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Examination of plasmid stability in a recombinant organism used in continuous fermentation

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EXAMINATION OF PLASMID STABILITY IN A RECOMBINANT ORGANISM USED IN CONTINUOUS FERMENTATION .

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ABBREVIATIONS

D-CPA	D-Chloro-propionic acid
L-Broth/Agar	Luria Broth /Agar
P^-	Plasmid-free cell
P^+	Plasmid-bearing cell
S2-Broth /Agar	Seed-2 Broth/Agar
Strep.	Streptomycin

ABSTRACT

The gene for the enzyme D-CPA dehalogenase has been cloned into a multi-copy plasmid to produce the vector pTB316. It was transformed into two strains of *Pseudomonas putida* , AJ1-23 and NCIB11767 in the hope of increasing production of this enzyme. The clones will be cultured in a continuous fermenter in a media not containing the antibiotic.

In the absence of antibiotic selection the plasmid is stable in the host but is unstable in continuous fermentation. The instability was identified as segregational using restriction mapping and Southern blots. It is caused by the high levels of constitutive gene expression in the clone; as a result a population of faster growing P^- segregants rise to predominance in the fermenter. Two fermentations were operated but were abandoned due to technical problems. Calculating the degree of plasmid instability was therefore done in repeated shake flask culture. From these results it was found that the cells lose the plasmid within 90 h. Strain 316NCIB11767 is very unstable and also develops natural antibiotic reistance to strep. very readily. The addition of the substrate analogue, MCA, to a shake flask culture of 316 AJ1-23 resulted in the stabilisation of the plasmid over a period of 190 hours. MCA is converted to glycollate, a toxic product in high concentrations. Conclusions regarding this observation are tentative since little is known of the organisms physiology but it may be possible that the MCA represses gene activity thereby stabilising the plasmid. Alternatively, either a glycollate-tolerant mutant has risen in the population, or a more stable variant has arisen, irrespective of the presence of MCA in the medium

It is suggested that the plasmid is probably best stabilised by the insertion of a regulatable promoter such as the temperature promoters $P_R and P_L$, of λ and the fermentation operated as a two-stage system separating the active growth phase from the active gene expression phase as suggested by Seigel and Ryu (1986) and Sayadi *et al* (1987).

Chapter 1: INTRODUCTION

1.1 RECOMBINANT DNA TECHNOLOGY AND INDUSTRIAL FERMENTATIONS

Recombinant DNA Technology involves the transfer and propagation of nucleic acid material, normally DNA, from one organism to another unrelated organism. The important factor is the propagation of the foreign DNA and this can only be achieved if the DNA can be effectively replicated and inherited. Therefore the DNA is transferred by means of a self-replicating vector such as a plasmid or a bacteriophage such as Lambda. The success in this field over the past decade has been considerable but mainly on a laboratory scale. The impact that these techniques are likely to have on industrial fermentations are only now being realised, not due to the unsuitability of the techniques but simply because the techniques had to be developed and found effective before applying them to the industrial pilot and production scale (Zabriskie & Arcuri 1986). Industrial fermentations such as antibiotic prodution, brewing, enzymes and drug manufacture are constantly seeking new higher producing strains which will increase production, decreasing costs and improve process efficiencies, making them competitive with the equivalent chemical synthesis processes.

Insertion of foreign DNA into a bacterium such as *E.coli* will result in the production of a new foreign protein at higher yields and lower costs. This novel product market has great potential. Since the first cloning of the human insulin gene in *E.coli* more products have arrived on the market. Insulin is now produced in the yeast *Saccharomyces cerevisiae* , human growth hormone, interferon and vaccines are produced in a wide variety of bacteria and fungi.

These manipulations make a great demand on cellular processes. The cell has to replicate and stably maintain the plasmid, therefore a heavy burden is placed on the cell's metabolism, especially if the process requires the amplification of an entire metabolic pathway; as a result a cell's metabolic intermediates can be rapidly depleted during overproduction. Finally the large scale on which many of these fermentations would be required to operate at would mean that the plasmid must be stable over many generations. Plasmid instability can create many problems for fermentations at small scale as well as large scale. Plasmid stability can be defined as the ability of a transformed cell to maintain a plasmid unchanged during their growth and manifesting their phenotypic characteristics (Immanaka and Aiba 1981). Since plasmids are invariably the vectors used in these processes their frequent instability can reduce product levels, have a negative impact on specific important proteins and increase costs on a large scale fermentations since the growth media is being consumed by non-productive cells (Ensley 1986).

1.2 PLASMIDS: STRUCTURE AND FUNCTION.

Plasmids are autonomous, self-replicating elements found in procaryotes and some lower eucaryotes such as yeast. These elements are invariably composed of dsDNA and contain genes for dispensible but advantageous characteristics such as antibiotic resistance, toxin production and the F factor which allows bacteria to conjugate, thus transferring genetic information. The pseudomonads also have a range of degradative plasmids which allow them to metabolise a wide range of organic compounds. Plasmids exist in the cell as either a single copy or as a multiple copy, this copy number is determined genetically by the Plasmid. Replication of the plasmid can be

independant of the genome, i.e. relaxed or under control of the genome, stringent. Low copy number plasmids (1-5 copies/cell) are normally stringent and intermediate (20-60 copies/cell) and high copy number (> 60 copies/cell) are normally relaxed.

Self-replication is regulated by an inhibition mechanism and plasmids are capable of correcting stochastic fluctuations by ensuring that copy number and replication rate in individual cells are inversely proportional (Novick 1987). Plasmids should be inherited with reasonably high fidelity; in the case of high copy number plasmids the distribution into daughter cells is attained by stochastic distribution of the plasmids. However in the case of the low copy number plasmids, where it is possible that the plasmid free cells can arise at cell division, distribution is achieved by a partitioning sequence, *par* a 270 bp sequence which acts in a cis location to ensure the even distribution of plasmids at cell division. Its function is entirely independant of replication (Meacock and Cohen 1980).

Since plasmids are dispensible elements, they can be unstable in an environment where there is no selective advantage to their maintenance. The instability of plasmids takes two forms: segregational and structural. Segregational instability is the failure of the plasmid to segregate at cell division whereas, structural instability is the loss of plasmid integrity due to insertion, deletion or rearrangement of the plasmid genes. Plasmid stability is best studied in continuous culture because maximum growth rate, (μ_{max}), and limiting nutrient uptake (Ks), the two most important parameters in an organism's physiology in a continuous fermenter, can be manipulated, thereby affecting the growth of the organism to produce one which is fitter and more adapted to survive (Primrose *et al* 1984) Batch culture can produce ambiguous results which could be attributed to organism growth but could also be attributed to nutrient

exhaustion. The failure of a plasmid to be successfully partitioned at cell division is due to internal factors such as host strain type, physiology and genotype and external factors such as availability of nutrients and dilution rate. The genetics of the plasmid also interact with the already mentioned factors to determine its stability.

1.3 SEGREGATIONAL INSTABILITY:

This form of instability as a function of the environment and as a prerequisite for genetic manipulation has been extensively studied in chemostat culture, first of all with the natural occuring plasmids and secondly with the recombinant plasmids. The bulk of the work has been done using *E.coli* Jones *et al* 1980; Jones and Melling 1984; Godwin and Slater 1979; Wouters *et al* 1980; Caulcott *et al* 1985. However experiments have also been done using *Pseudomonas putida* ; Keshavarz *et al* 1986 and *Bacillus subtilis* ; Pinches *et al* 1987.

1.3.1. Segregational Instability due to Differences in Growth Rates .

Cells containing high copy number plasmids, either recombinant or natural, when cultured in a chemostat with no selection pressures will start to lose plasmid; as a result a population of faster growing plasmid free " segregants " will arise. Growth rate is faster due to the reduced amounts of cellular DNA; allowing the energy from cellular metabolism to be channeled into active growth (Godwin and Slater 1979), (Engberg and Nordstrom 1979). High copy number plasmids such as the ColE1 type plasmids were cultured under various forms of nutrient limitation by Jones et al (1980). The segregative fidelity of these plasmids is expected to be high and indeed for several such as pDS1109 and RP1 this was the case, no plasmid free segregants were observed after 120 generations of growth. However the plasmids pMB9 and pBR322 were lost early

from the culture no matter what the kind of limitation. Agarose gel electrophoresis revealed that there had been a drop in copy number of these plasmids brought about by nutrient limitation, this had meant that the plasmid then had segregated incorrectly allowing a population of faster growing segregants to arise. It could therefore be stated that although maximum copy number is a function of the plasmid genotype the actual copy number may be a response to the phenotypic pressure of the environment. Further experiments on pBR322 related plasmids in a chemostat using glucose, phosphate and magnesium limitations demonstrated that phosphate limitation resulted in the greatest degree of plasmid loss, due to the copy number and plasmid replication being affected by the reduction in available materials such as dNTP's (Jones and Melling 1984). Altering the dilution rate in the chemostat can also affect the stability of the plasmid. At lower dilution rates D= $0.1h^{-1}$ the plasmid-bearing cells P^+ cells are less competitive than their plasmid free P^- counterparts leading to an increase in the rise of the plasmid free segregants in the population. Apart from considering the maximum specific growth rate it is also important to consider the competition for limiting nutrient, with plasmid bearing cells having a possible lower affinity for the limiting nutrient (Wouters et al 1980). The TOL plasmid of *P. putida* contains the genes for the degradation for several organic compounds, such as toluene and xylene. It is also capable of metabolising benzoate via the *meta* -cleavage pathway, there is also an alternative chromosomally controlled *ortho* -cleavage pathway. These organisms when grown under benzoate limitation produce a high frequency of TOL^- segregants, suggesting a selective advantage for the *ortho* pathway. Batch culture revealed that there was no specific growth rate advantage between the P^+ and the P^- cells. the authors suggested as one possible reason for this could be that there was a different regulation mechanism between the two paths, affinity for the limit-

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ing nutrient could be greater with the *ortho* path giving the segregants a selective advantage.(Keshavarz et al 1985). The stability of the plasmid and its copy number while affected by the environment are also determined by the host strain physiology plasmid construction and plasmid genotype. Plasmids of pBR322 such as pAT153 were notably stable for over 100 generations in *E.coli* strain K12 under all limitations yet it was later documented as being unstable in *E.coli* strain W5445 and stable in strain HB101 (Jones and Melling 1984)

1.3.2 Segregative instability due to defective partitioning.

Low copy number plasmids rely on the *par* sequences of the plasmids to direct proper partitioning at cell division, these sequences are not as necessary in high copy numbers due to the quantity of plasmid material. However copy number can very considerably depending on host cell growth and conditions of nutrient limitation, therefore it is not possible to rely on solely high copy number to ensure partitioning of cells (Engberg and Nordstrom 1979), (Jones *et al* 1980) Likewise plasmids which have been manipulated by insertion of foreign DNA can have disrupted *par* sequences which become unstable. (Caulcott *et al* 1985).

The TOL plasmids of *P. putida* also become unstable through defective partitioning when cultured under benzoate limitation and was one explanation offered by Keshavarz *et al* (1985) to explain the lack of specific growth rate advantage between the $P^+ and P^-$ cells.

Low copy number plasmids are affected by different nutrient limitations compared to high copy number ones. Caulcott *et al* (1987) noted that the plasmid pHSG415 (low copy number) was stable under conditions of glucose, magnesium and phosphate

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limitations, unlike high copy number ones. However this plasmid was unstable under sulphate limitation. Furthermore partitioning is not a completely random process since there are considerable variations in the kinetics of plasmid loss under the variety of culture conditions used. They suggest that the effect that some plasmids exert on their host metabolism is significant or, alternatively, there may be a relationship between nutrient conditions under which the host is grown and the copy number, as was observed by Seigel and Rhu (1985) who stated that copy number was inversely proportional to the host cell growth rate.

1.4. STRUCTURAL INSTABILITY IN PLASMIDS.

Structural instability is perhaps a more insidious form of instability since the important gene may be deleted but the marker will continue to function. Frequently the recombinant gene in question is deleted from the plasmid and it is thought to occur by recombination at regions of homology between small direct repeats. (Cohen *et al* 1978) (Hahn and Dubnau 1985). Deletions are not random but are made at specifically defined locations on the plasmid; pHV15-1, a hybrid of pBR322 and pC194-1 generates deletions through a Stem-Loop Structure which forms at the regions of homology. (Alonso and Trautner 1985). The length of homology is important in determining the incidence of deletion because single base substitutions reduce the extent of homology and thereby reduce the incidence of deletion. (Albertini *et al* 1982). Furthermore deleted plasmids can be positively selected for resulting in their stable maintenance in a population. Addition of chloramphenicol to a medium containing *Streptococcous pneumoniae* with the hybrid plasmid pJS37 results in deletions due to recombination in areas of short direct repeats. One of the resulting plasmids had

a deletion at a point which sequestered the ribosome binding site of the CAT gene (chloramphenicol acetyl transferase), rendering the this enzyme production constitutive hence the chloramphenicol resistant strain rose to predominance (Ballaster et al 1986).

The TOL plasmids of *Ps putida* also demonstrate structural instability due to deletions (Williams et al 1988) The deletions, generated during benzoate limitation result in the loss of the catabolic gene function, therefore the *ortho* path is utilised resulting in the positive selection of these faster growing cells.

1.5. A MATHEMATICAL MODEL FOR PLASMID INSTABILITY.

It is possible to use mathematical models to describe and predict plasmid stability. It can be assumed that

1 . with plasmid instability the phenotypic expression is lost

2 . The host cells are growing exponentially and

3 . Cells are deprived of the plasmid with a probability of p divisions per host cell.

This factor is affected by the factors already mentioned i.e. host genetic make-up etc. The coefficient α is the ratio of growth rates of $P^{+} and P^{-}$ cells ie. $\mu^{-} : \mu^{+}$ and represents the metabolic burden on the host cells. For values of α ¿1 the relative loss of plasmid increases. The fraction of P^{+} cells, designated F, over n generations can be related to α and p by the follwing equation

$$F_n = \frac{1 - \alpha - p}{1 - \alpha - p2^{n(\alpha-p-1)}}$$

1.1.

Taking n as 25 and plotting the relationship between F_n and α with p as the parameter

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the following relationship can be seen.
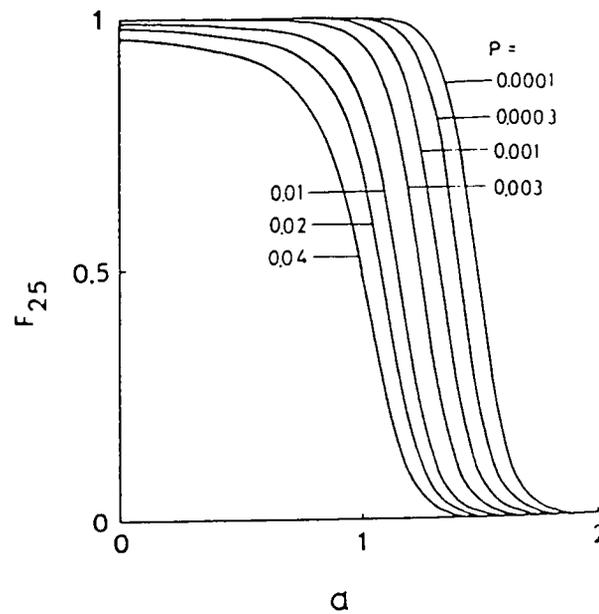
Fig.1.1 From Immanaka and Aiba (1981) Amount of P^+ cells plotted against growth rate ratio α with probability as the parameter

The number of P^+ cells rapidly deteriorates as α increases from 1-2 and is more marked at high p values (Immanaka and Aiba 1981). This model was expanded by Ollis and Chang (1982) to take into account product formation and substrate consumption. Solutions to their equations indicate the importance of the inoculum purity

$$\frac{P_0^-}{P_0^+}$$

especially at scale-up. The higher the inoculum purity i.e. $\frac{P_0^-}{P_0^+} = 0$ then the better the substrate consumption and the higher the product formation and the fewer number of plasmid free cells.

An alternative model has been recently developed by Cooper *et al* (1987) in which it is possible to calculate the form of the instability, segregational or instability due to differences in growth rate. These two parameters are denoted, R for segregational instability and is better described as the rate of conversion from which P^- cells arise from P^+ cells. $d\mu$ is the difference in growth rates $(\mu^- - \mu^+)$. Most models of plasmid instability when plotted will include a straight line section which if converted to the natural log, lnp , and plotted against number of generations will yield a more substantially straight section from which it is possible to calculate $d\mu$ and R. For values of $d\mu >> R$ it can be described as segregation due to differences in growth rate. For values of $d\mu < R$ it can be described as segregation due to faulty partitioning. This system is extremely sensitive and using a linear regression model can give values of $d\mu$ and R within 95% confidence limits.

1.6. METHODS OF INCREASING PLASMID STABILITY .

The causes of plasmid instability can be a complex interaction of factors invariably with one outcome, the rise of a plasmid-free segregant population. To prevent instability arising two approaches can be used:

1.Selective Controls   .

2. Non-selective controls.

1.6.1. Selective Controls .

Plasmids will remain stable as long as they are under conditions where they possess the selective advantage. Antibiotic resistance is a widely used selective marker in

shake flask and small scale fermentations and provided the organism does not develop antibiotic resistance at the genome level, selection should be strong. However the expense of supplying large quantities of antibiotic in a fermenter would be very high and the environmental implication of releasing such resistant organisms would also have to be considered. Furthermore residual antibiotic in the product might make it unacceptable and there would be possible allergen risks (Meyer 1986).

An alternative selective agent would be the use of an auxotrophic marker, i.e. the insertion of a gene into the plasmid which would complement a mutation of an essential gene on the host chromosome. This was the case in I.C.I.'s production of Single Cell Protein using *Methylophilus methylotrophus* . This organism normally utilises ammonia via the ATP-dependant glutamine synthetase pathway. A glutamate auxotroph was transformed with a plasmid containing the glutamate dehydrogenase gene from *E.coli* which is utilised in the ATP-independant glutamine synthase pathway, as a result the carbon conversion of the process increased 4-7%, which on a large scale is very significant. The plasmid was maintained because of the ATP saving advantage conferred on the recombinant organism, the antibiotic resistance markers however were rapidly lost (Powell and Byrom 1983).

1.6.2. Non-selective methods .

1.6.2.i. Addition of a *par* Sequence .

The function of the *par* element has already been outlined. Low copy number plasmids require it for effective partitioning however high copy number plasmids have theoretically no requirement for such a function. The insertion of this 270bp fragment into an unstable plasmid will increase the stability of the plasmid substantially.

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A *par* sequence in pBR322 carrying the trp operon resulted in a 3-10 fold increase in instability for the plasmid. Therefore a high copy number plasmid can benefit from these elements. (Skogman *et al* 1983). The effect in a low copy number plasmid is more marked ; an insertion into an R1 plasmid by Larsen *et al* (1984) gave a 100 fold increase in stability when the plasmid was kept at the low copy number temperature of 30 C.

1.6.2.ii. Control of Gene Expression

Recombinant DNA inserted into a multi-copy plasmid will produce an increased amount of product. (Ulin and Nordstrom 1979). Therefore to increase the production of a particular process a high copy number plasmid should be used. This however is not always the case; it can be due to the rate limiting step in the process being at transcriptional or translational level where the build up of gene product may affect the stability of the plasmid, or alternatively it could be that high copy number plasmids are unstable due to the metabolic burden placed on the cells. Tryptophan biosynthesis is under strictly regulated control, however attempts to amplify expression in*E.coli* by transferring a plasmid bearing the enzymes to a mutant lacking the repressor resulted in a 90% loss of plasmid under certain conditions (Herschfield *et al* 1974). A similar result was found by Rood *et al* (1980) using the tyrosine operon in a mutant lacking a *tyr* repressor. This apparently defeats the purpose of the whole recombinant DNA exercise, but a possible solution is to place a regulatory control mechanism on the process. This is done by using inducible promoters such as the *lac* or the *trp* promoters which are regulated by media manipulations or the P_L and P_R promoter of λ which are regulated by temperature (Ulin *et al* 1979). Active growth

can be separated from the production phase and that way there is unlikely to be any growth rate advantage offered to the cells. Seigel and Rhu (1985) found that derepression of the P_L promoter marked the starting point for the onset of segregational instability in their production of Tryptophan Synthetase. A two stage continuous fermentation was then used, the organism was grown to a suitable cell density at 30 C, at which point the promoter is repressed, the temperature was then raised to 42 C and the promoter was then derepressed and production went ahead. Similarly in the production of Met-prochymosin, Caulcott et al (1985) found the use of the trp promoter to be ineffective due to " leakiness " however, production improved markedly when the P_L promoter was used. The use of these promoters does not eradicate the instability. In fact with the two-stage fermentation derepression at the higher temperature did decrease the stability, however the activity in the fermenter was just as high and ran twice as long.

More recently this system has been improved by immobilisation of the cells in karageen. Recombinant cells are more stable when immobilised (Nasri et al 1987). Sayadi et al (1987) has taken the two stage fermentation system a step further by immobilising the cells at stage 1, the most unstable part of the process. The cells are still under a temperature control and are repressed at 30 C in the first stage. The dilution rate is very high; $3.2h^{-1}$ and the released cells are washed out of the fermenter and are therefore unable to divide, thereby preventing the rise of segregants in the population. Cells are released into the second stage which operates at 42 C and the system becomes derepressed and production commences. Sayadi used the xylE gene of Ps putida in E.coli and found that plasmid stability was 100% in stage 1 after 400 generations. Likewise stability at stage 2 was very high, 93% with activity 5 times

higher than at stage 1.

1.6.2.iii. Screening for Stable Variants .

This final alternative in non-selective methods is an adaptation of the technique of strain selection. A recombinant organism growing in culture under non selective conditions will lose the plasmid, however it has been observed that after several hundred hours, activity can increase due to the rise of a new more stable variant which will last longer in culture than the parent organism. *Pseudomonas putida* carries the genes from napthalene dioxygenase which when transformed into *E.coli* were used to make the textile dye, indigo. One of the advantages of this system from a genetic point of view is that stable colonies are blue, therefore screening for stable variants is easy. Colonies isolated and screened showed a marked increase in stability compared to the original culture even under adverse conditions of high expression. Certainly this method is more tedious than the others, especially if there is no selectable phenotype, however, it is nonetheless worth considering (Ensley et al 1983).

1.7 Dehalogenation of a chloro-organic acid using a recombinant organism

. Chloro-propionic acid (CPA) is a major precursor in the production of phenoxy propionic acid herbicides. This CPA is synthesised as a racemic mixture of D and L forms. However the active herbicide is synthesised only from the L-form, the D-form is discarded. Therefore conversion of the D-form to another compound with an alternative use would improve the economic and the environmental aspects of the project. The conversion of D-CPA can be done biologically using the bacterium *Pseu-*

domonas putida , one particular strain, AJ1, is capable of converting CPA to Lactate using dehalogenase enzymes. This wild-type organism naturally has dehalogenases to convert both forms, therefore a mutant was required which posessed only the D-dehalogenase for conversion of D-CPA only. The mutant strain AJ1-23 possesses only a D-dehalogenase giving the reaction:

$$D - CPA + H_2O \longrightarrow L - Lactate + HCl$$

The lactate and L-CPA can then be separated by chemical methods leaving the pure stereo-isomer, while the lactate can be used in other processes. However, the activity of the D-dehalogenase in the fermenter is extremely low and mutant reversion is possible, therefore to improve yields and to prevent reversion it was proposed to clone the dehalogenase gene, HAD-D, into a broad host range vector such as the pTB plasmids in the hope that the intermediate/high copy number plasmids would give increased gene expression. 3 vectors were constructed

1 pTB 316: a mid copy number plasmid (30 copies/cell).

2 pTB 322: A temperature sensitive plasmid which increases copy number when the temperature rises from 30-37 C.

3 pTB 509: a high copy number plasmid (> 60 copies/cell).

1.8 Construction of the pTB plasmids .

Plasmids pTB316 and pTB322 are 11.5kb in size and contain the genes for streptomycin (Strep) and carbemicillin. A restriction map of the plasmid can be seen at Appendix 1.

1.8.1 Cloning of the D-CPA dehalogenase gene into AJ1-23 .

A gene library was prepared from the wild-type AJ1. It was partially restricted with a Xho-I/Sal-I double digest and another HindIII digest and phage packaged into *E.coli* The probe was derived from the sequenced D-dehalogenase enzyme and 2 small 20-mer and 54-mer probes were used to screen for the gene. This was done using, first of all hybridisation of the AJ1 library to nitrocellulose filters and probing with the HAD-D gene. Secondly, positive clones were screened using Seed2/D-CPA agar plates (a minimal media with D-CPA acting as the carbon source.) Therefore only D-CPA positive clones will grow. Thirdly, positive clones were selected using plates containing strep and checking for resistant strains. From the screening strategies 8 clones were identified which fulfilled all the requirements already mentioned. D and L activities were both expressed in *E.coli* and *P. putida* and it was deduced that the D and L gene lay together on the chrormosome and operated from the same promoter. To produce a clone which was D^+L^- would require that inactivation of the L-gene. (see the restriction map of the D/L genes (Appendix 1) To inactivate the L gene a sub-cloning procedure was used, whereby the BamHI fragment of the HAD-D/L genes were deleted and subjected to partial digest with Sau-III. Fragments were religated to the remaining EcoRI/BamHI fragment until a suitable D^+L^- clone was established. The finished gene fragment contains all the HAD-D gene and a small inactive fragment of the HAD-L gene. This gene was then ligated to the pTB plasmid at the HindIII site. Plasmids were transformed into AJ1-23 and another *P. putida* strain NCIB11767 which still has D and L dehalogenase activity. Clones are designated 316 AJ1-23 and 316 NCIB11767.

Plasmids were transformed, initially into *E.coli* . However results were poor due to problems at the promoter level. In *P.putida* the results were more promising with

a significant increase in the activity of the dehalogenase. The plasmid was stable in the bacterial host but it was exteremely unstable in continuous culture. Samples taken from the fermenter revealed two colony sizes, small and large, when plated onto Nutrient Agar. Small colonies apparently retained the plasmids whereas, only a small proportion of the large colonies retained it. This apparently suggests a growth rate advantage on the cells lacking the plasmid; in fact large colonies were the dominant form after prolonged continuous culture (Immanaka et al 1980). This suggests there is a selective disadvantage in the high expression of the HAD-D gene (which is constitutive in the clones) due to a metabolic burden on the host, however there is also the possibility, suggested by sequence data, that the protein, a 46% hydrophobic protein without a leader sequence, may become located in the inner membrane, causing it to become "clogged". Plasmid pTB509 was found to be extremely unstable due to the high levels of expression it would induce. Further development on it was abandoned.

1.9. AIM OF THE PROJECT

The plasmid was unstable in continuous culture. Therefore what had first of all to be established was the actual form of the instability; segregational or structural. It was then proposed to run the two clones in a continuous fermentor and monitor the rate of plasmid loss over a period of several hundred generations. The ratio of small to large colonies could be used as a possible means of identifying plasmid bearing clones as well as using antibiotic selection and restriction mapping. The actual cause of the instability was unknown but this would hopefully be revealed from the fermenter results.

Two clones were used in order to ascertain which would be the more suitable and

more productive in the large scale culture.

Chapter 2: MATERIALS AND METHODS

2.1 BACTERIAL STRAINS

Mutant strains of *Pseudomonas putida* lacking the HAD-L gene were used. AJ1-23, NCIB 11767

2.2 PLASMIDS USED

pTB 244: A low copy number plasmid constructed without the HAD-D gene which is very stable and can act as a marker/control; pTB316: An intermediate copy number plasmid with the HAD-D gene inserted at the multiple cloning site; pTB322: Copy number is under temperature control. It has a low copy number at 30 C which can be increased when the temperature is raised to 37 C. See appendix 1 for restriction map.

2.3 RESTRICTION ENZYMES

The restriction enzymes were supplied by Boerhinger Mannheim along with the with the appropriate restriction buffers. The enzymes used were HindIII, BamH1 , Eco RV and Pst1

2.4 BACTERIOLOGICAL MEDIA

2.4.1. L-broth

Bacteriological tryptone	20 g
Yeast extract	10 g
Sodium chloride	10 g

The above materials are dissolved and made up to one liter with water and the pH adjusted to pH 7.5. L-agar was made by adding 6g of agar per 400 ml of media. L-Strep was made by adding streptomycin from a stock solution of 10 mg/ml to give a final concentration of 100 μg /ml

2.4.2. S2 BROTH

Phosphate buffer	20 ml
Ammonium sulphate	5 ml
Magnesium sulphate	0.5 ml
Ferric chloride	0.1 ml
trace elements.	1 ml

Make up to 1 litre with distilled water

2.4.3. FERMENTATION MEDIUM

Culture media was designed to support 30g/l of dry cell material

CONSTITUENT	g/l TAP WATER
Glucose	82
H_3PO_4	3.3
$FeSO_4$	0.1
$MgSO_4$	1.9
K_2SO_4	1.2
$CuSO_4$	0.3 mg/l Cu
$ZnSO_4$	1.3 mg/l Zn
$MnSO_4$	1.45 mg/l Mn
$CaCl_2$	42 mg/l Ca

Temperature was kept at 28 C, pH was 6.8 in an automated titration with NH_4OH

Agitation was at 1,000 rpm with aeration at 1,000ml/min air per litre of culture. The antifoam used was polypropylene glycol

2.5. SOLUTIONS AND BUFFERS

2.5.1. STET Buffer per 100ml

Sucrose 8% w/v	8g
tris-HCl pH 8 50mM	5 ml of 1M
Triton-X-100 5% v/v	5 ml
EDTA 50mM	10 ml of 0.5M

2.5.2. TES Buffer 10X Stock for 1 Litre

TRIS 0.5M	60.75 g
EDTA 0.05M	18.6 g
NaCl 0.5M	29.22 g

2.5.3. Gel Electrophoresis Buffer :

Tris Borate 10X Stock.

TRIS	216 g
Boric acid	110 g
EDTA	18.6 g

The above materials are dissolved in water and made up to 2 liters the pH should lie between 8.3 and 8.7. It should not be adjusted as the addition of chloride ions can affect the electrophoresis.

2.5.5. Denaturation Buffer

Final Concentrations	2.5 Litres
1.5M NaCl	219.25 g
0.5M NaOH	50 g

2.5.6. Neutralisation Buffer

Final concentrations	2.5 Litres
3M NaCl	438.25 g
0.5M TRIS	151.5 g

pH to 7 with HCl

2.5.7. SSC Solution

Final concentrations	2.5 Litres
3M NaCl	438.25 g
0.3M Na Citrate	220.5 g

2.5.8. Prehybridisation Solution

6x SSC	33 ml
SDS 0.5%	0.5 g
5x Denhardts Solution	0.1 g of each
Denatured salmon sperm DNA 100ug/ml	10 ml
Denhardts solution	500 ml of 50x strength
Ficoll 400	5 g
Bovine serum albumin (BSA)	5 g
Poly vinyl pyrrolidine	5 g

Salmon Sperm DNA was made from a stock of 0.1 g/100ml, dissolved over 2 hours

and microwaved for 5 min to denature.

2.6. AGAROSE GELS

Gels were made at a concentration of 0.7% w/v using the tris borate buffer diluted accordingly. The gel electrophoresis tanks used was the GNA-200 Pharmacia for the large slab gels.

2.7 PLASMID DNA EXTRACTION .

Plasmid DNA was extracted from the cells using the rapid boiling method of Holmes and Quigley (1981), with some variations. A large colony was resuspended in $150\mu l$ of STET buffer, $10\mu l$ of freshly made lysosyme(10mg/ml) was added and incubated at 37 C for 5 min. The lysosyme weakens the cell wall and allows for easier lysis. The samples were then transferred to a boiling water bath for 90 sec: the high temperature causes the cells to lyse and this was seen as a sticky precipitate at the bottom of the tube. In order to remove the cell debris the samples were centrifuged at 13,000 rpm for 10min , the cell debris,including the genomic DNA was pelleted at the bottom of the tube and the plasmid DNA remained in the supernatant. The supernatant was carefully decanted off into a fresh tube containing $100\mu l$ of iso-propanol and left on ice for 10 min. The iso-propanol precipitates the plasmid DNA. Centrifugation was repeated to pellet the plasmid DNA and the supernatant discarded . The plasmid DNA was resuspended in 70% ethanol, centrifuged again and then vaccuum dried for 15 min. The dried DNA could be stored indefinately at this stage, however for immediate restriction analysis it was resuspended in $20\mu l$ of sterile distilled water and appropriate restriction buffer and enzyme were added.

2.8. PREPARTION OF PROBE AND NICK TRANSLATION

2.8.1. Fragment Isolation

Restricted plasmid DNA samples were run on a 0.7% agarose gel, the desired band fragment was then cut from the gel and placed in a $500\mu l$ eppendorf tube along with some glass wool. A small hole was pierced in the bottom of the tube and placed in the larger 1.5ml eppendorf. The tubes were centrifuged at 13,000 rpm for 10min, extract from the gel fragment,including the DNA, passed into the larger tube. This extract was then added to $500\mu l$ of chloroform which removes unnecessary protein and impurities from the sample. The top aqueous layer containing the DNA was removed to a clean eppendorf to which was added $5\mu l$ of BSA, 1/10 volume of 3M sodium acetate/0.1M magnesium acetate pH=5.2 and 2 volumes of ethanol. This was left at -80 C for at least 30 min, again the ethanol at low temperatures precipitates the DNA which was subsequently pelleted by centrifuging at 13,000 rpm. The supernatent was discarded and the DNA washed in 70% ethanol, centrifuged and vacuum dried for 15 min.

2.8.2. Nick Translation

The dried pellet was resuspended in $50\mu l$ of water and boiled to denature the DNA. Then the materials for the nick translation were added, these include $2\mu l$ of the Klenow enzyme from DNA Polymerase-1 in $8~\mu l$ of Klenow buffer, $5~\mu l$ of a random hexamer primer, $2~\mu l$ each of dGTP, dATP and dTTP. $8~\mu l$of BSA and $1~\mu l$ of ^{32}P-dCTP. This mixture is incubated at room temperature for two hours, in that time the klenow enzyme in the presence of the primer, joins the free deoxy-nucleotides to the denatured strands of the plasmid DNA, inserting the labelled dCTP at the

appropriate point. After two hours the sample is boiled to once again denature the DNA.

2.8.3. Southern Blot

DNA bands can be transferred onto a nitrocellulose filter in a technique known as the Southern blot (Southern 1975). Samples were run overnight on a 0.7% agarose gel and then the gel was soaked in 15 ml/2L HCl for 10min to depurinate the DNA. The gel is then transferred to the alkaline denaturation buffer for 45 min, to denatures the DNA on the gel. It is subsequently immersed in a neutralisation buffer for another 45 min. A long strip of 3 mm filter paper is cut the width of the gel and placed on a glass plate , the ends dipping into a tray of 10x SSC solution, this paper will act as a wick. The gel is placed on top and the nitrocellulose filter, cut to fit the gel, is placed on top of the gel. Another 3 pieces of 3 mm filter paper the size of the gel and soaked in the 10xSSC solution were placed on top of the nitrocellulose filter, more absorbant paper on top of that and finally a heavy lead weight which compresses the layers and ensures that there is a constant flow of liquid between the filter papers sandwiching the gel and nitrocellulose. This is left standing overnight and the DNA bands are absorbed onto the nitrocellulose filter. The filter is then removed from the gel which can now be discarded, the filter is allowed to dry on 3 mm filter paper at room temperature for 5-10 min and then sealed in an envelope of 3 mm filter paper and dried for 2 h. in a vacuum oven at 80 C.

The nitrocellulose filter was then hybridised to the nick translated probe. Prior to the hybridisation the filter was sealed in a plastic bag containing 50 ml of the prehybriisa-tion buffer and incubated at 65 C for 2 h. The probe was then added to the the filter, the bag resealed and incubated overnight at 65 C. The hybridisation fluid was emp-

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tied from the bag and replaced with 200 ml of washing solution (0.1%SSC/0.5%SDS) and again incubated at 65 C for 2hours. After this the filter is checked with a geiger counter, high counts should register only in the areas where the probe has hybridised all other areas should show a minimal count. The filter was left to dry on 3 mm filter paper then sellotaped to the filter paper, marked with radioactive ink, placed in a plastic bag and put in a fim case. In the dark room the film was placed on top of the filter and resealed. The film is developed over a period of 2/3 days at -80 C.

Chapter 3: RESULTS

The clones used in the experiments were the 316 AJ1-23 clone, a mutant lacking HAD-L activity and carrying the HAD-D gene on the plasmid pTB316; the 316 NCIB11767 strain which possesses both HAD-D and HAD-L genes on the chromosome as well as having the HAD-D gene inserted on the plasmid. A qualitative and quantitative analysis of plasmid stability was carried out based on results from plates/flask culture, 2 fermenter runs of 316NCIB11767 and 316 AJI-23 and repeated shake flask culture of these two clones.

3.1. IDENTIFICATION OF PLASMID INSTABILITY

The actual form of the instability of the pTB316 plasmid was identified using restriction mapping. This technique involves restricting isolated plasmid DNA fragments and electrophoresing them on an agarose gel in a tris-borate buffer. The plasmid DNA separates out in the direction of the anode with the small fragments travelling the furthest. The bands are stained with ethidium bromide and visualised and photographed under UV light. Fragment size can be calculated using a standard band separation, in this case λ restricted with Pst-1, and the plasmid restriction map. (See Appendix 1). Restriction mapping was carried out on an inoculum from one of the fermentations with 316 NCIB11767. The inoculum was plated out on L-agar and small colonies isolated, a number of these colonies were sub-cultured again in S2 broth with 10 mM D-CPA as the carbon source. 48 hours later samples were replated onto Luria. This time a mixture of small and large colonies grew and once more a number of these small colonies were sub-cultured in the Seed 2 D-CPA media. These small colonies should contain the plasmid and by this repeated sub-culturing it is hoped to isolate a high expressing organism which may be also highly stable. Activity tests for the dehalogenase activity were measured on a chlorometer which detects chloride release fom the D-CPA. Of seven small colonies sub-cultured two had a high activity,

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these were designated F and G and they were plated out on L-Agar and incubated at 30 C and 37 C for 24 h. These plates were used to test for the presence of the plasmid. A number of colonies were picked off with a cocktail stick and streaked onto a L-Strep Agar plate which had been divided into squares (1 colony/square). These were incubated for 24 h. at 30 C and then assessed for growth. Two different growth patterns emerged, one was a thick mucoid growth achieved after 24 hr the second was a sparse dotted growth achieved after 24 hr or longer. (see Fig 3.1.) Some colonies did not grow at all.

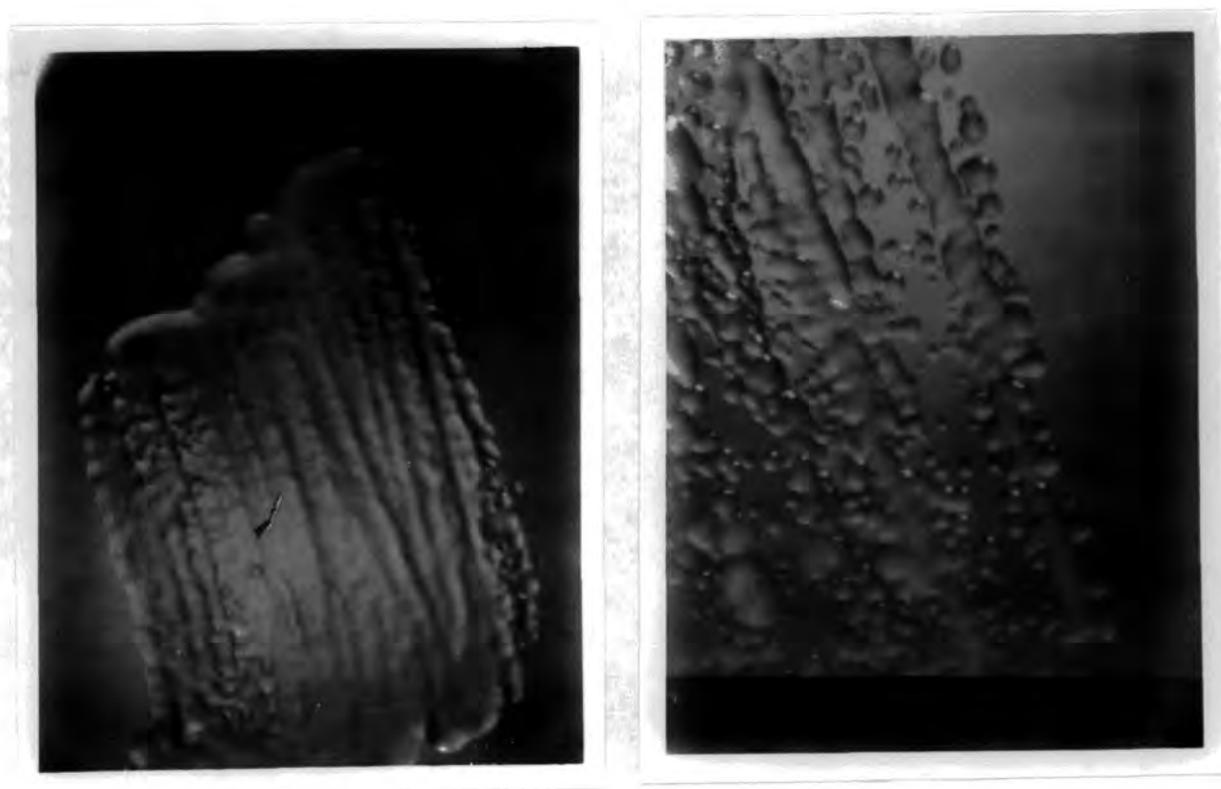Fig.3.1. Growth patterns of 316NCIB11767 on L-Strep. To test for plasmid presence samples were taken from the L-Strep plates, or in the case of the non growers, from the L-agar. DNA was extracted. restricted with different restriction enzymes and run on a 0.7% agarose mini-gel in tris-borate buffer containing ethidium bromide. The gel was photograped under U.V. light using a polaroid 545 land film type 52.

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3.1.1. Culture F

15 colonies were isolated onto the strep. media from the plate incubated at 30C After 24 h. on L-strep 3 colonies had grown well, 8 grew sparsely and 4 didn't grow. A further 24 h at 30 C saw one non-grower produce sparse growth. 20 colonies were isolated from the plate incubated at 37 C. 10 had been small in size 10 were larger. After 24 h. 1 had grown well, 4 sparsely and 15 not at all. 24 hr later 2 sparse growers had improved and 2 non growers were growing sparsely. Only one of the growing colonies was small in origin.

3.1.2. Culture G

Of the 16 colonies isolated from the 30 C plate , 10 were small 6 were large. 24 h. on L-strep showed 6 good growers, 5 sparse growers and 5 non growers. 24 h. later a futher 3 had improved to give thick growth,and only 2 had failed to grow. At 37 C Culture G showed a predominance of small colonies, 14 were isolated along with 6 large. 24 hr later 6 had grown well, 4 sparsely and 10 not at all. Further incubation showed little improvement apart from 3 non-growers growing sparsely. All the large colonies gave poor growth patterns.

3.1.3. Restricion analyses of Cultures F and G

Out of these 70 isolates 20 were used for restriction analyses. Results showed that only the colonies which gave the thick growth after 24 hr had any plasmid present, the other colonies had no plasmid present at all. (see figures 3.2) It would appear that the plasmid is lost from the cell and is therefore segregationally unstable.
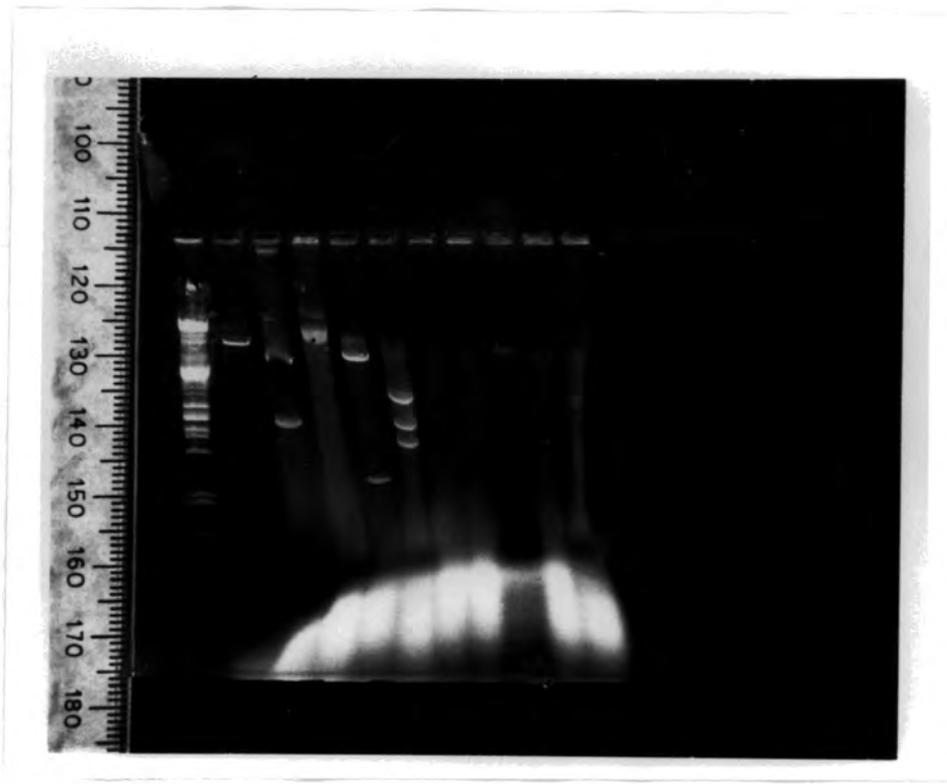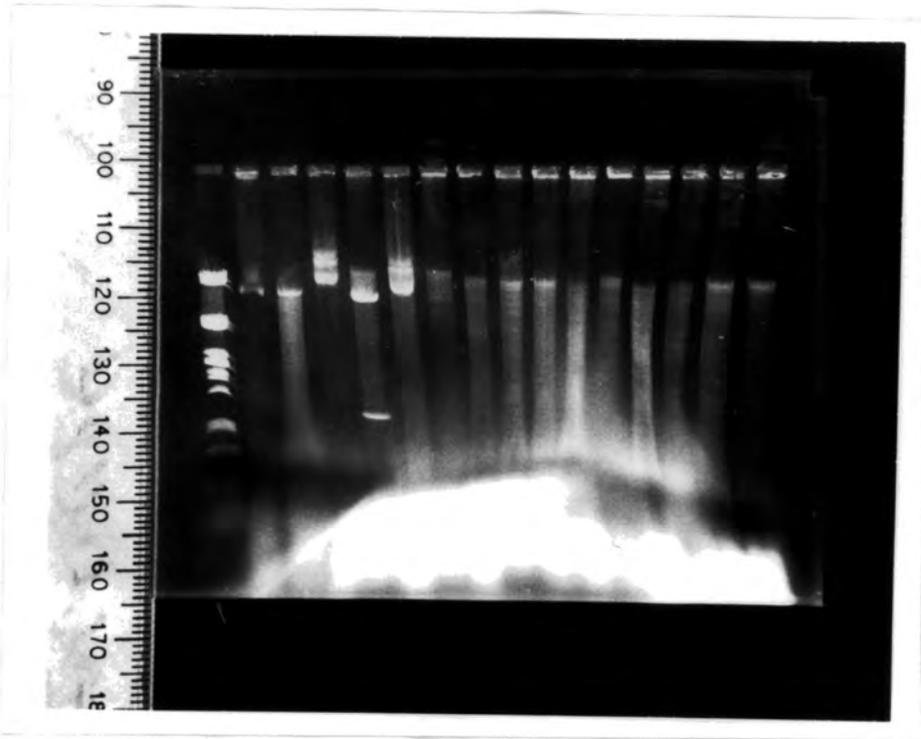
Figure 3.2 shows restriction digests of culture F (Top) and G (Bottom). Samples

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have been restricted with Hind III and Bam HI. Standard is λ restricted with PstI. The empty lanes at the end have no plasmid present.

There was also no apparent correlation between small and large colonies because it was hard to actually distinguish a real colony size difference on the Luria media and it should be noted that the original observations were made on nutrient agar. Luria agar may affect colony growth rate in a different way to the nutrient agar.

Although derived from the same strain 316NCIB11767 the G culture showed a higher degree of stability compared to the F culture both at 30 and 37 C. the sparse growth pattern on L-strep may be due to the subsequent development of natural antibiotice resistance in the P^- strains whereby these colonies grow at a slower rate than the P^+ colonies.

The plasmid is lost from the cell therefore the instability is segregational. What then is the degree of instability in the two clones? To answer this question the clones should be culture continously in a fermenter. This is because under continuous culture conditions it is possible to get an accurate estimation of the generation time and the instability is unlikely to be affected by nutrient fluctuations which can affect plasmid stability in their own right.

3.2. FERMENTATIONS F5 AND F15

Two 5 litre fermenters were inoculated with the clones 316 AJI-23 and 316 NCIB11767 and were designated F5 and F15 respectively. Batch growth lasted 18 hours and the depletion of glucose signalled the change to continuous culture under the following conditions.

1 dilution rate $D = 0.1h^{-1}$

2 inlet glucose at 80-85 g/l to give a dry cell mass of 30g/l

Samples were removed daily for microbiological evaluation, activity, dry cell weight

and supernatant mineral analysis. Glucose was the limiting substrate and remained at 0.1 g/l throghout continuous culture.

3.2.1. Fermentation F5: Clone 316AJI-23

This fermentation ran for approximately 300 hours and the activity over this period was 9m mol/h/g throughout, a lot lower than expected. The culture suffered a pH shock at the end of batch growth and temperature fluctuations throughout the first 24 hours of continuous culture. (See table 3.1)

SAMPLE NO	HOURS AFTER INOCULATION	CELL MASS gl^{-1}	ACTIVITY $mmolh^{-1}g^{-1}$
4	22.00	12.09	5.0
5	27.00	17.56	6.7
6	44.25	32.0	9.6
7	68.25	27.21	10.4
8	92.33	28.51	9.7
9	164.33	27.12	9.2
10	188.33	-	9.2
11	212.33	27.48	8.8
12	308.33	31.52	8.5

Table 3.1: Dehalogenase activity of clone 316 AJ1-23 over 308 h. with respect to cell mass .

The above table gives an indication of the activities of the clone in the fermenter.The calculated cell doubling time, or number of generations, Td, for this fermentation is

taken from the equation:

$$Td = \frac{\ln 2}{\mu_{max}}$$

The Td for this fermentation is taken as 45 with $\mu_{max} = D$

Samples 5,6 and 9 were given to be tested for plasmid stability. The samples were diluted down and plated onto L-Agar and incubated at 28 C for 24 hrs. A mixture of small and large colonies grew; a number of small colonies were noted and picked off with a cocktail stick and streaked onto a L-strep grid. The procedure was repeated with the large colonies. Plates were incubated for 24-48 h. and these colonies were then used in restriction analyses. At the same time the diluted samples also plated onto L-strep plates and the count compared to quantify the percentage plasmid lost.

In spite of the low activity, viable counts showed practically 100% stability throughout the sampled fermentation. 20 colonies taken from sample 6 had three non-growing colonies. Restriction analyses on these 20 colonies confirmed plasmid presence in the 17 growers and nothing in the 3 non-growers.

3.2.2 Fermentation F15 with 316 NCIB11767

This fermentation ran the same length of time as F5, and the activity was much higher, at 22-23 mmol/h/g. However after 160 hours on continuous culture the iron feed pipe blocked overnight resulting in iron limitation, Activity after that point became unstable falling to < 5 within 3 days. Samples were taken about the same time as F5 and the same procedures used.

SAMPLE NO	HOURS AFTER INOCULATION	CELL MASS gl^{-1}	ACTIVITY $mmolh^{-1}g^{-1}$
3	19	9.74	21.2
4	37	27.12	20.0
5	61	27.95	23.2
6	85	26.69	22.1
7	157	27.04	23.2
8	181	27.16	27.1
9	205	27.72	17.6
10	301	28.63	< 5
11	325	27.83	< 5

Table 3.2. Dehalogenase activity of 316 NCIB11767 over 325 h. with respect to cell mass.

Samples 4,5 and 7 were given for restriction analyses; here the culture started to lose plasmid at an early stage. The first viable counts from sample 4 showed a 14% loss of plasmid after 18 hours on continuous culture. This had increased to a 30% loss just prior to the blockage (no further samples were analysed). The restriction analysis of 20 colonies taken after 43 hours (sample 5), on continuous culture showed 2 to be lacking the plasmid and a later sample (7) taken for restriction at 157 hours showed 11 colonies out of 50 to be lacking the plasmid, a 22% loss of plasmid. The calculated number of generations of this culture is approximately 45. No more samples were submitted for testing after F15 went into iron limitation at 160 hours.

One more attempt was made to assemble the fermenter with the previously mentioned sub-cultured inoculum of 316 NCIB11767. One small colony was taken from culture G and inoculated into 50 ml of culture media and from there inoculated into a 5 litre

fermenter. The fermentation ran over the weekend before suffering a severe drop in pH. The culture never recovered and activity crashed to zero. One sample was taken and plated onto L-agar and L-strep. Counts revealed a 90% loss of plasmid. The colonies on L-agar showed no plasmid presence whereas the colonies on L-strep did. These plates were used as samples for the southern blot.

3.3. SOUTHERN BLOT OF CLONE 316 NCIB11767

A known stable sample from the previous aborted fermentation was restricted with HindIII and run on a 0.7% agarose gel. The 9.8 and 1.7 kb fragments were cut from the gel and the DNA eluted. This DNA was then denatured and nick translated to give a probe which would hybridise to the pTB316 plasmid. The test samples were composed of 5 known stable, 5 suspected stable samples and 10 known unstable samples. The known samples came from the aborted fermentation the suspected samples came from an F15 plate. Samples were restricted with HindIII and run on a 0.7% agarose gel. The first 10 lanes contained the stable samples and showed plasmid, the remaining 10 lanes with the unstable sample were apparently empty. The gel was blotted onto a nitrocellulose filter and probed with the radiolabelled probe. The film was developed over 2-3 days at -80 C. See figure (3.3). The blot shows the first 10 lanes as having the plasmid the remaining lanes are empty. (Fig. 3.3.)
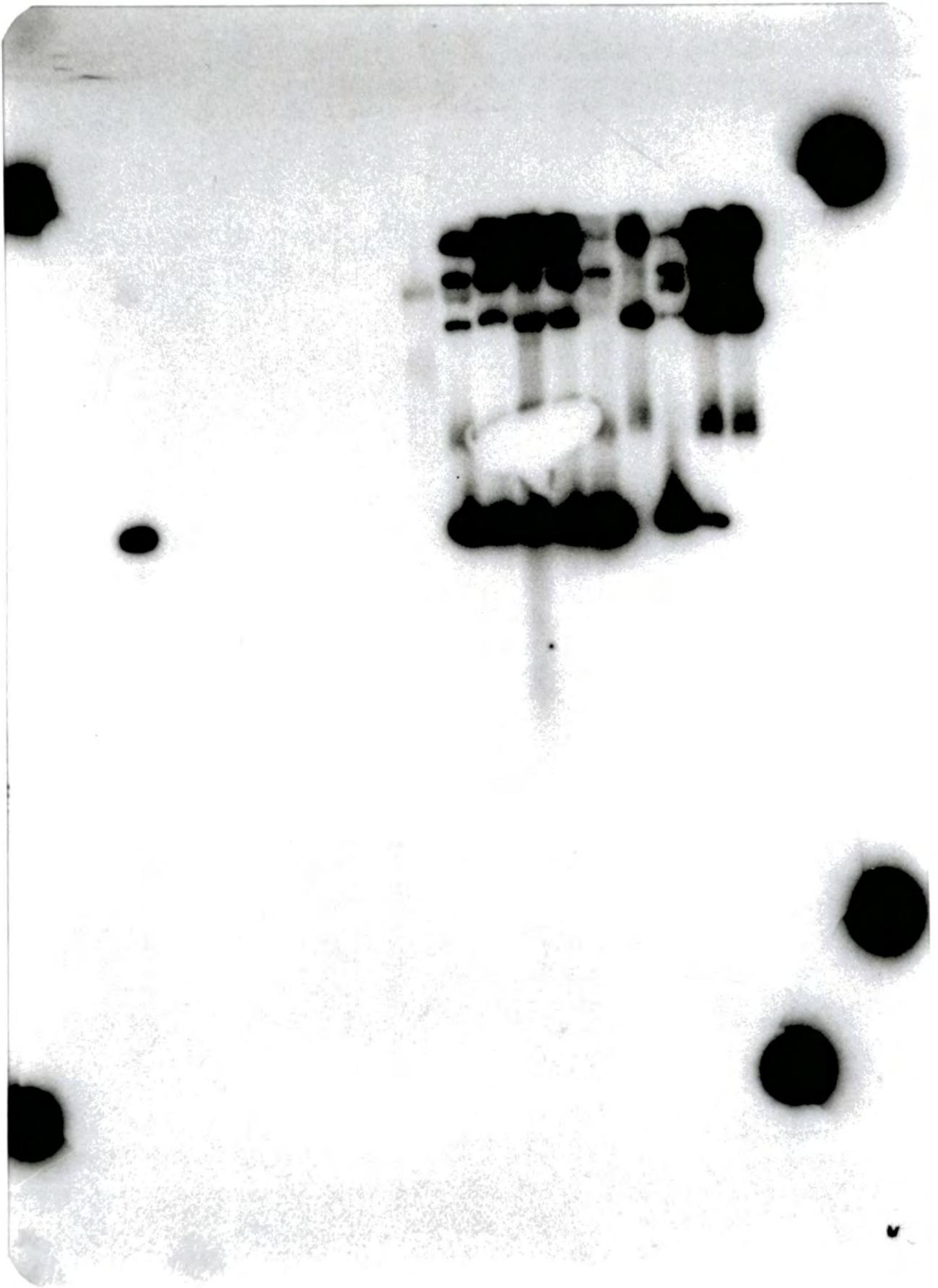
Fig 3.3 Southern Blot: NB.   Last 10 lanes have   plasmid;
First 10 are   empty.

3.4. RESULTS FROM SHAKE FLASK CULTURE

Having already established that the plasmid is lost from the cell, the next step is to ascertain the degree of plasmid loss and the time taken for the cell to lose the plasmid under non-selective conditions. Quantifying plasmid loss with respect to time requires that the cells are in exponential growth in as constant an environment as possible, where nutrient and oxygen limitation are not going to cause the loss of the plasmid. The best system for this as already mentioned in the introduction is Continuous Culture.

However this option was not available in the latter stages of the project therefore the quantification of plasmid loss was done in sub-cultured shake flasks. The principals are the same as Continuous Culture although in practice shake flask results will not give the identical or even the accurate results as would be obtained in a continuous system. This is due to temperature fluctuations in s nutrients and temperature, also inadequate buffering may impose a pH selection on the culture. Furthermore there is the inconvienience of sub-culture, unlike a chemostat which can be left for days and finally calculation of generation time is not only difficult but inaccurate. (Dwivedi et al 1982) (Primrose *et al* 1984)

Cells were inoculated into flasks. As cell numbers increase growth become exponential and will remain so until nutrient limitation eventually sets in causing cell starvation and death. Under these conditions plasmids become unstable and are lost from the cell (Pinches *et al* 1985). To prevent limitation the cultures are inoculated into fresh media as soon as they reach late log phase; flasks are baffled to prevent oxygen limitation and are shaken throughout to enhance oxygen transfer. The two organisms used in the shake flask culture were 316NCIB-11767 and 316AJ1-23 and

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information from their respective fermentations are also taken into account. Cells were inoculated into 250ml flasks containing 50ml of S2 media with pyruvate as the carbon source since although Glucose was used in the fermenter it cannot be used in shake flask since its metabolism results in a pH drop which inhibits growth. Flasks were grown during the day at 28 C and were then transferred at night to 20 C to slow down the growth rate and thus prevent limitation. At the weekend the cultures had to be stored at 4C and transferred to 28C at 4 am on Monday morning. Samples of 1 ml were taken twice daily, diluted and plated on L-agar and L-strep media for viable counts ; another 1ml sample was taken at the same time for restriction analysis to check for plasmid. Growth rate was calculated by measuring the cell optical density (OD) at 600 nm every hour. Graphs showing the changes in growth rate along with a table summarising the rates given in the text can be found in Appendix 2.

Results, where appropriate, are tabulated showing plasmid % loss along with viable counts. The calculation of generations can be calculated from the growth rates, however it is likely to be inaccurate due to the temperature changes, especially the weekend incubation at 4 C, therefore plasmid loss is shown with respect to time and a rough estimation of number of generations is given in the text.

3.4.1. 316NC1B-11767 IN SHAKE FLASK

Three colonies were inoculated into the S2/Pyr media, the colonies came from one of the initial samples of the F15 fermentation. Two were small in origin N1 and N2 the other was a large colony N3. All were capable of growing on Strep and all contained the plasmid. Cultures are designated N_1 , N_2 and N_3. These cultures were maintained over a period of 55 h. with 2 sub-cultures. Td or cell doubling time is

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calculated from the plot OD against Time where the time taken for the cell optical density to double is taken as the Td, from there μ max , the maximum specific growth rate, can be calculated

$$\mu_{max} = \frac{\ln 2}{Td}$$

Culture N_1 had a μ max $=0.22h^{-1}$ Culture N_2 had a μ max $=0.25h^{-1}$ and culture N_3 had a μ max $= 0.345h^{-1}$ These differences in growth rate was also reflected in colony size with N_3 producing the largest colonies on L-agar.

After 24 hours viable counts showed a 33% and 28% drop in plasmid bearing cells for N_1 and N_2 respectively, meanwhile N_3 had lost a total of 65% of its plasmid bearing poulation. However restriction analysis still showed the prescence of plasmid in all three cultures. 24 hours later the growth rates for N_1 and N_2 were very slow and did not reach log phase that particular day, N_3 had a marked increase in growth rate to $0.60h^{-1}$. The viable counts showed a total loss of 55% and 95% for N_1 and N_2, the count was too high for N_3 even the L-Strep plates had a high count. The following overnight culture saw the two cultures N_1 and N_2 in limitation and N_3 in late log phase , one viable count sample was taken on N_3 and this showed an apparent 39% loss of plasmid. However restriction analysis from day 2 and day 3 showed that there was no plasmid present in any of the three cultures.

This loss of plasmid can account for the increased growth rate of the three at the last 16 h. at 20 C but it does not explain the apparent ease with which N_3 grew on strep. A sample was taken from the L-strep plate and restricted and it revealed no plasmid presence. It was possible that the plasmid or streptomycin resistance part of the plasmid had been incorporated into the host genome. However this possibility was dimissed when a hundred colonies from the L-agar were picked off and plated on to a L-

strep plates divided into 25 sections and every single one of the colonies grew within 24 hours. Such a high transposition rate (100%)is extremely unlikely, therefore it could only be assumed that this particular strain had developed a natural resistance to strep. This resistance had probably developed after the cultures prolonged exposure to strep. A sample of the wild-type NCIB11767 was plated on L-Strep and it started growing within 72 h. It was then decided to use the original clone whch had been stored at -70 C and had not undergone any prolonged culturing.

3.4.2. CULTURE OF 316 NCIB11767: ORIGINAL CLONE

This culture was checked and found to contain the plasmid, it was then inoculated in to the S2 and pyruvate media and was cultured for approximately 120 h. this included a weekend at 4 C. Again an average of two plates and two DNA samples were taken per day. The growth rate for this organism ranged from $\mu_{max} = 0.31 - 0.345h^{-1}$ for 96 h. Samples taken over this period showed a gradual decrease in plasmid bearing cells to approximately 6% On the final day the growth rate doubled to $0.69h^{-1}$ and a final sub-culture over-night at 20 C had the organisms in stationary phase at 9am. Viable counts showed a high number of colonies growing on L-strep after 96 hr while restriction analysis was showing the lack of plasmid presence.(see table 3.3.)

TIME	LURIA COUNT	LURIA.STREP COUNT	% LOSS
hours	$\times 10^7 ml^{-1}$	$\times 10^7 ml^{-1}$	a
17	16	17	0
24	229	190	13
72	350	210	40
85	2.05	1.4	34
101	TMC	TMC	?
109	TMC	TMC	?

Table 3.3: This table denotes the number of colonies growing on L-agar and L-strep. From these numbers it is possible to calculate the amount of plasmid lost with time.

The estimated number of generations over this period was 37. This organism has once again developed a natural resistance to antibiotic. This property makes it an unlikely organism for future culture as it would not respond to antibiotic selection pressures and screening.

3.4.3. SHAKE FLASK CULTURE OF 316 AJ123

Returning then to the second strain used in the fermenter, this clone was cultured alongside the 316NCIB11767 original clone. The inoculum was taken from an early sample of the F5 fermentation and was found to be stable. The culture ran for 120 h. again including a weekend at 4 C. The growth rate was calculated at $0.23h^{-1}$ at 28 C and it remained so for the first 96 hours of the culture. however after this time the the growth rate doubled to $0.46h^{-1}$ viable counts showed a fairly rapid drop in

plasmid bearing cells and by the final 8 hours a total loss of 95% was detected and as in the case of the NCIB organism, the final sub- culture overnight had the AJ1 strain in limitation. Restriction analysis confirmed that there was no plasmid present after 96 hours. (See Table 3.4.)

TIME Hours	LURIA COUNT org. $\times 10^8 ml^{-1}$	LURIA.STREP COUNT org. $\times 10^8 ml^{-1}$	% PLASMID LOSS
16	1800	1080	40
22	3.35	1.11	66
74	2.5	0.5	80
79	0.16	0.034	80
112	0.207	0.0306	85
117	1310	70	95

Table 3.4: This table denotes the number of viable colonies growing on L-agar and L-strep. From these numbers it is possible to calculate the % of palsmid lost with time.

The estimated number of generations for this culture is 20.

3.4.4. 316 AJ1-23 WITH MONO-CHLORO ACETATE

After the instability demonstrated by the AJ1-23 in the S2 and Pyruvate a second culture was set up ,this time containing 1mM Mono-chloro acetate (MCA) MCA is a substrate analogue for the dehalogenase enzyme, it is dehalogenated to glycollate and chloride, the glycollate is not utilised by the AJ1-23 since it does not have the enzymes for the glyoxyllate path, therefore when the glycollate accumulates it has a toxic effect. 1 mM was a maximum dose which was suffficient to allow expression of

the HAD D gene without poisoning the bacteria. The culture ran for 192 h. and 9 sub cultures were made. Over this period the stability of the culture improved markedly, the plasmid was still present at the end of the 192 h. Growth rate remained on average at a steady $0.345h^{-1}$ with only an increase on Day 5 to $0.69h^{-1}$ where instability was apparent in the culture. Viable counts revealed an massive fall in plasmid bearing organisms to less than 10%, a subsequent upsurge in numbers arose after 106 h. Table 3.5. shows the plasmid loss and fig.3.4 shows the restriction pattern obtained from that days samples; lanes 2-5 are samples restricted at 106 hours the last three lanes 8-10, are samples restricted at 114 hours . Lane 7 contains the control plasmid pTB 244.

TIME hours	LURIA COUNT org $\times 10^8 ml^{-1}$	LURIA.STREP COUNT Org $\times 10^8 ml^{-1}$	% PLASMID LOSS a
16	16	15	6
24	1.7	1.68	2
42	5.8	4	31
48	420	200	53
106	TMC	59	90
114	0.64	0.55	14
136	1.15	0.86	25
143	TMC	TMC	b
164	850	610	28
181	72	48	34

. Table 3.5: This table denotes the number of viable 316AJ1-23 organisms growing on L-agar and L-strep. Plasmid loss can be calulated from the figures with respect to time.

Estimated generation time is 46. The addition of MCA has apparently stabilised the plasmid as it remained in the cell twice as long as the previous culture. However the MCA is of no value to the cell in fact its presence is toxic. Therefore perhaps another explanation is needed to account for the increased stability.

Fig.3.5. Restriction Pattern of Samples on Day 5 of 316 AJ1-23 on S2 and MCA. Lanes 2-5 contain the early samples, these lanes contain no plasmid. The lanes 8-10 contain the sample taken 8 h. later. Plasmid is present.
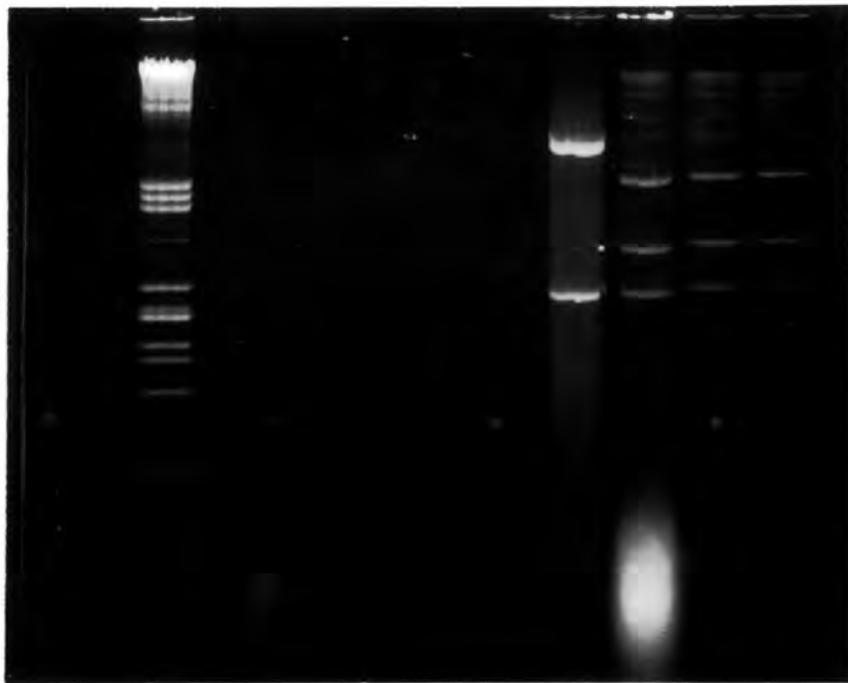
Chapter 4: CONCLUSION AND DISCUSSION

The problems which had to be resolved in this project were:

1 . identify the type of plasmid instability.

2 . discover the possible causes of the instability

3 . attempt to quantify the loss and compare stability between different strains of host organism.

4 . suggest possible mechanism to stabilise the plasmid in continuous culture.

4.1 Segregational instability of the pTB316 plasmids .

The form of the instability is segregational. The plasmid is lost from the cell at cell division and is spontaneous over a few generations. This was confirmed by restriction analyses and the southern blot which showed complete absence of plasmid from the cell (fig.3.3).

4.2 High levels of gene expression contribute to the instability . The loss

from the cell is due to the high levels of expression of the plasmid. Production is constitutive in the clones and at these high levels the strain on cellular resources, in the absence of selection pressure, results in the rise of a fast growing plasmid-free segregant. The two fermentations did not run for very long, therefore it was not possible to ascertain the actual level of instability in that time. However fermentation F15 had a higher level of expression than F5 and a higher level of instability, with a 14% loss after only 18 hours and rising to 30% ust prior to the end. F5, on the other hand, was 100% stable throughout the run with its activity running at a half of the F15. This low activity could be due to strain differnces, although the pH and

temperature shocks experienced by F5 at the beginning may well have caused the poor production. These results were corroborated by the analysis of the ratio of small to large colonies taken from the fermenter (results not printed). Samples taken from the F15 fermentation and plated onto L-agar showed a gradual rise in the proportion of large colonies with time. Early in the fermentation, sample 5, 25% of the population were large colonies whereas by the end, sample 10, 93.5% of the population were large. These large colonies are due to an increased growth rate (Immanaka et al 1980). Colonies taken from the F5 fermentation showed no discernable differences in colony size throughout the fermentation.

4.3. Growth rate advantage of the plasmid free segregants

Shake flask culture experiments confirmed the growth rate advantage for the plasmid free cells. The graphs of the growth rates (Appendix 2) show that growth rates virtually double by the final day when the plasmid is lost

Returning to the mathematical model of Immanaka and Aiba (1981) the ratio of growth rates denoted α can be expressed thus:

$$\alpha = \frac{\mu^-}{\mu^+}$$

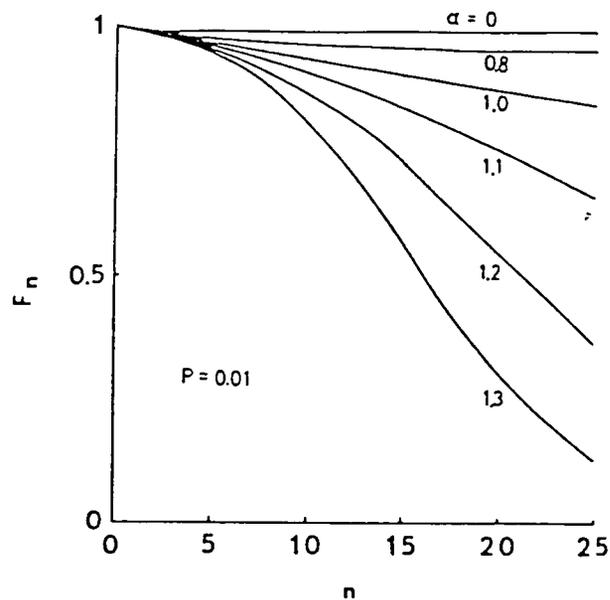. Plasmid stability decreases for values of $\alpha > 1$. See fig. 4.1.

Fig.4.1. Number of P^+ cells in a population over 25 generations at different values of α. As α values increase the plasmid stability decreases.

The graph shows the decrease in the number of plasmid bearing cells over 25 generations at α values ranging from 1-2. The α values for the shake flask cultures of 316NCIB11767 and 316 AJ1-23 are approaching 2, therefore the number of p^+ cells are likely to be very small. It was emphasised at the start of chapter 3 that shake flask culture gave an inaccurate estimation of generation time and that the rate of plasmid loss calculated from these results may be dubious. Therefore assumptions taken from this source regarding such parameters are done with some trepidation. However the

shake flask results did clearly show the notable differences in growth rates of P^+ and P^- cells and could then be regarded as valid.

4.4 Strain suitability .

The host strain is of considerable importance to the plasmid stability. The strain NCIB11767 gives high activity but is apparently the more unstable of the two clones; also it must be taken into account the organism's high resistance to strep. Streptomycin is an antibiotic to which resistance does develop very easily due to successive mutations at multiple loci after prolonged exposure to the antibiotic. (Franklin and Snow 1981).

Plating experiments confirmed that the resistance is probably the result of a spontaneous mutation at chromosome level rather than the action of a transposon. The resistance did not apparently rise till after the plasmid was lost, so if the plasmid was stabilised and expressing its own strep. resistance gene then this may prevent the development of resistance at the genome level. In its present unstable state it would prove to be an extremely difficult organism to culture due to its inability to respond to the antibiotic markers.

4.5. Stabilisation of the plasmid due to addition of MCA

The shake flask culture of the clone 316 AJ1-23 showed a high degree of instability with a 95% loss of plasmid after 20 generations. The addition of the inducer MCA resulted in the increased stability of the clone. Results show that the loss had plummeted to < 10% and within 8 hours the stability had increased back to 86%. As has already been mentioned the MCA is of no apparent use to the organism, it is not a glycollate utiliser since it does not have the glyoxyllate pathway enzymes. Early

experiments done using the wild-type strain, AJ1-23, show that the dehalogenase activity of a culture grown on MCA is substantially lower than when grown on the normal substrate D-CPA therefore the organism may not use the MCA as efficiently as the D-CPA. Why does the MCA, a substance with a toxic end-product, stabilise the plasmid? One suggestion is that there has been a rise in the population of an adaptive variant. The presence of the MCA in such a small amount has perhaps had no effect on the organism and its presence is purely incidental. Results show that the plasmid stability dropped to 10% and then rose to 86% in a matter of hours, (See fig 3.4), the resulting population lost the plasmid at a much slower rate than the original. This suggests a more competitive strain, capable of surviving in more adverse conditions. However the actual rate at which it rose to predominance may tend to cast doubts on the idea of it being a more stable variant.

Continuing the idea of variant in the population, it could be possible that the P^+ organism, due to its greater dehalogenase activity has developed an increased tolerance to glycollate and/or has mutated to produce the enzymes for its utilisation. As a result these glycollate utilisers have developed a selective advantage over their non-glycollate utilising counterparts and therefore rise to dominance.

An alternative explanation is the possibility that the MCA does play a role and it, or a metabolic product, acts as a repressor. If the MCA represses gene function then this would probably result in its increased stability. However it would not explain the massive drop in stability at the start of the experiment and the subsequent rise, repression would have started immediately and therefore no drop would have been noted. There is also the possibility that the MCA or a metabolite affects the action of the enzyme.

What these results certainly show is the need for a better understanding of this organism's physiology. The tolerance threshold for glycollate is not known nor is the mechanism of action on the cell and until these parameters are established there is little point in using the MCA in culture experiments ; it is possible that its stabilising effect is purely incidental. However if it does play a role there is really no point in developing this until its action on the cell is fully understood.

4.6. Suggestions for Future Developments

4.6.1. Insertion of a regulated promoter

The cause of the instability is the result of high levels of gene expression. Therefore as has already been outlined in the introduction, gene expression should be repressed during active growth and then derepressed once an appropriate cell density has been reached. This could be done by adding a temperature sensitive promoter such as the P_L or the P_R promoters of λ or a promoter such as the *lac* or *trp* promoters which require media manipulations. The cost effectiveness of which promoter to use will possibly depend on the scale of the fermentation; a small scale fermentation would perhaps be operated cheaply using the temperature regulated promoters whereas the large scale fermentations are probably cheapest operated using the media regulated promoters.

In conjunction with the regulation system, a two-stage fermentation could be used as done by Seigel and Rhu (1986). Furthermore, the immobilisation of the first stage may be worth considering given that it greatly increases plasmid stability.

4.6.2. Screening for a more stable varaint

The screening for a more stable variant is a time-consuming process which could be

expensive. However it is a technique which could possibly be used in conjunction with the development of the regulated clone; i.e. screen for a stable variant and use it to insert the regulatory genes.

As in the case of the naphthalene dioxygenase system, (Ensley 1983) this organism also has a selectable phenotype in that small colonies are more likely to be plasmid containing,which could make possible future screening easier.

4.6.3. Development of an Auxotrophic Mutant .

It is possible to put an auxotrophic selective pressure on the cells to increase plasmid stability. If the MCA/glycollate path does confer a selective advantage on the organism then this could be a suitable marker to use, however its effect on the media is not properly understood. The development of other mutants is possible but, as has been already mentioned, the organism's physiology has not been fully elucidated, therefore this technique is perhaps better left till a later stage.

SUMMARY

The plasmid pTB316 in strains AJ1-23 AND NCIB11767 is segregationally unstable due to the high levels of gene expression in the cell. Restriction analyses and the southern blot demonstrated the absence of the plasmid in unstable cells and this was confirmed by screening on L-strep plates. The levels of instability in the high activity F15 fermentation, containing 316NCIB11767, compared to the highly stable but low activity F5 fermentation of 316 AJ1-23 suggest that the instability is due to high levels of gene expression. The result of this is a strain on cellular metabolism and since there is no selection pressure, the plasmid-bearing population are at a competitive disadvantage compared to the plasmid-free segregants which ultimately rise in the population.

Shake flask culture of the two clones demonstrated that the growth rate of the P^- cells is greater by a factor of 2 compared to the growth rate of the P^+ cells. These experiments also revealed that the plasmid is extremely unstable in the absence of selection pressure with no plasmid detected after about 90 h in culture. Dehalogenase activity was not measured in the shake flask experiments, therefore the actual reason for plasmid loss is uncertain since under these conditions instability could have been a result of nutrient limitation rather than gene expression levels which were unknown.

The addition of the substrate analogue MCA, to the shake flasks stabilised the plasmid for nearly 200 h. The reason for this is not known since so little is known of the organism's physiology. Gene repression is one suggestion since this does normally stabilise plasmids. However since there were no activity measurements in the cultures

this can not be proved.

Alternatively there is the possibility that a mutant has arisen in the population which is glycollate tolerant, there is no evidence to confirm hypothesis. However there is some evidence to suggest a more stable variant has arisen in the population. The P^+ popu lation are never fully lost from a fermenter (Primrose *et al* 1984) and the upsurge in activity and improved stability suggests a more competitive and more stable strain.

Since it has been discovered that the plasmid is lost due to high expression levels of the gene, it seems appropriate at this stage to insert inducible promoters. This, coupled with suitable fermentation protocols should increase the plasmid stability and improve the efficiency of the process.

With regard to strain suitability, the better strain to use would be the 316 AJ1-23 because the 316 NCIB11767 is too prone to the development of natural antibiotic resistance and in it's present unstable state would be extremely difficult to work with.

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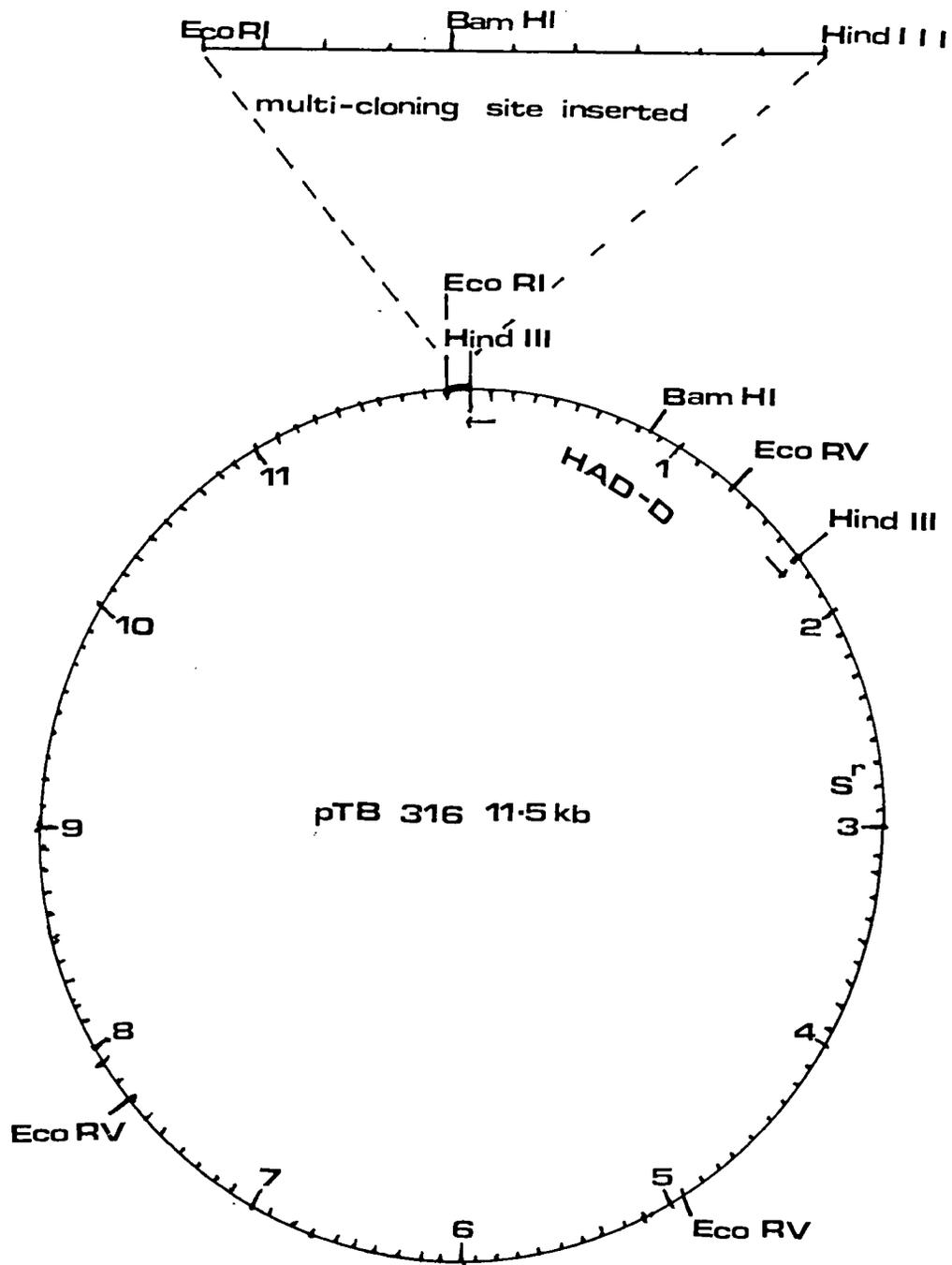
APPENDIX 1. RESTRICTION MAP OF pTB316

Fig. A.1. Restriction map of plasmid pTB316 showing HAD-D insert and appropriate restriction sites used in the project.
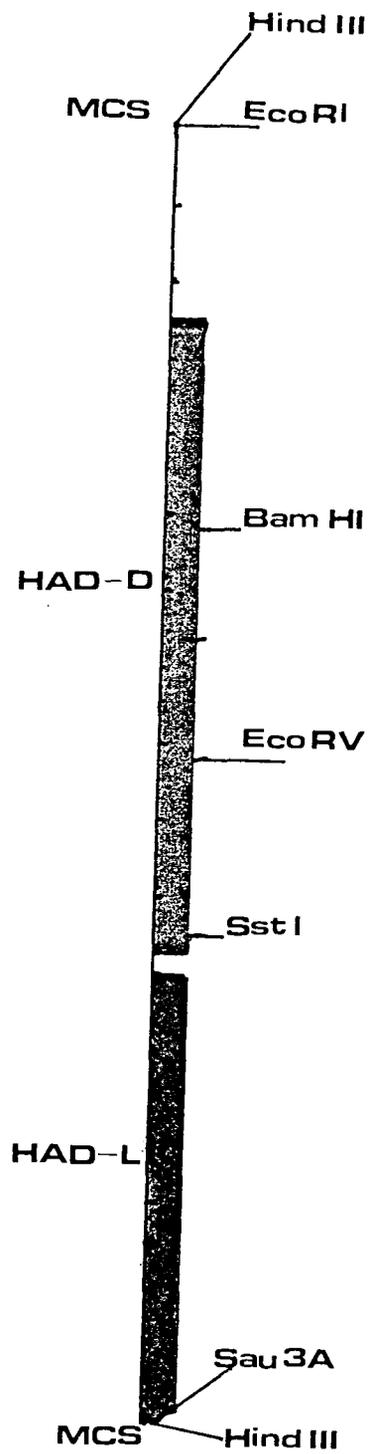
Fig. A.2 Restriction map of the HAD-D/HAD-L insert showing restriction sites of the enzymes used in the project.

APPENDIX 2: GROWTH RATES FROM SHAKE FLASK CULTURE

3 CLONES OF 316 NCIB11767 ON S2 AND PYRUVATE

Growth Rates (h^{-1})			
DAY	N_1	N_2	N_3
1	0.22	0.25	0.345
2	unknown	unknown	0.6

316 NCIB11767 ORIGINAL CLONE

DAY	Growth Rate (h^{-1}) 316 NCIB11767
1.	0.345
2.	0.31
3.	0.69

316 AJ1-23 ON S2 AND PYRUVATE : 316 AJ1-23 ON S2, PYRUVATE AND MCA

DAY	Growth Rate (h^{-1}) 316AJ1-23
1.	0.23
2.	0.23
3.	0.46

DAY	Growth Rate (h^{-1})
1.	0.345
2.	0.345
5.	0.6
6.	0.345
7.	0.345

Graph I

3 Clones of 316NCIB11767 in S2 and Pyruvate

OD (abs)

N1
N2
N3

Time (hours)

Graph 2

316 NCIB11767 Original Clone on S2 and Pyruvate

OD (abs)

Legend:
- Day 1
- □ Day 2
- × Day 3

Time (Hours)

Graph 3

Clone 316 AJ1-23 on S2 and Pyruvate
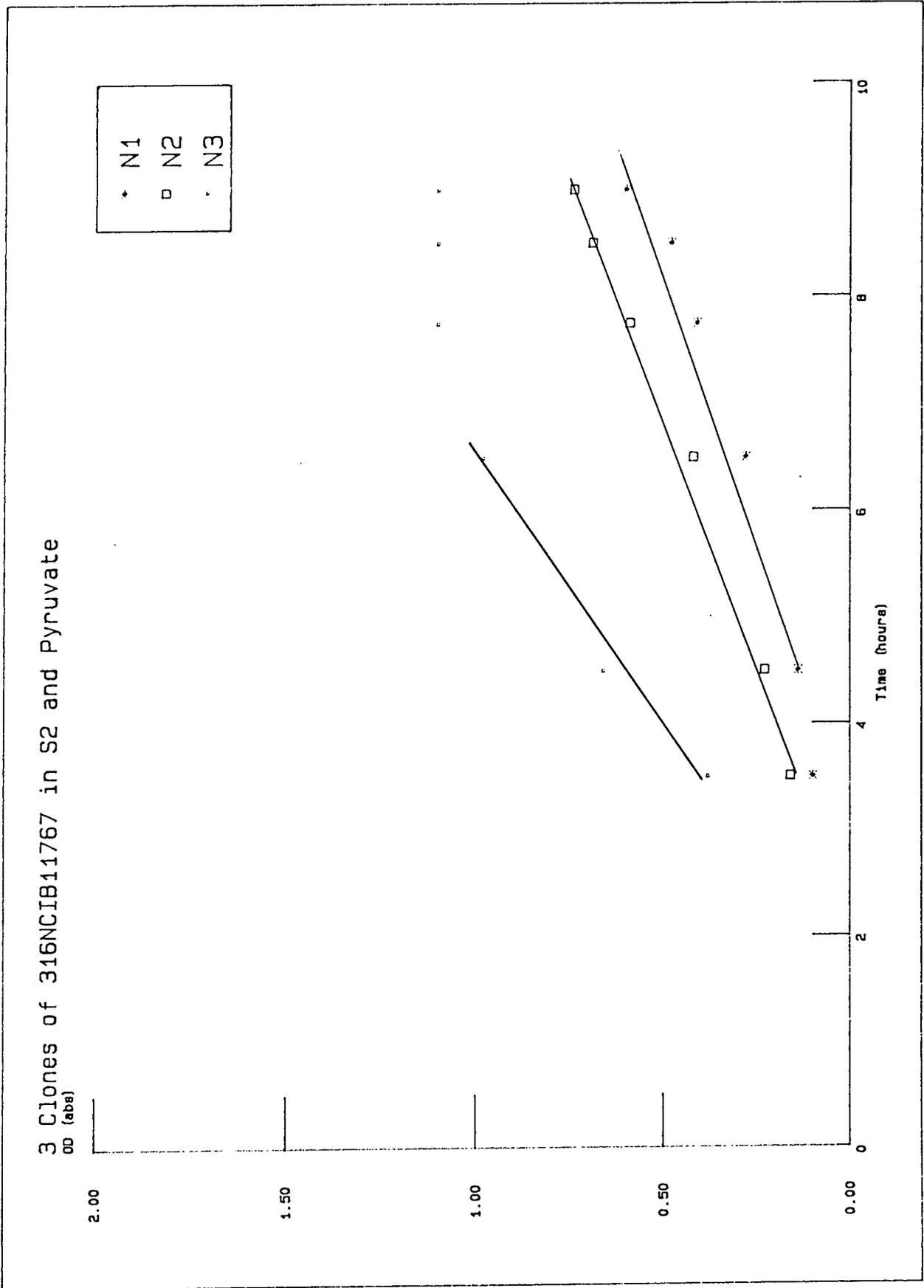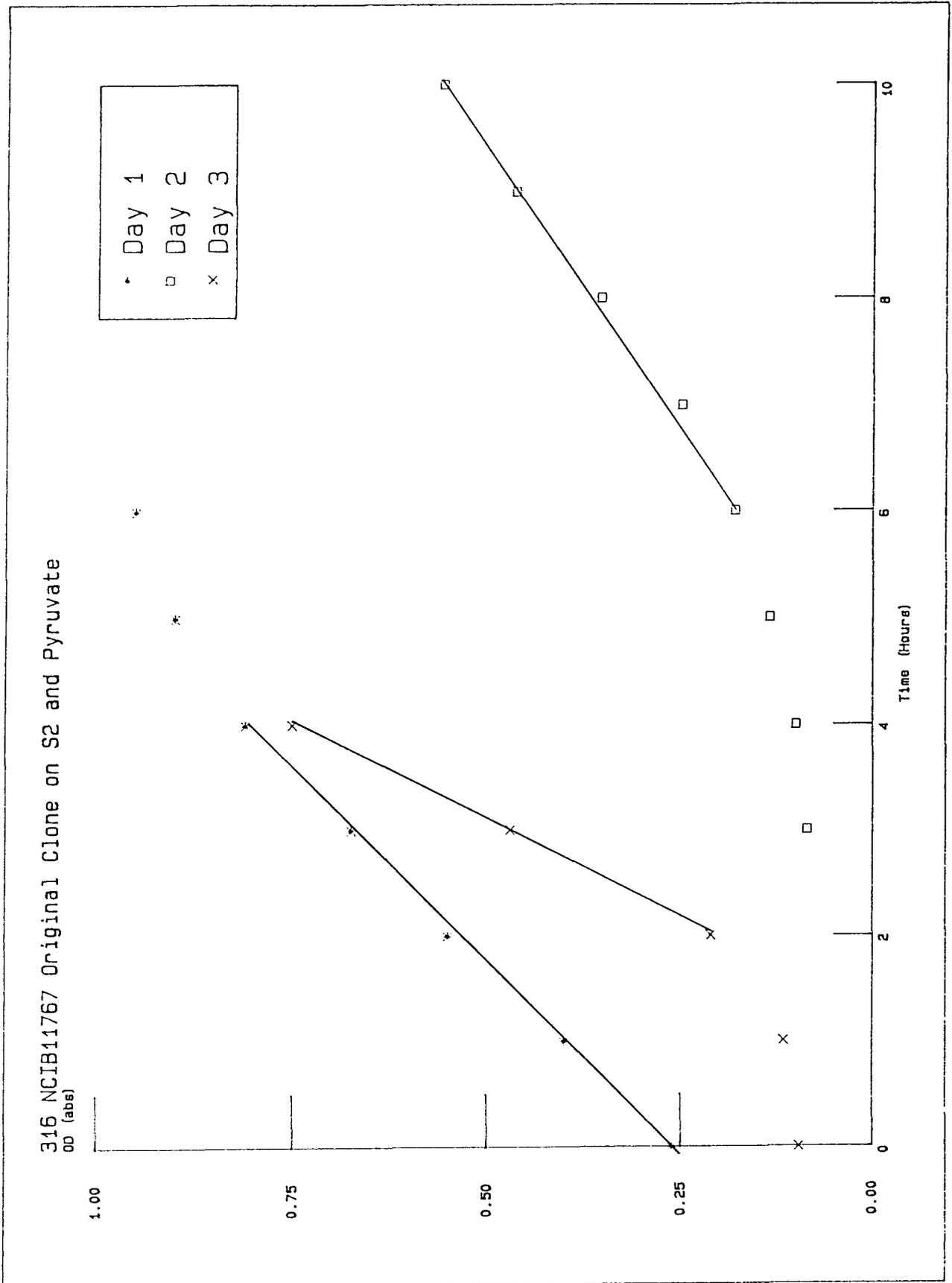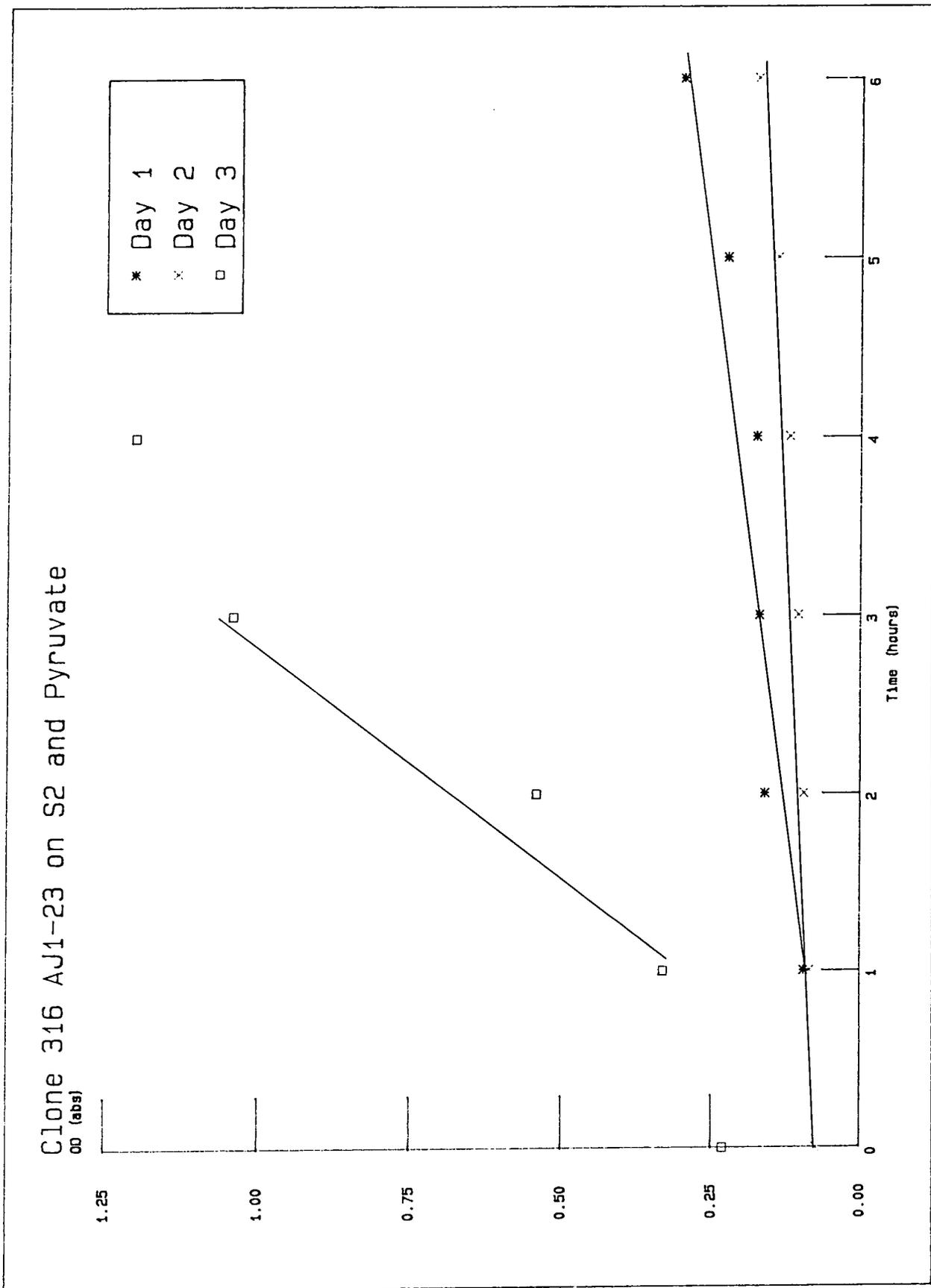
graph 4

316 AJ1-23 on Seed2, pyruvate with MCA

OD (abs)

Day 1
Day 2
Day 5
Day 6
Day 7

Time (hours)