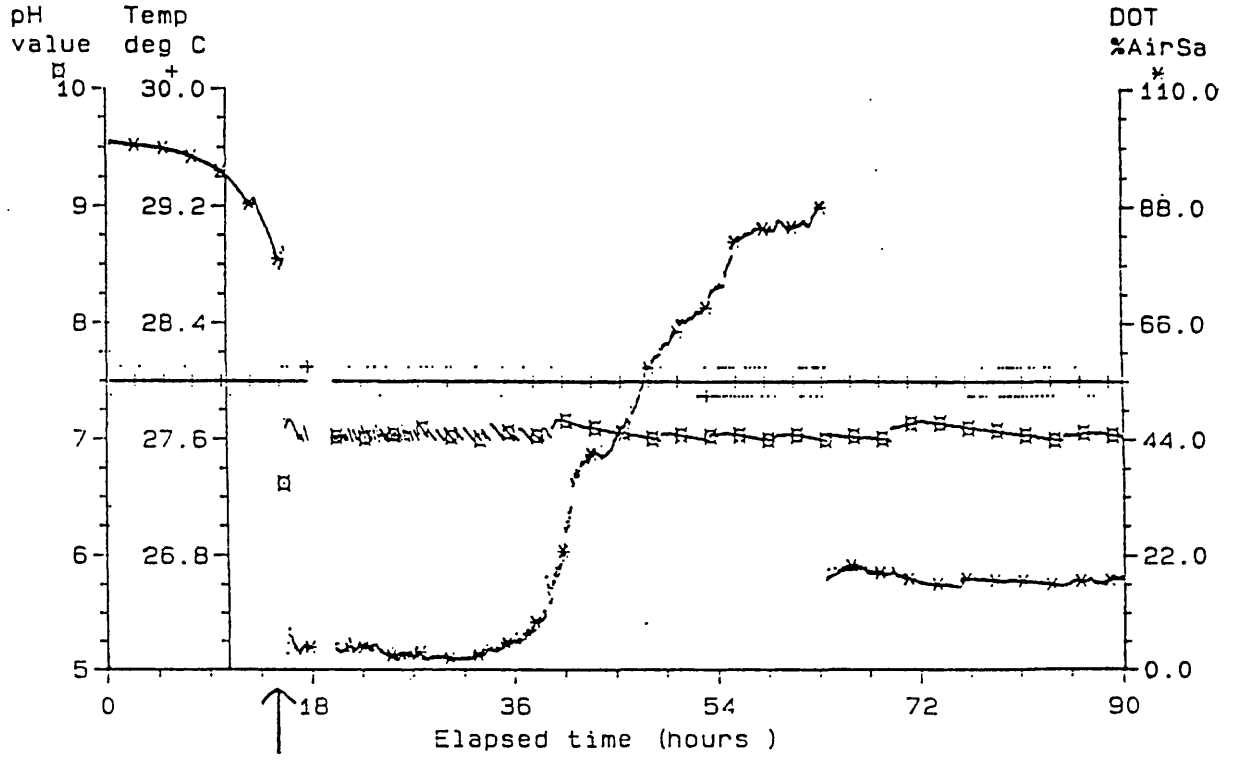

Figure 67 Fermentation parameter data of a MEP+10mMPO_4^{3-} 5L fermentation of *S. lividans* TK24 pIJ303 under DOT stress after 15h (marked with arrow). pH (⌑), temperature (✳) and DOT (+). N.B. DOT scale 0-100% from 1-15h, 0-25% from 15-63h and 0-100% from 63-88h.

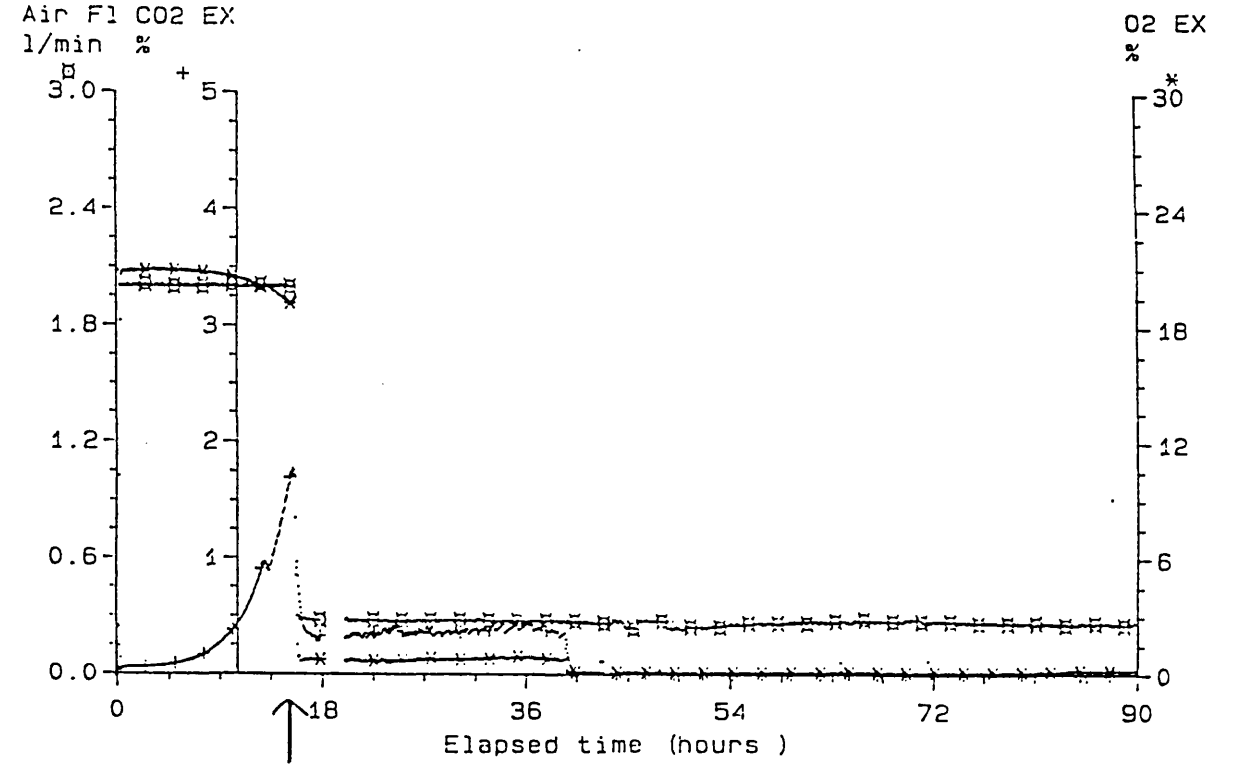

Figure 68 Gas analysis data of a MEP+10mMPO_4^{3-} 5L fermentation of *S. lividans* TK24 pIJ303 under DOT stress after 15h (marked with arrow). $\%CO_2$ (✳) and $\%O_2$ (⌑).

synthesis of fewer plasmid molecules but complete plasmid loss was not observed. Experiments where normal culture conditions were resumed after a period of stress were not conducted,. It would be expected that plasmid copy number would be restored to normal levels under these conditions.

Structural plasmid stability was checked at the beginning and end of all the fermentations described in Section 3.6.3. There was no evidence of plasmid structural re-arrangements since the expected size and number of *BclI* plasmid fragments were always obtained.

Plasmid pIJ303 did not therefore exhibit any major problems when recombinant cultures were increased in scale from shake-flasks to 5L stirred vessels.

3.7 Stirred vessel fermentations of *S. lividans* TK24 pIJ702.

3.7.1 Aim.

The aim of these experiments was to investigate the stability of the plasmid pIJ702 in 5L fermentations. The plasmid stability of pIJ702 in shake-flask fermentations was reported in Section 3.3 and it has been shown that the plasmid copy number increased during initial rapid growth. A maximum plasmid copy number of up to 500 was achieved at early stationary phase in shake-flask cultures. The plasmid copy number in 5L fermentations will be compared in this section.

Plasmid pIJ702 carries a cloned tyrosinase gene (Katz *et al*, 1983) and it is of interest to correlate the plasmid copy number with enzyme yield. Such studies will clarify whether merely measuring the level of cloned gene yield is sufficient to indicate the stability of the plasmid. Since this cloned gene is inducible, the effect of induction at different points in the culture's growth will also be studied. The cloned gene promoter, however, does not remain in a non-induced state even in the absence of inducer(s) (refer to Section 3.3.6.2), therefore a basal level of tyrosinase activity is always detected.

3.7.2 Protocol.

The experiments were conducted as two-stage batch fermentations with a vegetative "seed" inoculum used to initiate growth. All experiments were conducted as described in Section 2.3 with periodic samplings of biomass for plasmid copy number determination, biomass concentration, deoxyribose concentration and tyrosinase enzyme activity. All enzyme assays were performed as described in Section 2.3.13 and 2.3.14.

Tyrosinase enzyme induction was achieved by aseptically adding the Bactotryptone-copper sulphate nutrient mixture. The recipe is described in Section 2.1.3.2(f) (after Lerch and Ettlinger, 1972) and was added to the fermentation vessel *via* an addition port. In order to make direct comparisons with fermentation data derived from *S. lividans* plasmidless host and pIJ303-bearing *S. lividans*, MEP+10mMPO_4^{3-} was used as the fermentation medium.

As with the *S. lividans* pIJ303 fermentations, BIOi data logging was used to collect and retrieve fermentation parameter information. The mass spectrometer was used to analyse outlet gases, but due to frequent equipment and software malfunction complete sets of data were rarely

obtained.

3.7.3 Results of *S. lividans* pIJ702 stirred vessel fermentations.

3.7.3.1 Results of *S. lividans* pIJ702 5L fermentations using MEP+PO_4^{3-} medium without tyrosinase induction.

Two fermentations were conducted using *S. lividans* pIJ702 grown in MEP+PO_4^{3-} under standard fermentation conditions. Excessive antifoam addition problems described in Section 3.6.3.1 were also encountered during the first of these experiments, hence biomass concentration data was absent after the 23h sampling.

The plasmid copy number and %SC plasmid data from the first fermentation are shown in Figure 69 and the tyrosinase specific enzyme activity data are shown in Figure 70. As seen in Figure 69 the plasmid copy number of pIJ702 increased quickly during initial rapid growth and continued to increase until 48h when the maximum value was approximately 450. The plasmid copy number then declined rapidly during stationary phase and stabilized at about 280 during the latter part of the experiment. The %SC plasmid profile contrasted significantly from that of pIJ303 since a very high proportion of SC molecules were detected throughout most of the fermentation. Also, in contrast to pIJ303, the %SC declined steadily as the culture became older. The tyrosinase specific activity shown in Figure 70 also increased quickly during initial rapid growth to a maximum level of 212 units per mg protein after 39h. The subsequent decrease in enzyme activity was approximately proportional to the decrease in plasmid copy number.

The biomass and deoxyribose concentration data of the second fermentation are shown in Figure 72 and the plasmid copy number data in Figure 73. The %SC plasmid was 100% throughout the fermentation. Rapid growth occurred between 0h and 46.5h where a maximum biomass concentration of 10.5gL^{-1} was obtained. The plasmid copy number however increased further during stationary phase until a maximum plasmid copy number of over 700 was obtained after 71h. Figure 74 shows that at 46.5h the tyrosinase specific activity was 79 units per mg protein.

In comparing the two fermentations it is possible to see that the plasmid copy number profiles are similar except that in the second experiment only SC plasmid molecules were obtained. The enzyme activity pattern was also similar but the maximum values obtained were different.

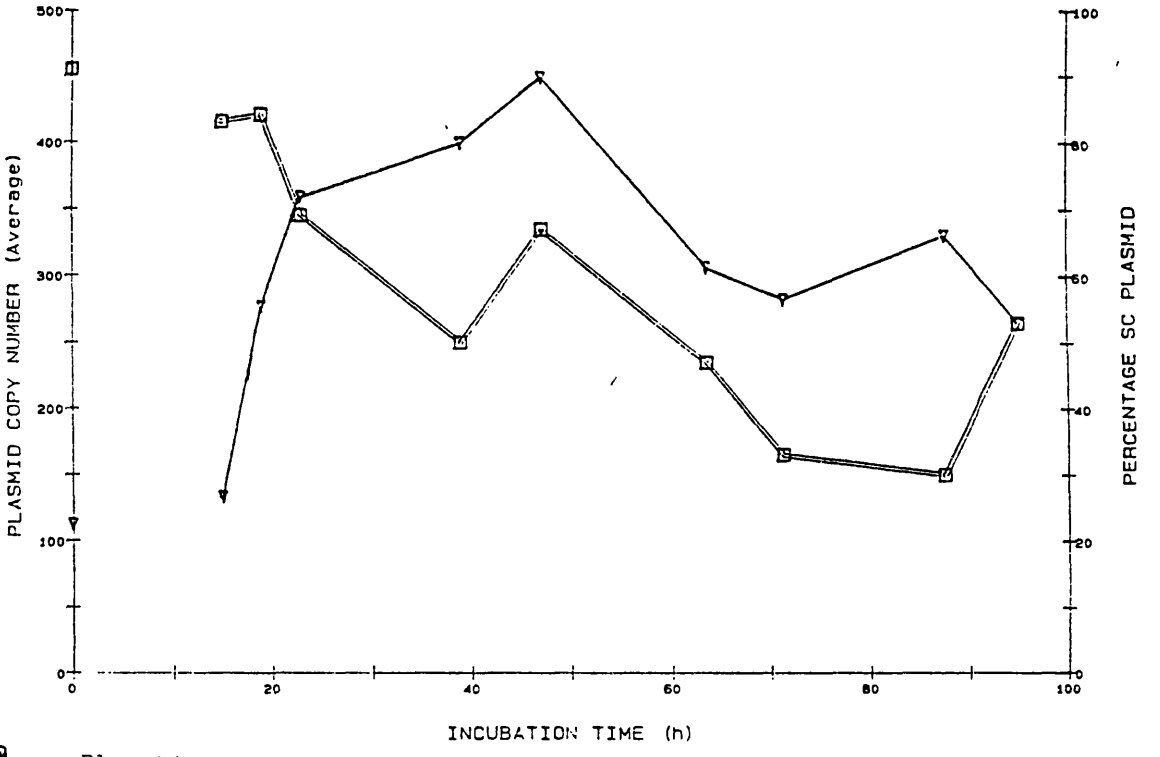

Figure 69 Plasmid copy number (▽) and percentage supercoiled plasmid (□) data of a MEP+10mMPO_4^{3-} 5L fermentation of *S. lividans* TK24 pIJ702.

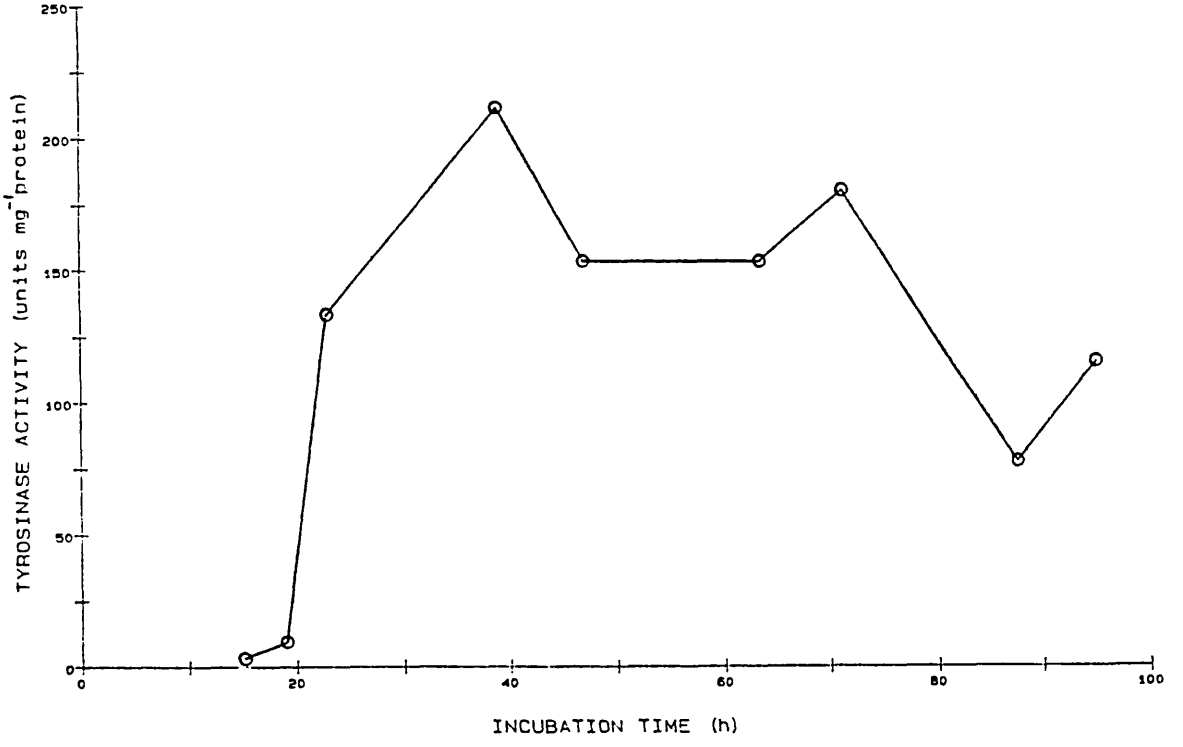

Figure 70 Tyrosinase specific activity data (○, units mg^{-1} protein) of a MEP+10mMPO_4^{3-} 5L fermentation of *S. lividans* TK24 pIJ702.

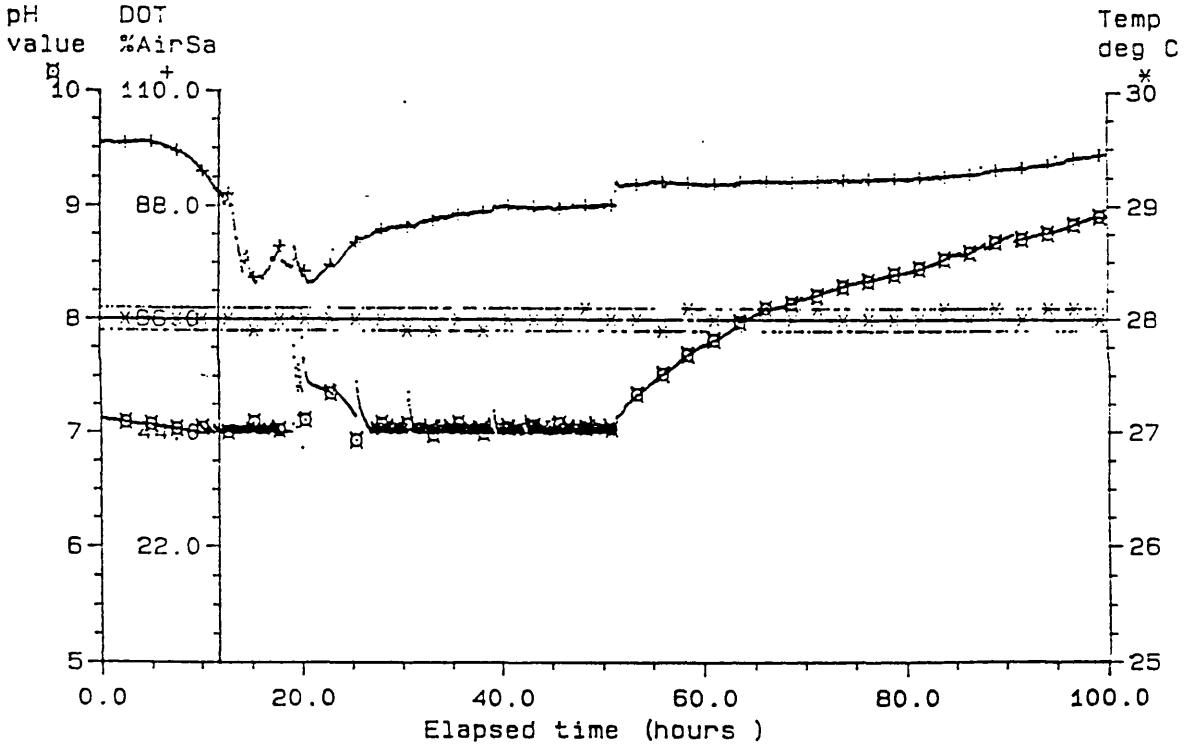

Figure 71 Fermentation parameter data of a MEP+10mMPO_4^{3-} 5L fermentation of *S. lividans* TK24 pIJ702. pH (⊠), temperature (✳) and DOT (+).

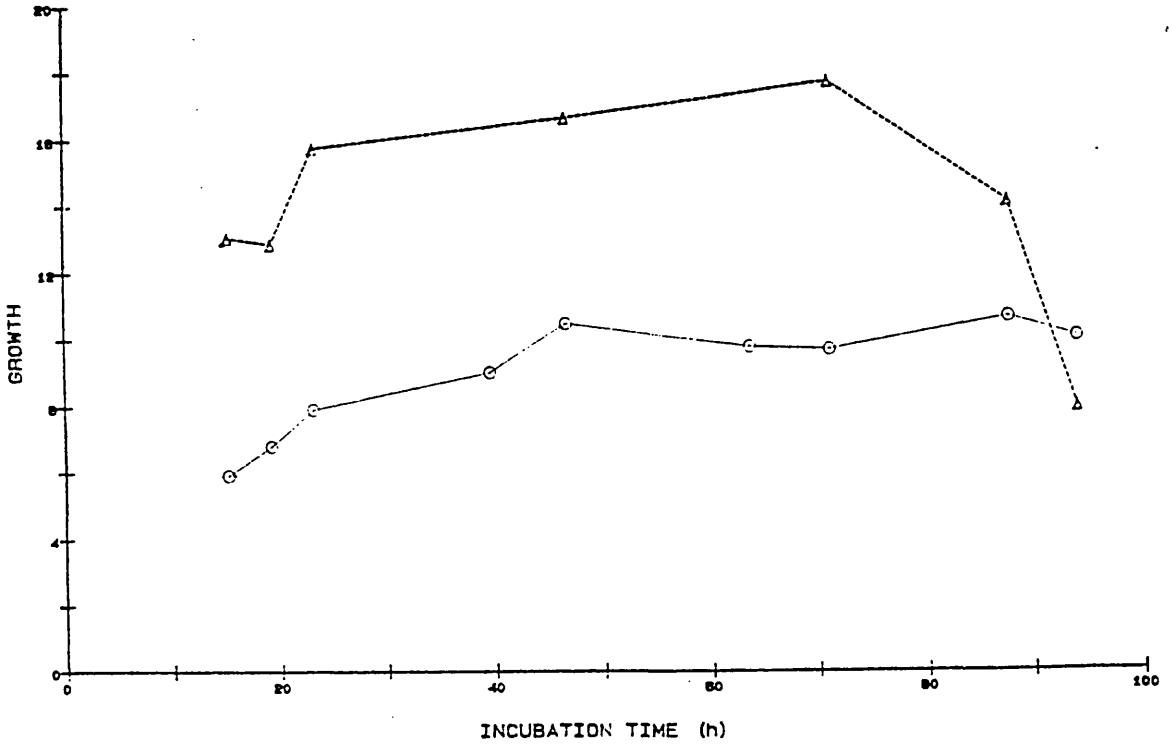

Figure 72 Biomass concentration (O, gL^{-1}) and deoxyribose concentration (Δ, μgmL^{-1}) data of a MEP+10mMPO_4^{3-} 5L fermentation of *S. lividans* TK24 pIJ702.

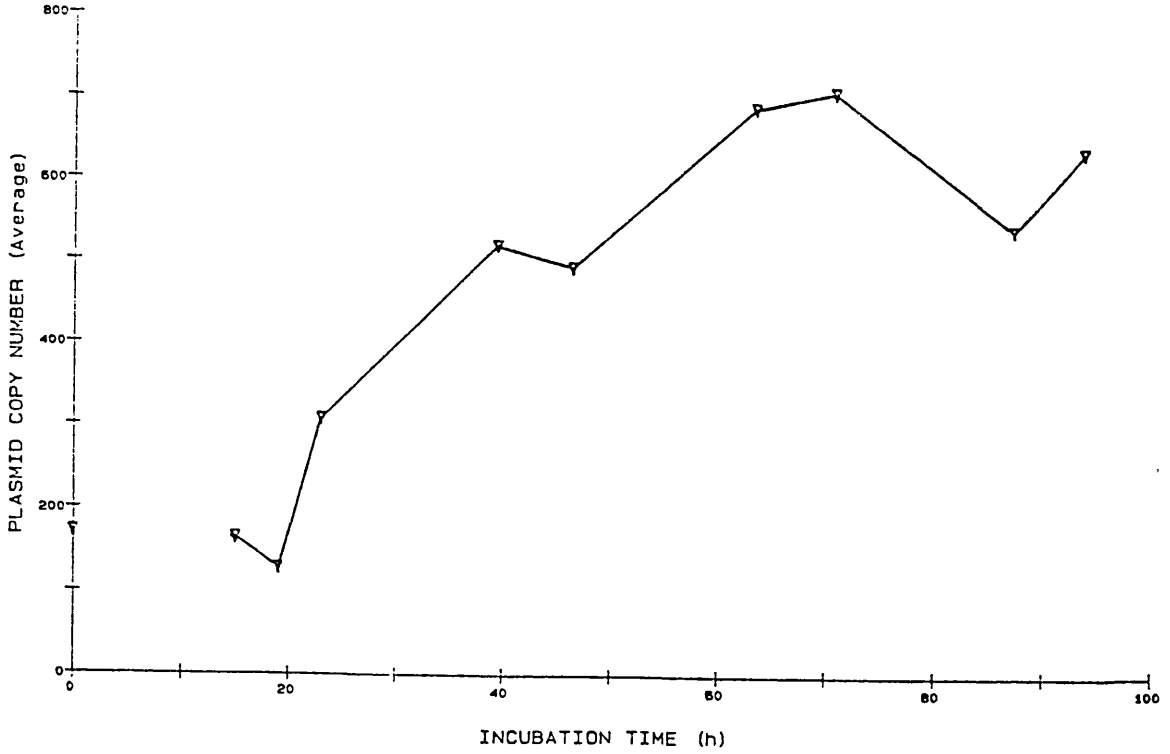

Figure 73 Plasmid copy number (▽) profile of a MEP+10mMPO_4^{3-} 5L fermentation of *S. lividans* TK24 pIJ702.

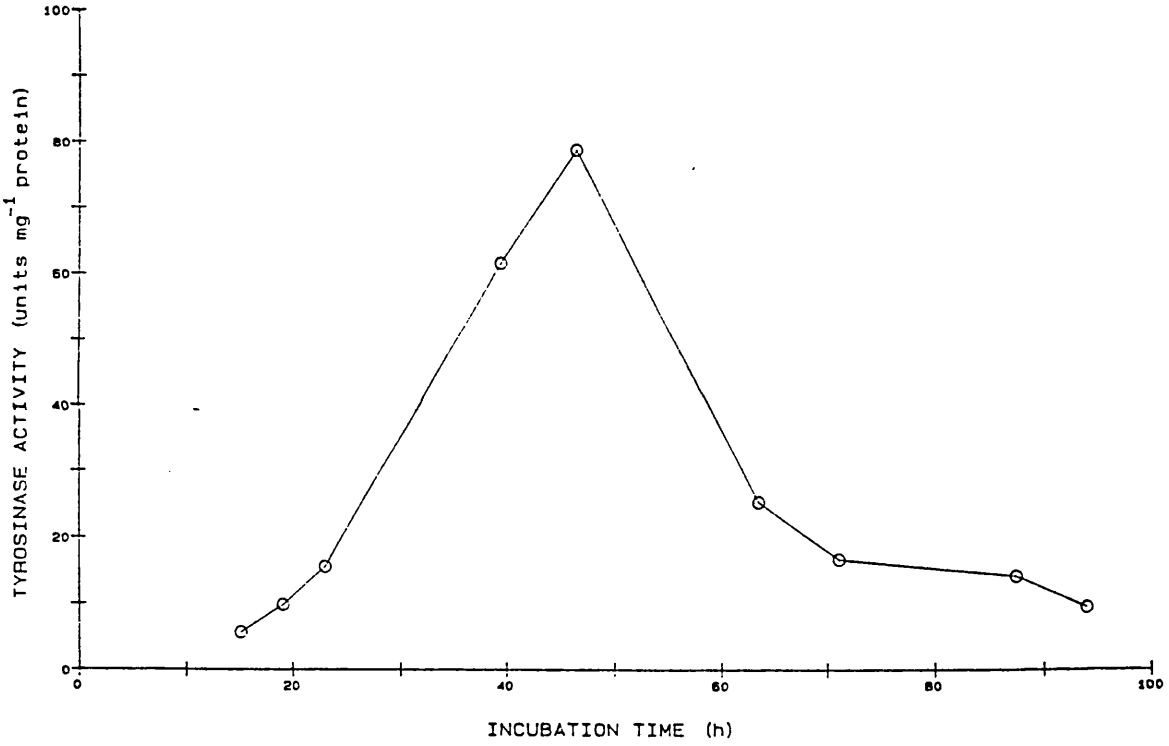

Figure 74 Tyrosinase specific activity data (○, units mg^{-1} protein) of a MEP+10mMPO_4^{3-} 5L fermentation of *S. lividans* TK24 pIJ702.

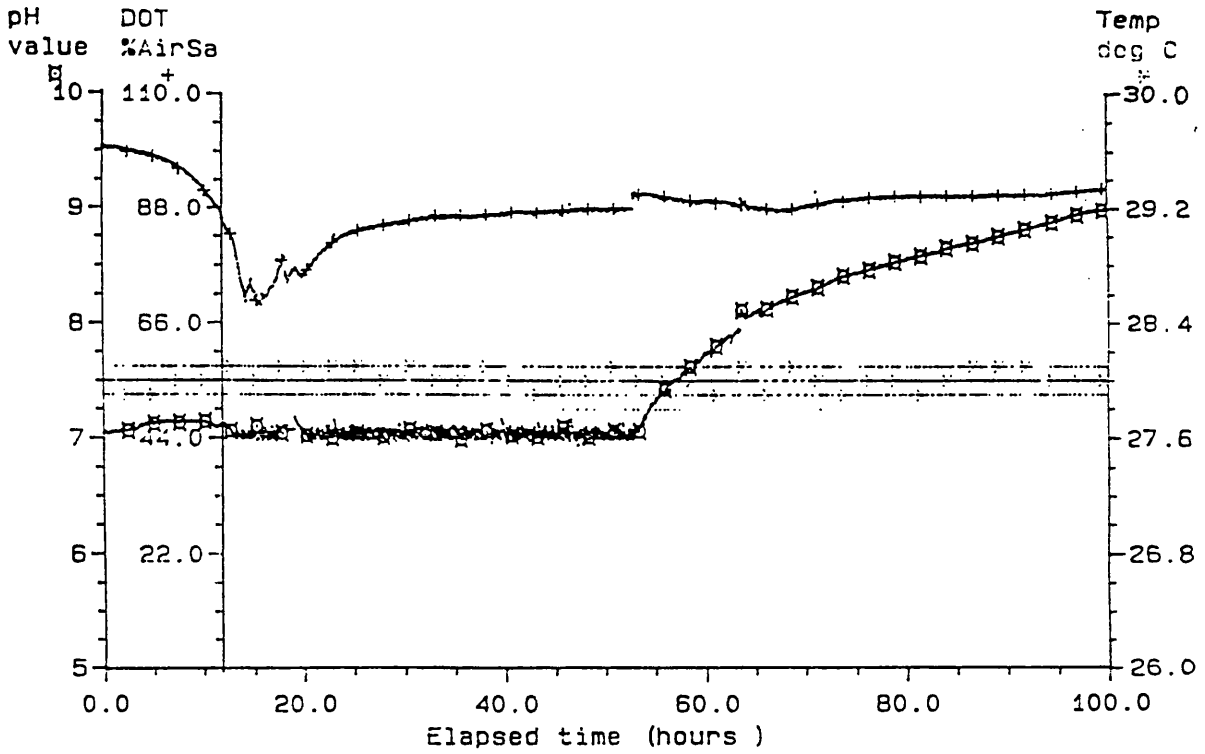

Figure 75 Fermentation parameter data of a MEP+10mMPO_4^{3-} 5L fermentation of *S. lividans* TK24 pIJ702. pH (⊠), temperature (✳) and DOT (+).

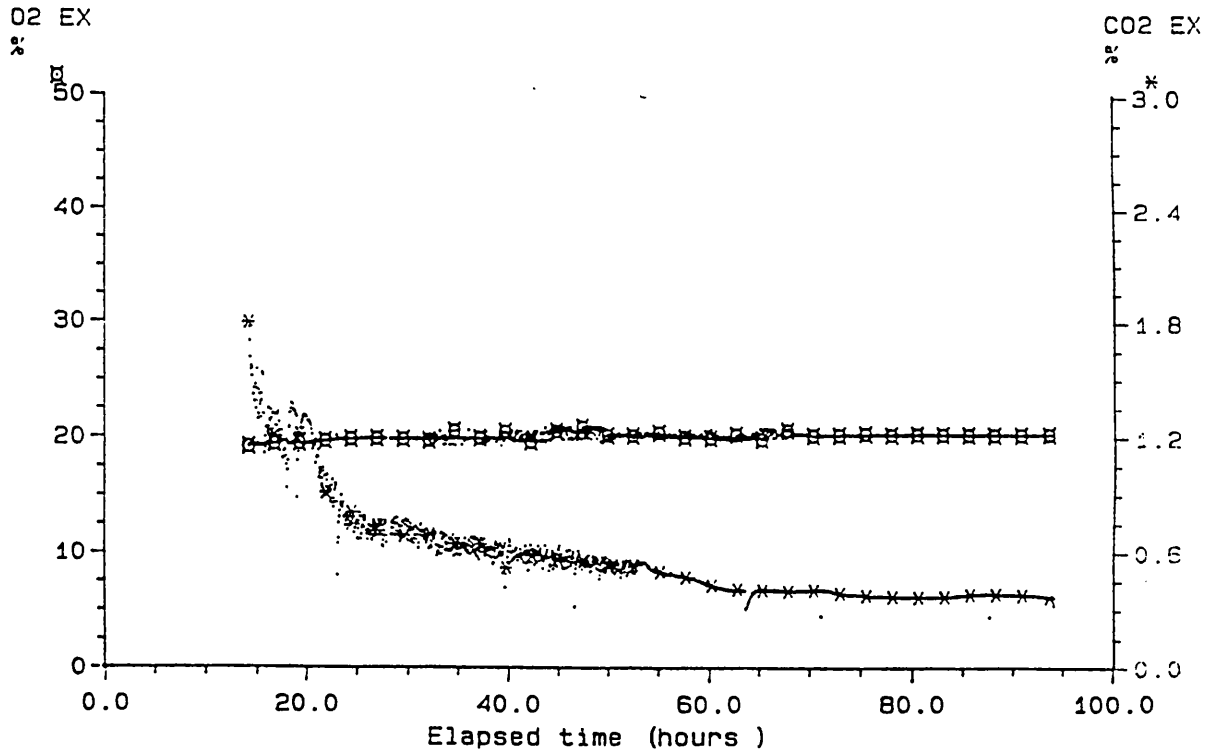

Figure 76 Gas analysis data of a MEP+10mMPO_4^{3-} 5L fermentation of *S. lividans* TK24 pIJ702. %CO_2 (✳) and %O_2 (⊠).

This difference is probably due to variable losses upon frozen storage since it was not possible to assay tyrosinase activity from fresh biomass samples.

Figures 75 and 76 show the logged fermentation data which were essentially similar to those of *S. lividans* plasmidless host (see Section 3.5.3.3) and *S. lividans* pIJ303 (see Section 3.6.3.1). Rapid uptake of DO occurred until 15h with only a gradual increase in DOT thereafter. The pH was controlled (average 4M NaOH addition 135mL=21.6g) until 50h indicating a period of extended active growth (a feature which is supported by the biomass concentration data of Figure 72). This amount of alkali was needed to neutralize 0.54 mole equivalents of organic acid. The increase in pH after 50h was also a typical feature of *S. lividans* fermentations which does not seem to be affected by pIJ702 replication and expression. The final pH value was *circa* pH9 after 100h incubation. The gas analysis data from the second fermentation is shown in Figure 76. Maximum CO_2 concentration was seen at late rapid growth phase (15h) where the RQ was calculated to be 0.97. Higher rates of metabolic activity may have occurred earlier but due to instrument malfunction, data were only available from 15h onwards.

3.7.3.2 Results of *S. lividans* pIJ702 5L fermentations using MEP+PO_4^{3-} medium with tyrosinase induction.

Two fermentations were conducted using *S. lividans* pIJ702 with cloned gene expression induced at different points during growth. In the first experiment tyrosinase expression was induced at mid-rapid growth phase (at 15h) and during the second fermentation expression was induced in late rapid growth phase (at 24h).

The biomass and deoxyribose concentration data of the first experiment are shown in Figure 77 and as seen in the graph normal growth proceeds until 23.5h (12.1gL^{-1}). However a stimulation of growth occurs due to the addition of nutrients in the "induction" mixture leading to a DCW of 17.5gL^{-1} at 71h. The plasmid copy number profile (only SC plasmid molecules were detected) is shown in Figure 78 and reveals a maximum value of *circa* 450 after 47h but a sharp decrease was observed after 64h with a second increase occurring thereafter up to a additional peak of *circa* 400 after 94h. Both biomass concentration and plasmid copy number decreased upon further incubation up to 159.5h (data not shown). The stimulation of growth

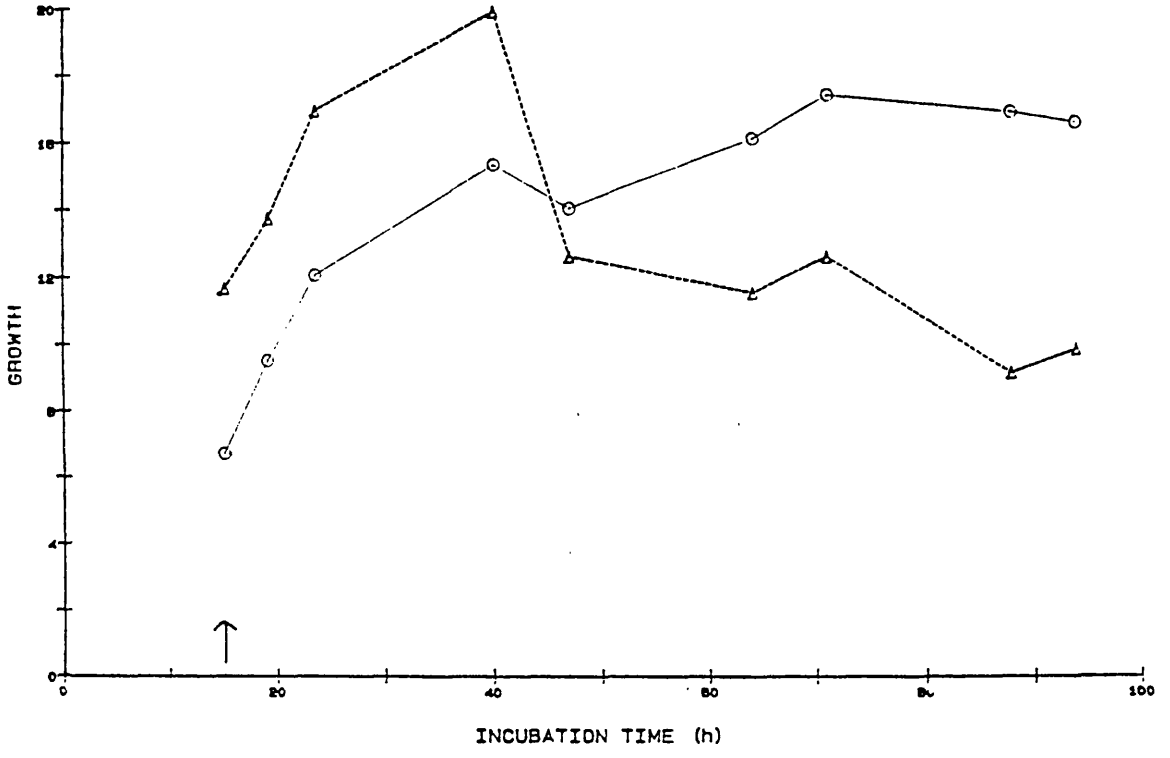

Figure 77 Biomass concentration (○, gL^{-1}) and deoxyribose concentration (△, μgmL^{-1}) data of a MEP+10mMPO_4^{3-} 5L fermentation of *S. lividans* TK24 pIJ702 (with "induction" after 15h, marked with arrow).

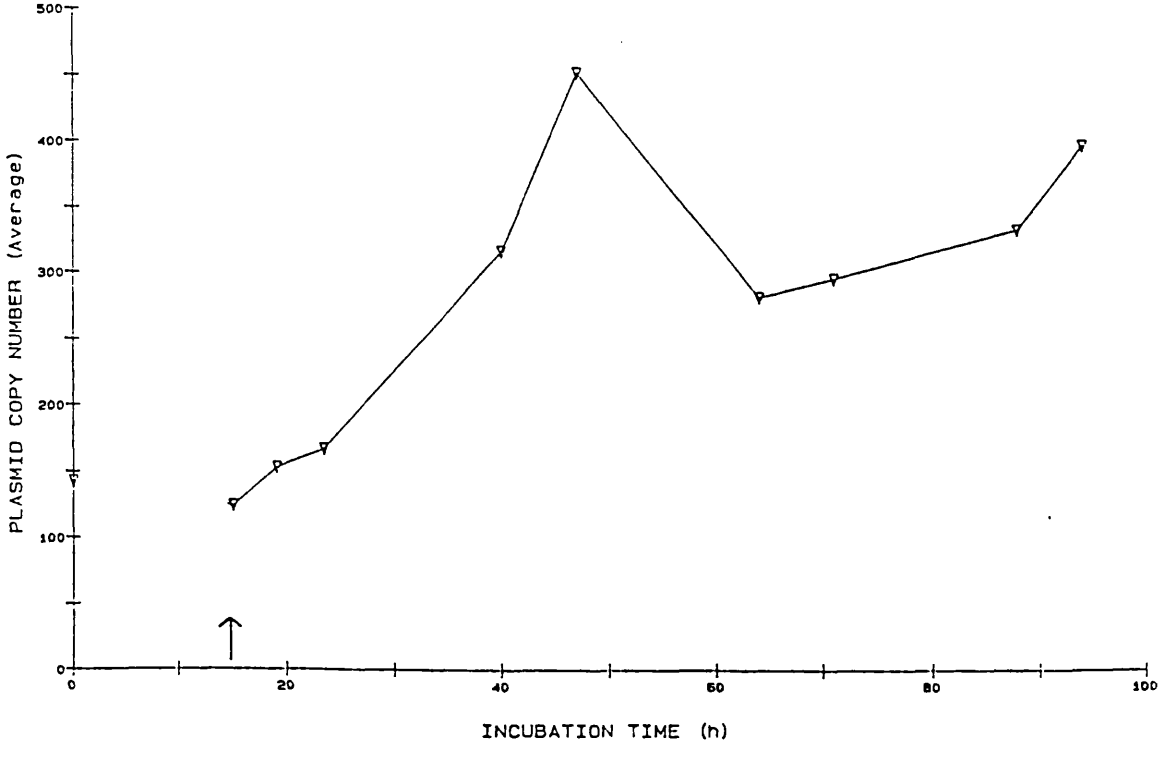

Figure 78 Plasmid copy number (▽) profile of a MEP+10mMPO_4^{3-} 5L fermentation of *S. lividans* TK24 pIJ702 (with "induction" after 15h, marked with arrow).

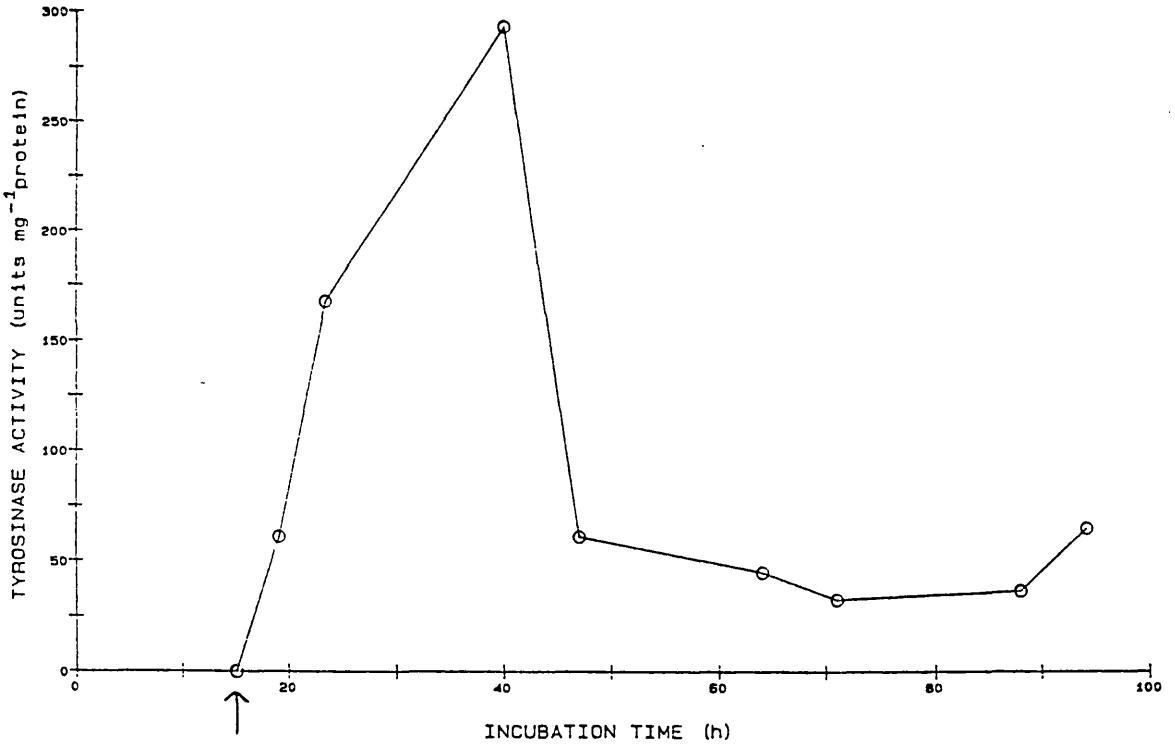

Figure 79 Tyrosinase specific activity data (O, units mg^{-1} protein) of a MEP+10mMPO_4^{3-} 5L fermentation of *S. lividans* TK24 pIJ702 (with "induction" after 15h, marked with arrow).

by adding the Bactotryptone-copper sulphate mixture resulted in the noticable pattern of plasmid copy number values in Figure 78 since the growth rate initially increased after addition of the induction mixture. Growth rate then subsequently slowed down in true stationary phase.

Tyrosinase enzyme production is shown in Figure 79 and seems similar to that of Figure 70 where no "induction" strategy was employed. The maximum specific activity in Figure 79 , however, was slightly greater at *circa* 300 units per mg protein. No data logging was obtained for this fermentation but the maximum pH was pH9.9 at 88h. Approximately 0.94 mole equivalents of organic acid was produced during the early part of the fermentation.

The biomass and deoxyribose concentration data from the second fermentation are shown in Figure 80. During this experiment a high level of biomass concentration was obtained with a maximum DCW of 15.1gL^{-1} occurred after 47h. The plasmid copy number profile (Figure 81) revealed the expected increase during initial rapid growth but this trend then continued throughout the whole fermentation, probably because of the stimulatory effects of the nutrient addition. The percentage SC plasmid molecules were not noted in the graph since only SC molecules were visible until 95h and 159.5h when the proportion was 83% and 69% respectively.

The tyrosinase enzyme activity (Figure 82) also increased during initial growth to a maximum level of 263 units per mg protein after 47h. This production compared well with that of Figure 79 suggesting that the time of induction does not affect the ultimate cloned gene yield, only shifting the point at which it is achieved. The tyrosinase level also decreased rapidly after the maximum point but approximately 100 units of tyrosinase per mg protein were detected after 159.5h.

The fermentation parameter data is plotted in Figure 83 although only data from 15h onwards is available. The observed rise in DOT between 15 and 20h suggests that the initial period of rapid growth has ceased. At 24h the induction mixture was added and since the cells were still very metabolically active, the DOT plummetted to *circa* 10% of saturation. Studies described in Section 3.6.3.3 suggest that *S. lividans* is not oxygen limited at this DO concentration and normal growth continues. The pH profile shows that control was required until 25h, but the pH increased gradually thereafter. A more characteristic pH rise then occurred after

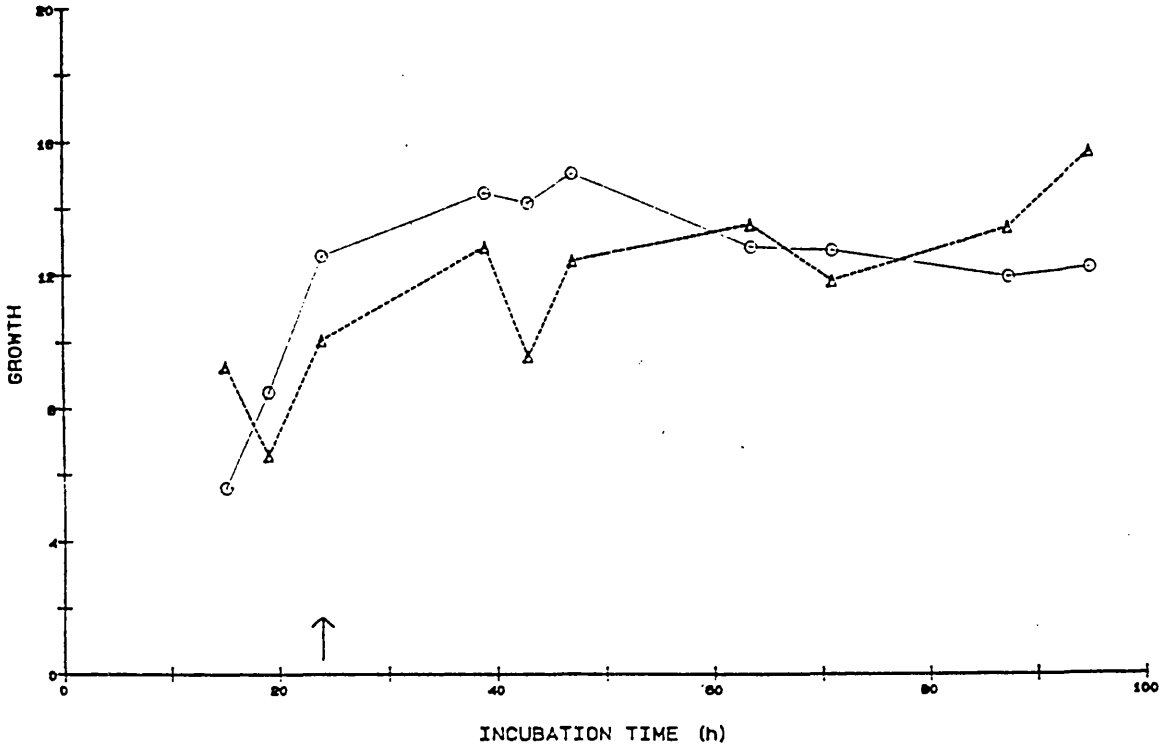

Figure 80 Biomass concentration (○, gL^{-1}) and deoxyribose concentration (△, μgmL^{-1}) data of a MEP+10mMPO_4^{3-} 5L fermentation of *S. lividans* TK24 pIJ702 (with "induction" after 24h, marked with arrow).

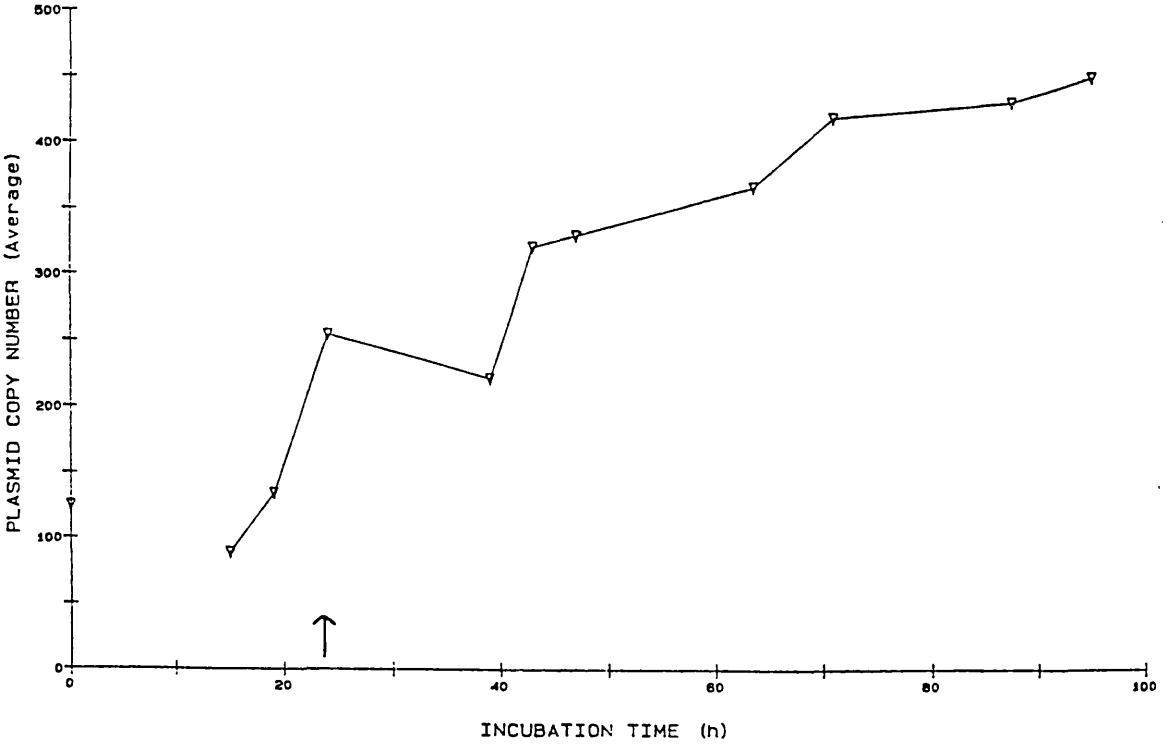

Figure 81 Plasmid copy number (▽) profile of a MEP+10mMPO_4^{3-} 5L fermentation of *S. lividans* TK24 pIJ702 (with "induction" after 24h, marked with arrow).

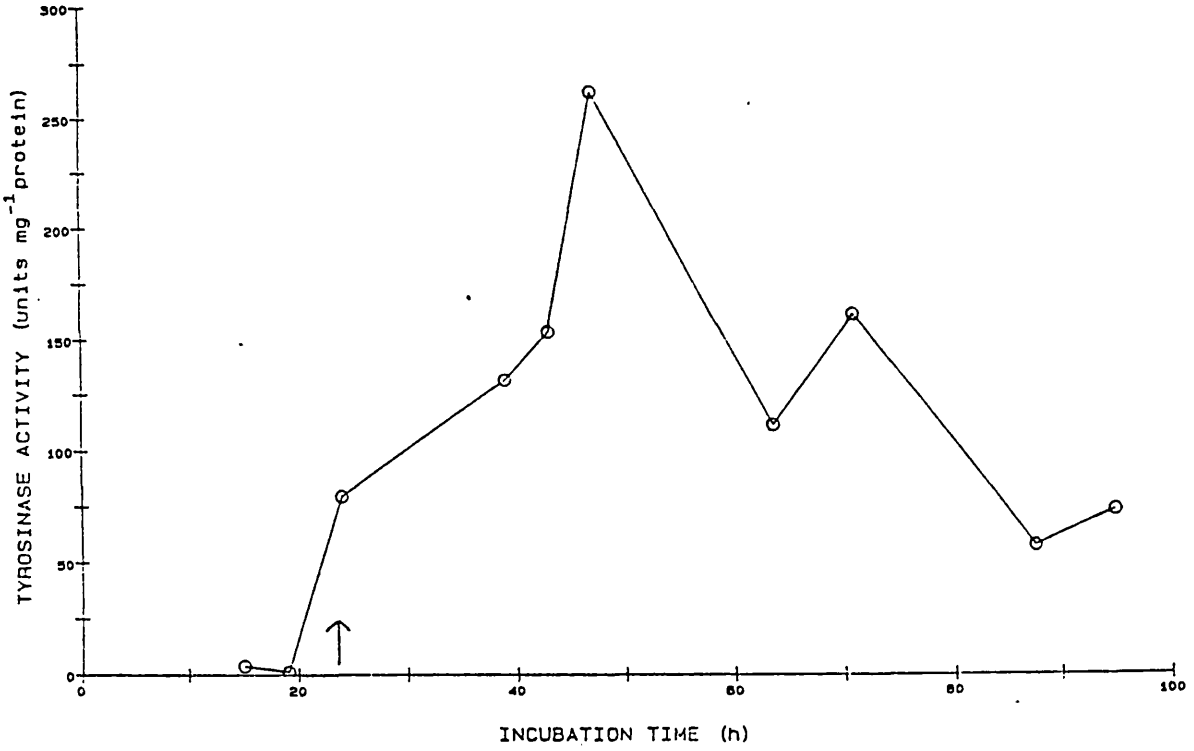

Figure 82 Tyrosinase specific activity data (O, units mg^{-1} protein) of a MEP+10mMPO_4^{3-} 5L fermentation of *S. lividans* TK24 pIJ702 (with "induction" after 24h, marked with arrow).

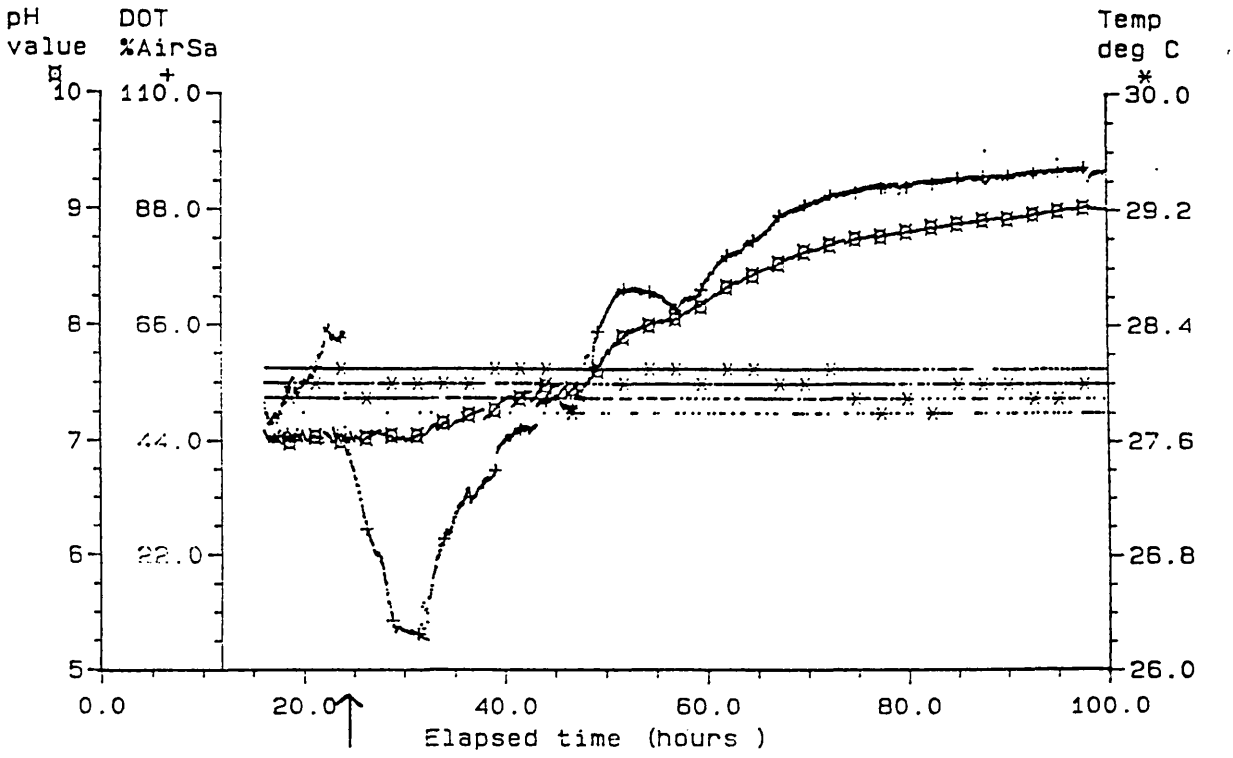

Figure 83 Fermentation parameter data of a MEP+10mMPO_4^{3-} 5L fermentation of *S. lividans* TK24 pIJ702 (with "induction" after 24h, marked with arrow). pH (⊙), temperature (✳) and DOT (+).

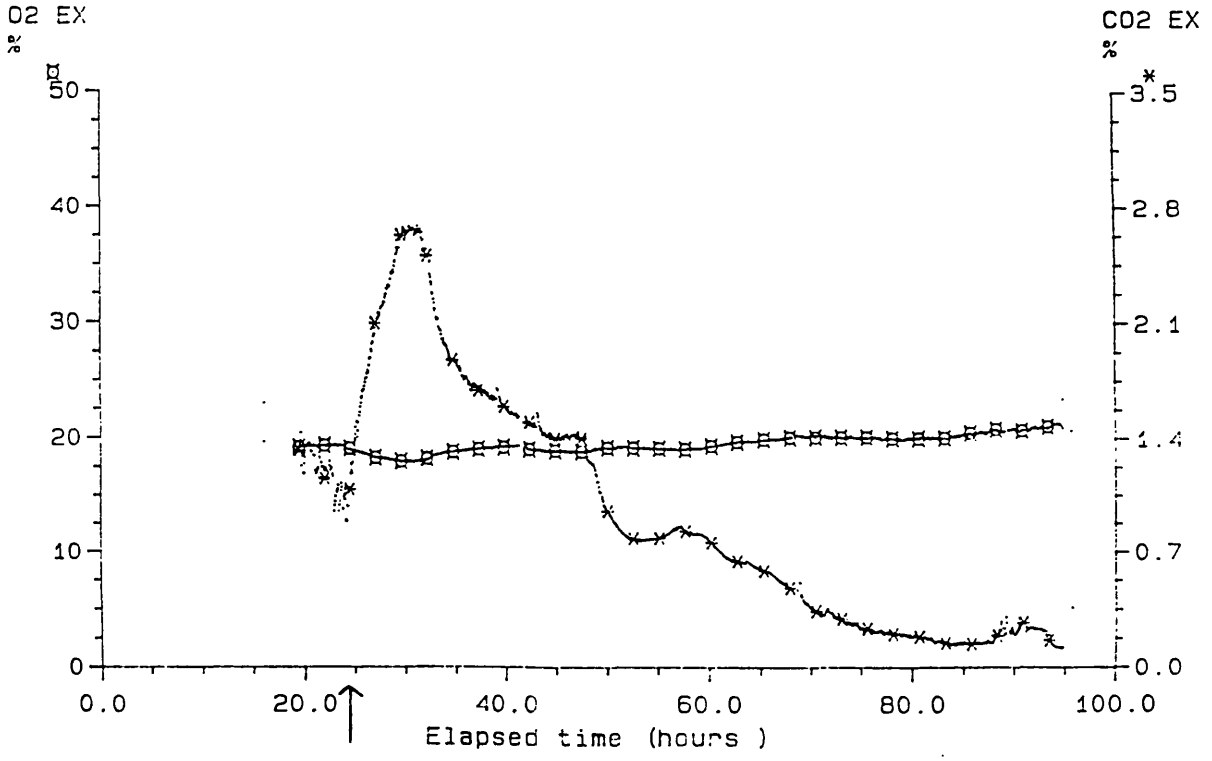

Figure 84 Gas analysis data of a MEP+10mMPO_4^{3-} 5L fermentation of *S. lividans* TK24 pIJ702 (with " induction" after 24h, marked with arrow). %CO_2 (✳) and %O_2 (⊙).

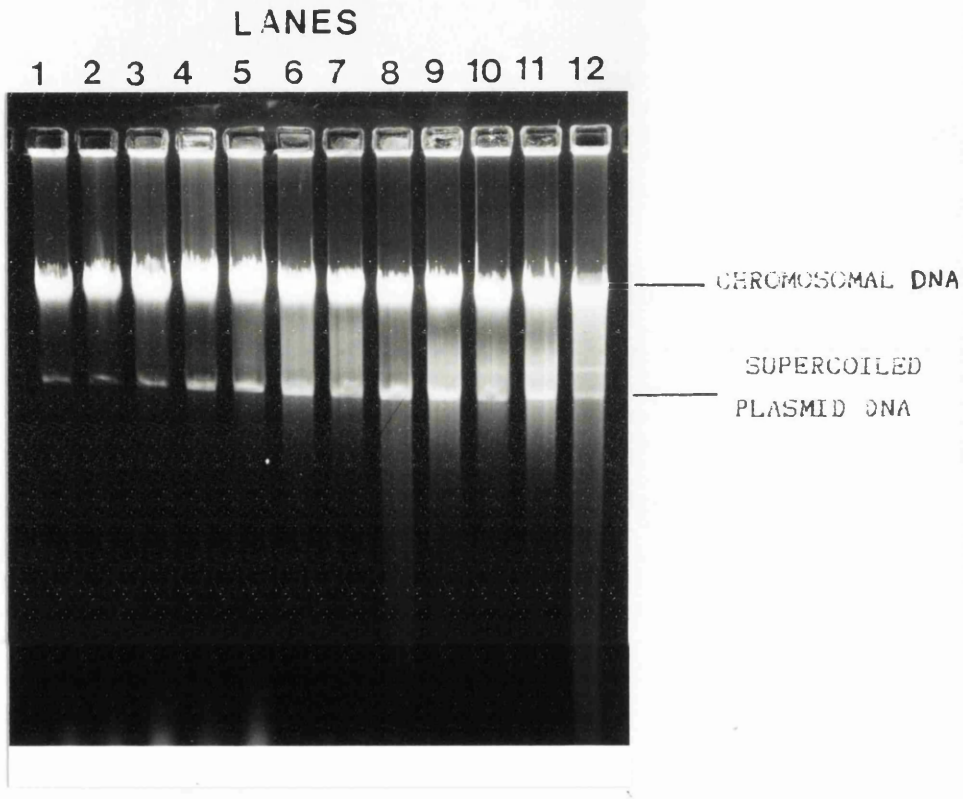

Figure 85 Total DNA extracts (at 20uL loadings) taken during final 5L fermentation of *S. lividans* TK24 pIJ702 with " induction" after 24h.
Lanes 1-11 are timepoints 0h-95h, lane 12 = 160h.

50h. Since pH control was only needed for a short period of time, only 35mL (5.6g) of 4M NaOH was added. Approximately 0.14 mole equivalents of organic acid was therefore produced.

Figure 84 shows the outlet gas analysis data of this fermentation. Again the growth stimulation effect of the Bactotryptone addition is clearly seen between 25h and 30h. The maximum RQ calculated (at 30h) was 1.15 compared with 0.97 from the fermentation data plotted in Figure 76 where no induction strategy was employed. A gel showing the total DNA extracts from this experiment is presented in Figure 85. Only supercoiled plasmid DNA bands were visible until a second plasmid band appeared in later samples (lanes 9-12). The identity of this band was not certain but it may be supercoiled dimeric plasmid or single stranded plasmid circles.

3.7.4 Remarks on the stability and expression of pIJ702 in stirred vessel fermentations.

As with pIJ303, pIJ702 showed similar plasmid copy number profiles in 5L fermentations compared with shake-flask cultures. Under some conditions maximum plasmid copy number values were even higher in 5L fermentations and such plasmid levels were maintained for longer at the larger scale. A major difference when comparing the plasmids is that pIJ702 cultures mostly yielded only SC plasmid molecules, while pIJ303 yielded a definite trend in the plasmid topoisomers being isolated.

Tyrosinase activity was improved with the addition of the Bactotryptone mixture but the point of addition did not seem to affect the ultimate yield only by shifting the time at which it was obtained. There is little evidence to suggest that this is a truely inducible promoter since high basal levels of tyrosinase were obtained even without induction. In general, the plasmid copy number profile did not follow the enzyme production profile since in some cases high plasmid levels were present but were not accompanied by high enzyme yields (see Figures 73 and 74). Adding the Bactotryptone nutrients stimulated metabolic activity resulting in very high RQ and low DOT levels and also, in one case, decreased the level of alkali needed to neutralize the broth.

Where structural stability was checked no differences between plasmids isolated at the start and end of the fermentation could be detected. Therefore there seems to be no deleterious effects upon plasmid

copy number when *S. lividans* pIJ702 fermentations are increased in scale.

4 DISCUSSION.

4.1 Discussion of copy number and structural stability methods.

As reported in Section 3.1.1.3, a linear relationship between Chromoscan peak integral and level of DNA per band was measured for the apparent range 0.15µg to 3µg DNA. Gels where higher levels of DNA were loaded (not shown) revealed that the linear relationship ceased at levels of DNA higher than 3.5µg. The reason why there was no further increase in DNA fluorescence beyond this point was that the DNA probably saturated the EtBr-stained agarose gel at such levels. It was also interesting to note that when peak height (rather than peak integral) was plotted against amount of DNA per band, the curve was seen to plateau at levels of DNA above approximately 2-2.5µg DNA (refer to Figure 6). Overloading of DNA in any given gel lane would therefore lead to a band broadening effect without any further increase in the level of DNA fluorescence.

A linear relationship between the level of DNA per band and fluorescence measured *via* photographic negatives was also found by Park *et al* (1990) in the range 9ng to 246ng. Projan *et al* (1983) also found a linear relationship existed between DNA and direct fluorescence peak integral in the range 15ng and 700ng and these workers quantified DNA using the diphenylamine reaction. The reported linearity ranges are different to those found in this work. The reason for this may be that the OD_{260} absorbance measurements used to quantify the concentration of DNA in these extracts was erroneous and led to an overestimate of DNA concentration in some preparations (refer to Section 3.1.2.4). Fluorescence linearity is therefore probably lost at levels of less than 3µg of pure DNA per band. However, since the DNA extracted for the linearity experiments was prepared in a similar manner to that used for plasmid copy number estimates the level of contaminating material was assumed to be equal and hence the actual concentration of DNA in preparations were assumed to be comparable. It is frequently not stated in literature reports how the DNA was quantified.

Another observation made in published reports is that different plasmid topoisomers fluoresced with varying intensity per µg DNA. Projan *et al* (1983) reported that linear or open circle plasmid DNA fluoresced approximately 1.36 times greater than supercoiled plasmid DNA (refer to Section 1.12). Other workers, Lin-Chao and Bremer (1986) also found a

difference between open circle and supercoiled plasmid fluorescence intensity was only 10%. Such a small difference may be accounted for by experimental error since Seo and Bailey (1985) found no difference in intensity between linearized and supercoiled DNA. Attempts during the course of this project to establish whether any difference in fluorescence linearity between open circle or linear plasmids and supercoiled plasmids failed since no plasmid DNA could be purified which contained only supercoiled molecules. Also the inadequacies of absorbance measurements to quantify DNA concentrations would also have hindered such a study. Since the published reports are conflicting and, in some cases, may be accounted for by experimental error, a correction factor to accommodate for such discrepancies was not employed during plasmid copy number calculations presented in this project. It is theoretically unlikely that a significant reduction in the relative fluorescence of supercoiled plasmid molecules would have occurred here since, at the concentration of EtBr used during electrophoresis ($0.5\mu gmL^{-1}$), complete unwinding of the DNA superhelix would have occurred (Maniatis *et al*, 1982). Plasmid migration rate was also minimal at this EtBr concentration. The argument for a relative decrease in fluorescence proposed by Projan *et al* (1983) was that the physical constraints of the superhelix limited the binding of EtBr molecules greater than if the plasmid was in an open circle or linear state. However, the unwinding of the supercoiled plasmid under the electrophoresis conditions used during this project (but not in Projan *et al's* work) probably avoids the proposed limitation.

Having established a linear relationship between peak integral and level of DNA per band in preparations of similar concentrations to those obtained from experimental samples, the assessment method was further tested by "reconstitution" of separately purified plasmid and chromosomal DNA, as reported in Section 3.1.2. A problem which was encountered during early electrophoresis procedures was the co-migration of open circle plasmid topoisomers and chromosomal DNA (a feature also reported by Projan *et al*, 1983), thus leading to an underestimate of the actual plasmid copy number. A novel method was investigated which may have overcome this problem. The novel procedure involved digesting total DNA extracts with a restriction enzyme which cuts the plasmid at a single site thus linearizing it. However, the resulting smear of digested chromosomal DNA resulted in a

less accurate method of plasmid copy number assessment (refer to Table 2). It was interesting to note that the effect of the background smear, at the plasmid band position, varied with the amount of plasmid DNA present (refer to Figure 10). At the highest level of linearized plasmid DNA, Chromoscans of identical lanes (with and without background smear) were virtually superimposable. As the level of plasmid DNA present decreased the effect of the background smear became progressively more additive. At the highest plasmid level the limits of fluorescence linearity had probably been reached so the expected cumulative effect of plasmid DNA, plus similar sized chromosomal DNA at that point, was not observed. Also no further increase in peak integral value was measured. Another, less likely, reason for this effect was that at the highest level of plasmid DNA, an exclusion effect may have occurred such that the fluorescent DNA at that particular point in the gel, was plasmid DNA rather than similar sized fragments of chromosomal DNA. As a result of these observations (and those reported in Section 3.1.3) it was decided that all further experimental plasmid copy number estimates were conducted using 0.7% agarose gels under fast electrophoresis conditions. This approach improved both the speed of measurements and also the separation of plasmid topoisomers away from chromosomal DNA thereby increasing its accuracy (refer to Section 3.1.4).

The results of reconstitution gels using the standard method (not involving chromosomal DNA digestion) showed an expected downward trend in plasmid copy number as the porportion of added plasmid DNA was decreased. What was unexpected, though, was the discrepancy between the observed and the expected copy number values. The probable reason for this was, again, the inaccuracy of using absorbance readings to quantify DNA preparations and these observations are discussed in Section 3.1.2.4. Despite the limitations of using OD_{260} absorbance measurements to quantitate DNA preparations, the novel method of "reconstituting" DNA of different sources to achieve an artificial copy number proved very useful in defining method sensitivity. There are probably no reports in the literature of a similar method being employed to test a plasmid copy number assessment protocol.

It was found that a mean plasmid copy number value of 9 gel lanes of one extract was accurate to within 10% (refer to Section 3.1.3). the variation of different extracts of the same culture sample (at the same age) was also found to be 10%. Consequently, since all experimental plasmid

copy number estimates were made by averaging two lanes each of two extracts, such mean values would be accurate to within 20% (since within-extract and between-extracts variation is cumulative). Projan *et al* (1983) also found that their plasmid copy number estimates were accurate to within 20% (standard deviation) of the mean.

The identity of various plasmid topoisomeric forms is not particularly important for plasmid copy number determinations, since all plasmid forms were included in calculations (although few, if any, literature reports actually state this). However, during the course of this study it was observed that certain topoisomers predominate at different points during cultivation. Such effects were particularly noted during *S. lividans* pIJ303 stirred vessel fermentations (refer to results presented in Section 3.6). It was therefore important to identify the different plasmid topoisomers, including dimers. The results of these experiments were reported in Section 3.1.4. It was possible to identify supercoiled pIJ303 monomers and dimers and open circle pIJ303 monomers and dimers. Fewer forms of pIJ702 were present and the supercoiled monomer and open circle monomer forms were identified, only faint dimer bands were visible. The results reported in Section 3.1.4 supported the assumptions that in total DNA extracts, open circle pIJ303 DNA migrated behind while supercoiled plasmid migrated ahead of chromosomal DNA under conditions of fast electrophoresis through 0.7% agarose gels. There are few published reports of attempts to identify different plasmid bands in this way (e.g. Projan *et al*, 1983) and few indications of attempts to optimize conditions in order to improve topoisomeric separations.

Plasmid structural stability was tested through restriction fragment analysis as reported in Section 3.1.5. This method could detect gross changes in plasmid structure but not subtle alterations or small deletions. No gross structural instability was found although pIJ702 has been shown to re-arrange when used as a cloning vector (Lee *et al*, 1986). Structural stability is also mentioned in Sections 4.6 and 4.7.

4.2 Discussion of pIJ303 stability in shake-flask experiments.

The plasmid copy number of pIJ101-derived vectors such as pIJ303 was already known to increase during early rapid growth up to maximum values of about 300 (Kieser *et al*, 1982). Results presented in Section 3.2 of this thesis also revealed that such plasmid copy number values could be obtained under similar shake-flask culture conditions. The slow-growing characteristics of *S. lividans* pIJ303 cultivated in MOPS medium showed that plasmid copy numbers of between 400 and 500 could be sustained over a period of 50h (refer to Figure 16). However with growth in a richer medium, such as TSB where rapid growth was followed by a stationary growth phase a pronounced peak of maximum plasmid copy number level was observed (refer to Figure 19). The maximum plasmid copy number measured in both one-stage fermentations were similar but this level was maintained for longer in the *S. lividans* pIJ303 MOPS culture. The culture morphology shown in Figure 17 yielded a homogenous broth desirable of seed cultures. However, biomass concentration was low in MOPS medium therefore MEP was chosen for subsequent seed cultures since similar morphology was obtained with a slightly improved biomass yield.

During two-stage fermentations both plasmid copy number and biomass concentrations were followed. The first sample from the second fermentation stage of a two-stage MEP fermentation revealed a dry cell weight concentration of $4gL^{-1}$ which when compared to subsequent samplings, was the maximum biomass concentration (refer to Figure 20). Rapid growth had therefore occurred before 24h and the first sampling was probably at early stationary phase. A steady decline in biomass concentration was seen from between 24h and 96h, an overall decrease of 50% was observed between those timepoints. Interestingly, a concomitant decrease in plasmid copy number was also observed during the same period of time. During the two-stage phosphate-supplemented MEP fermentation it was possible to obtain a mid-rapid growth phase sampling (refer to Figure 21). The plasmid copy number was again seen to closely follow the biomass concentration data and both eventually decreased to approximately 30% of the maximum values. The availability of extra inorganic phosphate increased the maximum biomass concentration to $8gL^{-1}$ (only $4gL^{-1}$ were obtained from growth in MEP medium). Slightly greater plasmid copy number levels were also obtained from growth in phosphate-supplemented MEP medium. The plasmid copy number

at transfer was measured at about 230. Although a similar value was measured at the first sampling it is likely that a decrease in plasmid copy number would probably have occurred during very early growth since rapid chromosomal DNA replication may have taken place during this period.

As seen in Figure 22 *S. lividans* pIJ303 growth in GYT medium revealed an early linear growth phase which continued until 80h incubation at 28°C cultivation temperature. Plasmid copy number was however much less than that obtained from growth in either MEP or phosphate-supplemented MEP media. When *S. lividans* pIJ303 was cultivated at the elevated temperature of 37°C, biomass concentration was reduced by 50% and plasmid copy number was reduced by 70%. The control GYT culture revealed a rapid decline in plasmid copy number between 50h and 80h incubation but similar values were measured in both 37°C and 28°C cultures at the last sampling.

The plasmid copy number profile obtained from growth in phosphate-supplemented MEP medium showed nearly complete plasmid loss after 24h growth at 37°C (refer to Figure 23). No cell lysis was observed since a steady biomass concentration of approximately 50% of the control culture was maintained throughout the experiment. The plasmid copy number in the control culture revealed the characteristic profile when grown in this medium, a noticable increase during rapid growth followed by an equally sharp decline during stationary phase. The rapid decrease in plasmid copy number, to apparently zero when cultures were grown at 37°C in rich medium suggests that plasmid replication initiation was probably arrested under these conditions. Also since plasmid bands were observed to disappear from total DNA extracts of cultures under environmental stress (data not shown), partial DNA degradation may also have occurred. It was noted that in 37°C-grown cultures the intensity of chromosomal DNA bands also decreased perhaps indicating concomitant degradation of both plasmid and chromosomal DNA.

The DNA gyrase inhibitor novobiocin is known to 'cure' plasmids from *E. coli* cells (McHugh and Swartz, 1977 and Wolfson *et al*, 1983). However, the same effect was not observed in recombinant *S. lividans* cultures since no reduction in plasmid copy number was observed (refer to Section 3.2.6). A one-stage TSB experiment with novobiocin added at 5μgmL^{-1} final concentration to the test culture revealed no preferential decrease in plasmid copy number. The control culture showed the typical plasmid copy

number profile of a one-stage TSB culture (compare Figure 19 and Figure 24). However the test culture did not reveal the expected decrease in plasmid copy number during stationary phase. In fact the copy number remained at the maximum level for an extended period of 25h and then decreased to the same value as the control culture after 88h incubation.

The second novobiocin experiment involved a two-stage fermentation in phosphate-supplemented MEP medium with novobiocin added at $5\mu gmL^{-1}$ and $25\mu gmL^{-1}$ after 17.5h second-stage growth. The plasmid copy number at seed transfer was low (about 20) therefore the maximum plasmid copy number in the control culture was only approximately 125. However, the typical trend in plasmid copy number was obtained (compare Figure 21 and Figure 25). The $5\mu gmL^{-1}$ novobiocin culture resulted in a maximum plasmid copy number of approximately 160 after 42h incubation. These plasmid copy number trends are similar but the novobiocin seems to have shifted the profile by about 40h. The highest novobiocin concentration culture ($25\mu gmL^{-1}$) did show a reduced plasmid copy number since the level remained at approximately 75 on average, for 70h. The plasmid copy number values at the highest novobiocin concentration is unlikely to indicate preferential inhibition of plasmid replication since the values were still greater than those of the control culture during stationary phase.

In both one-and two-stage experiments using novobiocin, its effect seemed to either delay the attainment of maximum plasmid copy number or to extend the period of high plasmid copy number. There was no significant difference between plasmid copy numbers in either test or control cultures from samples taken in late stationary phase, differences were observed in earlier samples. The novobiocin culture profile shown in Figure 24 was remarkably similar to that of Figure 16 which was the result of a one-stage MOPS culture. MOPS medium is not as rich as TSB medium, however the addition of novobiocin to the latter medium seems to have, perhaps, reduced the culture's growth rate. It was not possible to specify whether this explains the extended period of high plasmid copy number since no biomass concentration data were taken.

The effects of novobiocin addition at $25\mu gmL^{-1}$ during a two-stage fermentation resulted in a more gradual increase in plasmid copy number and no rapid decrease was seen during stationary phase (refer to Figure 25). There was therefore little or no evidence that novobiocin selectively

inhibits *Streptomyces* plasmid replication but that a general decrease in culture growth rate may have occurred resulting in an altered plasmid copy number profile. Novobiocin was known to affect biomass at these concentrations (refer to APPENDIX 3) but it probably cannot 'cure' *S. lividans* mycelium of plasmids in the same way as *E. coli* cells. The reason for this may be that it is the segregation of plasmids into daughter cells that may be affected by DNA gyrase inhibition thereby resulting in segregational plasmid loss from unicellular bacteria. Since *Streptomyces* are mycelial organisms such segregation is unlikely therefore preferential plasmid loss was not observed. Novobiocin did not exert an environmental stress in the same way as high incubation temperature, which resulted in a plasmid copy number of apparently zero.

When the influence of seed culture age at transfer was investigated it was found that the best plasmid copy number level was obtained from a culture where the seed was transferred after 39h incubation. Younger seed age transfers resulted in a long lag in the second-stage fermentation because of the low physiological status of the younger seed culture. The 39h transfer resulted in the best plasmid copy number level (attained after 24h) suggesting that the seed culture was more metabolically active after 39h incubation. The physiological status of the seed culture at transfer was therefore important and that the normal protocol of transferring the seed culture after 72h growth was both convenient and was of no disadvantage regarding the plasmid copy number level.

Many industrial fermentation protocols involve many stages in inoculum development due to the increase in scale from laboratory to production fermentations. The effect of multi-stage fermentation upon plasmid copy number was reported in Section 3.2.7.3. It was found that at each withdrawal/refill point the subsequent peak in biomass concentration never quite reached the previous maximum biomass level (refer to Figure 26). This may be due to the accumulation of toxins or other metabolites deleterious to growth. Despite the availability of fresh nutrients on three occasions, an increase in plasmid copy number was not observed. The plasmid copy number profile obtained is more typical of a two-stage phosphate-supplemented fermentation (compare Figure 21, 28°C curve of Figure 23 and Figure 26). A dilution effect upon plasmid copy number was observed at each

withdrawal/refill point. The reason for this could be that the organism's growth rate remained high due to the availability of fresh nutrients. Plasmid copy number tends to be greatest at late exponential growth or early stationary phase where the growth rate is slower than during rapid or exponential growth. It is therefore possible to dilute out plasmid molecules by repeated transfers if the culture is not allowed to proceed to stationary phase when the plasmid copy number is higher. This suggests that the number of stages in the inoculum development should be minimized and that after each transfer the culture should be allowed to grow until stationary phase. Also, perhaps, only a small volume of seed culture should be transferred to avoid the accumulation of deleterious metabolite. Plasmid instability in recombinant *E. coli* was overcome by Pierce and Gutteridge (1985) when 300L fermentation was intitated by 0.3µL of inoculum from an agar slope *i.e.* it was a one-stage production fermentation.

To summarize therefore, similar *Streptomyces* plasmid copy number values to published data were observed but a clearer study of transient changes in copy number during culture growth was made. An increase during intial rapid growth was seen in rich media during both one- and two-stage fermentations. Poorer media such as MOPS resulted in a period of sustained high plasmid copy number, a feature also observed at some novobiocin concentrations. In general, a decline in plasmid copy number to approximately the same level as at seed transfer was seen during some two-stage fermentations at stationary phase. Complete plasmid loss was observed in some media when cultures were grown at 37°C , however a concomitant decrease in chromosomal DNA band intensity was also seen. The use of multi-stage fermentation protocols should be avoided and seed cultures should be allowed to grow until stationary phase wherever possible. No structural plasmid instability was observed during these experiments.

Stueber and Bujard (1982) found that the copy number of pBR322 when hosted in *E. coli* increased 4-fold from early exponential phase to stationary phase. Such "autoamplification" was also found by Frey and Timmis (1985) when ColD-CA23 plasmid was hosted in *E. coli*. In fact, these authors reported a 10-fold increase in plasmid copy number during exponential growth.

The effect of reduced growth rate upon plasmid copy number by altering the growth medium of recombinant *E. coli* was reported by Seo and

Bailey (1985b). They found that plasmid copy number increased when the growth rate was reduced using poorer media. This result supports the observation reported here regarding the sustained high copy number in MOPS medium and the unexpectedly low copy number during the multi-stage experiment where growth rate was high. It may also help to explain why plasmid copy number was found to be maximal at late exponential growth since the growth rate at this point would be decreasing due to the exhaustion of nutrients.

Growth of recombinant Gram-ve organisms at non-optimal temperatures was also found by Bernardez and Dhurjati (1987) to increase plasmid instability. McLoughlin *et al* (1987) also reported decreased *B. subtilis* plasmid stability at higher growth temperatures. Roth *et al* (1985) reported that segregational plasmid loss from recombinant *S. lividans* grown in chemostat culture was greater at 36°C than at 28°C. Reinikainen *et al* (1989) however found that a pBR322-derivative was more stable in *E. coli* when the host was grown at 34°C rather than at 40°C which was the optimum growth temperature.

In general therefore, the phenomenon of large increases in plasmid copy number during exponential growth is common to both Gram +ve and Gram -ve bacteria. There are also some literature reports of increased plasmid loss at non-optimal growth temperatures.

4.3 Discussion of pIJ702 stability in shake-flask experiments.

As mentioned in Section 3.3, pIJ702 has a *sti*$^-$ phenotype and is hence prone to accumulate single stranded DNA at each round of plasmid replication (Deng *et al*, 1988). Due to this inefficient mode of plasmid replication the yield of double stranded plasmid DNA was therefore expected to be lower than from a *sti*+ plasmid such as pIJ303.

The results of a one-stage TSB experiment plotted in Figure 27, revealed an increase in plasmid copy number to nearly 200 after 48h incubation. Later in the stationary phase the plasmid copy number declined to approximately 50% of the maximum value. The results of an identical experiment using *S. lividans* pIJ303 are shown in Figure 19. The maximum plasmid copy number of pIJ303, approximately 425 was measured after 32h incubation. The level then decreased to about 150 during the latter part of the experiment. pIJ702, in contrast, did not reach its maximum plasmid copy number until 48h but the level then decreased to 50% of the maximum, compared to 40% for pIJ303. There was therefore a slight shifting of the peak by 16h and the copy number increase/decrease profile was not as pronounced for pIJ702.

The results of a two-stage MEP medium experiment are shown in Figure 28. As seen in the graph, rapid growth had occurred before the first sampling at 24h. Maximum plasmid copy number was also observed at this point *i.e.* early stationary phase, since only a slight increase in biomass concentration was seen after this point. The plasmid copy number at seed transfer was about 120 but a decrease probably occurred during early growth due to rapid chromosomal DNA replication. The plasmid copy number then increased 4-fold during rapid growth. A sharp decrease in plasmid copy number was observed throughout the remainder of the experiment. After 72h incubation only faint plasmid bands were visible, however this level was approximately the same as that at seed transfer. The plasmid copy number profile of pIJ303 under the same experimental conditions were similar to those of pIJ702 (refer to Figure 20). However, the relative decrease in pIJ702 plasmid levels was greater. Both cultures showed rapid growth had ceased by 24h, but *S. lividans* pIJ702 showed a slight increase in biomass concentration until 48h. A gradual decrease in biomass concentration during late stationary phase suggests some cell lysis which may account for the continued rapid decline in plasmid copy number perhaps due to increased

plasmid DNA degradation. A similar decrease in biomass concentration was seen in the *S. lividans* pIJ303 culture.

The results of a second two-stage fermentation, this time using phosphate-supplemented MEP medium, are given in Figure 29. Better biomass concentration was obtained from cultivation in phosphate-supplemented MEP medium ($6gL^{-1}$ compared with $3.8gL^{-1}$ in MEP medium). Initial rapid growth had again occurred before the first sampling point at 24h incubation. Unlike the MEP two-stage experiment no cell lysis was observed in the phosphate-supplemented MEP experiment. The plasmid copy number profile was also very different since a rapid increase during initial growth in MEP medium was not observed. A maximum plasmid copy number of about 250 was measured (compared with approximately 270 at seed transfer). The physiological status of the seed culture may have been different in this experiment and that may explain the relatively poor levels of plasmid copy number observed in the second stage fermentation. A sharp decrease in plasmid levels during the latter part of the experiment was not seen either and probably did not occur since no cell lysis was detected by biomass concentration measurements. Growth of *S. lividans* pIJ702 in phosphate-supplemented MEP medium therefore yielded decreased plasmid copy number values but the expected decrease during late stationary phase was less pronounced. The observed drop in plasmid copy number after seed transfer suggests that this probably happens in all two-stage fermentations after the transfer point. Normally, however, the plasmid copy number would be expected to increase above the level at transfer.

The results of pIJ702 plasmid stability at 37°C are shown in Figure 30. As seen in the graph there was little difference in the biomass concentrations from both 28°C and 37°C cultures. Both cultures grew rapidly between seed transfer and 16h incubation and then continued to grow slowly until 48h incubation when slight cell lysis was observed in the 37°C-grown culture since the biomass concentration decreased by $1gL^{-1}$. The control plasmid copy number profile was partly similar to that obtained from the two-stage MEP+PO_4^{3-} fermentation shown in Figure 29. Since the level decreased during early growth and then increased quickly to a maximum value of about 140 after 40h incubation. A slight decrease was seen thereafter. It should be noted that during this experiment the plasmid copy number level of the seed culture at transfer was attained. The plasmid copy number

values of the 37°C culture showed a gradual increase until 65h incubation but a sharp decline to apparently zero plasmid levels was seen towards the end of the experiment. The stability of pIJ702 was therefore seen to decrease under conditions of environmental stress. The reason for this may be that the rate of plasmid replication initiation was decreased under such conditions. Plasmid DNA degradation may also have occurred at elevated growth temperatures. In comparison to pIJ303, pIJ702 showed better stability at higher growth temperatures since plasmid DNA bands were still visible after 72h incubation. The increased stability may be accounted for by the difference in plasmid copy number profiles of the two vectors when cultivated in phosphate-supplemented MEP medium *i.e.* pIJ702 profile was less pronounced.

The pIJ702 results discussed in Section 3.3.6 involved the study of both plasmid copy number levels and the expression of the tyrosinase gene during shake-flask culture. The results of a two-stage GYM medium experiment with tyrosinase "induction" after 48h incubation are shown in Figure 31. As seen in the graph the plasmid copy number peaked at 50h incubation and then showed a rapid decline thereafter until 54h incubation. The "induction" mixture was very nutritious and hence stimulated growth at at point where the culture would normally have reached stationary phase. The stimulation of growth probably led to increased chromosomal DNA replication rates and hence a decrease in plasmid copy number. The tyrosinase activity trend revealed an increase from 150 units mg^{-1} protein to 1300 units mg^{-1} protein after 24h post-"induction". The tyrosinase activity trend was found to be completely different to that of the plasmid copy number. Some workers advise using cloned gene product level as a measurement of plasmid copy number (*e.g.* Satyagal and Agrawal, 1990). As seen from these results such assumptions can be incorrect (this was also reported by Seo and Bailey, 1985b who found that in recombinant *E. coli* the ratio of cloned β-lactamase activity to plasmid content decreased significantly at high plasmid copy number). It is unfortunate that tyrosinase activity cannot be "induced" by non-growth stimulating effector molecules.

The results of tyrosinase activity measurements made from cultures grown in a variety of media without "induction" were reported in Section 3.3.6.2. Also the effect of copper upon enzyme activity was also

investigated. No growth was obtained from the minimal medium with copper possibly due to the toxic effects of copper at 2.5mgmL^{-1}. Added copper in complex medium was probably chelated by macromolecules and was therefore not as toxic. It was found that the best tyrosinase activity was obtained from GYM+Cu cultures (see Table 4). Good levels of tyrosinase activity were also obtained from minimal medium-grown *S. lividans* pIJ702 cultures. This was unexpected since no inducers (*i.e.* amino acids, Hintermann *et al*, 1985) were supplied in the medium. It is therefore not possible to completely repress the operon even when the culture was grown in minimal medium. Since tight regulation of this operon cannot be obtained it is probably unsuitable for large-scale heterologous gene expression. Periods of maximum vector replication and expression ideally should occur at different points in order to optimize cloned gene production. A model described by Seressiotis and Bailey (1987) revealed that it is best to have environmental switching on/off of cloned gene expression where such expression is deleterious to cell growth.

To summarize therefore, pIJ702 plasmid copy number was lower than that of pIJ303. The reason for this is probably the inefficient mode of plasmid DNA replication due to the *sti*- phenotype. Maximum plasmid copy numbers were again obtained in late rapid growth phase or early stationary phase. Many cultures also showed rapid decline in plasmid level towards the end of the experiment. This decline could be partly alleviated by cultivating *S. lividans* pIJ702 in phosphate-supplemented medium, the reason for this is not clear. pIJ702 was less stable when grown at elevated temperatures, however complete plasmid loss did not occur as early as in pIJ303 cultures. Tyrosinase activity was detected in minimal medium-grown cultures suggesting that complete repression of the operon could not be achieved. However, a considerable increase in tyrosinase activity was detected when the "induction" mixture was added, but this effect may be partly growth-related and induction. Hintermann *et al* (1985) also reported that *S. lividans* harbouring pIJ702 formed melanin constitutively when grown on solid media (complete and minimal agar) with and without inducers.

Despite the observations by Lee *et al* (1986), Chen *et al* (1987) and Pigac *et al* (1988) that recombinant *sti*$^-$ plasmids are prone to DNA re-

arrangements, no structural instability was detected during any of these experiments. No dimer formation was detected in the pIJ702 culture experiments reported here.

4.4 Discussion of pMT605 and pMT608 stability in shake-flask experiments.

Both of these plasmids were known to be rapidly lost from *S. lividans* recombinant cultures due to the findings of Kendall and Cullum (1984). Thiostrepton was often added to culture media by these workers in order to improve plasmid retention. The results of a study to investigate the plasmid copy numbers of these vectors were reported in Section 3.4.

The results of a one-stage TSB experiment are shown in Figure 34. The copy number of pMT605 increased quickly during initial rapid growth to a maximum value of approximately 250 after 48h incubation which was the first sampling. A rapid drop in copy number was observed between 72h and 77h after which no further plasmid bands were visible. Plasmid pMT608 showed a similar trend but faint plasmid bands were still visible after 90h incubation. The profile measured is typical of that obtained during a one-stage culture in TSB medium (refer to Figure 19 and Figure 27. However, neither pIJ303 nor pIJ702 disappeared completely but no stabilization of pMT605 and pMT608 copy number levels was seen during late stationary phase.

The results of two-stage, shake-flask fermentations of the agarase plasmids were reported in Section 3.4.5. When grown in MEP medium the maximum pMT605 plasmid copy number was measured after 55h incubation and declined thereafter. The pMT605 plasmid copy number trend shown in Figure 35 was not found to be similar to those of either pIJ303 (Figure 20) or pIJ702 (Figure 28). pMT605 copy number peaked (at about 215) after 55h which was much later than either pIJ303 or pIJ702 (normally 24h incubation). It was not possible to measure pMT608 plasmid copy number from samples grown in MEP medium since plasmid DNA bands were not visible.

Good pMT605 plasmid copy number levels were obtained from growth in phosphate-supplemented MEP medium. As shown in the graph Figure 36, the maximum level of plasmid copy number (nearly 400) was measured after 31h incubation. A high level of plasmid copy number was maintained for a further 50h incubation with only a slight decrease seen towards the end of the experiment. Much better pMT605 levels were therefore obtained when recombinant *S. lividans* was grown in phosphate-supplemented MEP medium. A similar stabilization of plasmid copy number values in MEP+PO_4^{3-} medium was also seen with pIJ702 (Figure 29). It was again not possible to measure pMT608 plasmid copy number values since the bands were too faint.

Since the agarase enzyme is constitutively expressed and is

easily assayed, the level of cloned enzyme activity was measured for both pMT605 and pMT608 during two-stage fermentations. *S. lividans* pMT605 was found to be a poorer producer of agarase since only faint zones of clearing could be detected in all culture supernatant samples. The agarase enzyme levels from pMT608 were plotted in Figure 37. During the first experiment a fairly constant level of agarase activity was detected, but the second showed a decrease in activity after 56h incubation and no measurable zones of activity were detected after 72h incubation. Ironically, no pMT608 plasmid copy number values could be measured despite good levels of agarase activity, the converse was observed for pMT605 since good levels of DNA were measured but only faint zones of activity were detected. Only a few copies per chromosome of pMT608 seemed to be needed to yield measurable levels of agarase activity, therefore clone gene expression alone was not a reliable indicator of plasmid level.

Since no plasmid copy number values could be obtained for pMT608, plasmid segregational stability was followed by streaking out cultures taken from experiment samplings of fragmented MEP-grown cultures (refer to photomicrograph Figure 17). Agarase-producing colonies were visualized after flooding with Gran's iodine. It was found that up to 31.5h incubation in the timecourse experiment, all streaked colonies produced a surrounding halo of degraded agar. After 48h incubation more than 30% of colonies had lost the ability to produce cloned agarase enzyme. This suggests that pMT608 copy number had decreased to such a low value that the plasmid suffered segregational instability into mycelial fragments at this point in the experiment.

To summarize therefore, both pMT605 and pMT608 were found to be less stable than the parent vector pIJ702. The original workers, Kendall and Cullum (1984) intended the agarase plasmids to be used as vectors but they are obviously too unstable for this purpose despite detectable levels of cloned enzyme. The segregational stability results of streaked samples from MEP-grown *S. lividans* pMT608 also suggested defective plasmid partitioning into new mycelium fragments. No other plasmid investigated during the course of this study exhibited similar segregational instability. Where sufficient plasmid DNA could be isolated, no evidence for structural instability was found.

4.5 Discussion of *S. lividans* TK24 stirred vessel fermentations.

Initial fermentations (reported in Section 3.5.3.1) were conducted without automatic pH control. It was anticipated that the fermentation characteristics of *S. lividans* in stirred vessels could then be directly compared to those from shake-flask experiments, where there was no pH control either.

The results of the first experiment were reported in Figure 38 and from the graph it was seen that a maximum biomass concentration of $4.8gL^{-1}$ was attained after 24h incubation. A decrease in biomass concentration to 50% of the maximum was seen during the remainder of the experiment. The poor biomass concentration levels were thought to be due to the rapid increase in culture acidity which resulted in a final pH value of between 3 and 2. Growth under such conditions was probably severely hindered and hence led to the poor biomass concentrations obtained during this fermentation. In comparison to similar shake-flask experiments, using the same medium, the maximum biomass concentration in the stirred vessel fermentation was only 70% of that obtained from shake-flask experiments. pH values during shake-flask fermentations were never measured, but it is assumed that the medium did not become as acidic as that during stirred vessel experiments because of better aeration in fermenters. Since these levels of biomasss were unacceptably low all further stirred vessel experiments were conducted with alkali addition to neutralize the organism's acid production. No similar acid additions were made since the increase in pH towards the end of the experiments were both interesting and characteristic of *S. lividans* TK24 when grown under these conditions. Similar pH control strategies and profiles were reported by Martin and McDaniel (1975).

The results of a second phosphate-supplemented MEP medium experiment was reported in Figure 39. This experiment was conducted by manually adjusting the pH back to neutrality at each sampling point. An initial phase of rapid growth was observed between 7.5h and 23h with a further increase in biomass concentration seen between 23h and 47h incubation. The second phase of growth suggests that true stationary phase did not occur until beyond 47h. Deoxyribose concentration measurements taken from mycelium samples revealed a fairly constant level at $5\text{-}7\mu gmL^{-1}$. However, if the deoxyribose concentration was expressed as a function of

broth volume then there was only 36μgmL^{-1}L^{-1} of culture at 7.5h compared with 150μgmL^{-1}L^{-1} of culture 23h. The maximum biomass concentration achieved during this fermentation however was only marginally greater than that achieved from shake-flask fermentations using MEP+10mMPO_4^{3-}.

Since the period of maximum growth rate occurred between 7.5h and 24h it was desirable to obtain samples from this period of growth. Most subsequent fermentations were therefore conducted by sampling from 15h onwards since this corresponds to approximately late exponential growth. Automatic alkali addition to *S. lividans* TK24 when grown in phosphate-supplemented MEP medium resulted in a maximum biomass concentration of >10gL^{-1} dry cell weight (Figure 40). A continued phase of growth from 15h to 48h incubation was again seen. When the biomass concentration results of Figure 40 were compared with the logged data plotted in Figure 41, it can be observed that DOT levels decreased rapidly until 12h incubation. This coincided with the point at which pH control by alkali addition became necessary. Alkali addition then continued for a further 40h until the biomass concentration had reached its maximum level. The DOT level, despite a noisy signal, increased only gradually. This further supports the notion of a period of slow, secondary growth beyond the exponential phase. During the latter half of the fermentation the pH was observed to increase steadily until it reached *circa* pH8.8 at the end of the experiment.

The results of a MEP medium *S. lividans* TK24 fermentation were plotted in Figure 42 and Figure 43. During this experiment a less pronounced period of rapid growth was observed since the increase in biomass concentration was more linear. Essentially no difference in maximum biomass concentration obtained from MEP medium and the phosphate-supplemented version was found. These results contrasted with those of shake-flask experiments where the same two media were used. The reason for this could be that the phosphate available in MEP+10mMPO_4^{3-} medium provided a slight buffering capacity for the acid production during shake-flask cultures, where there was no other means of pH control. Such buffering might then permit better growth characteristics and ultimately a higher maximum biomass concentration. Evidence was again seen for a period of slow linear growth beyond the initial phase of rapid growth (which occurred up to 15h in this experiment). The intiation of pH control coincided with the end of the DOT level decrease at 15h and the period of pH control extended

for a further 35h. The characteristic rise in pH during the latter part of the fermentation was seen and the final value was 8.5.

To summarize therefore, the first stage of rapid growth was followed by a period of copious acid production. Without neutralization with alkali the resulting low pH was found to inhibit further growth. This period of acid production coincided with a steady increase in biomass production and both parameters were seen to cease simultaneously. Evidence from DOT measurements also support the notion of a period of secondary growth before true stationary phase. During stationary phase the pH was observed to increase, unchecked by acid addition, in a characteristic mode.

Streptomyces are known to produce quantities of organic acids. Ahmed *et al* (1984) discovered that *S. venezuelae* released pyruvic acid and α-ketoglutaric acid when grown under nitrogen limitation. Surowitz and Pfister (1985) found that aerial mycelium formation by *S. alboniger* was inhibited due to pyruvic acid production. Dekleva and Strohl (1987) reported that *S. peucetius* secreted pyruvate and 2-oxoglutarate when grown with glucose or fructose carbon sources. Payne *et al* (1990) attempted to analyse the acids produced by recombinant *S. lividans* during batch fermentations with glucose as carbon source. Gas chromoatography was used for analysis but significant quantities of pyruvate or α-ketoglutarate were not found. The authors were not able to identify the organic acid(s) produced by *S. lividans*. It was estimated that during the course of the fermentations reported in Section 3.5 that 0.5 mole equivalents of organic acid was produced. The significance and possible solutions to the copious acid production by *S. lividans* will be discussed later.

4.6 Discussion of pIJ303 plasmid stability during stirred-vessel fermentations.

The results of stirred vessel fermentations of *S. lividans* pIJ303 grown in phosphate-supplemented MEP medium were reported in Section 3.6.3.1. The change in the relative proportion of supercoiled plasmid monomer DNA bands was also reported in the same section since a definite pattern was observed. This parameter had not previously been noted in shake-flask experiments.

Maximum biomass concentration of $>10gL^{-1}$ were obtained during these experiments. Such concentrations compared favourably with those obtained for *S. lividans* plasmidless host grown under similar conditions (refer to results in Section 3.5.3.3). For this reason hosting the plasmid pIJ303 did not seem to affect the biomass concentration nor the growth rate during rapid growth phase since comparable maximum biomass concentrations were measured at similar timepoints. Where biomass data was available for pIJ303-hosted cultures in MEP+10mMPO_4^{3-} medium, further (slight) increases in biomass concentrations beyound 24-30h incubation were not detected, unlike *S. lividans* cultures (compare Figure 48 and Figure 40). There was some evidence of continued metabolic activity (if not biomass accumulation) from the DOT trend shown in Figure 50. The DOT level initially decreased during exponential growth to a minimum value of 75% of saturation. It then increased steadily during the remainder of the fermentation suggesting active uptake of dissolved oxygen from the medium. The pH profile of the pIJ303-hosted culture shown on the same plot is also very similar to that of *S. lividans* plasmidless host. Alkali addition for pH control was needed for a period of 35h after the end of initial rapid growth. Such a time period for pH control was also seen during *S. lividans* plasmidless stirred vessel fermentations (refer to Section 3.5.3.3). The increase in pH towards the end of the fermentation was also characteristic of both plasmidless and plasmid-hosting cultures. The average amount of alkali needed for pH control during *S. lividans* pIJ303 growth in MEP+10mMPO_4^{3-} indicated that 0.45 mole equivalents of organic acid was produced. This amount was only slightly less than that from *S. lividans* plasmidless fermentations *i.e.* 0.5 mole equivalents *versus* 0.45. The effect of pIJ303 upon the fermentation characteristics of *S. lividans* when grown in MEP+PO_4^{3-} were found to be insignificant. One reason for the lack of so called "burdening" effects of

hosting the plasmid was that no overexpression of pIJ303 genes was achieved or desired. This is therefore evidence that merely maintaining a plasmid *via* DNA replication probably does not, in itself, significantly alter the growth characteristics of the host organism. However, when cloned gene overexpression is desired and achieved then this may prove a greater metabolic strain upon the host organism and hence lead to instability and suboptimal culture behaviour.

The plasmid copy number profiles of stirred vessel fermentations were closely investigated and revealed interesting results. When compared to shake-flask copy number data (Section 3.2.4.2) it was found that similar copy number profiles were obtained. Maximum values were, in general, different since values up to 550 were achieved in shake-flasks but such values were not measured in stirred vessel fermentations. Maximum plasmid copy number values in 5L fermentations using phosphate-supplemented MEP were typically 200-250, again measured at about 24h post-transfer. The reason for the difference may be that chromosomal DNA replication is enhanced during stirred-vessel fermentations through better aeration thereby decreasing the relative amount of plasmid DNA present.

Another interesting point is that the relative proportion of supercoiled monomeric plasmid DNA increased during the course of all the fermentations, even though total plasmid copy number was sometimes declining. This proportion was seen to increase on average from between 0%-20% to >50% during the course of fermentations. The relative change in plasmid topoisomers can be clearly observed in the gel shown in Figure 51 where the proportion of open circle plasmid decreased and supercoiled plasmid increased (supercoiled dimers failed to separate from chromosomal DNA therefore were excluded from calculations). No other reports of such transient changes in plasmid topoisomers exist in the literature. The high proportion of open circle (nicked) plasmid molecules measured early in the fermentation were probably replication intermediates. Such molecules may have been nicked enzymatically in order to initiate plasmid replication. As the culture ages plasmid replication rate decreased and hence fewer open circle molecules were isolated, however an increasing proportion of non-replicating, supercoiled molecules were observed.

The results of *S. lividans* pIJ303 grown in MEP medium were reported in Section 3.6.3.2. Unlike the phosphate-supplemented MEP results

a period of continued biomass concentration was observed. The maximum biomass concentration compared favourably with that obtained when extra phosphate was used and also better than that of parallel shake-flask experiments. From the DOT logged data (Figure 54) it can be seen that rapid growth ceased after 12h and was followed by a period of extended metabolic activity since dissolved oxygen was still being taken up from the medium. This period also coincided with alkali addition for pH control and lasted approximately 40h, which is similar to that of *S. lividans* plasmidless host (refer to Figure 43). An average volume of alkali was added which corresponds to the production of 0.55 mole equivalents of organic acid. This level of organic acid was hence slightly higher than that of the plasmidless host grown in MEP medium. Both fermentations showed the characteristic rise in pH value towards the end of the fermentation. Again, the effect of hosting pIJ303 upon *S. lividans* fermentation characteristics in MEP medium did not seem to be significant.

The plasmid copy number data of these two identical experiments were rather different (refer to Figure 53 and Figure 57). The first experiment showed the expected rise in plasmid copy number during initial rapid growth up to a maximum value of nearly 400 after 24h. Plasmid copy number then declined to an average level of 250 for the remainder of the experiment. This profile was different from that of MEP+10mMPO_4^{3-} media since the maximum level was higher but it was achieved at a similar point during the experiment. Plasmid copy number was maintained at this level for a longer period of time than when *S. lividans* pIJ303 was grown in phosphate-supplemented medium. In the first experiment the proportion of supercoiled plasmid molecules also increased during the course of the fermentation (a feature also observed from growth in phosphate-supplemented MEP medium).

The results of the second experiment were rather different since a rise in plasmid copy number probably occurred between transfer (when it was measured at about 160) and 15h when the value was over 400. The plasmid copy number remained at this high level for the duration of the experiment. The proportion of supercoiled plasmid molecules was also high (approximately 50%) for the whole fermentation. An observation that may account for this was that all the visible plasmid DNA at seed transfer was in the supercoiled form, whereas only about 20-30% would normally be

supercoiled at the end of the seed culture stage.

Growth with reduced inorganic phosphate availability therefore resulted in higher plasmid copy number values which were maintained for longer. The growth characteristics of both plasmidless and recombinant *S. lividans* in this medium were similar. When compared to *S. lividans* pIJ303 growth in MEP+PO_4^{3-}, similar biomass concentrations were obtained but a period of secondary growth, before true stationary phase, was more evident. Both growth characteristics and plasmid copy number profiles were different when compared to those derived from MEP shake-flask experiments.

The effect of temperature stress upon plasmid stability in shake-flask cultures was reported in Sections 3.2.5 and 3.3.5). It was more feasible to investigate the effect of anaerobic stress upon plasmid stability in stirred vessel fermentations than in shake-flasks and the results of this study were reported in Section 3.6.3.3. No deleterious effects upon fermentation characteristics were observed when the DOT level was maintained at 5% of saturation. Copious acid production was still evident (refer to Figure 62) and the characteristic rise in pH was seen towards the end of the fermentation.

The plasmid copy number under low DOT conditions showed a maximum plasmid copy number value similar to that obtained under normal (control) fermentation conditions. However, the level dropped to an average value of about 75 for the remainder of the experiment. Virtually all the plasmid DNA isolated was of the open circle form suggesting a high proportion of plasmid replication intermediates.

The second experiment with nitrogen gas blending of the inlet air was designed to stress the organism even further in order to inhibit growth as far as possible. The results were shown in Figures 65-69 and as seen from the biomass concentration data, growth did not seem to be seriously affected by the imposition of anaerobic conditions after 15h. The plasmid copy number level was less but increased towards the end of the experiment. Again, no complete plasmid loss was observed and only open circle plasmid topoisomers were isolated.

Both low DOT experiments resulted in the production of an average of 0.45 mole equivalents of organic acid. This level of organic acid suggests that the organism's growth was virtually unchanged despite anaerobic conditions. Another curious observation was the apparent

'increase' in DOT level towards the end of the experiment (refer to Figures 62 and 67). Gas analysis data revealed that virtually complete anaerobic conditions existed within the fermenter, however a signal was produced from the DOT probe. It is feasible that a small, volatile molecule could mimic the signal of dissolved oxygen. The redox potential of the culture was not measured but the environment may have been sufficiently reducing to yield such a false signal. The reliability of DOT probes in *S. lividans* fermentations under conditions of low DO is therefore questionable.

To summarize, slightly poorer pIJ303 plasmid copy number levels were measured in MEP+PO_4^{3-} medium stirred vessel fermentations compared to shake-flask results. However, this observation, in itself, probably would not affect fermentation performance since good plasmid levels could still be obtained in stirred vessels. The plasmid copy number obtained from MEP growth was both better than that from phosphate-supplemented media and shake-flask experiments. The relative proportion of supercoiled plasmids increased during the course of phosphate supplemented MEP medium. No noticable differences between the fermentation characteristics of *S. lividans* and *S. lividans* pIJ303 cultures were observed. Copious acid production was still evident.

Plasmid copy numbers under conditions of low DOT were less than normal but complete plasmid loss was not observed. This observation contrasts with those made by Hopkins *et al* (1987) who found considerable segregational instability in a recombinant *E. coli* culture under conditions of low DOT. Plasmid pIJ303 was therefore found to be very stable in stirred vessel fermentations and no evidence of structural re-arrangements was found.

4.7 Discussion of pIJ702 stability and expression in stirred vessel fermentations.

The results of fermentations conducted using *S. lividans* pIJ702 grown in MEP+PO_4^{3-} medium were reported in Section 3.7.3. From these experiments it was possible to study plasmid copy number (and % supercoiled plasmid in some cases), growth and tyrosinase enzyme activity with or without inducers present.

The results of the first experiment showed a rapid increase in plasmid copy number during initial growth which reached a maximum value of about 440 after 48h growth. Unlike the parallel shake-flask experiment using the same medium, a much more pronounced plasmid copy number profile was obtained from the stirred vessel fermentations (compare Figure 69 with Figure 29). Although maximum plasmid copy number was achieved after 48h in both experiments, much better plasmid copy number levels were obtained in the 5L fermentations (440 *versus* 250). The reason for this difference is not clear. The copy number profile obtained was similar to the pIJ303 plasmid copy number profile shown in Figure 49 where the maximum level was also reached after 48h, with a decrease observed later in the fermentation. A feature not observed in any previous experiments was the apparent decrease in the proportion of supercoiled plasmid molecules isolated. This observation contrasts completely with those made with pIJ303 results (refer to Section 3.6.3) and the reason is not clear but may reflect differences in plasmid replication *i.e. sti*$^+$ and *sti*$^-$ phenotypes.

The tyrosinase activity pattern during this experiment closely followed that of the plasmid copy number, although maximum activity (about 210 units mg^{-1} protein) was measured after 40h incubation. A decrease to 50% of the maximum level was measured towards the end of the experiment. Such a decrease may have been due to the concomitant fall in plasmid copy number, although this is unlikely. More feasible, perhaps, was increased protease activity or decreased metabolic status, leading to reduced protein synthesis levels. This may explain the fall in tyrosinase enzyme activity after 70h incubation. The logged data of the first pIJ702 5L fermentation are shown in Figure 71. As seen from the plot, DOT level decreased rapidly between 5L and 15h, but increased slowly to the original level. This suggests a period of slow growth, after initial rapid growth, for a further 35h. However, since no biomass concentration data was

available then dry cell weight measurements could not be compared. Like both *S. lividans* and *S. lividans* pIJ303 5L fermentations, a period of pH control from 15h-50h incubation was seen where approximately 0.54 mole equivalents of organic acid was produced. The increase in pH beyond 50h is also a typical feature of *S. lividans* fermentations and is not affected by pIJ702 replication and expression. The increase in pH towards the end of the experiment also coincided with decreased tyrosinase activity and such alkaline conditions may have affected cloned enzyme activity.

A complete set of data was obtained from the second *S. lividans* pIJ702 MEP+10mMPO_4^{3-} fermentation as was plotted in Figures 72 to 76. An extended period of slow growth beyond initial rapid growth was again witnessed and is supported by the DOT data (Figure 75) and CO_2 analysis (Figure 76). The plasmid copy number data was different since, although a level of about 500 was measured after 40h incubation, the plasmid copy number continued to rise even further to a maximum level of nearly 700 after 70h. Therefore the plasmid copy number during 5L fermentations was again better than that measured in parallel shake-flask experiments.

Another difference between the two fermentations was the tyrosinase activity pattern since a much more acute peak was witnessed during the second experiment. However, the maximum activities did occur at the same point in growth but a 4-fold decrease in specific activity was seen. These differences may be due to the effects of frozen storage upon mycelium samples or may be due to batch variability. The logged data plots for both experiments were remarkably similar therefore both cultures exhibit identical behaviour during these two experiments. There was again little evidence that pIJ702 replication and expression affected the maximum measured biomass concentration or the growth rate (compare Figure 72 and Figure 40). The fermentation characteristics of *S. lividans* pIJ702 were therefore found to be very similar to those of the plasmidless host. The expression of the cloned gene did not seem to affect the fermentation performance but a distinct peak effect in optimum tyrosinase enzyme activity was observed.

A second series of fermentations where the effects of tyrosinase enzyme "induction" upon *S. lividans* pIJ702 fermentation performance were studied and are reported in Section 3.7.3.2. The effect of "induction" at different points in the fermentation was also investigated.

The biomass concentration data of the first stirred vessel "induction" experiment were plotted in Figure 77. As seen from the graph normal growth was observed until 20h incubation but a stimulation of growth was seen thereafter probably due to the effect of adding the nutrients in the "induction" mixture. This effect yielded a final maximum biomass concentration of $17.5gL^{-1}$ after 71h incubation. The plasmid copy number level showed a maximum value of about 450 after 50h with a decrease thereafter. However the plasmid copy number was then seen to increase subsequently towards the end of the fermentation probably due to the stimulation effect, upon growth, of the "induction" mixture. The proportion of supercoiled molecules was again measured as 100% throughout the experiment.

The tyrosinase activity pattern of the same experiment again revealed a pronounced peak of activity at 40h. The maximum tyrosinase level in comparison to a non-induced culture was not considerably greater (compare Figure 78 and Figure 70). This suggests that the effect was probably not a true "induction" and is more than likely growth-related. A surprising 0.94 mole equivalents of organic acid was produced which was nearly 2-fold greater than during any other fermentation.

The second experiment involved "induction" after 24h and again revealed a stimulation effect upon growth. A maximum biomass concentration of $15.1gL^{-1}$ was measured after 47h, a level slightly less than that of the first experiments. The plasmid copy number profile revealed a continued rise in values throughout the fermentation and mostly supercoiled plasmid molecules were isolated (refer to Figure 85). The identity of the second plasmid band is uncertain but it may be a supercoiled dimer or single stranded circle.

The tyrosinase activity profile was similar to that obtained during the first "induction" experiment, but maximal levels were shifted by about 8h *i.e.* the same difference in time as there was between the points of induction. From the logged fermentation data shown in Figure 83) it can be seen that the observed rise in DOT between 15 and 20h suggests that the initial period of rapid growth had ceased. The induction mixture was added at 24h and hence the DOT level was seen to plummet to <10% of saturation. Data from results in Section 3.6.3.3 indicate that the culture was not oxygen starved at the level of dissolved oxygen and that normal growth

could continue. Interestingly, only 0.14 mole equivalents of organic acid was produced which was much less than even the production in control experiments. The stimulation of growth after 24h therefore reduced the amount of alkali needed for pH control and hence the amount of organic acid produced. The gas analysis data shown in Figure 84 clearly shows the growth-stimulation effect with an almost instantaneous increase in the output of CO_2 after the addition of the bactotryptone-copper sulphate mixture.

To summarize, pIJ702 plasmid copy number was generally higher in stirred-vessel fermentations indicating an improvement in performance. A large proportion of the plasmid molecules isolated were invariably supercoiled in nature, however in some cases the electrophoresis conditions used meant that other topoisomers failed to separate from chromosomal DNA. Some total DNA extracts (particularly from older samples) revealed a band which migrated between the chromosomal and supercoiled plasmid bands which was at first thought to be the open circle form. The probable identity of this plasmid form is not known but it may be a supercoiled dimer or a single-stranded plasmid circle.

No massive increase in tyrosinase specific activity was seen upon "induction" (unlike the shake-flask results). Also a pronounced peak of activity was detected and different induction strategies merely shifted the maximum level in time. Both induction at 15h and 24h produced comparable enzyme activities however the fermentation characteristics *e.g.* organic acid production, was drastically changed. A sharp peak effect in cloned enzyme production was also reported by Erpicum *et al* (1990). No evidence for structural plasmid re-arrangements was found.

4.8 Recombinant *S. lividans* fermentation characteristics and possible improvements.

The plasmid stability of pIJ303 and pIJ702 in *S. lividans* was studied extensively in both shake-flask and stirred vessel fermentations. In general, maximum plasmid copy number was observed at early stationary phase when the culture growth rate had probably slowed down. Previous sections included discussions of the results obtained during the course of this project and this section will deal with some of the problems encountered in using these host-vector systems.

The main plasmids studied in this project showed remarkable stability with measurable copy numbers under nearly all the culture conditions examined. Plasmid instability may arise, however, when high cultivation temperatures or low DO levels are encountered. Such potential situations should be minimized by employing adequate fermenter cooling and aeration/agitation systems.

It is important to note that good levels of plasmid copy number were obtained during stationary phase since it is during this period that most secondary meabolites are produced. Therefore if these vectors were to be utilized for the purposes mentioned in Sections 1.11.1 and 1.11.2 where cloned gene expression is desired in conjunction with host secondary metabolite production, then no major problems of poor plasmid copy number should occur.

Plasmid pIJ702, despite its wide use, is not currently suitable as a cloning vector due to its *sti*$^-$ phenotype and its tendency to structurally re-arrange when used for gene carriage (refer to Section 1.10.1). One solution to this problem might be the re-introduction of the *sti* region thereby possibly improving the plasmid replication characteristics and its structural stability during cloning experiments. pIJ702 could also be improved by incorporating a strong, inducible promoter rather than utilizing the existing *mel* gene promoter.

Antibiotic selection using thiostrepton was not necessary since the plasmids were sufficiently stable. Few literature reports exist which mention the use of antibiotic selection for recombinant *S. lividans* cultures. Steiert *et al* (1989) showed there was little difference in the maximum yield of a cloned phophotriesterase enzyme from recombinant *S. lividans* cultures, with and without thiostrepton. Both cultures exhibited a

sharp peak of activity typical of many cloned enzymes carried on the pIJ702 plasmid vector. Similar peaks of cloned gene levels were also reported by Erpicum *et al* (1990).

Results from this project showed that tyrosinase production trends did not directly match plasmid copy number profiles. Relatively low levels of tyrosinase activity were often obtained when plasmid copy number was still high. Plasmid instability could therefore not explain these observations. Although protease activity was not measured during this project, Erpicum *et al* (1990) reported that protease activity did not account for their observed inactivation of a cloned β-lactamase when hosted in *S. lividans* TK24.

Inactivation of the tyrosinase enzyme when pIJ702 was hosted in *S. antibioticus* was reported by Gardner and Cadman (1990). They found that tyrosinase deactivation was detected during all phases of batch culture growth with higher specific deactivation rates observed during the stationary phase. A strategy was developed which increased tyrosinase productivity by enriching the growth medium (mid-way during the fermentation) and reducing the culture temperature to 25°C which helped alleviate the proposed problem of thermal deactivation. It was difficult however to conclude that the increased tyrosinase activity was due to improved expression rather than better growth since specific (mg^{-1} protein) enzyme activity was not measured.

During the course of this project it was found that *S. lividans* produced copious amounts of an unknown organic acid. Such production normally occurred from mid-rapid growth phase onwards. A large volume of strong alkali was needed to neutralize the acid produced during this period. The organism seemed to re-assimilate the acid later during the fermentation, probably when all the primary C-source (glycerol) had been utilized. Such observations suggest rapid initial glycolytic activity but relatively slow turnover of tricarboxylic acid cycle intermediates. As glycolysis slows down, due to the utilization of the primary C-source, the secreted acid is probably then re-assimilated and the organism remains metabolically active until the onset of true stationary phase. The culture pH was observed to rise during stationary phase since no pH control was implemented during this stage. The pH value normally increased to *circa* pH 8.5-9 towards the end of the fermentation, such an alkaline environment

could be deleterious to the activity of a cloned enzyme and therefore ideally both the copious acid production and subsequent increase in pH towards the end of the fermentation should be avoided.

Streptomycetes are known to produce a large amount of organic acid (refer to Section 4.5). Payne *et al* (1990) reported that in recombinant *S. lividans* cultures, increases in the initial C-source (glucose) level led to reduced pH. An initial glucose concentration of $52gL^{-1}$, in shaken culture, led to a minimum pH value of 5.5. The authors then attempted pH-controlled growth in a batch fermenter with slight modifications to the medium, however both cloned gene production and biomass concentration were not much improved.

Although Payne *et al* (1990) did not observe a subsequent rise in pH at late stationary phase, this phenomenon has been recorded by other workers in addition to the results of this project . Erpicum *et al* (1990) noted that recombinant *S. lividans* TK24 revealed a pronounced increase in pH when grown in Lennox broth medium, an increase from pH7.5 to pH9.2 after 100h growth in shake-flask culture was measured. The authors suggested that the rise in pH was perhaps due to amino acid catabolism, after rapidly metabolizable carbon sources were exhausted. The authors proposed that both the initial decrease and subsequent rise in pH value could be minimized by incorporation of an organic buffer (BES) in the medium. It was also found that when Bactotryptone (a medium component also used in the course of this project) was replaced by undigested proteins, a considerable improvement in both biomass concentration and cloned enzyme production was obtained. The inclusion of both glucose and undigested protein (to provide a slow progressive supply of amino acids) with an organic buffer in the medium alleviated the problems of vast pH fluctuations during non-pH controlled shake-flask fermentations. Cloned gene production (during stationary phase) was optimized by these authors in this way.

The use of organic buffers for pH control of *S. clavuligerus* was also reported by Aharonowitz and Demain (1977) who found that MOPS provided adequate buffering so that the pH remained at *circa* pH7.0. It was also found that adding potassium phosphate at 100mM provided a similar buffering capacity. At lower levels of phosphate the pH was seen to drop to pH5 after 100h incubation in shake-flasks using a chemically defined medium.

The difference between the biomass concentration results of

shake-flask experiments using MEP and phosphate-supplemented MEP medium during the course of this project may therefore be explained by the buffering capacity of the added phosphate in the latter medium. It is unfortunate that no pH readings were made of broth samples from these cultures. Considerable pH fluctuations in *Streptomyces* cultures have therefore been witnessed by other workers and several suggest the use of organic buffers or phosphates to overcome this problem. The use of such buffers in fermentations larger than laboratory-scale is expensive and impractical. Also since many fermentations are pH-controlled the underlying causes of such phenomena as organic acid production or amino acid catabolism may be overlooked although such metabolic behaviour may be deleterious to cloned gene expression. It is therefore necessary to minimize these effects and possible solutions are discussed below.

Organic acid production was observed during all of the stirred vessel fementations conducted during this project. Typically, levels of *circa* 0.5 mole equivalents of organic acid were produced which suggests some degree of metabolic imbalance during initial rapid growth. The use of buffers at this scale is both expensive and impractical. The problem could therefore be overcome by other means *e.g.* through physiological manipulations. Some workers found altering the medium constituents overcame the problem, however very often complex, slowly utilized media are used for industrial-scale *Streptomyces* fermentations therefore this option is not feasible.

During the second tyrosinase "induction" (at 24h) stirred vessel fermentation experiment of this project, only 0.14 mole equivalents of organic acid was produced. The experiment was conducted in a crude fed-batch mode after 24h growth and this factor seems to have influenced the organism's metabolism so that less organic acid was produced. The use of a true fed-batch system where medium components are added continuously after an initial batch growth mode might therefore alleviate the problem and also improve biomass concentration and cloned gene product yield. Results from this project show that plasmid copy number levels would probably remain high provided a slow feed rate was maintained in order to keep the growth rate relatively low. A slow feed rate might also help control the flux of intermediates through glycolysis without the accumulation of organic acids.

The use of fed-batch systems to overcome problems of organic acid

production. poor biomass concentration and cloned enzyme production in recombinant *S. lividans* was also suggested and investigated by Payne *et al* (1990). A six-fold increase, over a similar batch fermentation, of cloned enzyme production was obtained with continuous feeding of glucose and tryptone. Acid production was successfully controlled since only 1.2 $mmolL^{-1}$ of NaOH was needed for pH control during fed-batch operation (while 7.2 $mmolL^{-1}$ was needed the batch operation). It was also found that by slowly supplying (after 22h) both carbon (glucose) and nitrogen (tryptone) sources the production of the cloned enzyme was further enhanced. Plasmid copy number measurements were not conducted by the authors.

It is therefore evident that fed-batch operation could improve cloned gene production in recombinant *Streptomyces* and that there is scope for future work in this area. No plasmid stability data is available for *S. lividans* fed-batch fermentations, it is important to define the optimum growth rate which sustains high plasmid copy number. Fed-batch culture is already used for antibiotic production by *S. fradiae* (Gray and Vu-Trong, 1987) and it is therefore feasible to utilize cloned genes in addition to secondary metabolite production by the host when grown as a fed-batch fermentation.

In conclusion therefore, the potential use of recombinant *Streptomyces* to produce new products is large and they may, ultimately, prove better hosts than *E. coli* or *Bacillus* where a prokaryotic host organism is suitable. *Streptomyces* are also routinely grown at production-scale and no plasmid instability problems are foreseen if currently available plasmid vectors are used.

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APPENDIX 1.

Statistical analysis of biomass concentration determination by dry cell weight.

A large volume of *S. lividans* pIJ303 culture was obtained by inoculating a "seed" culture as described in Section 2.3.2 and transferring an aliquot of this culture, after 3 days' growth, into a large volume of MEP+10mMPO_4^{3-} medium. The second culture was incubated for a further 2 days and a portion of the broth was used for the statistical analysis of the dry cell weight determination method (see Section 2.3.4).

Twenty aliquots of 10mL each of culture were filtered, dried and weighed. The results were as follows;

0.077g, 0.077g, 0.075g, 0.078g, 0.080g, 0.078g, 0.076g, 0.079g, 0.079g, 0.078g, 0.079g, 0.079g, 0.077g, 0.073g, 0.074g, 0.074g, 0.072g, 0.075g, 0.072g, 0.073g.

The weights were converted to biomass concentration *i.e.* gL^{-1} and subjected to statistical analysis. The mean value was 7.61gL^{-1} and 95% confidence limits of the mean was 0.12g (calculated according to Parker, 1979, method for small samples). Therefore for this culture the mean biomass concentration was measured as 7.61gL^{-1}+/-0.12gL^{-1} or 7.61+/-1.6%.

The biomass concentration determination method was therefore found to be accurate to within <2%.

APPENDIX 2.

Statistical analysis of deoxyribose concentration determination using the Hanson and Phillips (1981) method.

A large volume of *S. lividans* pIJ303 culture was obtained by inoculating a "seed" culture as described in Section 2.3.2, an aliquot of this culture was used, after 3 days' growth, to inoculate a large volume of MEP+10mMPO_4^{3-} medium. The second culture was incubated for a further 2 days and a portion of the broth was harvested (Section 2.3.5) for statistical analysis of the deoxyribose concentration method (Section 2.3.12).

Ten aliquots of 0.2g wet weight biomass were subjected to perchloric acid digestion and the released deoxyribose was determined as follows;

10.68μgmL^{-1}, 10.08μgmL^{-1}, 9.84μgmL^{-1}, 11.88μgmL^{-1}, 10.68μgmL^{-1}, 9.84μgmL^{-1}, 9.24μgmL^{-1}, 9.84μgmL^{-1}, 12.84μgmL^{-1}, 10.44μgmL^{-1}.

The mean value was 10.54μgmL^{-1} and 95% confidence limits of the mean was 0.77μgmL^{-1} (calculated according to Parker, 1979, method for small sample). Therefore the mean deoxyribose concentration was measured as 10.54μgmL^{-1}+/-0.77μgmL^{-1} or 10.54μgmL^{-1}+/-7.3%.

The deoxyribose concentration determination method was found to be accurate to within <8%.

APPENDIX 3.

S. lividans growth inhibition by novobiocin.

A "seed" culture of *S. lividans* was grown as described in Section 2.3.2 and an aliquot was used, after 3 days' growth, to initiate second stage cultures in MEP medium. Novobiocin antibiotic was added to each culture at between 0.5μgmL^{-1} (final concentration) and 500μgmL^{-1}. All the flasks were re-incubated for 3 days and the biomass concentration of each culture was determined (see Section 2.3.4). The results are shown in graph Figure 86.

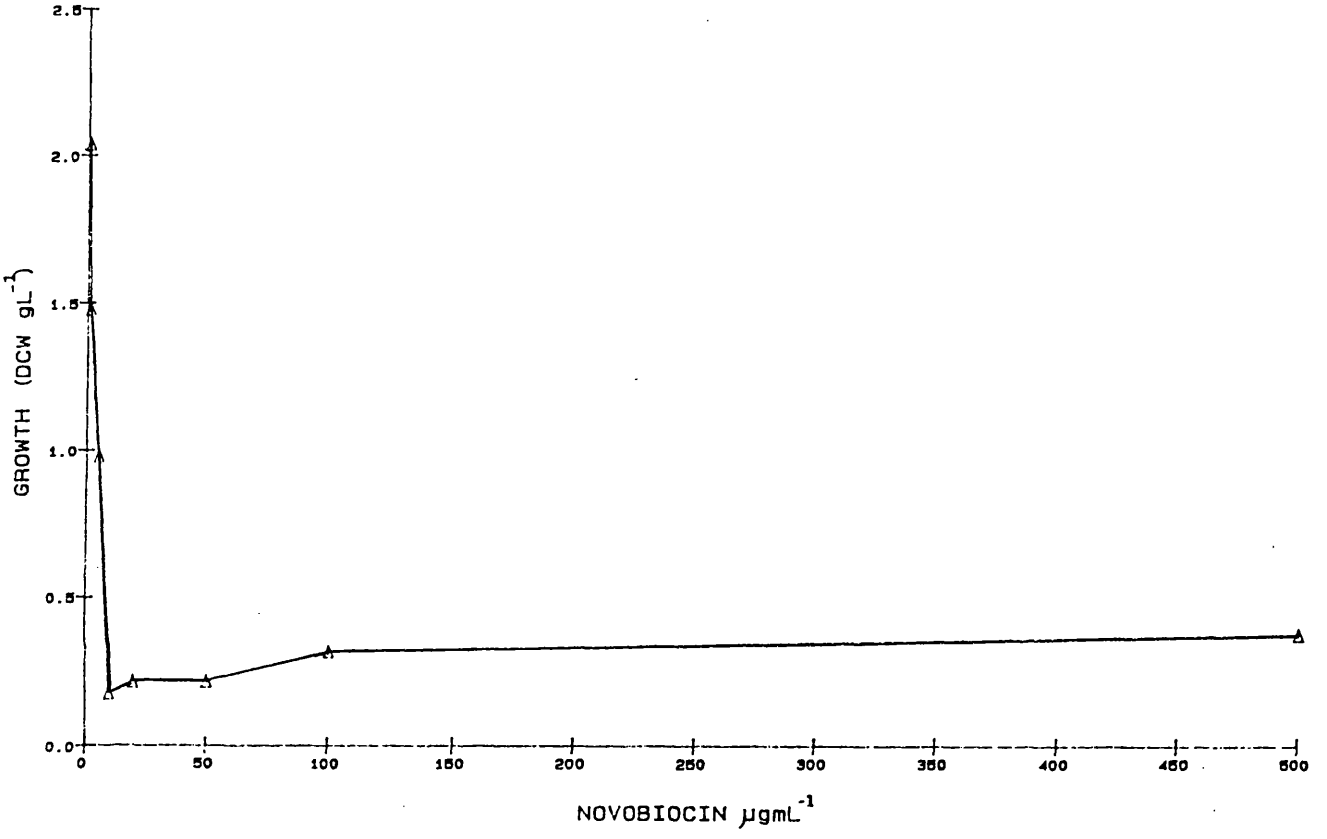

Figure 86 Inhibition of *S. lividans* TK24 growth by various concentrations of novobiocin antibiotic using MEP medium.