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DYNAMICS OF PLASMID CONTAINING CELLS IN A CHEMOSTAT UNDER TRANSIENT CONDITIONS

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ABSTRACT

DYNAMICS OF PLASMID CONTAINING CELLS
IN A CHEMOSTAT UNDER TRANSIENT CONDITIONS

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One of the major concerns in the utilization of plasmid-containing organisms in fermentation processes is the stability of the plasmid. In general, it is observed that two parameters, the probability of plasmid loss due to faulty segregation and the difference in the specific growth rate between the recombinant and reverted cells, govern the dynamics of the reactor. These two parameters are affected by a number of factors: the genetic make-up of the host cells and the plasmid and the reactor operating parameters such as temperature, pH, dilution rate, and growth medium. It is speculated that by manipulating these factors it may be possible to enhance the stability of the plasmid-containing population.

In this work the effects of well-defined perturbations in reactor conditions on plasmid maintenance and gene expression of a population of Escherichia coli K12 strain carrying the vector plasmid pBR322 grown in a chemostat with a non-selective medium were studied. In a set of experiments the cultures were exposed to square-wave oscillations in the dilution rate. Under these conditions the cultures were capable of maintaining a mixed population of plasmid-

containing and plasmid-free cells for a longer period of time than a culture grown under a constant dilution rate. The effect of the perturbations on plasmid copy number and expression were also studied. The data was dependent on the amplitude and frequency of the dilution rate oscillations. In addition, the experiments indicate that adaptation of the culture to the transient conditions will reduce or totally eliminate any advantage created by the perturbations.

An unstructured model, evaluating the probability of plasmid loss and the differences in the specific growth rate between the plasmid-containing and plasmid-free cells grown under dilution rate oscillations, was developed to describe the experimental data. The results indicate that the transient conditions provide a favorable environment for the plasmid-containing population.

It was further observed, through the use of minimum inhibitory concentration (MIC) experiments, that the plasmid-containing cells existed as a heterogeneous population with respect to the plasmid copy number. The population distribution could play an important role in plasmid maintenance and expression.

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The most important things are the hardest to say. They are the things you get ashamed of, because words diminish them - words shrink things that seemed limitless when they were in your head to no more than living size when they're brought out.

— Stephen King

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Ap^r	Ampicillin resistant
C_b	Intensity of chromosome DNA
C_s	Chromosome size
D	Dilution rate (hr^{-1})
k_s	Constant
OD	Optical density
p	Probability of plasmid loss
P_c	Intensity of closed-circle plasmid DNA
P_o	Intensity of open-circle plasmid DNA
P_s	Plasmid size
p_1	Rate of population shift from high copy number sub-population to lower sub-population
p_2	Probability of plasmid loss of low copy number sub-population
p_3	Probability of plasmid loss of high copy number sub-population
s	Substrate concentration
s_f	Feed substrate concentration
t	time
x	biomass concentration
y	Fraction of cells
Y	Biomass yield
μ	Specific growth rate (hr^{-1})
$\delta\mu$	Difference in specific growth rate

Subscripts

f	Plasmid-free sub-population
m	Intermediate copy number sub-population
s	High plasmid copy number sub-population
o	Initial conditions
T	Total

Superscripts

+	Plasmid-containing cells
-	Plasmid-free cells

CHAPTER 1

BACKGROUND AND RATIONALE

1.1 Introduction

Recombinant DNA techniques have revolutionized both biological research and applications in the past decade. The ability to direct which genes are used by the cells has led to the development of microorganisms that produce new products, existing products more efficiently, or large quantities of otherwise scarce products [43]. Recombinant DNA technology has been used to synthesize important eukaryotic products in bacterial strains. These products include somatostatin [104], human growth hormone [74,200], human leukocyte interferon [157,224], human insulin [29,73], and viral vaccines [44,138,256] in bacterial strains. Genetic engineering can also be used to develop organisms that are themselves useful [65]. These microorganisms have a great potential in the fields of agriculture, food technology, and detoxification of organic pollutants [52].

In most cases, the above objectives are accomplished by introducing into the host cells plasmids carrying the genetic code for the specific protein of interest. The use of plasmid-containing cells creates new problems and considerations in the design and operation of bioreactors such as selection of the host and cloning vectors, expression of

the cloned gene, stability of the plasmid, and purification of the product.

1.2 Host Strains

One of the most important factors to be considered in the production of recombinant proteins is the selection of a suitable host. In large-scale production it is advantageous to use fast growing cells with simple nutritional requirements. The cell should not produce toxic substances or be pathogenic. In addition, when considering product yield it is desirable that the cells can be grown to high densities and the recombinant product be excreted from the cell [65].

A variety of microorganisms are currently used as host strains in recombinant DNA studies. The benefits and drawbacks of each organism must be taken into account when selecting a suitable host cell. The more generally used organisms are described below.

Escherichia coli is the most commonly used bacterium in genetic-engineering. Because of the vast amount of information on its molecular biology and physiology this is the best understood bacterial cell. In fact, studies using E. coli have been crucial in the elucidation of protein synthesis. In addition, there are a number of expression systems that can be efficiently employed in E. coli. There are some potential problems, however, that can occur when working with this strain. These include pyrogen production,

improper folding of the protein, protein insolubility, and the inability to perform certain post-translational modifications, for example glycosylations of eukaryotic proteins [257]. In spite of these problems, E. coli has been successfully used in large-scale production of many recombinant products [144]. For this and the reasons mentioned previously, E. coli was chosen as the host cell in this work.

Bacillus subtilis and Bacillus stearothermophilus are increasingly being used as host strains [14,51]. B. subtillis is a non-pathogenic soil organism. It has a simple cell envelope which can simplify product recovery and purification [98]. B. stearothermophilus is a thermophile which produces thermostable enzymes. A disadvantage of these cells as host strains is the production of proteases that cause the degradation of the desired recombinant proteins.

Saccharomyces cerevisiae is employed in genetic engineering research for many reasons. First, as in the case of E. coli, is the existence of the quantity of information concerning its molecular biology and physiology. Also, the organism is safe, able to glycosylate proteins, and secrete recombinant proteins [102]. Problems that have been observed are the low levels of the cloned gene product, the cells resistance to disruption methods, and incorrect protein glycosylation [257].

The development of plant and animal cells as host cells is relatively recent. The Ti plasmid of Agrobacterium

tunefaciens and plant viruses are commonly used as vectors for the plant cells [207]. Insertion of the foreign DNA, through the use of a virus, into the genome is the current technique being used in animal cell cloning. Development of extrachromosomal vectors is also being investigated [251]. Extensive work must still be done before these cells can be used commercially as host cells.

1.3 Cloning Vectors

An important characteristic of a cloning vector is the ability to replicate in the host cell. Based on this qualification, viral and plasmid DNA molecules are used as cloning vectors. Although both vectors will be discussed, the emphasis will be on the plasmid as a cloning vector as it was used in this work.

The majority of the phage vectors used originate from the bacteriophage lambda. Two types of lambda vectors, insertion or replacement vectors, are used in studies with E. coli [98]. Some of the advantages and disadvantages of phage vectors are: 1) The cloned gene is stably inherited with chromosome replication as the viral DNA is incorporated into the chromosome; 2) The size of the cloned DNA sequence is limited; 3) The recombinant DNA can be easily isolated from phage particles; 4) A gene dosage effect cannot occur as only one copy of the viral DNA per chromosome exists. Lysis of the host cell results when phage growth is induced; 5)

Recombinant DNA can be packaged in vitro. This DNA can be introduced into E. coli cells with a 10 to 100 fold higher efficiency of transformation; 6) Finally, the type of host strain is limited because of the high specificity of interaction between the phage and the host cells [98].

Plasmids are nonessential extrachromosomal DNA that replicate autonomously of the cell's chromosome. Plasmids range in size from one to several hundred kilobases (kb) and in copy number, the number of plasmids per cell, from one to several hundred per cell [166,167]. Plasmids can be classified into two general types. The first type is characterized by a high copy number and a relaxed form of replication control. In addition, these plasmids are nonconjugative. The second type of plasmid are usually larger in size, maintained at a low copy number, and are under a stringent replication control. Generally, these plasmids are self-transmissible. This classification system, however, is not stringent [98].

Some of the desired characteristics of a plasmid for use as a cloning vector include: 1) The plasmid should be small as the transformation efficiency decreases as the size of the plasmid increases above 15 kb; 2) The restriction enzyme cleavage sites, gene location, and nucleotide sequence of the plasmid should be known; 3) The vector should contain a selection marker used to determine the transformed from the nontransformed cell; 4) In some systems it is desirable to

have a vector that can be easily amplified, usually by drugs or temperature changes; 5) The plasmid should have a number of unique restriction sites. This would provide a flexibility in terms of cloning different vectors; 6) The plasmid should be capable of being transferred to a range of host cells; 7) Lastly, and one of the most important criteria, the plasmid should be stably maintained, through faithful replication and accurate segregation of the plasmid, in the host cell [98,188].

1.4 Regulation of Plasmid Replication

An important determinant in the use of recombinant organisms in bioreactors is the stable maintenance of the plasmid in the host cell. Two factors are paramount in plasmid maintenance. The first factor is a reliable replication system that ensures plasmid replication at least once during the cell cycle. Regulation of replication, therefore, is primarily responsible for ensuring a constant copy number in each cell. As plasmid replication is an essential component in plasmid maintenance some thought should be given to the replication mechanism. The second factor is an accurate partitioning of the plasmids for stable inheritance [201]. A more detailed discussion of the partitioning of the plasmid is presented in Chapter 6.

The replication mechanism is not the same for every plasmid. There are at least six different systems [201] that

have been intensively studied. Since it is not within the scope of this work to discuss all these systems the focus will be on the replication mechanism of the plasmid used in this work.

1.4.1 Replication of ColE1-type Plasmids

Plasmid pBR322 is a ColE1-type plasmid. It is a member of a group of small plasmids that are able to replicate in the absence of protein synthesis [220,228,241]. The plasmids are nonconjugative and replicate as a multiple copy pool [219]. In the presence of chloramphenicol, which inhibits protein synthesis and initiation of chromosome replication of the bacterial host, the ColE1-type plasmid will continue to replicate [37]. It is therefore possible to amplify the plasmid with respect to the bacterial chromosome [36,61].

Replication of the ColE1 group has been studied by a number of investigators [6,21,134,209,233]. In general, replication of ColE1-type plasmids is unidirectional from a specific origin site [103,232]. Initiation of the replication is the result of a number of successive steps [105,142]. It depends on the formation of an RNA primer by the RNA polymerase of the host and RNAase H [16,232]. Synthesis of the RNA II or the primer transcript initiates at a site 555 base pairs upstream from the replication origin. The transcript forms a hybrid with the template DNA near the origin. This RNA-DNA hybrid is cleaved by RNAase resulting

in an RNA primer for DNA synthesis by DNA polymerase I [105,202]. Two additional points should be added. First, only a nascent RNA II transcript can be used in replication initiation [105]. Second, it has been found that RNAase H is not necessary for DNA replication [119,158].

Replication of ColE1-type plasmids is controlled by negative regulation [89,209]. Primer formation by RNA II is inhibited by a plasmid specified RNA, referred to as RNA I [122,229,231]. The RNA I transcript is approximately 108 nucleotides long [153]. Transcription of RNA I begins 445 bp upstream of the origin, proceeds in the opposite direction of RNA II synthesis, ending near the initiation site of RNA II [105,225]. A region specified by both RNA transcripts is involved in the determination of the incompatibility property and copy number of the plasmid [45,155,216,225].

RNA I binds to RNA II preventing the formation of a secondary structure of RNA II necessary for the RNA-DNA hybrid and plasmid replication [229,230]. The process and rate of binding of the RNA transcripts have been studied extensively [122,225,226,234]. It has been found when the nascent RNA II is shorter than 100 or longer than 360 nucleotides plasmid replication will proceed normally. This is because the RNA I cannot bind to RNA II molecules shorter than 100 nucleotides and will not affect primer formation of molecules longer than 360 nucleotides [228]. Therefore, not only is the pairing between the two RNA transcripts critical for

replication regulation, but the rate of binding of RNA I to RNA II is also essential.

An additional region downstream from the replication origin has been found to affect replication of ColE1-type plasmids [217,237]. The site encodes a 63-amino-acid protein. It was initially believed that the protein, termed Rop (repressor of primer), inhibited primer transcription [27]. However, further experimentation demonstrated that the protein does not effect RNA II transcription [123,227,234]. Instead, it affects the interaction of the RNA transcripts. Generally, the protein increases the rate of binding of RNA I to RNA II and the degree of inhibition of primer formation by RNA I [6,228,234]. Based on this, the protein was renamed Rom, RNA one inhibition modulator.

The above provides a brief overview of the regulation of ColE1-type plasmid replication. It should be noted that the mechanism involves three transcriptional events, the synthesis of RNA I, RNA II, and mRNA for the *rom* protein, and the interaction of these transcripts. Any disturbance of this system would affect plasmid replication and subsequently plasmid copy number and maintenance.

1.5 Problems Encountered using Plasmid-Containing Cells in Fermentation Processes

The use of recombinant organisms in fermentation systems has resulted in the development of new challenges.

This section will deal with some of the more prevalent of these problems and the approaches being employed to resolve them.

1.5.1 Cloned Gene Expression

Gene cloning is a powerful tool in the production of specific genes and gene products. One of the major problems encountered, however, is a low yield of the desired product. This is generally corrected by controlling either the plasmid copy number or the cloned gene expression [91].

One method of increasing the gene product yield is to use a multicopy plasmid [93]. To a point the amount of the cloned gene product increases with copy number, referred to as a gene dosage effect [239]. However, at high copy numbers this linear relationship breaks down [9,48]. A possible cause could be due to product toxicity or instability of the system [257].

Amplification is another technique used to increase the plasmid copy number. This can be achieved by increasing the strength of the promoter at the replication origin. A promoter is a specific DNA sequence where gene transcription is started [190]. Promoters used can be chemically or thermally induced [13,124,183]. A common problem with this system, however, is runaway-replication which is lethal to the host cell [238,240].

A second approach to increasing the gene product yield

is through the control of the gene expression. This is also achieved through the use of a promoter [25,62]. The promoter used determines whether the gene expression is constitutive or controlled [257]. The β-lactamase promoter of E. coli [12] and the glyceraldehyde phosphate dehydrogenase [143] and enolase promoters [102] of S. cerevisiae are examples of constitutive promoters. In constitutive systems the addition of chemicals or temperature shifts are not needed to induce gene expression. This makes the system simple and attractive from the stand point of fermentation systems. In fact, the constitutive gene expression has been used with success in the production of cloned products [12,30,143]. Constitutive systems are not problem-free, however. A constant production of the gene product could be detrimental to the cell [59,210]. It can lead to a decreased growth rate or even cell death [71]. It can also affect segregational instability [257]. In view of these potential problems, it would appear advantageous to utilize a system that can be controlled. Promoters that are commonly used to control cloned gene production are the *lac* [34,74], the *trp* [75,100], the *tac* promoter [5,46] and the bacteriophage lambda P_L or P_R promoters [156,187,214,222].

The *lac* system has been studied extensively and used in the production of eukaryotic proteins such as human growth hormone [74]. It is negatively regulated by the *lac* repressor

protein. The addition of isopropyl-β-D-thiogalactoside (IPTG) causes the derepression of the *lac* promoter by preventing the *lac* repressor from binding to the *lac* operator [71]. For high gene expression a mutation of the *lac* promoter, *lac*UV5, is often used [63]. An even higher gene expression occurs when the *lac*UV5 promoter is placed before a constitutive promoter [159].

The *trp* promoter is also negatively regulated. It has been used in production of interferon [75] and other eukaryotic gene products. Control is affected by a complex between tryptophan and the *trp* repressor. The addition of 3-indoacrylic acid (IAA) prevents repression of the *trp* promoter [71]. It has been demonstrated that the use of three *trp* promoters, instead of one promoter, increases the yield of the recombinant protein [76].

The *tac* promoter consists of regions from both the *lac* and *trp* promoters [46]. It is repressed by the *lac* repressor and derepressed by IPTG. Studies have shown that the *tac* promoter is at least five times more efficient than the *lac*UV5 promoter [5].

The bacteriophage lambda promoter, P_L, is negatively regulated by a temperature-sensitive cI repressor protein. At temperatures 28-30°C the cI repressor inhibits transcription. When the temperature is shifted to 42°C the cI protein is inactivated and transcription can occur. The gene for the cI repressor can be on the same plasmid as the P_L

promoter, a separate plasmid, or on the bacterial chromosome [71]. As temperature is one of the easiest environmental parameters to control this system can be very beneficial. One disadvantage of this system, however, is that incubation at 42°C also induces the nonspecific synthesis of protein, i.e., proteolytic enzymes, in E. coli. These enzymes increase the rate of degradation of the product.

There are other disadvantages in using a controlled gene expression. The promoters can be difficult to regulate, especially in high copy number systems [26]. The plasmid stability is also affected. It has been found that as the expression of the cloned gene increases the rate of formation of plasmid-free cells also increases [99,242]. In general, promoters employed in large-scale production should possess a high maximum level of transcription, be tightly regulated, and the method used to initiate protein synthesis should not effect the physiology of the cell [65].

The use of promoters is not the only technique to increase gene expression [98]. One method is to improve the Shine-Dalgarno (SD) sequence by rearrangement of the sequence or replacement with a chemically synthesized sequence [70,109]. The SD sequence, the ribosome binding site, is important for initiation of translation in prokaryotes. Stabilization of the mRNA would also affect the gene expression. It has been observed that the level of interferon

α-5 synthesized in E. coli is proportional to the half life of its mRNA [174]. Another method would involve the removal of the attenuator in the biosynthesis of amino acids [253]. In the absence of this site the expression of the bacterial operon could be enhanced.

1.5.2 Expression of Eukaryotic Gene Products in Prokaryotes

Although amplifying the cloned gene product is an important step in the scale-up of recombinant fermentation processes, it is futile if the product is unstable in the bacterial host. This is one of the primary difficulties in large-scale production of some eukaryotic products [44,104,224]. It is speculated that the eukaryotic foreign peptides are degraded in E. coli as they are treated as abnormal proteins [78,132]. Different techniques have been employed to circumvent this problem. Stabilization of the protein by overproduction is one method utilized [31]. The disadvantages of this technique, as mentioned earlier, are possible toxicity to the cell and increased instability. In addition, it has been shown that overproduction of foreign proteins resulted in an increase in the level of the proteases La [77]. A more common approach is to combine the gene of the desired product to a host structural gene [208]. This would result in a hybrid polypeptide with a "native" portion recognized by the bacterial host. This technique has been used to produce several eukaryotic proteins and viral vaccines

[29,74,185,212]. Because the product is only a small fraction of the hybrid polypeptide a decrease in product yield and additional difficulties in purification are two drawbacks of this method. A reduction of the prokaryotic portion generally resulted in an unstable product [185]. Another approach is to clone multiple copies of the desired gene in tandem on the plasmid [85,127]. This has been successfully done in the production of human alpha interferon and proinsulin [127,208].

Proteolysis can be affected by the growth environment. For example, proteolysis increased under amino acid limited or carbon source limited conditions [235]. In glucose and oxygen limited environments, a common problem in large-scale high density fermentations, recombinant interferon αA was rapidly degraded [112]. One proposed technique to reduce protein degradation is to culture cells at suboptimal temperatures [169]. However, further work is necessary to determine the optimal cultivation conditions for product stability.

1.5.3 Plasmid Stability

Plasmid stability, defined as the ability of a transformed cell to maintain the plasmid unchanged during growth, thereby manifesting the phenotypic characteristics [99], is one of most important factors in the scale-up of recombinant fermentation processes. Plasmid instability is

caused by the loss of the entire plasmid from the host cell, referred to as segregational instability, or by the alteration of the plasmid DNA, termed structural instability [168]. In either case the desired product is ultimately lost.

Plasmid stability is a function of a number of factors: the genetic make-up of the host cell and the plasmid, effect of the gene product on the host cell, and the reactor operating parameters such as temperature, pH, and the growth medium [257]. By manipulating these parameters it might be possible to enhance the stability of the plasmid. Some of the methods investigated to increase plasmid stability are: 1) insertion of genes into the plasmid which could encode for a partition function [145,170], couple plasmid proliferation to cell division [171], or improve the growth rate of the host cells [15,53], 2) elimination of transposable genetic elements, 3) deletion of nonessential DNA from the plasmids as the extra DNA can burden the host cell or increase the probability of alteration of DNA in vivo [98], 4) application of environmental pressure that would select for the plasmid-containing cells, i.e. plasmids carrying antibiotic resistant genes, [192,197,250], and 5) improvement of the host strain [173] and optimization of the plasmid copy number [100].

Although the preceding methods are used to increase stability, they are not always completely effective. For example, the use of a partition function has been demonstrated to increase plasmid stability, but after long term

cultivation, as observed in this work (Chapter 6), plasmid-free cells are detected.

There are also disadvantages to some of these techniques. Antibiotic pressure is one of the most common techniques used to ensure a culture of plasmid-containing cells. However, in large-scale production the use of antibiotics can be very costly [180]. In addition, degradation of the antibiotics is possible under cultivation conditions. In one study, the recombinant culture used excreted enough β-lactamase to degrade the ampicillin being used as a selective agent [180]. Furthermore, the antibiotic must be removed at the end of the fermentation process. Another method for overcoming plasmid instability is to use plasmids that code for bacterial toxins that are lethal to plasmid-free cells [125]. The toxins, however, can also be harmful to the plasmid-containing cells.

1.6 Operational Strategies for Plasmid-Containing Cultures

Two major objectives in the design of fermentation systems utilizing plasmid-containing cells are to minimize plasmid stability and maximize gene product yield. Unfortunately, these two factors are not mutually exclusive. As mentioned earlier, it has been observed that as the expression of the cloned gene product increased the rate of appearance of the plasmid-free cells also increased [99,194]. The approach to the problems of stability and product yield, on a molecular level, has been presented in Section 1.5. This

section will deal with these problems on an engineering level.

The objectives, high volumetric productivity, product concentration, selectivity, and yield at a low cost, for recombinant fermentation systems are the same for other fermentation systems. The goals can be achieved by high growth rates and product formation, high cell densities, and increased product stability. However, considerations to the unique characteristics, plasmid stability and expression of the cloned gene, of the plasmid-containing culture must be made in the implementation of an optimal reactor system. Immobilized cell [49,98] or biomass recycling reactors [172] are two examples of novel reactor configurations proposed for plasmid maintenance.

A more traditional approach is the use of a fed-batch process. Fed-batch reactors are commonly used in fermentation processes. Although fed-batch cultures have been used to achieve a high cell density and large amounts of certain cloned gene products [211,258], plasmid stability has not been studied under these conditions.

When long-term cultivation and low capital costs are considered, continuous fermentation is a more feasible operational scheme. One promising strategy is a two-stage continuous culture system [194]. In this scheme the cell growth and gene expression phases are separated to obtain a high cloned gene expression for a prolonged period in spite of plasmid instability caused by the increased expression

[213]. This is achieved by using a plasmid with a regulated gene expression. Briefly, cells are grown in a repressed state in the first stage. The culture is then continuously transferred to the second fermenter where the expression of the gene is induced by a temperature shift.

Seressiotis and Bailey theoretically examined a similar system by adding another stage [206]. In this model, as in the two-stage reactor, cell growth occurred in the first stage. In the second stage, however, the plasmid of the culture was amplified by a temperature shift. The cloned gene expression was then chemically induced in the third reactor. One of the major challenges of this system is the optimization of the residence times in each reactor.

Environmental parameters such as dissolved oxygen concentration [96], temperature [181], and medium composition also affect recombinant production and plasmid stability. An increase in the formation of interferons and other recombinant proteins occurred in the presence of small concentration of ethanol, which usually inhibits growth, or metal ions [11,112].

These results demonstrate the complexity in the optimization of fermentation systems involving plasmid-containing cultures and the need of further study.

1.7 Research Objective

The one common factor between the operational strategies presented is the emphasis on product yield. The problem of plasmid stability, although considered in these reactor systems, is not resolved. As plasmid stability is one of the major determinants that influences the productivity of recombinant populations the understanding of the various factors affecting plasmid maintenance is of both fundamental and practical importance.

One approach is to study the effects of well-defined transient conditions on a plasmid-containing culture grown in a chemostat. This would provide a better comprehension of the relationship between reactor parameters such as temperature, pH, dissolved oxygen, and nutrient concentration, and plasmid stability and expression. In this work the effect of square-wave perturbations in the dilution rate on plasmid maintenance, copy number, and gene expression were studied.

In addition, an unstructured model, evaluating the rate of plasmid loss and the differences in the specific growth rate between plasmid-carrying and plasmid-free cells, under transient reactor conditions might lead to an understanding of the mechanisms governing plasmid stability. These results could provide a window of opportunity to be exploited for the design of novel bioreactor operation to yield better performance.

CHAPTER 2

MATERIALS AND METHODS

2.1 Bacterial Strain and Plasmids

Escherichia Coli RR1, a K-12 mutant (*F^- supE44 LacY1 LeuB6 proA2 hsd20 mcrA (r^-m^-) ara-14 galk2 xyl-5 mtl-1 rpsL20 $recA^+$ thi-1*) served as the bacterial host throughout this work. This organism was chosen for many reasons [257]. These include the extensive research and information available for this cell and more importantly, the ease in growing and maintaining the strain. The host cell, without the plasmid, was obtained from American Type Culture Collection (ATCC #31343).

Three plasmids were used in this work. The plasmid pBR322, containing the genetic markers Ap^r and Tc^r, was used in most experiments [17]. A map of the plasmid can be seen in Figure 2-1. The plasmid-containing cell was from ATCC (#37017).

A derivative pBR322 plasmid was also used in this work. In this plasmid the partition or *par* locus, from the plasmid pΔ37-*par*, was inserted into the pBR322 plasmid (ATCC #37017). The plasmid pΔ37-*par* was a gift from Dr. Soberón. Escherichia coli K-12 was used in all transformations. The restriction enzymes and T4 DNA ligase were obtained from Sigma. The enzyme digestions and ligations were performed as recommended by the supplier or as described in Maniatis et al. [140]. A

map of the plasmid, referred to as pBR322-*par*, is shown in Figure 2-2.

The plasmid pDM247, generously supplied by Dr. Judith Campbell, was the third plasmid studied [154].

2.2 Media and Culture Conditions

Standard aseptic techniques in a normal aerobic environment were used in all procedures. The cells were obtained from ATCC in a freeze-dried form. They were reconstituted in a Luria Broth (LB) medium supplemented with glucose and the appropriate antibiotics. The medium was prepared by autoclaving a solution containing 10 g bactotryptone (Difco 0123), 5 g bactoyeast extract (Difco 0127), 10 g NaCl, and 2 g glucose per liter of distilled water. The pH of the medium was kept at 6.8-7.0. The medium was autoclaved for approximately 30 minutes at a temperature of 240°F. All antibiotics were filter sterilized using a presterilized 0.2 μm filter (Gelman). The addition of antibiotics was governed by the drug resistance accorded by the plasmid. 25 mg/l tetracycline (Sigma No. T-3258) and 100 mg/l ampicillin (Sigma No. A-9393) were used for the strain carrying the pBR322 plasmid. 200 mg/l streptomycin (Sigma No. S-6501) was added to the medium used in maintaining the host strain. In all cases the antibiotics were not added until needed. Tetracycline and streptomycin solutions were made

fresh and not stored longer than 2 weeks at 4°C. Ampicillin was stored in a salt solution at -20°C and thawed when needed. In this form the solution was stable for approximately three months.

The bacterial strain was activated by adding 0.35 ml of the LB medium. After mixing thoroughly the cells were transferred to a test tube containing 5 ml of the LB solution supplemented with glucose and ampicillin at the concentrations mentioned previously. The cells were incubated at 37°C in an Orbit Environ Shaker until growth was apparent. The last few drops of the cell suspension was transferred with a sterile inoculation loop to agar slants. The agar slants consisted of a modified LB solution. The slants or stabs were made by autoclaving a solution consisting of 10 g tryptone, 5 g yeast extract, 2 g glucose, and 6 g bactoagar (Difco) per liter of distilled water. The medium was autoclaved as before. After the medium had cooled to at least 55°C ampicillin and tetracycline were added to the final desired concentrations. The solution, approximately 5 ml, was then poured into 10 ml screw-cap vials. The vials were stored for 24 hours at room temperature.

After the reconstituted cells were stripped on the agar slants the vials were incubated at 37°C, until growth was visible. The vials were then wrapped in parafilm and stored

at 4°C. The cells transferred to the test tubes were also stored in agar slants after exponential growth had started.

Serial propagation was employed for strain maintenance. A flame sterilized loop was used to transfer a few loops of cells from the old agar slant to a 250 ml flask containing 100 ml LB medium supplemented with glucose and the appropriate antibiotics. During exponential phase 20 - 100 µl of cells were transferred to another flask. This procedure was repeated for a total of at least four transfers. Cells from the last flask were stripped on the new agar slants. The vials were stored as before. The cultures maintained in this way were used as inocula for subsequent experiments.

One observation should be made at this point. That is the importance of antibiotics in the maintenance of the plasmid-containing cells. It is necessary to ensure that all the antibiotics the plasmid is resistant to are present and at the correct concentrations.

2.3 Experimental Equipment and Procedures

All experiments were performed in a constant stirred reactor. A motorized stirrer, at an agitation of 400 rpm, was used to insure a well-mixed culture. The reactor was maintained at 37°C with a built in temperature controller. Oxygen was supplied by bubbling sterilized air into the reactor at a rate of 1 l/min. The air was sterilized by first

passing through a column of packed fiber glass then through two steam sterilized 0.2 µm filters (Gelman). The pH was measured with a combination electrode (Ingold Inc.) and controlled at 7.0±0.05 with the addition of a 3N KOH solution by a pH controller (Chemcadet). Antifoam A (Sigma No. A-5633) was added to the reactor when needed. The medium used for all reactor experiments was LB with 0.5 g/l glucose. The pH of the medium was adjusted to 4.0. This acted as a second pH control and helped prevent the growth of contaminants in the feed medium. In all experiments the reactor ran for 20 hours before inoculation to ensure the sterility of the system.

2.3.1 Inocula Preparation

A loopful of stock culture, cells stored in the agar slants, was transferred to a 250 ml shaker flask containing 100 ml LB medium plus 2 g/l glucose. In the case of plasmid pBR322 ampicillin and tetracycline were added to final concentrations of 100 mg/l and 25 mg/l, respectively. During exponential phase a small aliquot of culture, 20 - 100 µl, was transferred to another flask. After a minimum of five transfers a 10 ml sample was inoculated into the fermentor. After inoculation, cultures were grown in a batch mode for approximately two hours before the start of the continuous experiment.

2.3.2 Continuous Experiments

Continuous experiments were carried out in a 1 liter ferementor (VirTis) with a 0.5 liter working volume. A schematic diagram of the experimental apparatus is shown in Figure 2-3. The medium flow rate was controlled by peristaltic pumps (Masterflex) and was monitored daily by measuring the effluent from the reactor. In the control experiments the flow rate, therefore the dilution rate, was kept constant. In the cycling experiments the culture was exposed to forced perturbations of the dilution rate. This was accomplished by the addition of an extra feed pump. The pump was automatically controlled by a timer (Intermatic Master Control). The dilution rate for each experiment is detailed in the results section. Samples of the fermentor broth were taken twice a day to determine the percentage of plasmid-containing cells and every other day to analyze the enzyme activity and the plasmid copy number. Samples were also stored in 50% sterile glycerol at -20°C for subsequent tests.

2.3.3 Continuous Optical Density Measurement

The optical density (OD) of the fermentation broth was monitored periodically for several hours during the course of the cycling experiments. The OD was measured at 540 nm using a spectrophotometer (Sequoia-Turner Model 340). The

spectrophotometer was interfaced to an IBM PC AT microcomputer for automated data acquisition using an A/D converter (Data Translation). In addition, data were collected with the use of a strip chart recorder (Omniscribe). As the optical density of the fermentation broth under CSTR conditions was very high (about 7 optical units), a reduced optical path length was not able to bring the measurement into the linear range [126]. Therefore, a constant stream of the sample was diluted by an appropriate amount of sterilized distilled water before flowing through the optical cell. It was found that a dilution of 1 ml of culture/ 4.1 ml of water was needed for an OD between 0.2-0.6. A diagram of the dilution apparatus and the experimental set-up are shown in Figures 2-4 and 2-5, respectively.

2.4 Analytical Techniques

2.4.1 Plasmid Stability Analysis

The percentage of plasmid-containing cells was determined by their ability to form colonies in ampicillin supplemented plates. The plates consisted of LB medium containing 2 g/l glucose, 15 g/l agar, and when desired 100 mg/l ampicillin. Three methods were employed to determine the plasmid-containing population. In the first method, diluted samples from the fermentor were spread, using a glass rod, on LB agar plates, with and without ampicillin. Each plate was

triplicated to reduce experimental error. The quantity of cells on the plates, after incubation for 12 hours at 37°C, was compared to determine the percentage of plasmid-carrying cells. This technique is referred to as spread plating [69].

The second technique is referred to as replica plating [188]. In this method a velvet pad is used to transfer 100-200 colonies from an LB plate without ampicillin, the master plate, to a plate containing 100 mg/l ampicillin. The cells that cannot form a colony after a fixed period of time, plates were examined 12 hours after the transfer, on the ampicillin plate are assumed to be plasmid-free. This procedure was performed twice, using two different master plates, and the average was used to calculate the percentage of plasmid-containing cells.

The last method is a form of replica-plating and is referred to as stick replication. In this method a sterilized applicator stick was used to transfer colonies, one per stick, from a master plate to plates with and without ampicillin. These plates were marked with a grid labelled 1 to 60. A total of 60 colonies were then transferred to the corresponding space. After the plates were incubated at 37°C for 12 hours they were checked for cell growth. In general, all 60 cells grow on the plates without ampicillin. As such, these plates were used as the new master plate. Cells that were not able to form a colony on the ampicillin plates were

then transferred, using stick-replication, from the new master plates to the corresponding space on the ampicillin plates. For example, if there is no apparent growth after 12 hours of the initial transfer then the cell colony in space number 3 on the new master plate is again transferred to space number 3 on the ampicillin plate. This is referred to as the second application and was done to ensure that the absence of growth on the ampicillin plate was due to the lack of the drug resistance capability conferred by the plasmid in the cell and not to faulty transfer, i.e. absence of cells on the stick during cell transfer. After the second application, the plates were incubated for an additional 12 hours at 37°C before the number of colonies was scored.

2.4.2 Distribution Analysis

Stick replication was used to determine the distribution of the plasmid-containing population. Colonies were transferred to a series of plates containing different ampicillin concentrations. The concentrations were 25, 50, 100, 500, and 2000 mg/l ampicillin and 25 mg/l tetracycline. The minimum inhibitory concentration (MIC) for ampicillin for the plasmid pBR322 falls in the range of 4000-6000.

2.4.3 β-lactamase Analysis

As β-lactamase is the plasmid gene product for the plasmid pBR322 the activity of this enzyme is indicative of the cell's plasmid content. The β-lactamase activity was determined by the iodometric method [196,199]. This method is based on the reduction of iodine by penicilloic acid, the product of penicillin hydrolysis by β-lactamase. The reduction of iodine results in a colorless solution. This decrease in optical density can be used to determine the β-lactamase activity.

Cells (10 ml) were harvested from the reactor and placed on ice for 10 - 20 minutes. The cells were then centrifuged for 20 minutes at 3400 rpm (Adams Tabletop Centrifuge). The cells were washed with 5 ml (per 10 ml culture) of a 50 mM phosphate buffer, pH 7.0, containing 0.14 M NaCl, 10 mM $MgSO_4$, and 100 ml of 0.1 M phosphate buffer diluted to 200 ml with distilled water, and centrifuged for 5 minutes at 3400 rpm. After repeating this step the cells were suspended in 20 ml of 0.1 M phosphate buffer. The buffer contained 39 ml of a 0.2 M KH_2PO_4 and 61 ml of a 0.2 M $Na_2HPO_4 \cdot 7H_2O$ diluted to 200 ml with distilled water. Cell lysis was performed by sonication (Model W-225, Ultrasonics Inc.) at 30 second bursts for a total of 2 minutes. The lysate was centrifuged for 30 minutes at 15,000 rpm and 4°C in a Sorvall RC-5B centrifuge.

A fixed-time assay, the iodometric test, was used to determine the β-lactamase activity. Test tubes containing 0.1 M phosphate buffer, pH 7.0, and 0.5 ml of 10,000 benzylpenicillin (Sigma No. PEN-K)/0.5 ml distilled water were equilibrated at 37°C. An appropriate volume of cell lysate supernatant, usually 0.3 or 0.5 ml, was added to the test tubes. The final volume of the solution was adjusted to 3.5 ml by the addition of the phosphate buffer. In addition to the reaction tubes two controls were needed. Control or blank A contained only 3.5 ml of 0.1 M phosphate buffer. Blank B contained the phosphate buffer and penicillin to which the enzyme was not added until after incubation and the iodine reagent had been added. The volume of the phosphate buffer and enzyme added in blank B depended on the volume used in the reaction tubes.

After the enzyme was added to the reaction tubes the solution was incubated at 37°C for an appropriate time, usually for 0-10 minutes. The reaction was stopped by adding 5 ml iodine reagent to the tubes. The iodine reagent was prepared by adding 5 ml of stock iodine solution to 95 ml of acetate buffer, pH 4.0. The stock iodine solution contained 0.32 N iodine and 1.2 M potassium iodide diluted to 500 ml of distilled water. The solution was stored in a brown bottle at 4°C. The acetate buffer consisted of 80 g anhydrous sodium acetate adjusted to pH 4.0 with acetic acid and made up to

2 l with distilled water.

The tubes were stored at room temperature for 30 minutes after the reaction had been stopped. The optical density of each tube at A_{490} was measured using a Baush & Lomb Spectronic 1001. Each assay was duplicated. The β-lactamase activity was defined as the quantity that hydrolyzes 1 μmole of benzylpenicillin per minute at 37°C and was calculated using the following formula:

β-lactamase activity (μmole/ml min) (2-1)

$$= \frac{\triangle O.D.}{O.D.\text{-blank A}} \times \frac{40}{F} \times \frac{1}{T} \times \frac{1}{V}$$

Where $\triangle$O.D. = O.D. of blank B minus O.D. sample and O.D.-blank A is the O.D. of blank A. F is the consumed moles of iodine per moles of hydrolyzed substrate. The value of F for benzylpenicillin G is 4. T is the reaction time in minutes and V is the volume (ml) of enzyme solution added to the assay.

2.4.4 Protein Analysis

The Lowry assay was used to determine the protein concentration of the cell sample. This assay is one of the most sensitive and is valid up to 300 μg of protein. Two additional important points must be made about this assay. First, the biggest source of error in this assay is the

incomplete mixing of the reagents. Each tube must be thoroughly and rapidly mixed as soon as each reagent is added. Second, the assay is slightly nonlinear. This problem was eliminated by proper dilutions of the standards and samples. The correlation coefficient of each standard curve was also kept at 0.99.

Cells were harvested as described in the previous section. Two series of tubes were prepared. The first set served as the protein standard. An albumin (human) and globulin solution (Sigma No. 540-10) or albumin (bovine) solution (Sigma No. P-6529) was used as the protein standard. The set consisted of 5 tubes each containing 1.2 ml of solution. The series contained 1.2 ml water, this was used as the blank, 60, 120, 240, and 300 µg of protein standard per tube. The second series of tubes contained the cell lysate supernatant. The set consisted of 5 tubes containing either 0.6, 0.7, 0.8, 0.9, and 1.0 ml of lysate at the beginning of the experiment or 0.2, 0.3, 0.4, 0.5, and 0.6 ml of lysate throughout the rest of the experiment. All samples were brought to a final volume of 1.2 ml by the addition of distilled water.

4.0 ml of fresh alkaline copper reagent was added to each tube. The reagent was prepared by adding, in order, 1 ml of 1% $CuSO_4 \cdot H_2O$, 1 ml of 2% sodium tartrate, and 98 ml of 2% alkaline carbonate solution (2% Na_2CO_3 in 0.1 N NaOH). Each

tube was mixed immediately and thoroughly with a vortex mixer (Thermodyne 37600) after the addition of the reagent. After incubation, for 10 minutes at room temperature, 0.3 ml of a 2 N Folin-Ciocalteu phenol reagent (Sigma No. E-9252) was added and immediately mixed to each tube. The tubes were incubated at room temperature for 30 minutes. The absorbance of the standard and assay tubes were read at A_{650} against the blank. For each analysis the protein assay was duplicated.

2.4.5 Chromosomal and Plasmid DNA Analysis

A modified method of Frenkel and Bremer was used to determine the relative plasmid copy number [61]. 4 $O.D._{460}$ units of cell culture were harvested and centrifuged for 10 minutes at 15,000 rpm. 0.4 $O.D._{460}$ units were used for analysis of the chromosomal DNA. An $O.D._{460}$ unit is defined as:

$$O.D.\ unit = vol\ (ml) \times Abs\ at\ 460 \qquad (2\text{-}2)$$

Without disturbing the pellet the supernatant was removed. The pellet was then washed with 0.5 ml of ice cold STE buffer. The buffer contained 0.1 M NaCl, 10 mM Tris Cl, pH 7.8, and 1 mM EDTA and was stored at room temperature. The suspended pellet was centrifuged a second time and the supernatant was removed. The pellet was then frozen at -20°C

for 5 hours or longer. After thawing for 10 minutes at 37°C the cells were treated with 150 µl of a EDTA-RNase-Lysozyme solution for 15 minutes at 37°C. The solution was made fresh and contained 10 mM EDTA, pH 8.0, 40 µg/ml of RNase (Sigma No. R-5503), and 150 mg/ml lysozyme (Sigma No. L-6876). 150 µl of 2% $NaDodSO_4$ (SDS) was added for cell lysis. The mixture was heated and thoroughly mixed for 20 minutes at 65°C. The lysate was stored at 4°C for approximately 12 hours.

Gel electrophoresis was used to analyze the chromosomal and plasmid DNA. 75 µl of a loading buffer (60% sucrose and 0.5% bromophenol blue, stored at 4°C) was added to the lysate. 30 µl aliquots of this solution were loaded into 1.5 x 5 mm wells in a 0.8% horizontal agarose gel in a TAE buffer. The gel was made by adding 0.27 g of agarose (Sigma No. A-9539) to 33.75 ml (6.75ml solution/thickness of gel) boiling TAE solution. The mixture was cooled to 65°C before ethidium bromide (EtdBr) was added to a final concentration of 1 µg/ml. Ethidium bromide was also added to the TAE buffer at the same final concentration. 300 ml of the buffer was used in each electrophoresis cell. The TAE buffer contained 4.84 g Tris, 1.14 ml glacial acetic acid, and 2 ml 0.5 M EDTA, pH 8.0, diluted to a final volume of 1 liter with distilled water.

Electrophoresis was performed for 1.5-2.0 hours at approximately 70 V (approximately 9 v/cm gel length). A

Foto/Phoresis I system from Fotodyne was used. After destaining in distilled water for 8 hours the fluorescent DNA bands were photographed over a 300 nm transilluminator. Polaroid 665 B&W Positive/Negative film was used. The negative of the resulting gel was scanned by a densiometer (E-C Apparatus Corp.). The area of each peak was used to calculate the relative plasmid copy number by a procedure outlined in Lewington and Day [130]. The relative copy number was determined from the following equation:

$$\text{copy number} = \frac{C_s \times [(P_c \times 1.41) + P_o]}{P_s \times C_b \times \text{dil}} \qquad (2\text{-}3)$$

where C_s = chromosomal size, 3900 Kb for *E. coli* [133]. The size of the *E. coli* genome has been estimated to be within the range of 3500 to 4700 Kb [135]. P_c = the brightness or area of the covalently-closed-circle (CCC) plasmid, P_o = the brightness of the open-circle (OC) plasmid, P_s = plasmid size, 4.3 Kb for pBR322, C_b = brightness of the chromosome, and dil = dilution factor of the chromosome lysate, factor was 10 for all experiments. 1.41 is the conversion factor to convert a CCC band to its linear equivalent as the CCC band does not bind to the EtdBr dye as well as its linear form. There are many factors that effect the accuracy of the data. These include: trapping of the plasmid in chromosomal, staining and destaining, linearity of fluorescence signal as a function of

the amount of DNA, effect of the molecular topology on the EtdBr binding to the DNA, intensity of the chromosomal DNA fluorescence which can bleach the negative making measurement on the same scale as the plasmid very difficult, and loss of sample in the submerged gel. However, as all samples were treated identically, or as close as possible, and an average of three samples, both plasmid and chromosome, were used to calculate the plasmid copy number relative to the chromosome content, some of the problems caused by the above factors are minimized. It should be pointed out that in this work the interest is more in the behavior or trend of the plasmid content during the experiment and not in absolute numbers.

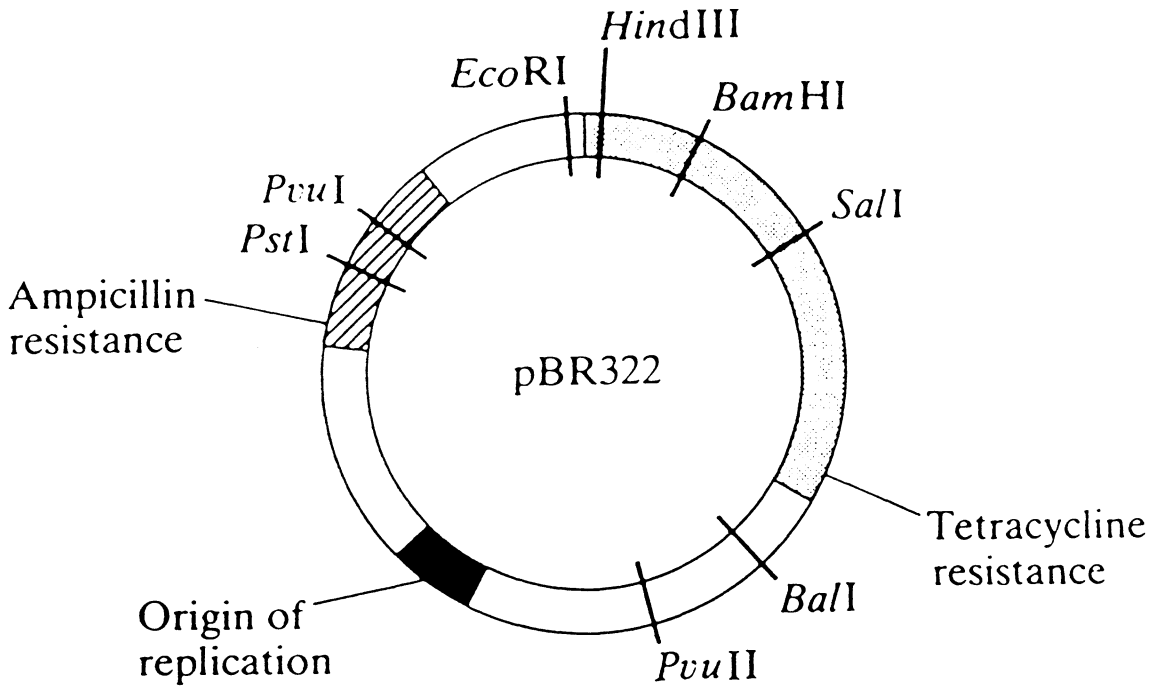


Figure 2-1. Map of the plasmid pBR322 showing the origin, antibiotic resistance genes, and some of the unique restriction enzyme sites, adapted from Bailey and Ollis [10]. The complete sequence of the plasmid has been documented. The construction and a more detailed desription map of the plasmid is described by Bolivar et al. [17].

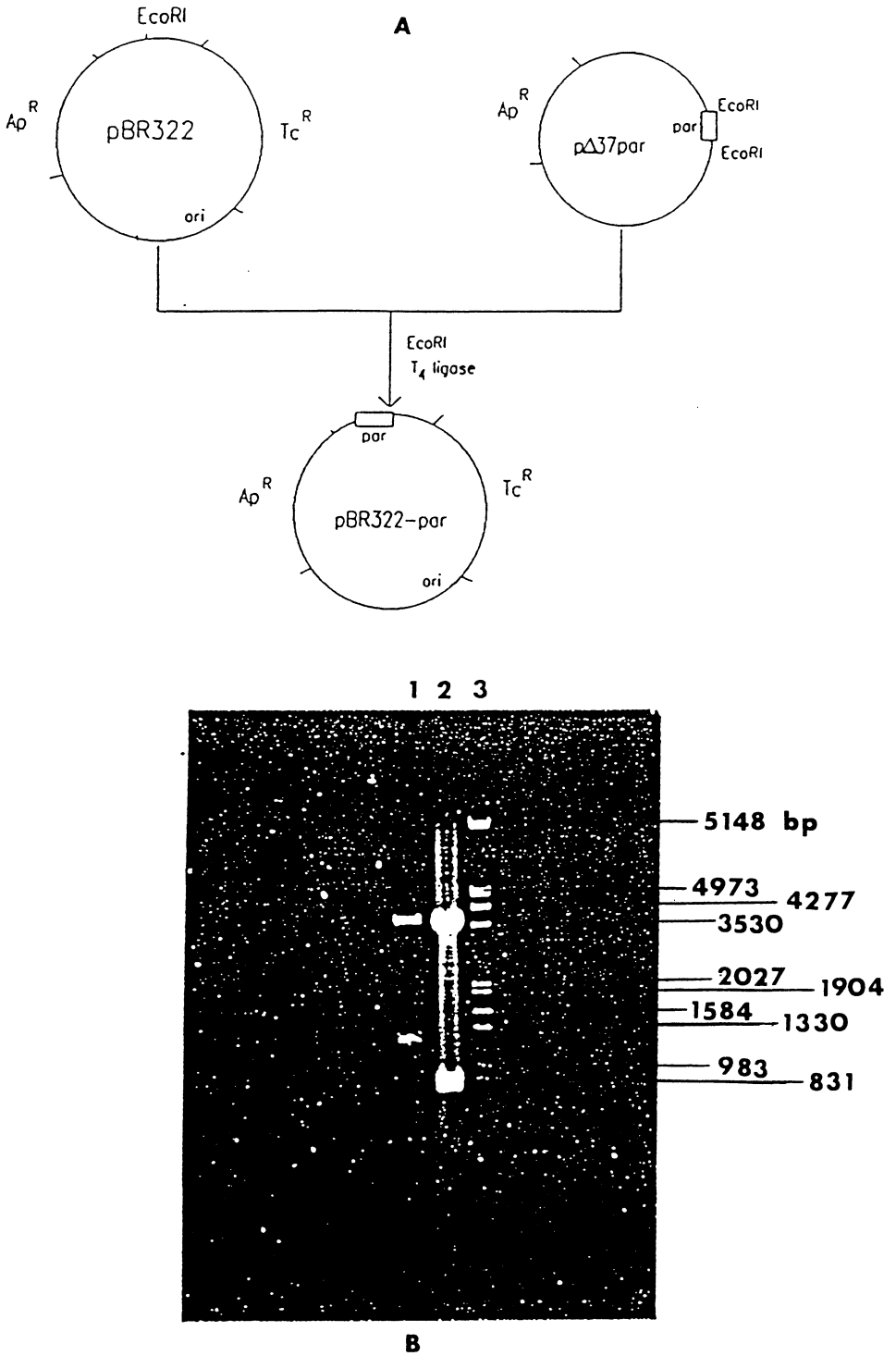


Figure 2-2. (a) Construction of the plasmid pBR322*par*. (b) Verification of the plasmid by gel electrophoresis. Lane 1 is pBR322*par* digested with PstI and HindIII. Lane 2 is pBR322 digested with the same enzyme. Lane 3 is the molecular weight marker.

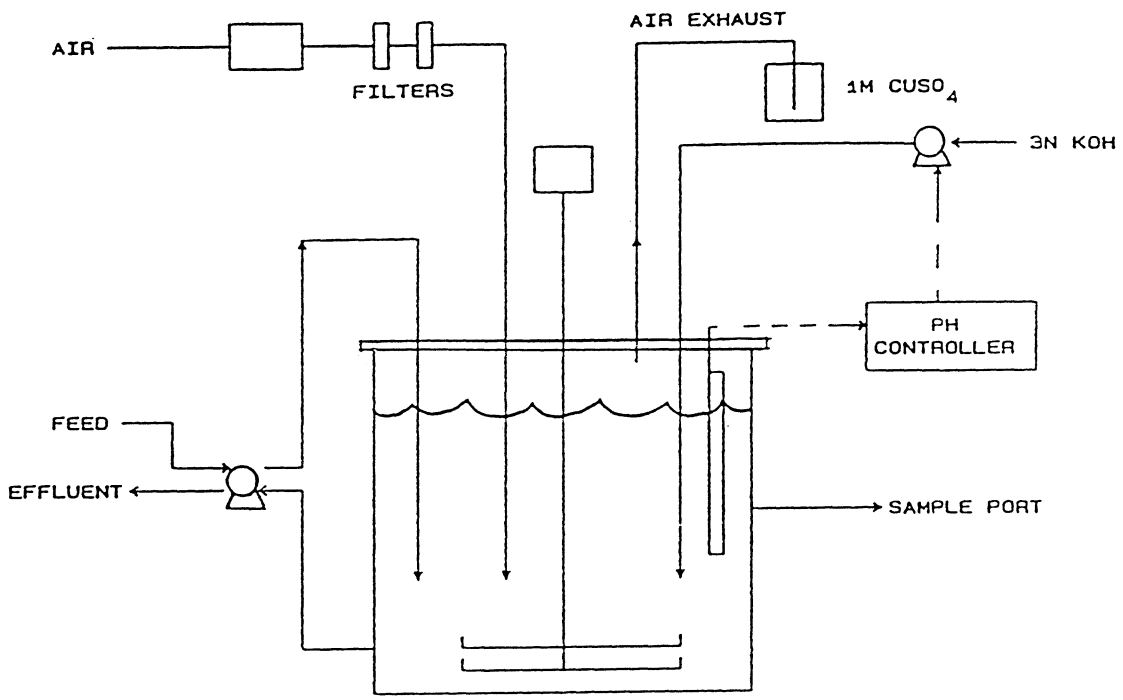


Figure 2-3. A schematic diagram of the experimental apparatus used in the constant dilution rate experiments. A single pump with two pump heads was used for both the medium feed and the effluent.

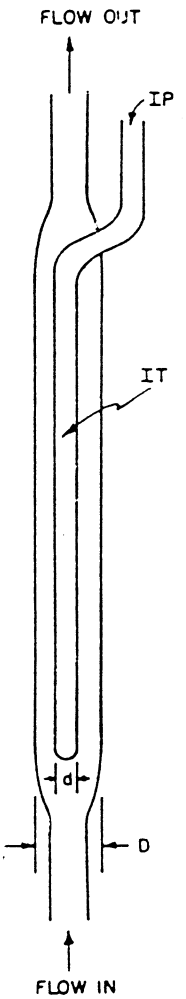


Figure 2-4. Diagram of effective dilution device used for the on-line measurement of the OD of the effluent during continuous experiments. The diameter of the inner tube, d, is 6mm. The diameter of the flow cell, D, is 13mm

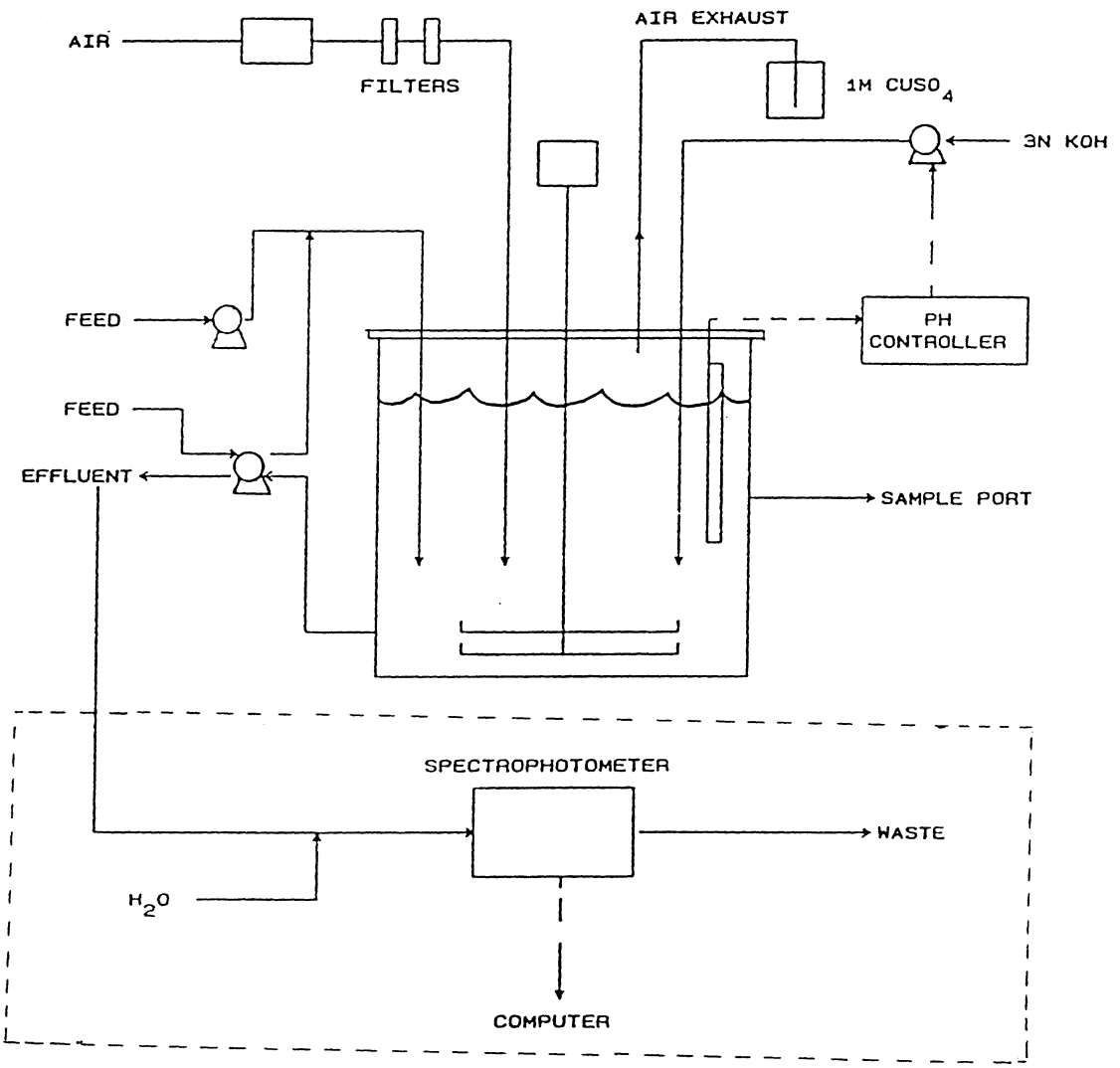


Figure 2-5. A modified version of the experimental apparatus. The additional pump was used to create a cycling effect of the dilution rate. The effluent was diluted with sterile distilled water before the OD of the culture was measured.

CHAPTER 3

PERSISTENCE AND EXPRESSION OF THE PLASMID pBR322 GROWN IN A CHEMOSTAT UNDER CONSTANT DILUTION RATES

3.1 Introduction

Plasmid stability is one of the potential factors that affects the productivity of the recombinant populations. Therefore, the understanding of various determinants affecting plasmid maintenance is of both fundamental and practical importance. The question of plasmid stability has been studied by a number investigators using either batch or chemostat cultures [2,42,92,114,161]. In general, it is observed that two major parameters, the probability of plasmid loss due to segregation during cell division and the difference in the specific growth rate between the recombinant and the reverted cells, govern the dynamics of the reactor. These two parameters, in turn, are affected by a number of factors: the genetic make-up of the host cell and the plasmid and the reactor operating parameters such as temperature, pH, and the growth medium [257].

The focus of this chapter will be on the effect of these factors, specifically growth in a complex medium and the structure of the plasmid, on the maintenance and expression of a plasmid-containing population grown in a chemostat. In addition, a procedure is developed to provide a quantitative

assessment of the two major parameters.

3.2 Effect of Medium Composition on the Maintenance of the pBR322 Plasmid

The percentage of plasmid-containing cells in a population of *E. coli* was determined during continuous culture with a complex medium. The dilution rates were kept constant at either 0.15, 0.31, 0.45, or 0.61 hr^{-1}. The results are shown in Figure 3-1. Except for the culture at the dilution rate of 0.15 hr^{-1} all experiments were repeated. The data between the runs generally showed good agreement.

The basic behavior of the culture is similar to that reported in the literature. The percentage of plasmid-carrying cells drops after a long lag period. However, the population becomes more stable , i.e. the lag time increases, as the dilution rate decreases. This result is quite different from those obtained with cells grown in defined medium with a limiting substrate, either under glucose or phosphate-limited conditions. Wouters, et al., [254] found that in a phosphate-limiting reactor the rate of plasmid loss decreased with an increase in the dilution rate. Moreover,it was observed that cells under phosphate limited growth showed a more rapid loss of plasmid than cells grown under glucose-limited conditions [72,111]. The disagreement between results is possibly due to the medium in which the plasmid-

containing cells are cultured. In a defined medium with limiting substrate, the lower the concentration of the growth-limiting substrate, that is at low growth rates, the greater the growth advantage of the plasmid-free cells [254]. In a rich medium, as used in our experiments, this is not the case. In fact, as summarized in Table 3-1, the lower the growth rate the smaller the growth advantage of the plasmid-free cells. This would indicate that the rate of plasmid loss is affected by the medium composition of the culture [246,254].

An additional observation can be made about the data from these experiments. That is a persistence of plasmid-containing cells at a relatively low percentage, specifically at D = 0.31 and 0.45 hr^{-1}. Similar observations have been reported in the literature data [72,149,254]. It is unclear why the plasmid-carrying cells are stably maintained at these levels when normally the uncompetitive populations should be eliminated. It is possible this phenomenon is an artifact of the system resulting from nonhomogeneous conditions of the reactor or wall growth. Addition of cells from the wall into the stirred liquid acts as a second nonsterile feed and can prevent washout of the plasmid-containing cells [24,97,172]. Wall growth was observed in most runs during the later stages of the experiment. It is also possible that a mechanism may exist controlling the persistence of low levels of plasmid-containing cells. This would be necessary for the long-term

survival of the population.

As mentioned previously, the loss of plasmid-containing cells generally occurs only after a lag period. This period corresponds to the time it takes before a population of competitive plasmid-free cells become detectable. It is possible to shorten this lag time, as demonstrated by inoculating an aliquot of plasmid-free cells into the reactor. At approximately 48 and 120 hours 1 and 5 ml aliquots of host cells were inoculated into the fermentor, respectively. The plasmid-containing cell reverts back to the host cell by completely shedding its plasmid. The host cell used in this experiment was isolated from a set of replica plates in which the original bacterial strain, the plasmid-containing cell, was not able to grow on the agar plates containing ampicillin. The lack of plasmid in the isolated host cell was further confirmed with gel electrophoresis experiments.

The introduction of plasmid-free cells on the control, $D = 0.31\ hr^{-1}$, compared to the previous run is shown in Figure 3-2. This observation is consistent with the results obtained by Jones et al. [110]. It was observed that the difference in specific growth rates between the plasmid-carrying cells and their naturally arising plasmid-free cells is smaller than that for the isogenic cells. However, we found this same discrepancy in our system in which a naturally arising plasmid-free segregant was used as an inocula. This is

evident in the differences between the specific growth rates shown in Table 3-1. Therefore, it would appear as though the occurrence is independent of the source of the plasmid-free cells. There is, however, some other mechanism that produces a lesser degree of competitiveness in the nondisrupted system than in the system where plasmid-free cells are added. Further discussion of this phenomenon will be presented in Chapter 5.

In most systems the plasmid-free cells arise due to the instability of the plasmid. There are two major types of plasmid instability [54,168]. The first, segregational instability, refers to the loss of the complete plasmid due to defective partitioning during cell division [145,170]. The second, structural instability, is caused by a change in plasmid structure due to the insertion, deletion, or rearrangement of the DNA, which can result in the loss of the desired gene function or plasmid replication [79,185]. The probability of the loss of plasmid is an important parameter when considering the stability of a plasmid-containing system.

Once the plasmid-free cells appear competition between these cells and the plasmid-containing cells is started. Competition is one method of interaction between two different species of cells [10,23,90]. This type of interaction is based on the competition of the two species for a common factor such as medium, oxygen, light, or other limiting supply. It has been shown, through experimental results and

mathematical models, that the species with the faster growth rate will dominate the culture as it can utilize the limiting source quicker than the other species. The competitive exclusion principle states when two species compete in a common environment one species will reach a limiting population size while the other species will eventually be washed out [10]. In the case of a continuous culture the dilution rate, in addition to the growth rate of the organisms, appears to be an important factor in determining the washout rate and which species will dominate and which will disappear from the culture [147]. In a culture containing plasmid-containing and plasmid-free cells the plasmid carrying-cells generally demonstrate a growth disadvantage under non-selective environments. This growth disadvantage results in the washout of the plasmid-containing cells.

Many theories have been proposed to explain the relative growth advantage of plasmid-free over plasmid-containing cells. One possible cause, is the additional burden on the host cell to synthesize plasmid genes and their products. This could produce a difference in the maximum specific growth rates for the two cell populations [72]. In an experiment performed by Zünd and Lebek, out of 101 resistant plasmids tested approximately three quarters reduced the growth rate of the host cell [259]. Another speculation is that the addition of the plasmid might also alter the nutritional

requirements resulting in changes in the specific growth yields [114].

To date, there has been little attempt to quantitate the magnitude of either the probability of plasmid loss, due to only segregation instability, or the difference in the specific growth rate between the plasmid-free and plasmid-containing cells. It is possible, based on a model developed by Imanaka and Aiba [99], to provide a quantitative assessment of these two parameters for experimental data.

3.3 Data Analysis of Plasmid Maintenance in a CSTR

3.3.1 Problem Formulation and Analysis

The scheme proposed by Imanaka and Aiba [99] describing a mixed-culture system with plasmid-carrying and plasmid-free cells is given by the following three "reactions". This scheme is valid only for non-conjugational plasmids. That is, the plasmid is not capable of being transferred from one cell to another.

$$X^+ \xrightarrow{\mu^+} (2 - p)X^+ \qquad (3\text{-}1a)$$

$$X^+ \xrightarrow{\mu^+} pX^- \qquad (3\text{-}1b)$$

$$X^- \xrightarrow{\mu^+} 2X^- \qquad (3\text{-}1c)$$

X^+ and X^- denote the plasmid-carrying and plasmid-free species, μ^+ and μ^- are the specific growth rate of the plasmid-carrying and plasmid-free cells, respectively, and p is the probability of plasmid loss. According to this scheme, the dynamics of X^+, X^-, and the concentration of the substrate in a CSTR can be obtained by simple mass balance and are given by a set of ordinary differential equations:

$$\frac{dx^+}{dt} = (\mu^+ - D)x^+ - p\mu^+x^+ \qquad (3\text{-}2a)$$

$$\frac{dx^-}{dt} = (\mu^- - D)x^- + p\mu^+x^+ \qquad (3\text{-}2b)$$

$$\frac{ds}{dt} = D(s_f - s) - \frac{\mu^-x^-}{Y^-} - \frac{\mu^+x^+}{Y^-} \qquad (3\text{-}2c)$$

where x^+, x^-, and s are expressed in grams per liter, D is the dilution rate in reciprocal hours, Y^+ and Y^- are the substrate yields, and s_f is the inlet concentration of the limiting substrate.

The equation governing the dynamics of the concentration of total cell mass in the reactor, x_T, defined as

$$x_T = x^+ + x^- \qquad (3\text{-}3)$$

can be obtained by adding equation 3-2a to 3-2b:

$$\frac{dx_T}{dt} = \mu_+x^+ + \mu^-x^- - Dx_T \qquad (3\text{-}4)$$

It should be pointed out that the total cell mass in the reactor may not be a constant, especially when the cell yields

for the two populations are different. Such a phenomenon has been reported in the literature and has also been observed in our work. In this case, the fraction of plasmid-carrying cells, y^+, defined as

$$y^+ = \frac{x^+}{x_T} \qquad (3\text{-}5)$$

cannot be obtained by simply dividing equation 3-2a with x_T. Instead, the time derivative of equation 3-5 has to be determined. The result, after algebraic manipulations, is

$$\frac{dy^+}{dt} = y^+[(\mu^+ - \mu^-)(1 - y^+) - p\mu^+] \qquad (3\text{-}6)$$

$$= y^+[\delta\mu(1 - y^+) - p\mu^+]$$

where

$$\delta\mu = \mu^+ - \mu^-$$

is the difference in the specific growth rate between the plasmid-containing and plasmid-free cells. A negative value of $\delta\mu$ indicates the growth advantage of plasmid-free over plasmid-carrying cells.

In general, equation 3-6 cannot be solved analytically. However, it is of interest to note that the dynamic behavior of y^+ is dictated by the sign of $\delta\mu$. If $\delta\mu > 0$, a nontrivial coexistence steady-state exists. Under these conditions, the fraction of plasmid-carrying cells is given by

$$y^+ = 1.0 - p\mu^+/\delta\mu \qquad (3\text{-}7)$$

On the other hand, if $\delta\mu \leq 0$, the plasmid-carrying cells will eventually be displaced by the plasmid-free cells [99].

In this work, only the case in which $\delta\mu \leq 0$ will be considered. If it is assumed $\delta\mu$ and $p\mu^+$ remain relatively constant during the course of the experiment, equation 3-6 can be integrated analytically, yielding a closed-form solution. The result is given by

$$y^+/(\delta\mu - p\mu^+ - \delta\mu y^+) = [y_o/(\delta\mu - p\mu^+ - \delta\mu y_o)] \times e^{(\delta\mu - p\mu^+)t} \qquad (3\text{-}8)$$

where y_o is the initial fraction of plasmid-containing cells in the inoculum.

In most experiments, the inoculum contains 100% plasmid-containing cells, i.e., $y_o = 1$. Therefore, equation 3-8 can be simplified further to

$$y^+/\delta\mu - p\mu^+ - \delta\mu y^+) = -[1.0/p\mu^+]e^{(\delta\mu - p\mu^+)t} \qquad (3\text{-}9a)$$

or

$$y^+/[1.0 - y^+ - (p\mu^+/\delta\mu)] = -[\delta\mu/p\mu^+]e^{(\delta\mu - p\mu^+)t} \qquad (3\text{-}9b)$$

Equation 3-9b provides a relationship between the experimentally observable quantity y^+ and the two parameters $\delta\mu$ and $p\mu^+$. Because of the nonlinearity involved, it is not a trivial task to obtain an estimate for these two parameters from the experimental data, y^+.

However, for a relatively stable plasmid system, the rate of plasmid loss is much smaller than the difference in the specific growth rate. That is,

$$-\delta\mu \gg p\mu^{+} \qquad (3\text{-}10)$$

When the quantity of plasmid-free cells in the reactor is appreciable, the following inequality will be valid:

$$1.0 - y^{+} \gg - p\mu^{+}/\delta\mu \qquad (3\text{-}11)$$

and equation 3-9b can be approximated by the equation

$$y^{+}/(1.0 - y^{+}) = y^{+}/y^{-} = -[\delta\mu/p\mu^{+}]e^{(\delta\mu - p\mu^{+})t} \qquad (3\text{-}12)$$

As a result, the slope and the intercept from a semilog plot of y^{+}/y^{-} vs. time will provide an estimate for the parameters $\delta\mu$ and $p\mu^{+}$. Specifically, the following relationships hold:

$$\delta\mu = \text{slope}/[1.0 + e^{-\text{intercept}}] \qquad (3\text{-}13a)$$

$$p\mu^{+} = \delta\mu - \text{slope} \qquad (3\text{-}13b)$$

3.3.2. Simulation Studies

A series of computer simulation studies were performed to evaluate the proposed data reduction algorithm. In these simulations, the set of differential equations 3-2, with the initial conditions given in Table 3-2, were integrated numerically. The specific growth rate of both populations in equations 3-2a and 3-2b is assumed to be of Monod type with

the corresponding parameters given in Table 3-2. The results are shown in Figure 3-3, in which $\ln(y^+)$ is plotted against time. Three different sets of simulations, with various rates of plasmid loss, ranging from 0.001 to 0.00001 hr^{-1}, are presented. The general behavior of the simulations is similar to that shown in Figure 3-1. The percentage of plasmid-containing cells drops after a long lag time. As expected, the higher the rate of plasmid loss, the faster the appearance of plasmid-free cells. This is evident by the difference in lag time. It should be pointed out that even at the later stage of the experiment, when the plasmid-containing cells are diminishing at a rapid rate, the plot is not a straight line.

The simulated results are then plotted as $\ln(y^+/y^-)$ against time in Figure 3-4. It must be noted that equation 3-12 is not valid when y^+ is equal or close to 1. In fact, the expression y^+/y^- is undefined when $y^+ = 1$. For simplicity, an arbitrary large number is used as the upper limit when the data fall within this range. Furthermore, this portion of the data set will be ignored in the calculation of the slope and intercept from Figure 3-4. The linear portion of the curves in Figure 3-4 was fitted with a standard least-squares program yielding a "best" estimate for the intercept and the slope. These results and the "true values", the values used initially to derive the simulated plots, are

compared in Table 3-3.

The proposed model, as demonstrated by the preceding results, provides an estimation of the value of $\delta\mu$ within 5% error. The accuracy of the other parameter value, the rate of plasmid loss, seems to depend on its magnitude. The most accurate estimate occurs when the rate of plasmid loss is the lowest. This trend is expected as one of the assumptions in the proposed scheme is that $\delta\mu >> p\mu^+$. Nevertheless, the largest discrepancy is still within 15%.

3.3.3 Applications to Experimental Results

The proposed method is applied to a culture of *E. coli* containing the plasmid pBR322 grown in a chemostat containing nonselective medium. The plot of the percentage of plasmid-containing cells against time at two different dilution rates, $D = 0.31$ and $0.46\ hr^{-1}$, is shown in Figure 3-5. The exact cause for the lack of smoothness for the data set at $D = 0.46\ hr^{-1}$ is unclear. It may be due to some unintentional perturbation in the reactor system. Similar oscillations have been observed when the reactor system is under forced perturbations [247].

A plot of the same set of experimental data, $\ln(y^+/y^-)$ vs. time, is shown in Figure 3-6. It can be seen that the data show a linear trend after 150 hours. The linear section of the curves was fitted with a least-squares algorithm to

obtain the best estimate of the intercept and the slope. The two parameters, the rate of plasmid loss and the difference in the specific growth rate, for the two dilution were then calculated by using equations 3-13a and 3-13b. The results are summarized in Table 3-1. The difference in the specific growth rate between the plasmid-containing and plasmid-free cells in the chemostat is rather small, with the high dilution rate, D = 0.46, producing a slightly larger value. It is of interest to note that such a small increase in the growth advantage is enough to cause a much faster rate of displacement of the plasmid-containing population by the plasmid-free cells, as seen in Figure 3-4. In addition, although the rate of plasmid loss is similar in the two dilution rates, both are in the order of 10^{-4} hr^{-1}, the difference between the two values produces a difference in the lag time. The results, however, indicate that the plasmid system is rather stable, substantiating the approximation made in equation 3-12.

Figures 3-7a and 3-7b show the experimental results and model prediction of plasmid maintenance in a chemostat for the two dilution rates. The model prediction was obtained by integrating equation 3-6 with the parameter values calculated previously, Table 3-1. In both cases, the model prediction provides an excellent fit to the experimental data.

The sensitivity of the model to the variations in model parameters is also examined. In this study, the simulated system in which the parameters deviate from the experimental parameters is compared with the experimental results.

The results, with a 10% deviation of the parameter $\delta\mu$ from the experimental value, are shown in Figure 3-8. It can be seen that a small error in this parameter leads to quite a large disagreement between the model prediction and experimental data. A 10% error in the growth advantage resulted in a curve that suggests either a much faster or a much slower disappearance rate of the plasmid-containing cells. On the other hand, the model is quite insensitive to a small error in the rate of plasmid loss as shown in Figure 3-9. However, when this parameter value is off by an order of magnitude, a large discrepancy between the model prediction and experimental data can be observed, Figure 3-10.

The results from the preceding sensitivity study indicate that a good agreement between the model prediction and experimental data requires an accurate estimate of the parameter $\delta\mu$. The accuracy of the other parameter, $p\mu^+$, must be better than one order of magnitude. In general, the two-parameter model together with the estimated parameter values, provide an excellent fit to the experimental data.

3.4 Effect of the Dilution Rate on the Cloned Gene Product

In addition to plasmid stability the expression of the cloned gene product must be considered in the utilization of a plasmid-containing system. There are many factors that affect the expression of the cloned gene. On the molecular level these include: promoter efficiency, ribosome binding efficiency, stability and location of the product in the host cell, the codons used to encode the product, the metabolic state of the cell, and the stability and copy number of the plasmid [71]. In turn, these factors are effected by the environment, such as temperature, pH, and medium, and consequently the growth rate of the culture. The focus of this section is on the relationship between the growth rate and the cloned gene activity.

The enzyme synthesis of a microbial culture will respond to a change in growth or dilution rate in five ways [148]. There will either be 1) no change in the enzyme activity, 2) the enzyme activity will increase with an increase in dilution rate, 3) the activity will decrease with an increase in the dilution rate, 4) the activity will pass through a maximum at a certain dilution rate, or 5) the enzyme activity may pass through a minimum at a particular dilution rate. Previous work has shown that the plasmid product activity does vary with the dilution rate [186,205]. An optimum dilution rate at which a maximum product concentration existed was observed.

The specific activity of β-lactamase, based on the total soluble protein present, as a function of time at various dilution rates is shown in Figure 3-11. Despite the culture containing 100% plasmid-containing cells during the initial phase of the experiment the specific activity never attains a steady-state. Instead it reaches a maximum value near 100 hours. Although this phenomena has been observed in other studies [7,160], it is still surprising. If the presence of the plasmid poses a burden on the cells, as is generally believed, in the absence of antibiotic pressure it is expected that the β-lactamase activity would remain constant or even decrease over a period of time, not increase.

It can be observed that the specific activity does depend on the dilution rate. Within the first 200 hours the highest activity occurs at D = 0.26 hr^{-1}. After this period the specific activity shows a rapid decrease for all dilution rates except at 0.31 hr^{-1}. This decrease is mainly due to a drop in the percentage of plasmid-containing cells. As the culture grown under the dilution rate of 0.31 hr^{-1} is more stable, Figure 3-1, it is expected that the activity will drop more slowly.

A comparison of the total expression of the β-lactamase enzyme by the culture at various dilution rates is complicated by the fact that the steady-state cell concentration in a chemostat differs at each dilution rate. A reasonable unit,

defined as β-lactamase activity/ml culture/OD, is used for further comparative purposes. This unit can be related to the activity of β-lactamase per equivalent dry cell mass. The result with respect to time is shown in Figure 3-12. The overall behavior is similar to that seen in Figure 3-11.

A comparison between the stability of the plasmid-containing culture and the β-lactamase activity is shown in Figure 3-13. It can be seen that the decrease in the percentage of plasmid-containing cells lags behind the decrease of β-lactamase activity. A more detailed discussion of this result is given in Chapter 5.

3.5 Effect of the Dilution Rate on Plasmid Copy Number

The plasmid copy number is mainly a function of the genetic make-up of the plasmid [154,162,201], but can also be strongly affected by the host cell physiology and growth conditions. It has been reported that the copy number decreased as the growth rate increased for the plasmid R1 in an *E. coli* host [57]. Similar results using different plasmids have also been reported [203]. As the results were obtained by employing different media to simulate a change in in the growth rate it was not certain whether the change in copy number was due only to a change in the growth rate or a result of the nutrient effects. However, later experiments [186,205] performed with a chemostat culture supported the results. Similar studies performed with the plasmid pLP11 in

the host, B. stearothermophilus, showed contradictory results [120]. It is speculated that this discrepancy is due to a difference between the plasmid replication mechanisms.

The work presented in this section is on the effects of different dilution rates on the plasmid pBR322 in E. coli. A comparison of the relative plasmid copy number, plasmid DNA/chromosome DNA, for three dilution rates is shown in Figure 3-14a. The plasmid copy number has also been reported on a per cell basis, Figure 3-14b, or a per cell mass basis, Figure 3-14c [133]. The behavior shown in all three graphs is similar. The results in Figure 3-14c are not unforeseen. On a per cell mass basis, it has been demonstrated that at dilution rate lower than D = 0.60 hr^{-1} the plasmid copy number is not greatly influenced by the dilution rate [186]. In this work the relative plasmid copy number will be based on the chromosome DNA. This is for comparative purposes with respect to cells grown under transient conditions.

In general, our data agree with previously reported behavior. The copy number appears to be affected by the dilution rate. However, due to the error involved with this procedure the results are not conclusive. The change in copy number with dilution rate could be caused by the dilution effects of its replication inhibitor RNAI and preprimer RNAII [133]. Lin-Chao and Bremer reported the activities of the RNAI and RNAII promoters and the efficiency of the RNAI

inhibition of plasmid replication, are dependent on the growth rate of the culture. Similar results involving the copy number control gene *copB* from plasmid R1 have been reported by Light and Molin [131].

The decrease in copy number as time progresses should also be noted. This decrease resembles the trend seen previously in the stability analysis in which a culture grown under a dilution rate of 0.31 hr^{-1} were more stable.

A comparison between the percentage of plasmid-containing and relative copy number is shown in Figure 3-15. It is easily observed that as the percentage of plasmid-carrying cells decrease so does the average copy number. These results suggest that plasmid instability is due to segregational effects rather than structural changes in the gene expressing the enzyme β-lactamase. In addition, gel electrophoresis experiments indicated that there was no substantial structural changes in the plasmid.

There is an obvious relationship between the plasmid copy number and the cloned gene activity. It has been shown that as the copy number increased the product also increased [239]. This is often referred to as the gene dosage effect. In general, the correlation between the copy number and cloned gene product activity in LB medium can be described by a model proposed by Seo and Bailey [203]. The rate of product formation, r_p, is calculated as a function of the plasmid copy

number per cell, n_p, as shown in equation 3-14

$$r_p = k_p n_p (1 - c n_p) \qquad (3\text{-}14)$$

where k_p and c are constants.

At low copy numbers, $cn_p << 1$, the relationship is linear. This has been observed for other plasmids [239]. It is postulated at high copy numbers the correlation between copy number and productivity is invalid. This is probably due to the limitations on transcription and translation. In addition, the host cell can become nonviable when the plasmid content, as in runaway systems, becomes too high [238].

The dependence of the specific β-lactamase activity on the plasmid copy number is shown in Figure 3-16a. A linear trend is observed for both dilution rates. As the data are time dependent this behavior indicates that the transcriptional and translational efficiency of the plasmid does not change substantially during this period. It should be noticed that the trend predicted by the data does not pass through the origin. In this system since the plasmid is the only source of the β-lactamase activity it is expected that the data should pass through this point. It is possible that the behavior at low copy numbers, approximately smaller than 10, is different than that shown in Figure 3-16a. The data within this region could follow a linear trend, with a different slope, or more likely, the data would show a gradual

decrease as demonstrated in Figure 3-16b. Further experiments are needed to make more conclusive comments about the behavior of a culture under these conditions.

In summary, the copy number is primarily determined by the genetics of the plasmid and the growth conditions and influenced by the host cell physiology. In addition, the cloned gene product is affected by the plasmid copy number. Within a limit, cells with high copy numbers are more productive than cells containing low plasmid copy numbers. This, however, may pose a problem when optimizing the system as the plasmid stability also depends on the plasmid copy number. High copy number cultures are usually more stable, with respect to the lag time, than low copy number systems. This is due to a decrease of the probability of plasmid loss. However, the higher the plasmid content the greater the burden imposed on cells resulting in a greater growth disadvantage of these cells. Therefore, with the appearance of a plasmid-free sub-culture a more rapid displacement of the plasmid-containing cells will occur. When considering the utilization of plasmid-containing cells in long-term fermentation systems it is therefore necessary to take the plasmid copy number, stability, and desired productivity into consideration.

3.6 Comparison of the Plasmid Stability of pDM247 and pBR322 Plasmids

To this point the focus has been on the effect of growth

rates on the stability of plasmid-containing cells. It has been shown, however, that different plasmids in the same host exhibit stability differences [110,111]. This section will examine the effect two different plasmids, pDM247 [154] and pBR322, have on plasmid stability, cloned gene product, and plasmid copy number. Plasmid pDM247 was kindly supplied by Dr. Campbell. As both plasmids have similar origins of replication [17,154], differences are assumed to be due more to plasmid copy number and structure than mechanisms of replication.

Experiments were performed in a chemostat with complex medium at a dilution rate of 0.45 hr^{-1}. The percentage of plasmid-containing cells for a culture containing pDM247 is compared to a culture containing the pBR322 plasmid in Figure 3-17. Under these conditions plasmid pDM247 is more stable. This could be due to the difference in the copy number between the pDM247 and pBR322 plasmid. However, the exact mechanism is not known as the two plasmids also differ in structure. As seen in Figure 3-18 the relative plasmid copy number for pDM247 is smaller than that for pBR322. According to the random segregation model, it is expected that a higher plasmid content tends to be more stable, i.e. the probability of plasmid loss due to imperfect segregation during cell division is small. A cell with a smaller plasmid content, therefore, would have a higher probability of plasmid loss. These cells,

though, are expected to be more competitive with the plasmid-free population due to a decrease in the burden imposed by the plasmids. This results in a much slower displacement of the plasmid-containing cells. It is difficult to see in Figure 3-17, but the pDM247 culture demonstrates a very slow decrease in the plasmid-containing population.

The results shown in Figures 3-18, 3-19a and 3-19b suggest the rate of plasmid loss for the pDM247 system is less than that of the pBR322 culture. The copy number and the specific β-lactamase activity for the pDM247 culture is fairly constant through the first two hundred hours while the plasmid content and expression for the pBR322 culture decrease during this period. It also appears, Figure 3-20, as though the pDM247 system is just beginning to show a displacement of the plasmid-containing population. As demonstrated earlier in Figure 3-15 a loss of plasmid-containing cells is preceded by a decrease of the plasmid content. Unfortunately, the experiment was prematurely stopped before this could be observed in the pDM247 culture.

The efficiency of the pDM247 plasmid was also examined. The result is compared with efficiency of the pBR322 plasmid in Figure 3-21. Unfortunately, the pDM247 data could not be plotted against the pBR322 data at the same dilution rate. Based on the assumption that the efficiency for the pBR322 plasmid grown under a dilution rate of 0.45 hr^{-1} falls between

that of 0.31 and 0.61 hr^{-1} the efficiency of the pDM247 plasmid is greater. This is probable as cells with a higher plasmid copy number may express their plasmid genes less efficiently due to competition with the host cell for the needed precursors and metabolic energy.

3.7 Conclusions

To conclude, the most important observations from the control experiments are:

1. The stability of a plasmid-containing population is effected by a number of factors including the growth medium and dilution rate. The rate of plasmid loss decreases with decreasing dilution rate in complex medium. This is different from results obtained with cells grown in a defined medium where plasmid loss was observed to decrease with increasing dilution rate.
2. In all experiments a lag period existed before the plasmid was eventually lost from the population. The lag period could be shortened by the addition of plasmid-free cells.
3. Two major parameters, the probability of plasmid loss due to faulty segregation during cell division and the difference in the specific growth rate between the recombinant and reverted cells, govern the dynamics of the reactor. Based on a previously derived model it is

possible to provide quantitative information on these two parameters.

4. The plasmid gene product is affected by the growth rate. The specific activity varies with the dilution rates with the highest activity occurring at 0.26 hr^{-1}. For all experiments the specific activity never attains a steady-state. Instead it reaches a maximum value near 100 hours. The subsequent decrease may be due to the appearance of plasmid-free cells.
5. The plasmid content is also affected by the growth rate. The relative plasmid copy number increases as the dilution rate decreases. This is consistent with the model prediction.
6. A decrease in the plasmid content and percentage of plasmid-containing cells indicate that plasmid instability is mainly due to segregation effects rather than structural changes in the plasmid gene expressing the product enzyme. In addition, a decrease in the plasmid content and product usually precedes a decrease in the percentage of plasmid-containing cells. The possible causes of this phenomenon will be explored in Chapter 5.
7. Efficiency, the relationship between the plasmid copy number and plasmid expression, of the plasmid is influenced by the dilution rate. A linear relationship

between the copy number and activity indicates that the expression efficiency of the plasmid does not change over time.

8. Stability and gene expression are affected by plasmid structure and copy number. This is illustrated by comparing cultures containing two different plasmids and is consistent with literature data.

Table 3-1. Estimated values for parameters $\delta\mu$ and p from experimental results using equations 3-13a and 3-13b.

Dilution rate (hr^{-1})	Growth advantage (hr^{-1})	Rate of plasmid loss (hr^{-1})
0.15	0.010	3.0×10^{-4}
0.31	0.023	0.3×10^{-4}
0.46	0.025	1.0×10^{-4}
0.61	0.027	1.3×10^{-4}
0.31 + host cells	0.028	0.31×10^{-4}

Table 3-2. Parameter and initial conditions.

Initial Conditions:		
	x_0^+	0.25 g/l
	x_0^-	0.00 g/l
	s_0	0.0005 g/l
Parameter Values:		
	D	0.40 hr^{-1}
	μ_m^+	0.80 hr^{-1}
	μ_m^-	0.84 hr^{-1}
	k_s^+	0.0005 g/l
	k_s^-	0.0005 g/l
	Y^+	0.50 g biomass/g substrate
	Y^-	0.55 g biomass/g substrate
	s_f	0.50 g/l

Table 3-3. Comparison of "true values" with estimated values.

"True Value"		Estimated Value	
Rate of plasmid loss (hr^{-1})	$\delta\mu$ (hr^{-1})	Rate of plasmid loss (hr^{-1})	$\delta\mu$ (hr^{-1})
10.0×10^{-4}	0.02	8.410×10^{-4}	0.021
1.0×10^{-4}	0.02	0.920×10^{-1}	0.020
0.1×10^{-4}	0.02	0.095×10^{-4}	0.020

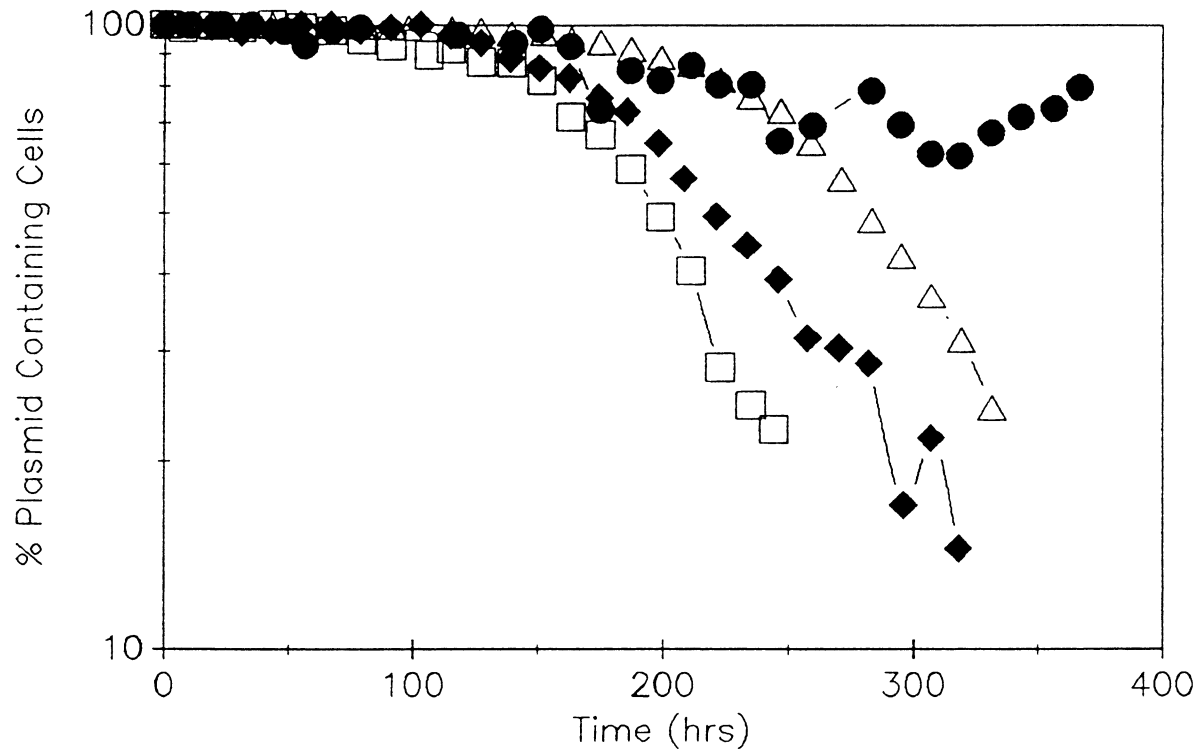


Figure 3-1. Percentage of plasmid-containing cells (pBR322) grown in complex medium at dilution rates of 0.15 (●), 0.31 (△), 0.45 (◆), and 0.61 (□) hr^{-1}.

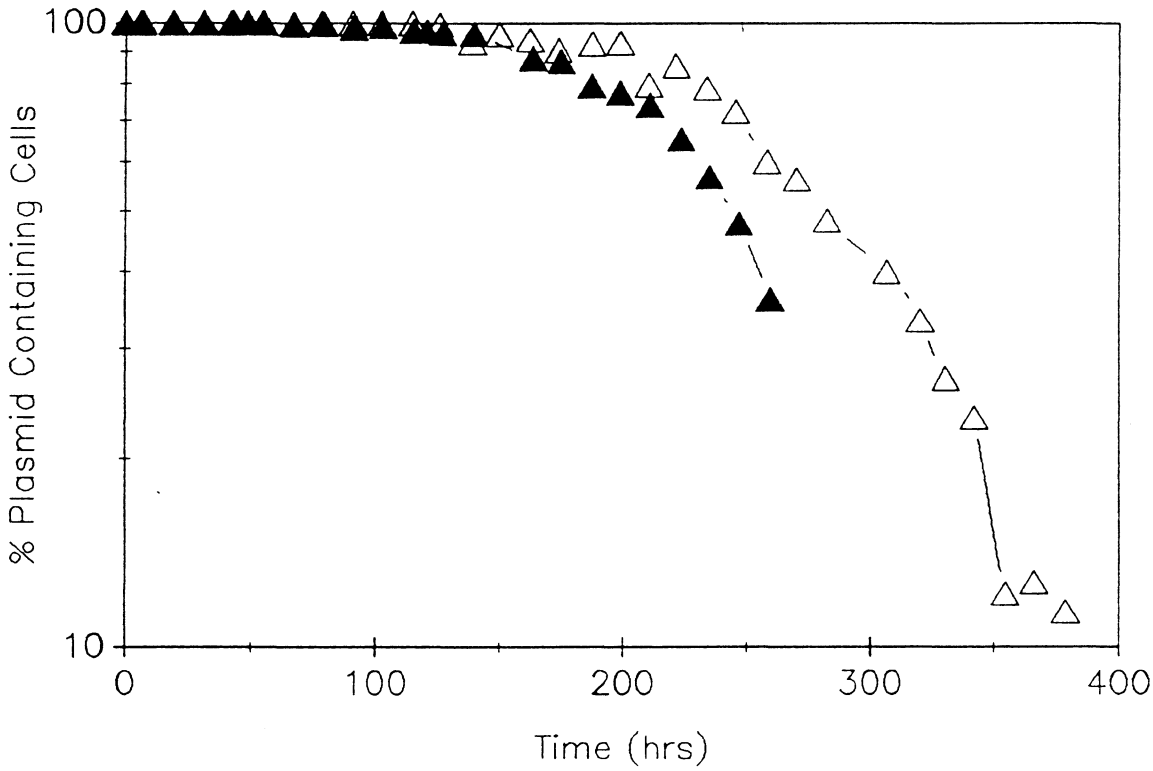


Figure 3-2. The effect of an inoculation of plasmid-free cells on a culture under constant dilution rates, $D = 0.31\ hr^{-1}$, with (▲) and without (△) the addition of host cells.

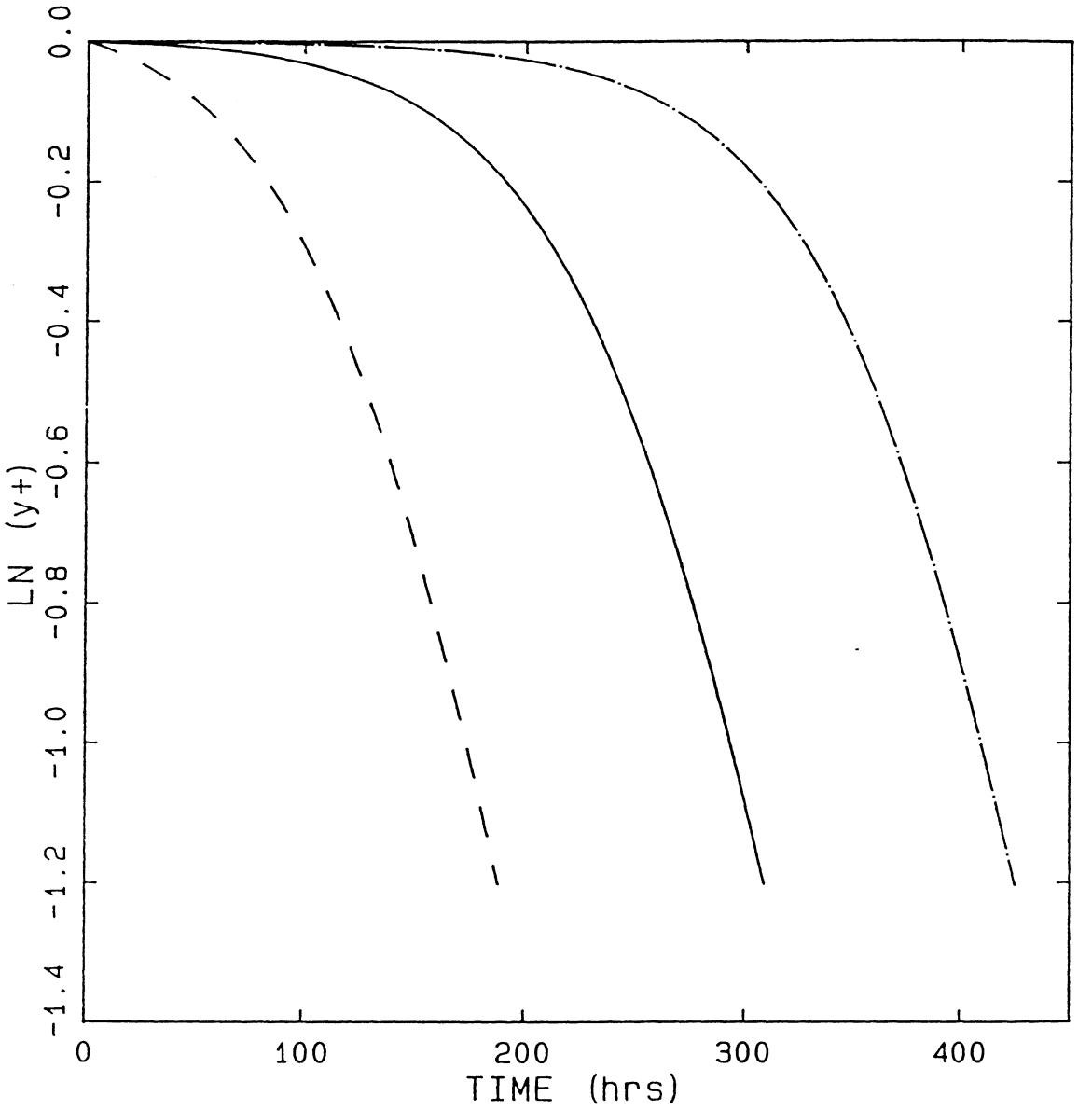


Figure 3-3. $\ln(y^t)$ with time. Simulation studies with different p; p = 0.001 (— —), 0.0001 (——), 0.00001 (-·—·-).

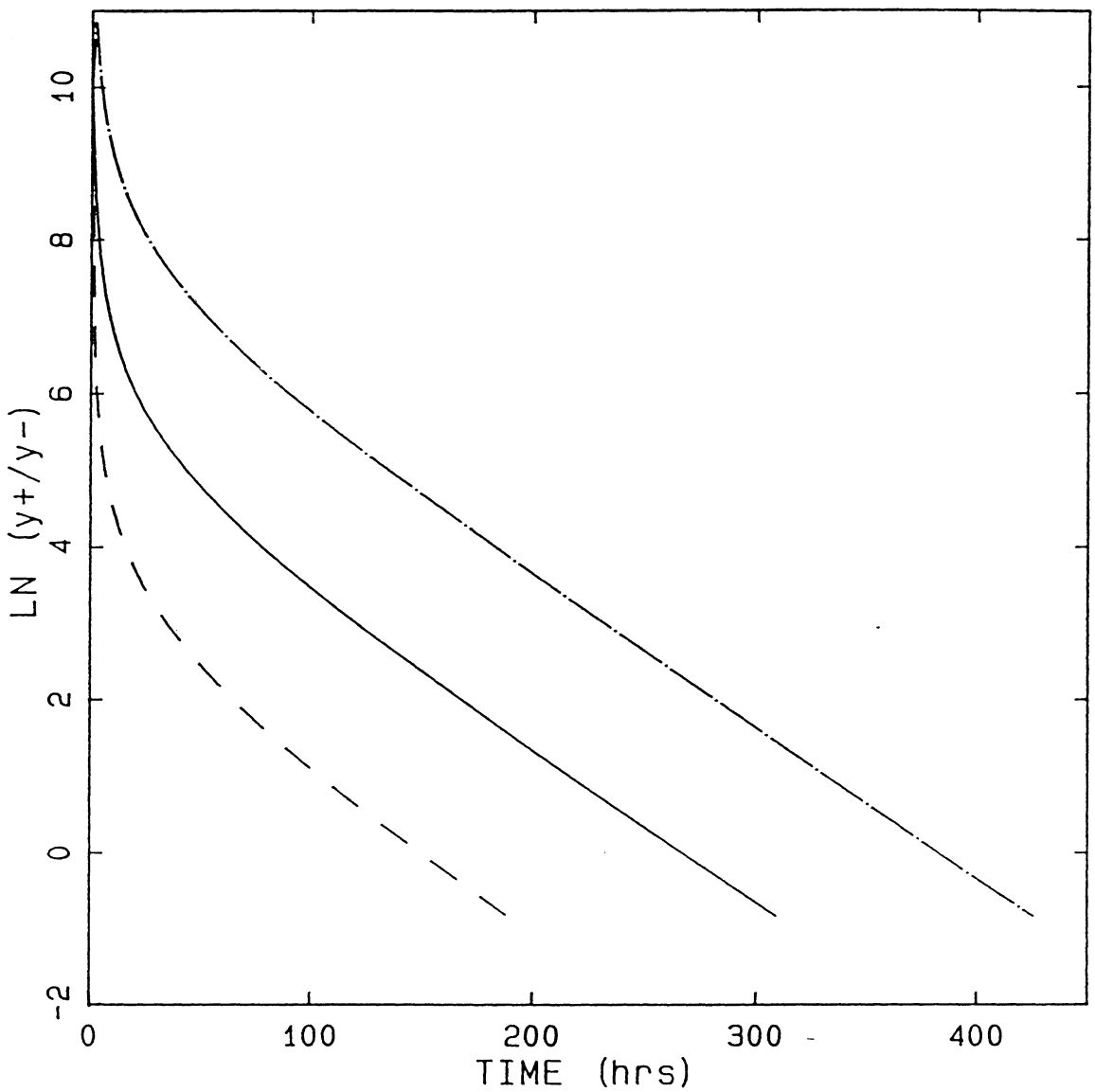


Figure 3-4. $\ln(y^{+}/y^{-})$ with time. Simulation studies with different p; p = 0.001 (— —), 0.0001 (——), 0.00001 (— — —).

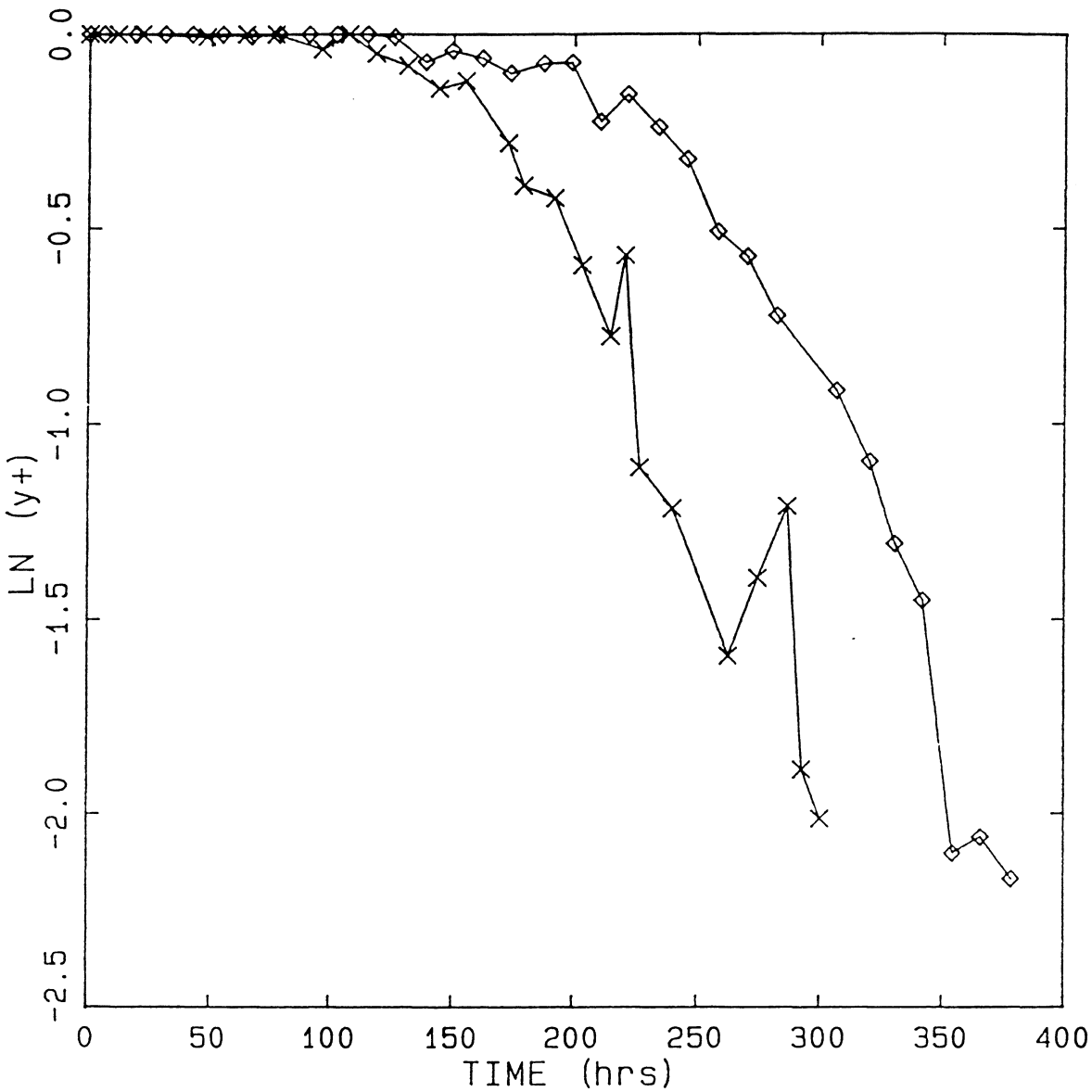


Figure 3-5. $\ln(y^t)$ with time. Chemostat experiments with plasmid-containing cells (pBR322) grown in complex medium at dilution rates of 0.31 hr^{-1} (◇) and 0.46 hr^{-1} (**X**).

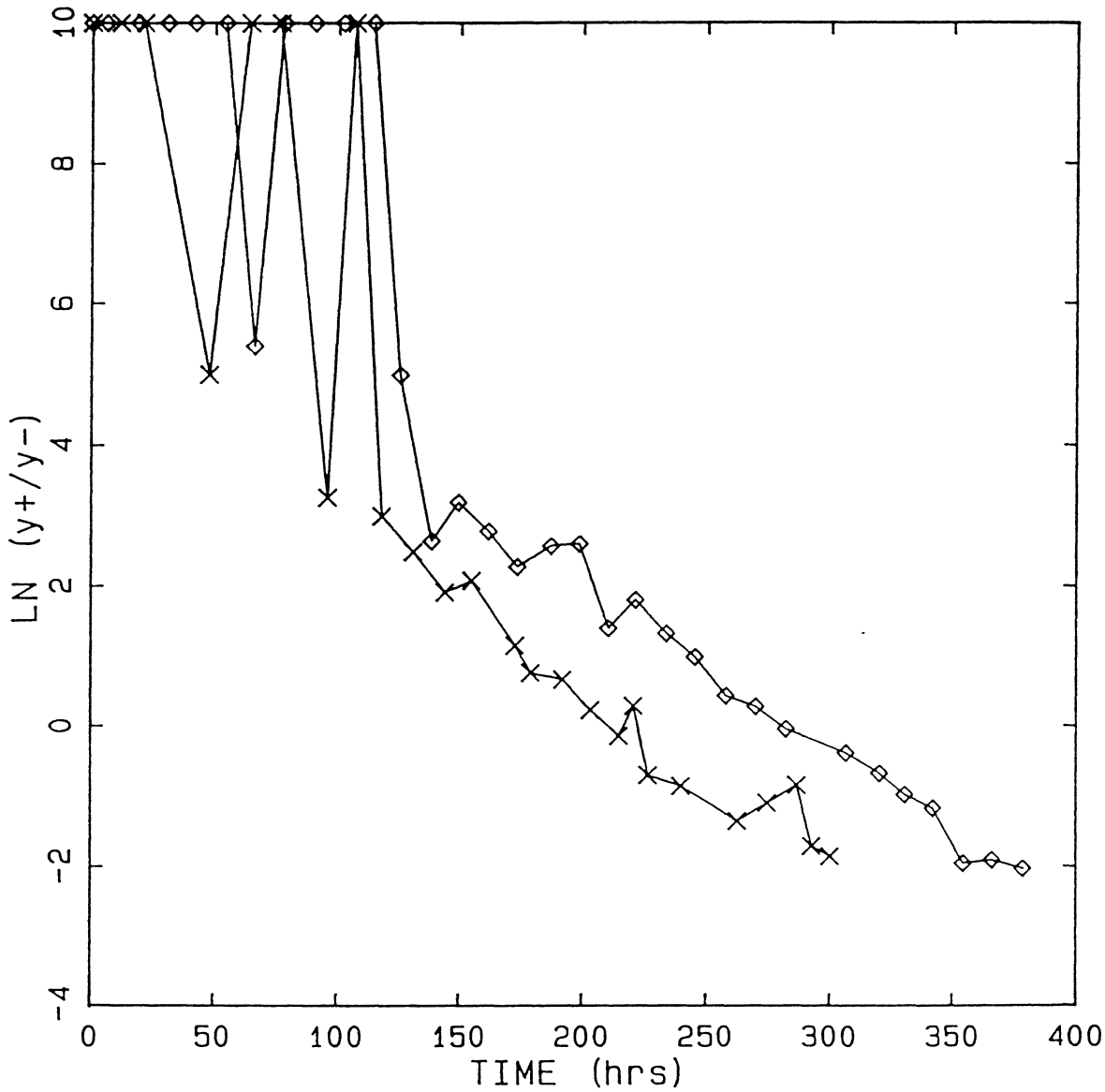


Figure 3-6. $\ln(y^+/y^-)$ with time. Chemostat experiments with pBR322 at D = 0.31 hr^{-1} (◇) and 0.46 hr^{-1} (**X**).

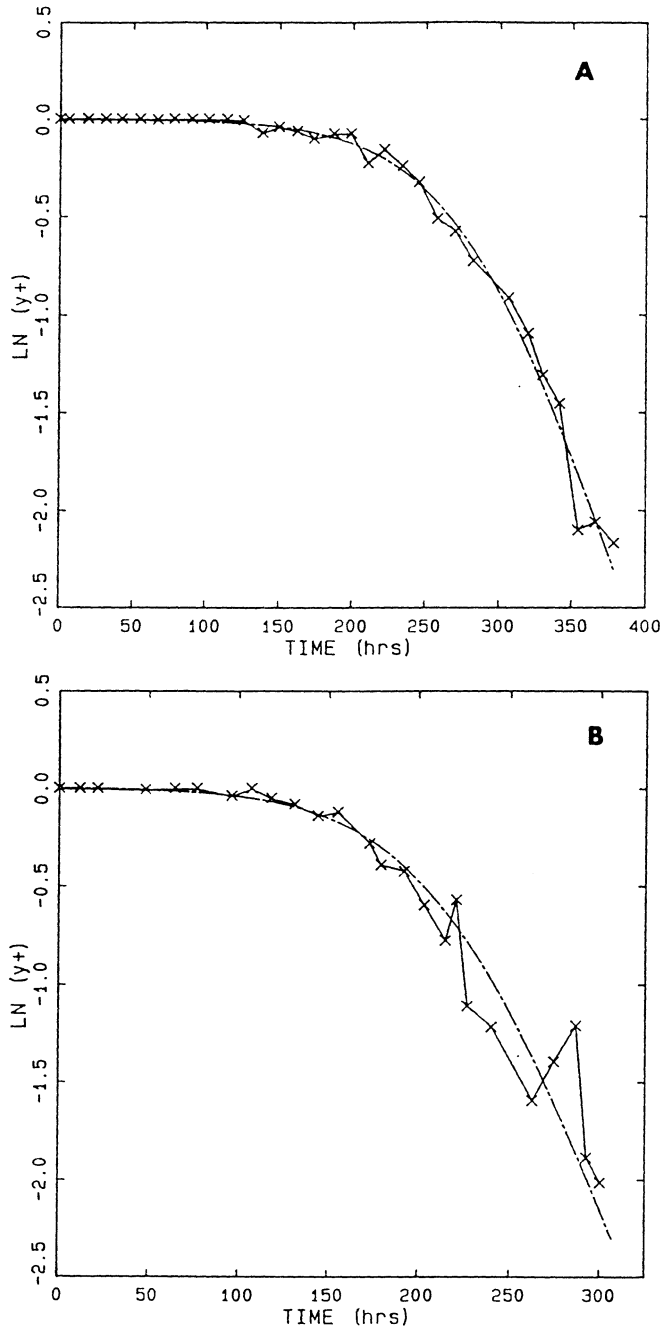


Figure 3-7. (a) Comparison of model prediction with experimental results D = 0.31 hr^{-1}; experimental data (x - x) and the model prediction for $\delta\mu$ = 0.023 and $p\mu^{+}$ = 0.3 x 10^{-4} (— — —). (b) Comparison of model prediction with experimental results D = 0.46 hr^{-1}; experimental data (x - x) and the model prediction for $\delta\mu$ = 0.025, $p\mu^{+}$ = 1.0 x 10^{-4} (— · —).

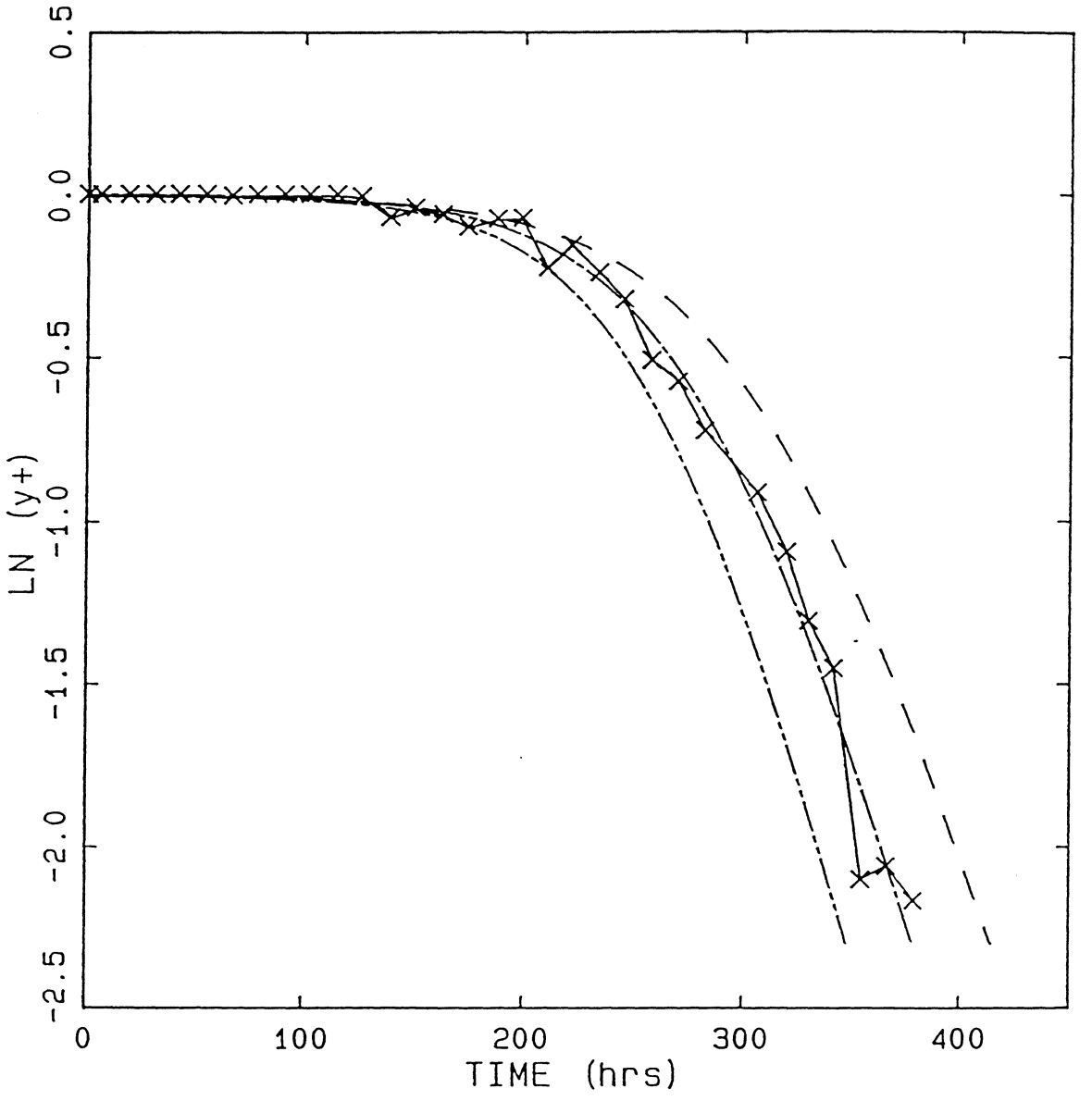


Figure 3-8. Sensitivity of model to parameter variations, ± 10% error in $\delta\mu$ at D = 0.31 hr^{-1}. Experimental data (x - x) and model predictions for $\delta\mu$ = 0.023 and $p\mu^{+}$ = 0.3 x 10^{-4} (—--—), $\delta\mu$ = 0.0253 and $p\mu^{+}$ = 0.3 x 10^{-4} (—·--·—), and $\delta\mu$ = 0.0207 and $p\mu^{+}$ = 0.3 x 10^{-4} (— —).

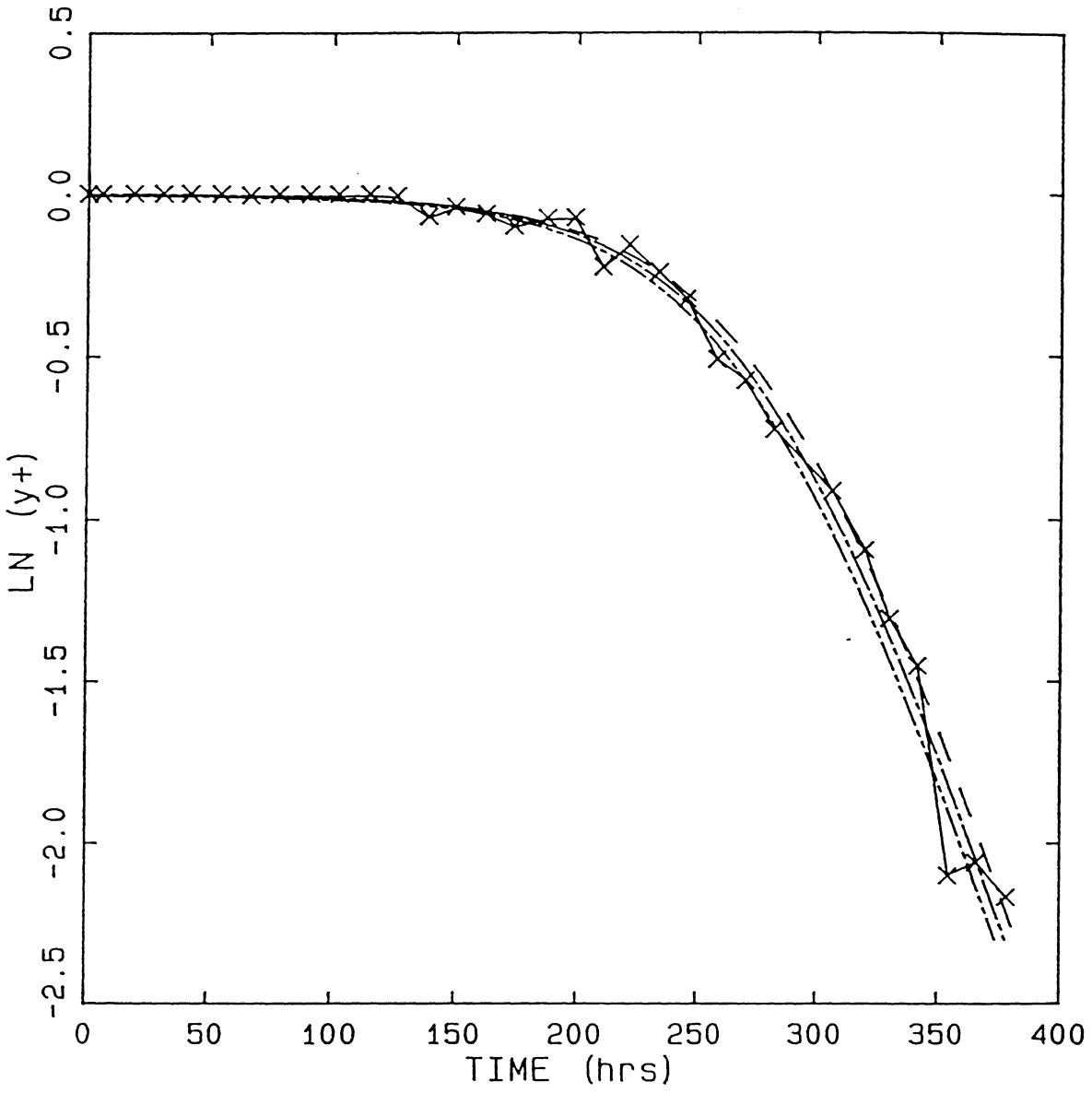


Figure 3-9. Sensitivity of model to parameter variations, ± 10% error in $p\mu^+$ at D = 0.31 hr^{-1}. Experimental data (x - x) and model predictions for $\delta\mu$ = 0.023 and $p\mu^+ = 0.3 \times 10^{-4}$ (— — —), $\delta\mu$ = 0.023 and $p\mu^+ = 0.33 \times 10^{-4}$ (— · · · —), and $\delta\mu$ = 0.023 and $p\mu^+ = 0.27 \times 10^{-4}$ (— —).

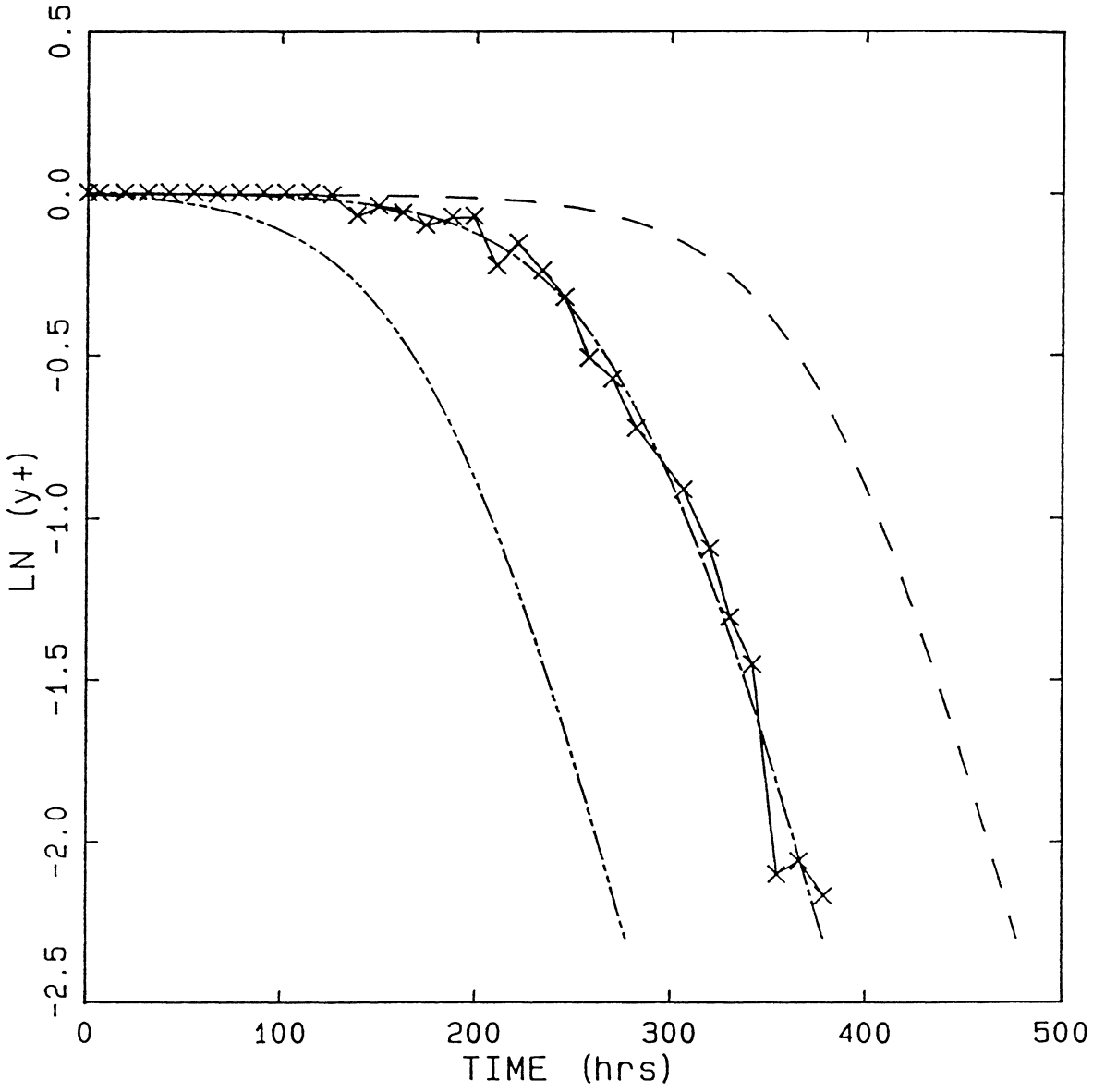


Figure 3-10. Sensitivity of model to parameter variations, one order of magnitude off from estimated $p\mu^*$ at $D = 0.31\ hr^{-1}$. Experimental data (x - x) and model predictions for $\delta\mu = 0.023$ and $p\mu^* = 0.3 \times 10^{-4}$ (—·—), $\delta\mu = 0.023$ and $p\mu^* = 3.0 \times 10^{-4}$ (—··—), and $\delta\mu = 0.023$ and $p\mu^* = 0.03 \times 10^{-4}$ (— —).

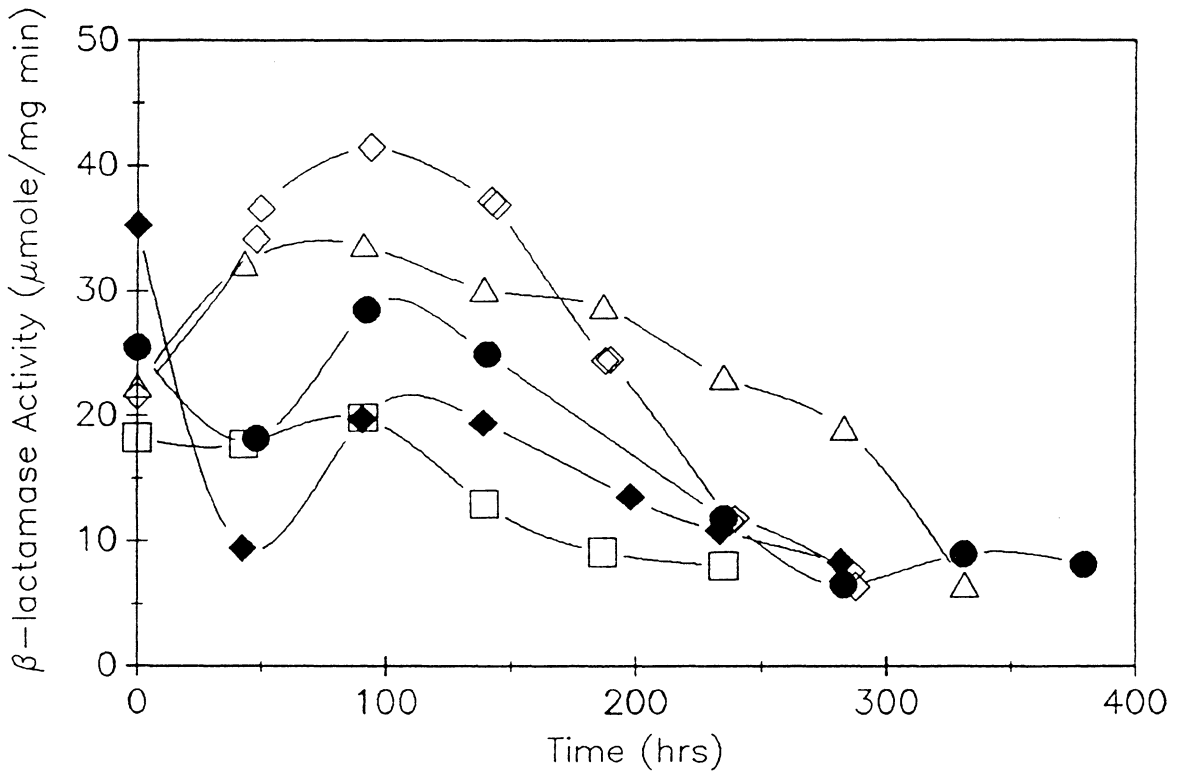


Figure 3-11. Specific β-lactamase activity, based on the total protein present, for a culture of plasmid-containing cells (pBR322) grown in complex medium at dilution rates of 0.15 (●), 0.26 (◇), 0.31 (△), 0.45 (◆), and 0.61 (□) hr^{-1}.

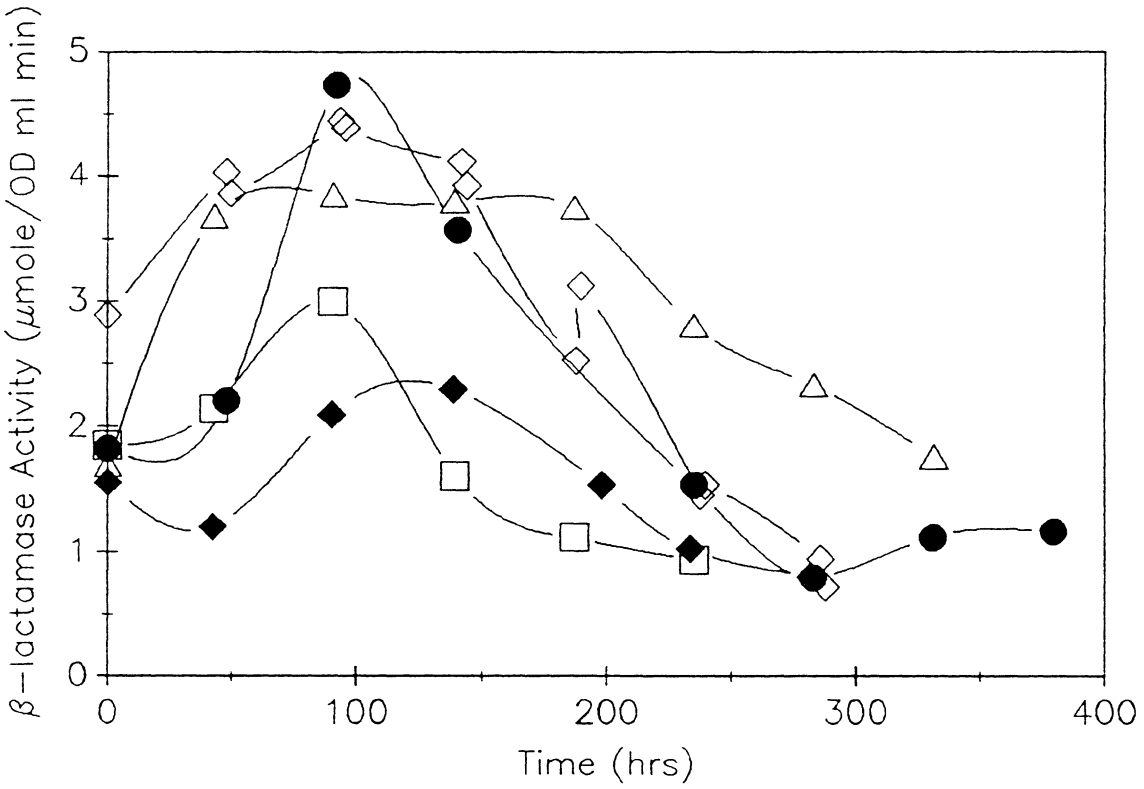


Figure 3-12. β-lactamase activity/ml culture/OD for a culture of plasmid-containing cells (pBR322) grown in complex medium at dilution rates of 0.15 (●), 0.26 (◇), 0.31 (△), 0.45 (◆), and 0.61 (□) hr^{-1}.

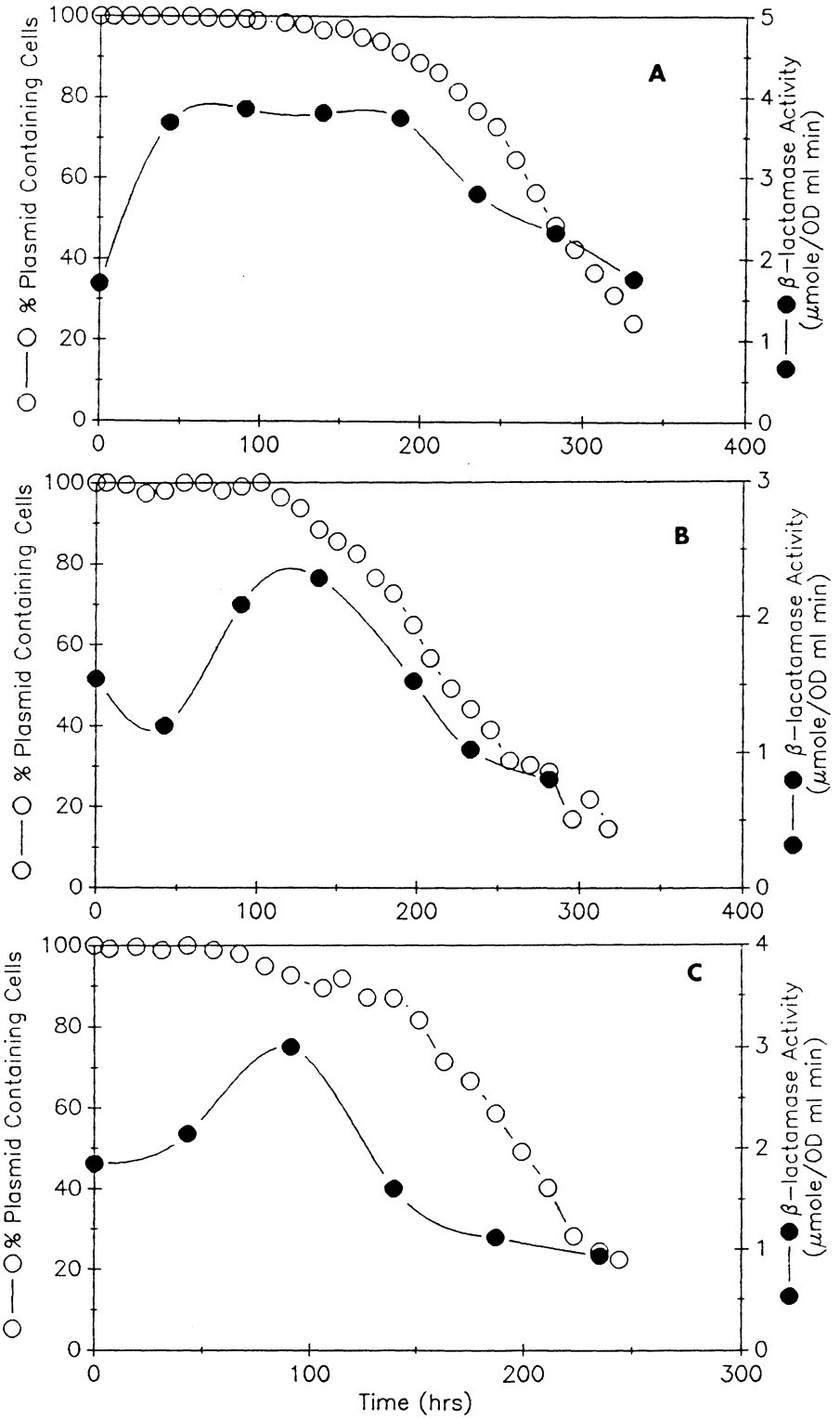


Figure 3-13. Comparison of the percentage of plasmid-containing cells (pBR322) and the β-lactamase activity for (a) D = 0.31, (b) 0.45, and (c) 0.61 hr^{-1}.

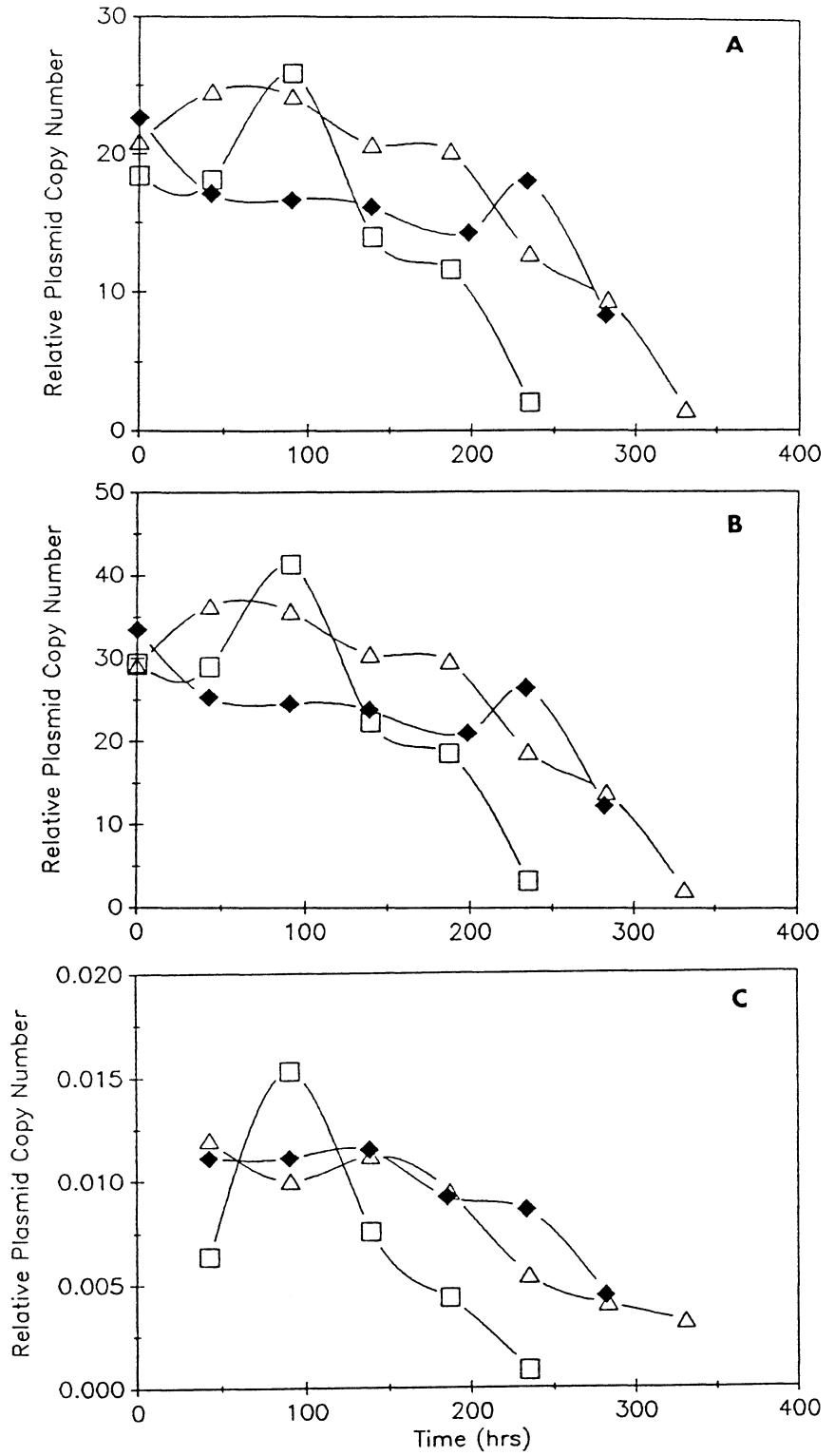


Figure 3-14. Relative plasmid copy number (a) plasmid DNA/chromosome DNA (b) plasmid DNA/cell and (c) plasmid DNA/OD for a culture of plasmid-containing cells (pBR322) grown in complex medium at dilution rates of 0.31 (△), 0.45 (◆), and 0.61 (□) hr^{-1}.

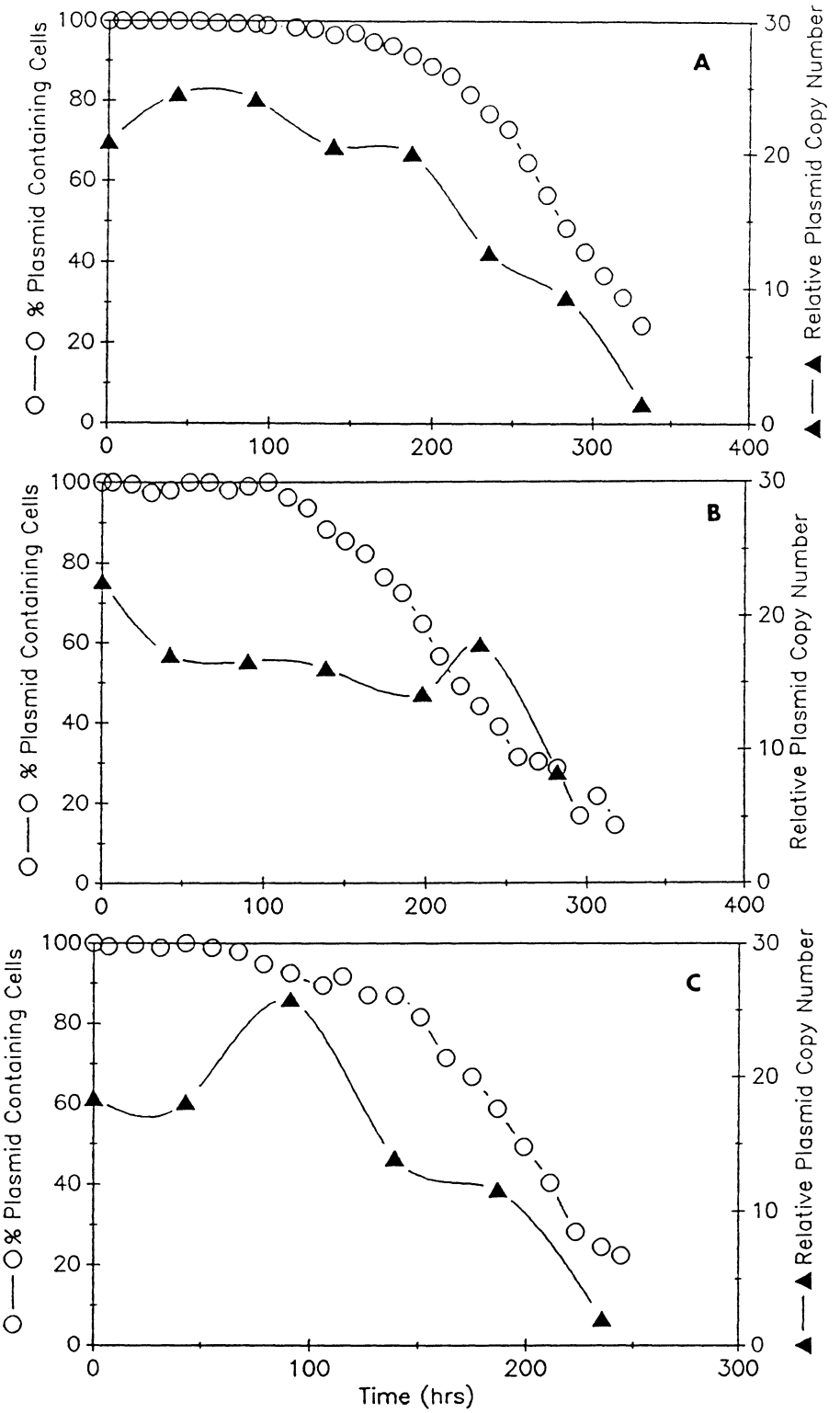


Figure 3-15. Comparison of the percentage of plasmid-containing cells (pBR322) and the relative plasmid copy number, plasmid DNA per chromosome DNA, for (a) D = 0.31 hr^{-1}, (b) D = 0.45 hr^{-1} and (c) D = 0.61 hr^{-1} .

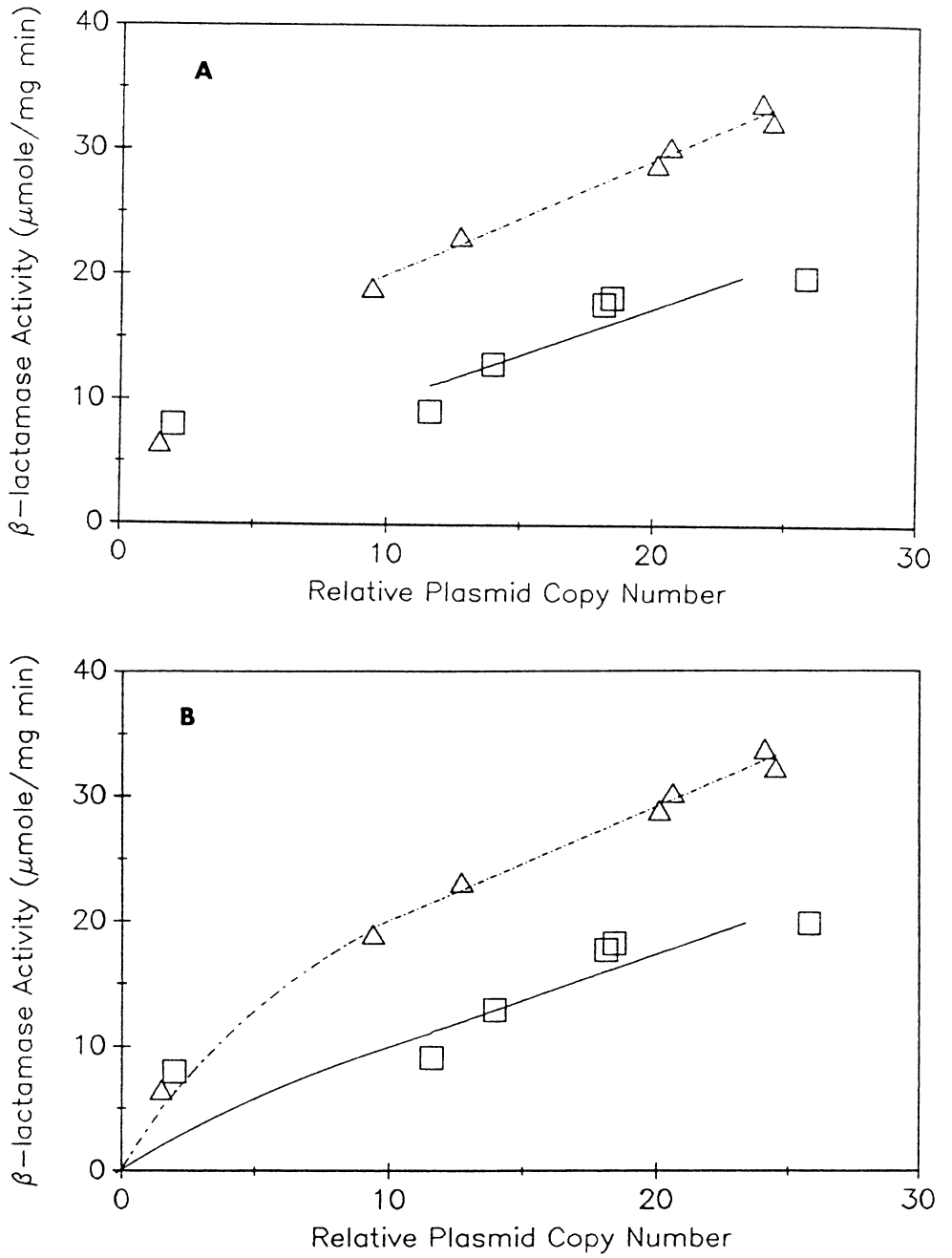


Figure 3-16. (a) Relationship between the cloned gene product, β-lactamase activity, and the relative plasmid copy number for plasmid-containing cells, pBR322, grown in complex medium at D = 0.31 (△) and 0.61 (□) hr^{-1}. (b) Predicted relationship between cloned gene product and the plasmid copy number at copy numbers below ten.

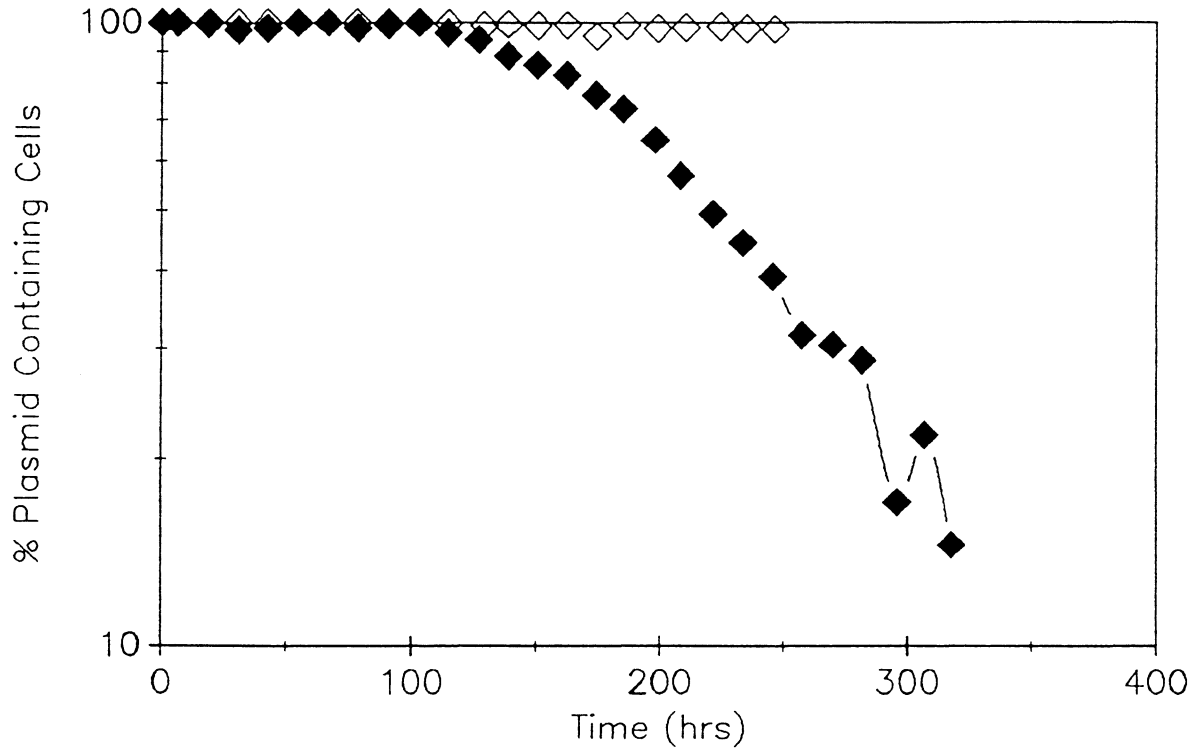


Figure 3-17. Comparison of the rate of plasmid loss for a culture containing the plasmid pDM247 (◇) vs a culture containing the plasmid pBR322 (◆) grown in a chemostat at D = 0.45 hr^{-1}.

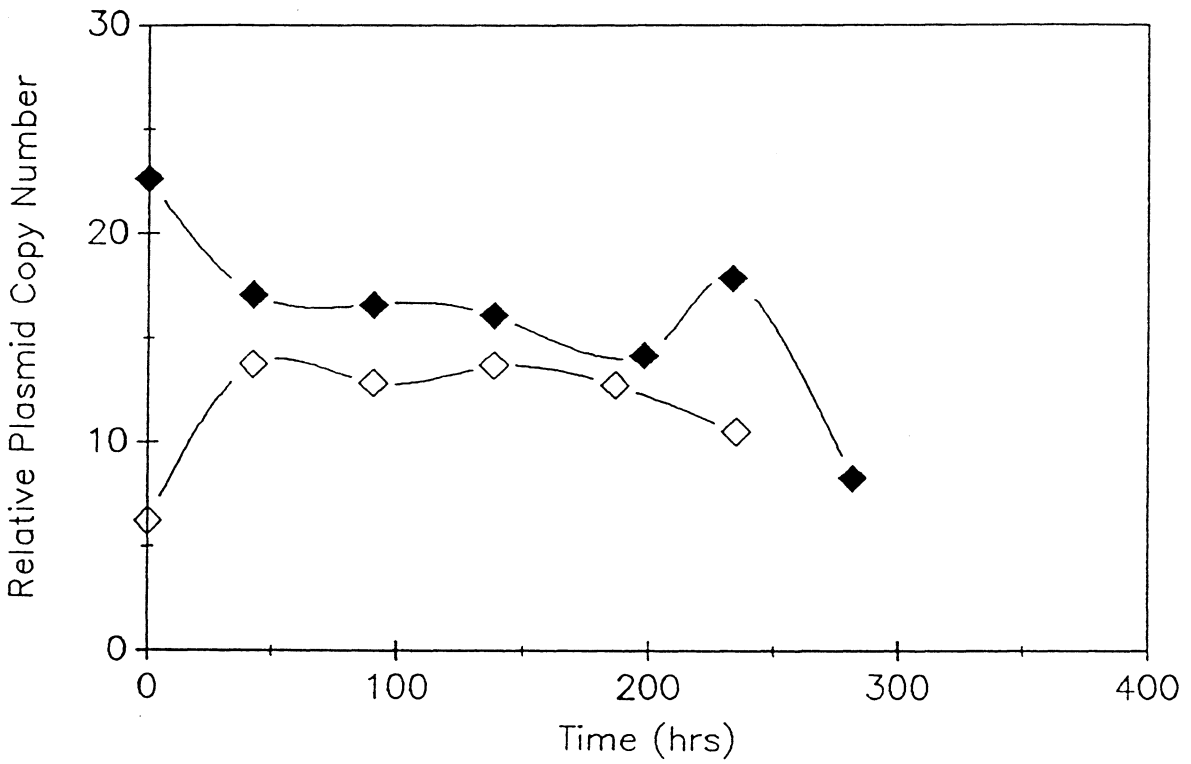


Figure 3-18. Relative plasmid copy number, plasmid DNA per chromosome DNA, of pDM247 (◇) and pBR322 (◆) for a culture grown in a chemostat at D = 0.45 hr^{-1}.

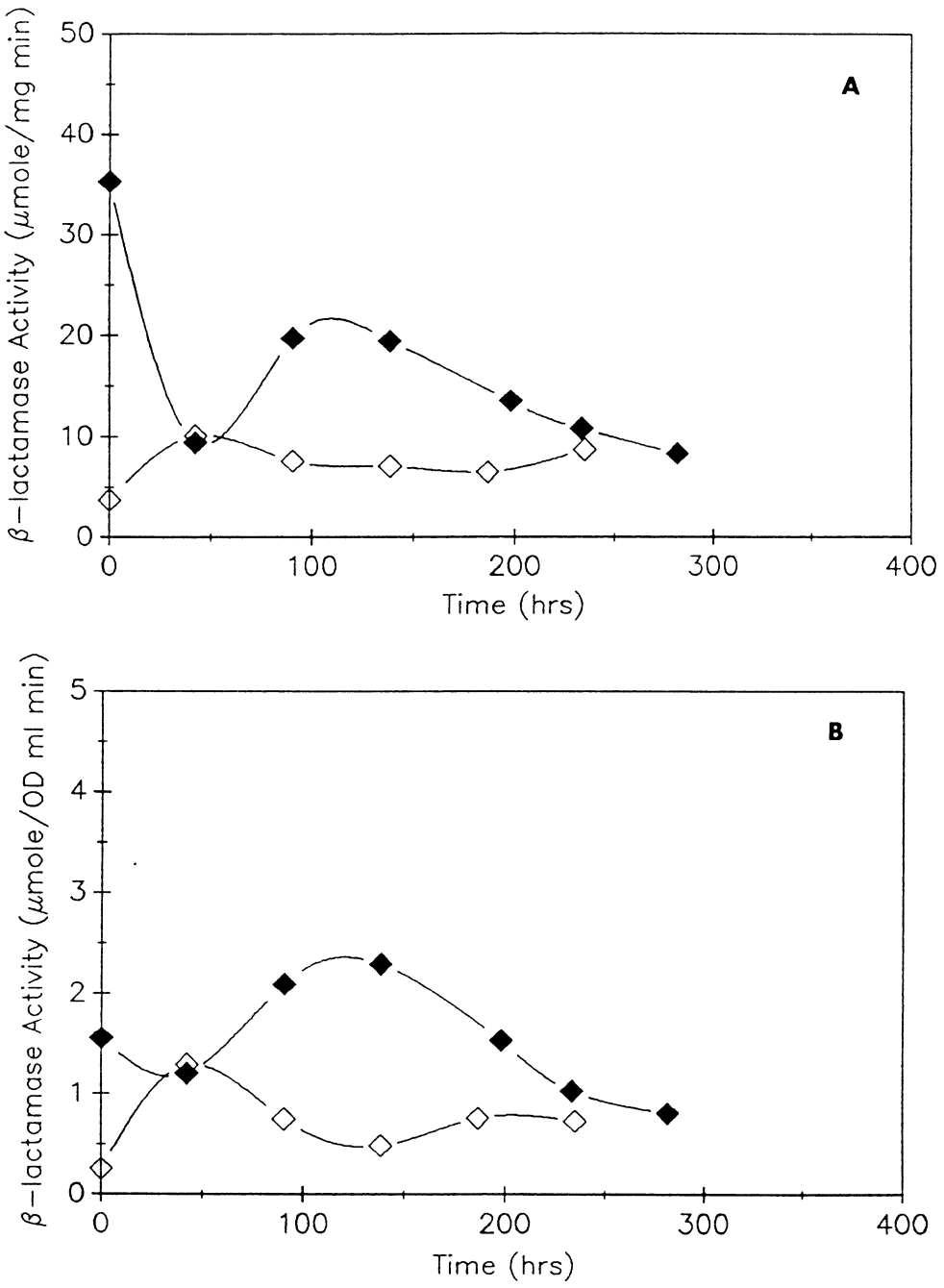


Figure 3-19. (a) Specific β-lactamase activity, based on the total protein present, for a culture of plasmid-containing cells pDM247 (◇) or pBR322 (◆) grown in complex medium at $D = 0.45\ hr^{-1}$. (b) β-lactamase activity/ml culture/OD for the plasmid pDM247 (◇) or pBR322 (◆).

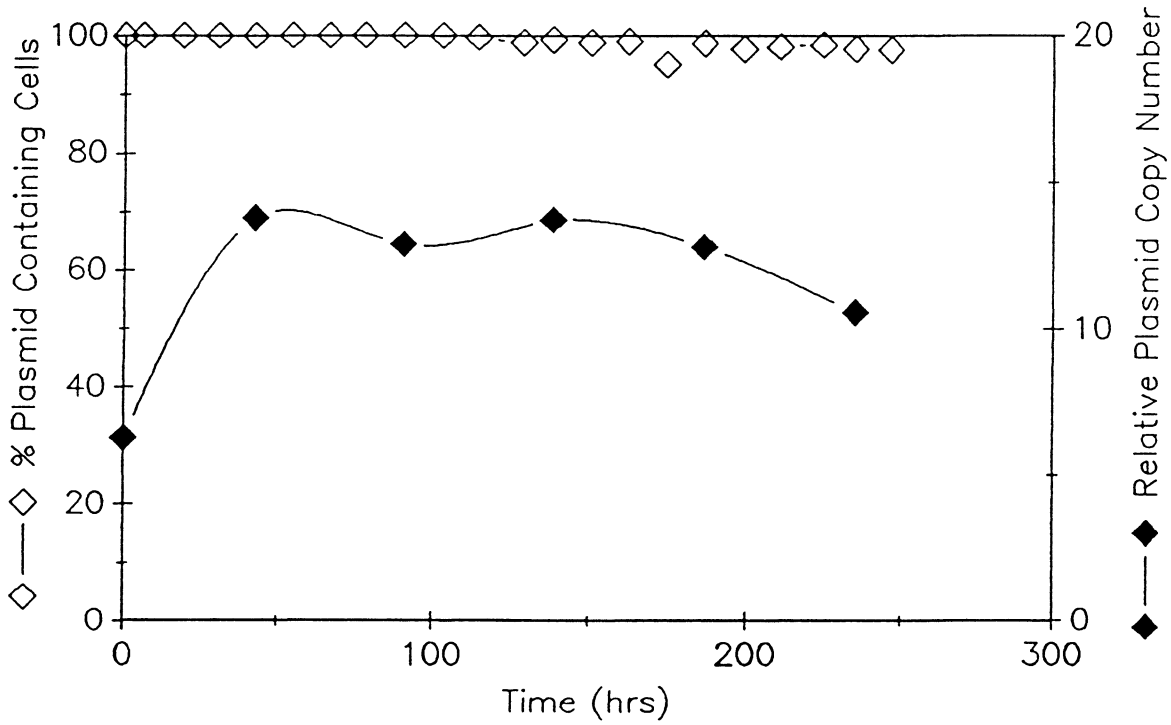


Figure 3-20. Comparison of the percentage of plasmid-containing cells (pDM247) and the relative plasmid copy number, plasmid DNA per chromosome DNA, at D = 0.45 hr^{-1}.

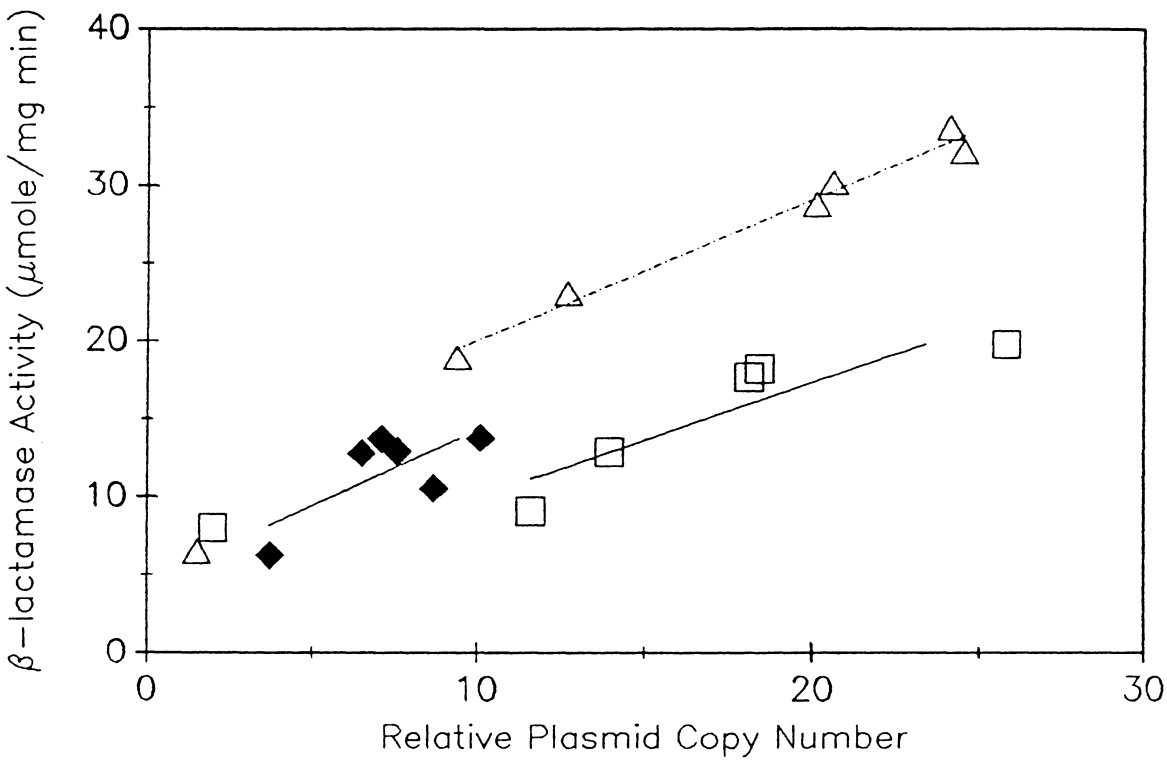


Figure 3-21. Relationship between the cloned gene product, β-lactamase activity, and the relative plasmid copy number for plasmid-containing cells, pDM247 (◆) at D = 0.45 hr^{-1} and pBR322 at D = 0.31 (△) and 0.61 (□) hr^{-1}.

CHAPTER 4

ENHANCED PLASMID MAINTENANCE IN A CHEMOSTAT UPON SQUARE-WAVE OSCILLATIONS IN THE DILUTION RATE

4.1 Introduction

The majority of work involving continuous-culture systems has been under steady-state conditions. However, it might be more interesting or even beneficial to study the behavior of microorganisms in a continuous culture under nonsteady state or transient conditions. Transient behavior created by well-defined perturbations has been long used as a tool to understand the mechanisms regulating growth and metabolism of microorganisms [40,116,118]. In addition, the introduction of transient behavior can be used to study naturally occurring systems [146]. Microorganisms growing naturally are continually exposed to a changing environment. This usually leads to a diversity of the original organism. Finally, the study of the effects of transient conditions is important when considering the operation or scale-up of fermentors. In large-scale fermentation systems, microorganisms circulating the reactor vessel may be subjected to variations in temperature, pH, and nutrient concentration due to imperfect mixing. In a work studying the characterization of mixing in fermentors, a limiting nutrient was found to fall below a concentration necessary for growth and metabolism at certain

times during circulation [22]. Therefore, fluctuations in fermentation conditions could have significant effects on culture behaviors and productivity.

Transient conditions in continuous cultures can be caused by interactions of organisms in a mixed population under a relatively constant environment or by changes in the environment [41]. The majority of literature studies have been on transient behavior due to environment changes, specifically nutritional and temperature variations. As the experiments performed in this work involved perturbations in the dilution rate the focus of the following discussion will be on the effect of nutrient changes on microbial behavior.

Changes in the nutrient supply, resulting in transient conditions, are generally caused by: step changes in the dilution rate, a pulse addition of one or more nutrients, or a change in the feed medium concentration or composition [41]. Although a number of studies have been performed using these systems, only experiments involving dilution rate changes will be discussed further.

The effect of dilution rate changes in a nitrogen-limited chemostat on E. coli was studied by Maletes [139] and Ryu [193]. It was found that cells adjusted quickly to the new growth rate with small changes in the dilution rate ($< 0.2\ hr^{-1}$). A large increase in the dilution rate ($> 0.2\ hr^{-1}$), however, resulted in a lag period of several

hours before the new growth rate was achieved. The results from this work indicate that the Monod model, a commonly used model to describe microbial growth, is not valid under a transient environment. In fact, the model predicts a faster adjustment to environmental changes than actually is observed. This indicates that the mechanism governing the growth limitations is not the same under steady-state and transient conditions [41].

In addition to the magnitude, the direction of the shift change is also an important factor in studying the response of a microbial culture to transient conditions. The metabolic response of a cell to a shift-up or shift-down in the nutrient concentration has been found to be asymmetric. A microbial population stops growing faster when exposed to a shift-down than it starts growing to a shift-up [178]. This appears to be caused by a physiological change in the cells grown in a suddenly substrate enriched environment. An increase in the number of metabolic sites in the cells or an increase in the rates of the involved reactions must occur for a microbial culture to increase its metabolism in response to a substrate concentration shift-up. It has been speculated that a steady-state population maintains a reserve of inactive ribosomes that can rapidly and efficiently respond to a nutritional shift-up necessary for the synthesis of additional proteins [115]. Experiments with both prokaryotic and eukaryotic cultures support this idea [4,28,118,244]. This change is not

needed in a shift-down of the nutrient concentration.

Pickett and Bazin [178,179] examined the effect of varying frequencies and amplitudes in the nutrient supply, glucose, on the growth and composition of Escherichia coli ML30. The growth yield and macromolecular composition changed with the frequency of the nutrient cycling. The yield decreased by approximately 10% as the cycling times decreases from 6.0 to 0.25 hr. The protein, RNA, and DNA concentration varied with the cycling time with a maximum near a time of two hours. A change in the cycling amplitude did not effect the cell yield, based on the glucose concentration. However, the concentrations of protein, RNA, and DNA generally increased with increasing amplitude. This is indicative of an increase in the metabolic activity of the cells. Based on these results it is postulated that through the use of well-defined transient conditions it is possible to select for a desired product or composition of the cell. It has been demonstrated that operating a fermentor under cyclic conditions might be beneficial in the production of intermediate compounds [50].

It should be noted that these transient systems studied involved normal cells. Until recently [247] the effect of well-defined transient conditions on genetically-engineered cells has not been studied. Theoretical models have been developed to show that plasmid-carrying and plasmid-free cells can coexist under certain cycling conditions [221]. A transient environment might prove advantageous for plasmid-

containing cells in competing with the plasmid-free cells. In addition, based on changes observed in the DNA, RNA, and protein content under transient conditions [178], perturbations in the dilution rate could result in an increase in plasmid content or expression. This could provide a window of opportunity to be exploited for the design of novel bioreactor operation to yield better process performance.

The effects of square-wave perturbations in the dilution on plasmid maintenance and gene expression were studied through a set of four experiments. In the first three experiments, the dilution rate was switched between 0.31 and 0.46 hr^{-1}. The period of switching was two hours in the first cycling experiment, 2/2 run. The first and second number refer to the high and low dilution periods, respectively. In the second experiment, 2/1 run, the cycling period was two hours for the higher dilution rate and one hour for the lower dilution rate. The third experiment was a random cycling system. The dilution rate was oscillated in one of the following four frequency patterns: 2/2, 2/1, 3/1, and 1/1. There was no standard pattern to the combination of the frequency periods, but the overall average cycling frequency was kept between 2/2 and 2/1. The fourth experiment was to determine the effect of the cycling amplitude on plasmid stability. The dilution rates were switched between 0.31 and 0.60 hr^{-1} at a period of two hours.

4.2 Effect of Square-wave Perturbations in the Dilution Rate on Plasmid Maintenance and Gene Expression

The effects of square-wave perturbation in the dilution rate on plasmid maintenance and gene expression were studied by comparing the transient experiment to the control or steady-state experiments [Chapter 3]. In the control experiments the dilution rates were constant at 0.31 and 0.45 hr^{-1}. In the transient experiment the dilution rate was switched between 0.31 and 0.46 hr^{-1} at a frequency of two hours. This frequency corresponded to the cycling time in which a maximum concentration of protein, DNA, and RNA occurred [178].

The percentage of plasmid-containing cells in the fermentor as a function of time for the control and cyclic experiments are shown in Figure 4-1. Unlike the control runs, where the culture drops precipitately from a population of 100% to 10% plasmid-carrying cells, the culture exposed to cyclic variations in the dilution rate is observed to maintain a mixed population of plasmid-containing and plasmid-free cells for a longer period of time. Two possible mechanisms are proposed to explain this phenomenon. First, under transient conditions the recombinant cells respond better than plasmid-free cells to environmental changes, thereby, diminishing the relative growth advantage of the plasmid-free cells. Second, under these highly transient conditions the

cells are induced to maintain a higher copy number. This increase in plasmid copy number might lead to a lowering of the probability of plasmid loss due to random partitioning. Preliminary results, presented in this chapter, indicate the existence of both mechanisms.

The relative plasmid copy number, number of plasmid copies per chromosome DNA, for the cyclic and control experiments are compared in Figure 4-2. Four points should be noted. First, the plasmid content appears to be greater under transient conditions. Second, the plasmid number is more oscillatory when compared to the control experiments. Third, there was no significant difference between the copy number at the end of the low cycle when compared to a sample taken at the end of the high cycle. Finally, the time difference between the loss of plasmid content for the transient experiment and the control experiments corresponds to the increased stability period created by the dilution rate perturbations.

It would appear as though a transient environment is beneficial to plasmid-containing cells. This is supported by the increase in plasmid stability and plasmid content. However, the cloned gene expression does not show such an increase, Figure 4-3. It can be observed that the β-lactamase activity of the plasmid-bearing cells under cyclic conditions falls between the two control experiments. This agrees with the theoretical study by Peretti [176]. Based on a single-

cell model it was demonstrated that there were no major benefits when the promoter strength was periodically changed. There were times, however, when the product level in a cyclic system was greater than in a steady-state culture.

The plasmid efficiency is lower under cyclic operation as compared to steady-state cultures, Figure 4-4. It is not clear why an apparent increase in the plasmid copy number occurs while the plasmid expression does not increase. Peretti and Bailey [177] found that at high copy number levels the gene-specific transcription rate continues to increase, however, the stability of the plasmid mRNA decreases sharply. Their results indicate that the productivity of the plasmid in not limited by the biosynthetic capacity of the cell, but by macromolecular stability. Further experiments are needed to understand these phenomena.

The stability of the plasmid-containing culture under dilution rate perturbations is compared to the gene expression and plasmid copy number in Figure 4-5. The overall trend is similar to that observed in the control experiments. The decrease in activity and plasmid content precedes a loss of plasmid-containing cells. This indicates a gradual decrease in plasmid copy number before the appearance of plasmid-free cells. A more detailed discussion will be presented in the following chapter.

It was previously pointed out that the behavior of copy number, and to some extent the β-lactamase activity, was

oscillatory. Because the time between samples is rather large the behavior of the plasmid content and expression could be slightly enhanced or dampened. Regardless, it should be noted that the oscillations stopped when the percentage of plasmid-containing cells dropped. At this time, approximately 220 hours into the experiment, it was also observed, through continuous OD measurements, that the culture did not respond as rapidly to perturbations in the dilution rates. The cells appear to have adapted to the oscillations. At this point the culture behaves as though it is under a constant dilution rate. It is speculated that once the culture "follows" the oscillation any potential advantages created by perturbations in the dilution rate has disappeared. Additional experiments were performed to test this hypothesis.

4.3 Effect of Varying Frequencies on Plasmid Stability

Based on the previous oscillatory behavior, it is postulated that a constantly changing environment should be maintained to avoid any adaptation by the culture. Therefore, two more experiments with different variations in the period of oscillation were performed. It is argued if the period is not symmetrical, as in the previous experiments, the culture will take a longer time to adapt. In turn, the stability of the reactor will be slightly enhanced.

In the first experiment, the dilution rate was switched between 0.31 and 0.46 hr^{-1}, for one and two hours, respectively. The results are compared to the previous cycling experiment in Figure 4-6. It can be observed that a change in cycling frequency does affect the stability of the culture.

The change in cycling frequency is also reflected in the behavior of the plasmid copy number, Figure 4-7. Although there is no apparent increase in the plasmid content, when compared to the 2/2 experiment, the oscillation period of the plasmid number seems to have been extended. It appears as though the increase in this period of oscillation corresponds to the increase in plasmid stability as evident in Figure 4-8b. It should be noted that the oscillation of the copy number dampens with time. This might also be due the adaptation of the culture to the cycling scheme.

As shown in Figure 4-9 the change in frequency does not create a measurable difference in the cloned gene product. The only effect is a shift in the oscillation of the activity. This agrees with the observations from the plasmid copy number results. In addition, the oscillation of the activity stops with the appearance of plasmid-free cells, Figure 4-8a. The same behavior was seen in the 2/2 cycling experiment.

Based on the plasmid number and β-lactamase activity data, it is assumed that the plasmid efficiency of the 2/1

experiment would be similar to that of the 2/2 experiment. However, as demonstrated in Figure 4-10 this is not the case. The data from the 2/1 experiment is too scattered to deduce any relationship between the plasmid content and activity under these particular conditions. This is probably caused by different response time constants of plasmid replication and protein expression to the transient conditions. The exact cause of this phenomena, however, is uncertain. The results from the two cycling experiments, 2/2 and 2/1, suggest that a population of plasmid-containing cells can be stably maintained as long as the culture is exposed to a constantly changing environment. However, as soon as the culture adapts to the changes, any advantages created by the transient environment is eliminated. At this point the culture acts as if it is in a pseudosteady-state environment. The resulting decrease in plasmid-containing cells, copy number, and plasmid product parallels that observed in the control runs where the dilution rate was kept constant.

The third experiment was performed to further test this speculation. To reduce the ability of the cells to adapt to the perturbations a chaotic environment was generated by a randomized cycling scheme. The dilution rates were switched between 0.31 and 0.46^{-1}. The overall cycling frequency was kept between 2/2 and 2/1 for comparison purposes. The plasmid maintenance under a random cycling scheme is plotted in Figure

4-11. The results are rather interesting. Initially the behavior resembles that of the 2/2 experiment. However, instead of continuing in this trend, the cells approached a plateau where they were able to stably maintain a mixed population of plasmid-containing and plasmid-free cells for approximately 100 hours. At this point the rate of plasmid loss parallels that of the other experiments. The loss of plasmid-carrying cells indicates the adaptation of the culture to the cycling conditions. At this time, the insensitivity of the culture to variations in the dilution rate was observed from the optical measurements, Figure 4-12. Nevertheless, the reactor under this highly chaotic environment is the most stable among the three experiments.

The results of the plasmid content analysis are also interesting. From previous experience it is assumed that the increase in plasmid stability corresponds to an increase and oscillation in the plasmid content. As shown in Figure 4-13, this does not occur. In fact, except for the first 100 hours, the plasmid copy number is lower and more stable than the other experiments. This stability of the plasmid copy number, in which there is no drastic increase or decrease, coincides with the period where a mixed culture is stably maintained, Figure 4-14. One possible explanation is the decrease in plasmid content has allowed the plasmid-containing cells to become more competitive, i.e., by reducing the growth rate advantage, with the plasmid-free cells. However, if this were

the case this type of behavior would be expected in all the experiments. Based on previous data this does not occur. Therefore, the chaotic environment must benefit the plasmid-containing cells. How it affects the stability and why it occurs after an initial decrease in the population of plasmid-containing cells is still unknown.

The data from the β-lactamase analysis are shown in Figures 4-15 and 4-16. In the previous cycling experiments the activity decreased with respect to the lower control experiment. In addition, the enzyme activity showed an oscillatory behavior. The β-lactamase activity in the random experiments, however, did not exhibit this type of behavior. Also, the activity did not decrease with respect to the control runs, Figure 4-16. In fact, in the later part of the random cycling experiment, starting at approximately 250 hours, when the activity in the control or fixed period cycling runs dropped, the activity in this experiment not only began to increase it was maintained for 100 hours. This corresponds to the period when the plasmid-containing and plasmid-free cells stably coexist, Figure 4-14a. The drop in the activity, at 200 hours, appears to precede the drop in the percentage of plasmid-containing cells. This resembles the trend observed in the plasmid copy number, Figure 4-14b.

Based on the behavior of the plasmid copy number, in which a rapid drop in number and then a small increase was observed, a slight increase of the activity might be expected.

However, the activity not only increases, it reaches the original level before a decrease in plasmid content or plasmid-containing cells was detected. The cause of this phenomena in the β-lactamase activity is unclear. It does, however, prove interesting and could be beneficial in the design and operation of a fermentation system. The increase in activity in the later part of the experiment has significant effects on the plasmid efficiency. It appears that there is a linear relationship between the plasmid number and product, Figure 4-17a. However, it is suspected that two different efficiency values exist during the experiment. The first value is generated during the earlier part of the experiment. The second value occurs during the later part of the experiment. This value is greater than the efficiencies from the other cycling and control experiments. This is due to the high activity during this period. The mechanism behind this phenomenon cannot be explained at this time. It does, however, appear to be caused by the response of the culture to the transient environment.

To this point, the two mechanisms proposed earlier in this chapter, explaining the enhanced plasmid stability under transient conditions, appear valid. The slight increase in plasmid copy number observed might extend the lag time before the plasmid-free cells appear. This would explain the increase in plasmid stability in the three cycling experiments. The decrease in the relative growth advantage

of the plasmid-free cells would result in a stably maintained period where the plasmid-containing and plasmid-free cells coexisted, as observed in the random cycling run. A model describing the culture behavior under transient conditions was developed to further provide discriminatory information about the possible underlying mechanisms.

4.4 Data Analysis of Plasmid Maintenance in a CSTR under Square-wave Perturbations in the Dilution Rate

4.4.1 Model Development

A more detailed development of the following model can found in Chapter 3. To summarize, the dynamics of a mixed culture system can be described by a set of ordinary differential equations.

$$dX^+/dt = (\mu^+ - D)X^+ - p\mu^+X^+ \qquad (3\text{-}2a)$$

$$dX^-/dt = (\mu^- - D)X^- + p\mu^+X^+ \qquad (3\text{-}2b)$$

$$dS/dt = D(s_f - s) - \mu^+X^+/Y^+ - \mu^-X^-/Y^- \qquad (3\text{-}2c)$$

The model, together with the estimated parameters, Table 3-1, provides an excellent fit to the experimental data. However, for the case where the cells are exposed to a square-wave oscillation in the dilution rate, the parameter $\delta\mu$ is a function of the cycling time. Therefore, equation 3-6 is rewritten as:

$$dy^+/dt = y^+[(\delta\mu(t))(1 - y^+) - p\mu^+] \qquad (4\text{-}1)$$

4.4.2 Determination of Model Parameters

If the culture responds instantaneously to step changes in the dilution rate and behaves the same under both "steady-state" and oscillatory environments, previously determined parameters from the control experiments can be used in the modified model, equation 4-1, to predict the dynamics of plasmid-containing cells grown under forced oscillations in the dilution rate. The simulated results are shown in Figure 4-18. It can be readily observed that the simulated results do not provide an adequate estimation to the experimental data. This indicates that the microbial culture behaves differently under an oscillatory environment.

If the rate of plasmid loss and the growth difference between the recombinant cells and the plasmid-free cells remain relatively constant throughout the experiment, it is possible to estimate the time averaged parameters using a previously derived data analysis algorithm [195]. Notice that if $\delta\mu(t)$ is approximated by an averaged value over the period of an oscillation period, equation 4-1 becomes

$$dy^+/dt = y^+[(\overline{\delta\mu})(1 - y^+) - p\mu^+] \qquad (4\text{-}2)$$

The parameters $\delta\mu$ and p can be determined from the slope and intercept of a semi-log plot of y^+/y^- versus time. The values for the 2/2 cycling experiment were estimated to be

-0.023 and $9.63e^{-6}$, respectively. Figure 4-19 shows the results from the simulation. The good agreement between the experimental results and the model seems to suggest that the stabilization of plasmid-containing cells under oscillatory conditions is mainly due to a decrease in the probability of plasmid loss as $\delta\mu$ is approximately the same in the control and cyclic experiments. It should be cautioned that the parameter estimation scheme assumed that both $\delta\mu$ and p remain relatively constant throughout the experiment.

Using the same scheme, the time averaged parameters, $\delta\mu$ and p, for the 2/1 cycling experiment were -0.022 and $5.9e^{-6}$, respectively. The simulated result is plotted in Figure 4-20. As seen, it provides a fairly good fit to the experimental data.

It should be noted that the composition of the cell culture is always changing. Therefore, the reactor never reaches a steady-state implying that the reactor conditions are also always changing. Subsequently, the growth kinetics of the microbial culture may also vary throughout the experiment. Since there is no existing technique which allows a direct measurement of the parameters $\delta\mu$ or p an indirect experiment is performed to test the validity of the above assumption. If it is assumed that no significant interactions exist between the two populations other than competing for the same nutrient, the magnitude of $\delta\mu$ can be estimated by two

different experiments with a pure culture under the same oscillation conditions. In the first experiment, the optical density of a culture with 100% plasmid-containing cells exposed to square-wave perturbations in the dilution rate was monitored. The experiment was repeated with a plasmid-free culture. The dynamics of a microbial culture in the chemostat can be described by the following equations:

$$dX^+/dt = (\mu^+ - D)X^+ \qquad (4\text{-}3a)$$

$$dX^-/dt = (\mu^- - D)X^- \qquad (4\text{-}3b)$$

Integrating the above equations from the beginning to the end of a half cycle, results in:

$$\ln(X_f^+/X_o^+) = \int_{t_o}^{t_f} (\mu^+(t) - D(t))dt \qquad (4\text{-}4a)$$

$$\ln(X_f^-/X_o^-) = \int_{t_o}^{t_f} (\mu^-(t) - D(t))dt \qquad (4\text{-}4b)$$

The time average of the difference in the specific growth rate at each half cycle can be obtained by subtracting equation 4-3b by 4-3a. For the same variations in the dilution rate in both experiments, the final result can be simplified and is given below in equation 4-5.

$$\overline{\delta\mu}(t_f - t_o) = \ln \left[\frac{X_f^o X_o^-}{X_o^+ X_f^-} \right] \qquad (4\text{-}5)$$

The subscript refers to the biomass concentration at the beginning and the end of each half cycle, respectively. A representative value for the difference in the specific growth rate, after 2 days into the fermentation, for the high dilution rate oscillation period, D = 0.46 hr^{-1}, was -0.0082. The value for the low dilution rate period, D = 0.31 hr^{-1}, was calculated to be -0.0120. It should be pointed out that the optical density data were rather noisy and as the values involved are rather small, the results obtained might not be very accurate. Nevertheless, these results do suggest that the growth rate difference between the plasmid-carrying and plasmid-free cells is smaller than in the control runs. The result also seems to support that the assumption the plasmid-containing cells respond quicker to environmental changes than the plasmid-free cells. Moreover, under the conditions studied, $\delta\mu$ in both periods was negative. This implies that transient conditions, though favoring the recombinant cells, are still not enough to enable the recombinant cells to outgrow the plasmid-free cells.

In Figure 4-21 the model prediction, based on these values and the rate of plasmid loss determined from the control runs, is compared to the experimental results. The model overestimates the stability of the system. The misfit could be explained as follows. First, the probability of plasmid loss parameter used in the simulation was obtained

from experiments performed under constant dilution rates. It is possible that the parameter may change under oscillatory conditions. However, an attempt to fit the experimental data using different values of the rate of plasmid loss failed, as demonstrated in Figure 4-22. This is due to the fact that the probability parameter plays a bigger role in influencing the lag time, the time before the appearance of an appreciable amount of plasmid-free cells, and not the rate in which the plasmid-free cells "take over" the reactor. This rate corresponds to the slope of the experimental results and is greatly influenced by the growth advantage the plasmid-free cells have over the plasmid-containing cells.

The second possible reason is that the difference in growth rates is not constant throughout the experiment. This may well be the case since the estimated $\delta\mu$ is quite different from the time averaged value. In addition, it has been observed that after a certain period of time, approximately 220 hours in this particular experiment, the cells adapt to the dilution rate oscillations. Therefore, the behavior of the culture in the later stages of the experiment differ from that in the beginning of the experiment. Based on this speculation, the model is again modified to include different values of the growth rate parameters. The simulated result, using two values for $\delta\mu$ before and after 220 hours, is shown in Figure 4-23. This time was chosen from the continuous OD measurements. It can be seen after this modification the

model provides an excellent fit to the experimental data.

4.5 Effect of Varying Amplitudes on Plasmid Stability and Expression

Variations in the oscillation amplitude were studied for two purposes. First, in a plasmid-free culture, large differences in the macromolecular composition of the cells resulted from a change in the cycle amplitude [179]. Similar behavior might occur in a plasmid-containing population, thereby effecting the plasmid content and product. Second, it is speculated that the magnitude of the perturbations would have an effect on the reactor stability. A larger variation in the dilution rate could create a bigger perturbation and should preferentially favor the plasmid-carrying population.

In this experiment the dilution rate was switched between 0.31 and 0.60 hr^{-1} at a cycling period of two hours. The dilution rate was kept constant at either 0.31 or 0.61 hr^{-1} in the control runs. The stability of the culture is compared with the previous 2/2 cycling experiment and the two control experiments in Figure 4-24. Although, there is no significant improvement of plasmid maintenance when compared to the lower control and previous cycling experiments the stability is enhanced with respect to the higher dilution rate. In this regard the transient condition is somewhat beneficial. The culture, however, appears to adapt faster to the perturbations

than the other cycling experiments. This observation agrees with the continuous OD measurements, the plasmid copy number, and the activity data, Figure 4-27. After 150 hours, the oscillation of the plasmid content and expression stops. Similar behavior was observed in earlier experiments.

The behavior of the plasmid-containing culture in this experiment could be the result of a number of causes. It is possible that the 0.61 hr^{-1} dilution rate creates an environment that is not advantageous to the plasmid-containing population. It has already been shown that cultures grown in this dilution rate are the least stable. In addition, Meyer et al. found a high acetate concentration existed in complex medium at high dilution rates [150]. Acetate accumulation in the reactor has been shown to be detrimental to cell growth and productivity.

The plasmid copy number and β-lactamase activity respond to the larger perturbation in a manner similar to the previous 2/2 cycling experiment, Figures 4-25 and 4-26. In comparison with the control experiments the copy number is larger and the activity falls between the values from the two experiments. The plasmid content and product also demonstrate an oscillatory pattern.

However, some differences between the two oscillation experiments do exist and should be examined. Comparatively, as seen in Figure 4-25, the copy number of the larger

amplitude experiment, D = 0.31 and 0.60 hr^{-1} ($\delta D = 0.29\ hr^{-1}$), is lower than the smaller amplitude, D = 0.31 and 0.45 hr^{-1} ($\delta D = 0.14\ hr^{-1}$), experiment. This could have resulted in a decrease in plasmid stability as observed in the higher amplitude experiment.

However, in the first 150 hours the β-latamase activity from the larger amplitude ($\delta D = 0.29\ hr^{-1}$) experiment is greater than in the smaller amplitude run. In the later part of the experiment the enzyme activities are virtually equivalent. Although, the cloned gene product demonstrates a more rapid drop in this experiment. This is probably due to the quicker onset of plasmid-free cells, Figure 4-27a.

Because of the variations in the response of the culture to the different oscillation amplitudes it is expected that the plasmid efficiency between the two experiments would also differ. This is seen in Figure 4-28. Although the data from the larger amplitude experiment shows more scattering, the efficiency is higher, except for the low control run, than the other experiments.

Based on these results it would appear as though a decision between plasmid stability and productivity must be made when considering a transient versus a steady-state environment in the design and implementation of bioreactors. Under transient conditions, as seen in the 2/2 and 2/1 frequency experiments, it is possible to increase plasmid

stability at the expense of the cloned gene expression. However, under long-term fermentation this might be desirable. When considering nonsteady-state operation only a random cycling scheme, of the systems studied, appears to increase both plasmid stability and productivity.

4.6 Conclusions

To briefly summarize the more important points presented in this chapter:

1. Well-defined square-wave perturbations in the dilution rate affect the plasmid maintenance, copy number, and expression. The amplitude and frequency of the oscillation are important factors when considering nonsteady-state operation.
2. Out of the systems studied a random cycling at a low oscillation amplitude proved to be the best scheme with respect to stability, plasmid content, enzymatic activity, and plasmid efficiency.
3. In all perturbation experiments the loss of the plasmid-containing cells appears to be the result of an adaptation of the culture to the oscillations. It is speculated that once the culture "follows" the oscillation, any potential advantages created by the perturbations in the dilution rate have disappeared. At this point the culture acts as though it is under a steady-state environment.

4. The relationship between the plasmid copy number and activity and dilution rate oscillations is not known. However, two mechanisms are proposed to describe the observed enhanced stability. First, under transient conditions, the recombinant cells respond quicker to the environmental changes, thereby diminishing the relative growth advantage of the plasmid-free cells. Second, under transient conditions, the cells are induced to maintain a higher copy number. The increase in copy number might lead to a lowering of the probability of plasmid loss due to random partitioning.
5. A model describing the culture behavior under transient conditions was developed to provide discriminatory information about the possible underlying mechanisms. The system can be described by 1) time averaged values of $\delta\mu$ and p and 2) when the experiment is divided into two stages with different $\delta\mu$ values for each stage.

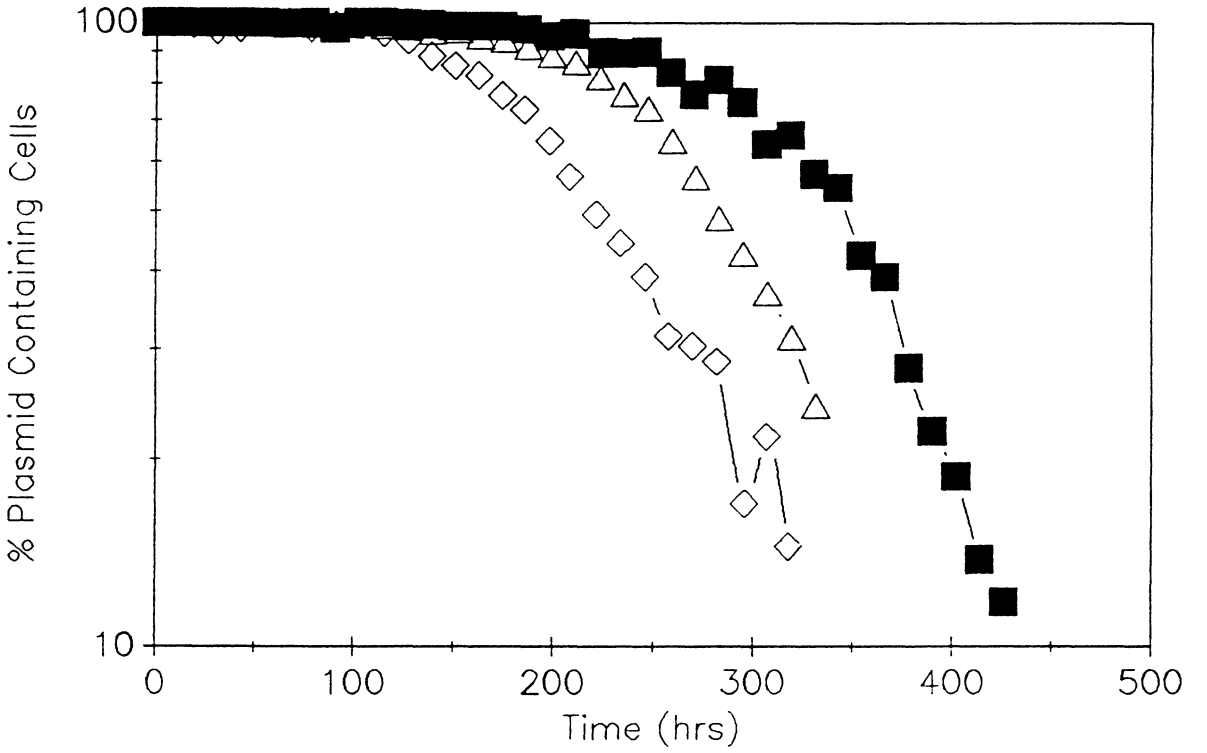


Figure 4-1. Rate of plasmid loss under cyclic and constant dilution rates. D = 2/2 cycling, dilution rate was switched between 0.31 and 0.46 hr^{-1} at a frequency of two hours (■), 0.31 (△), and 0.45 (◇) hr^{-1}.

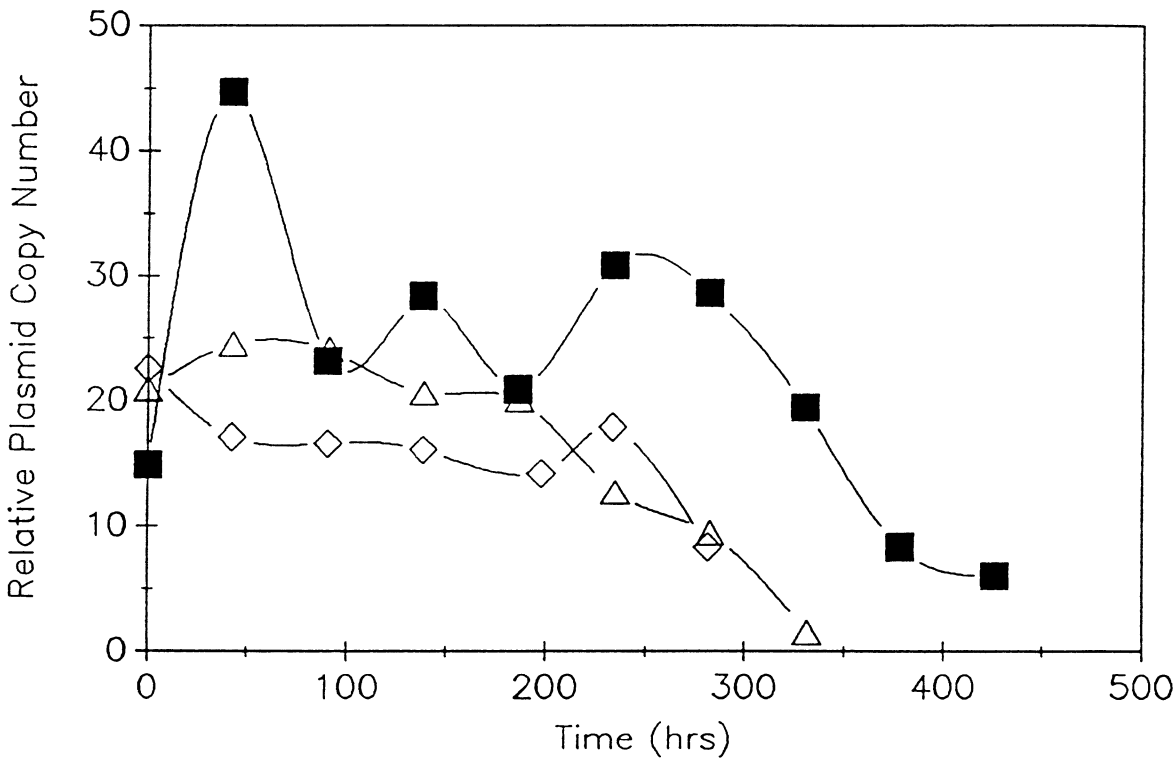


Figure 4-2. Comparison of relative plasmid copy number, plasmid DNA per chromosome DNA, for 2/2 frequency cycling (■) and control experiments, D = 0.31 (△) and 0.45 (◇) hr^{-1}.

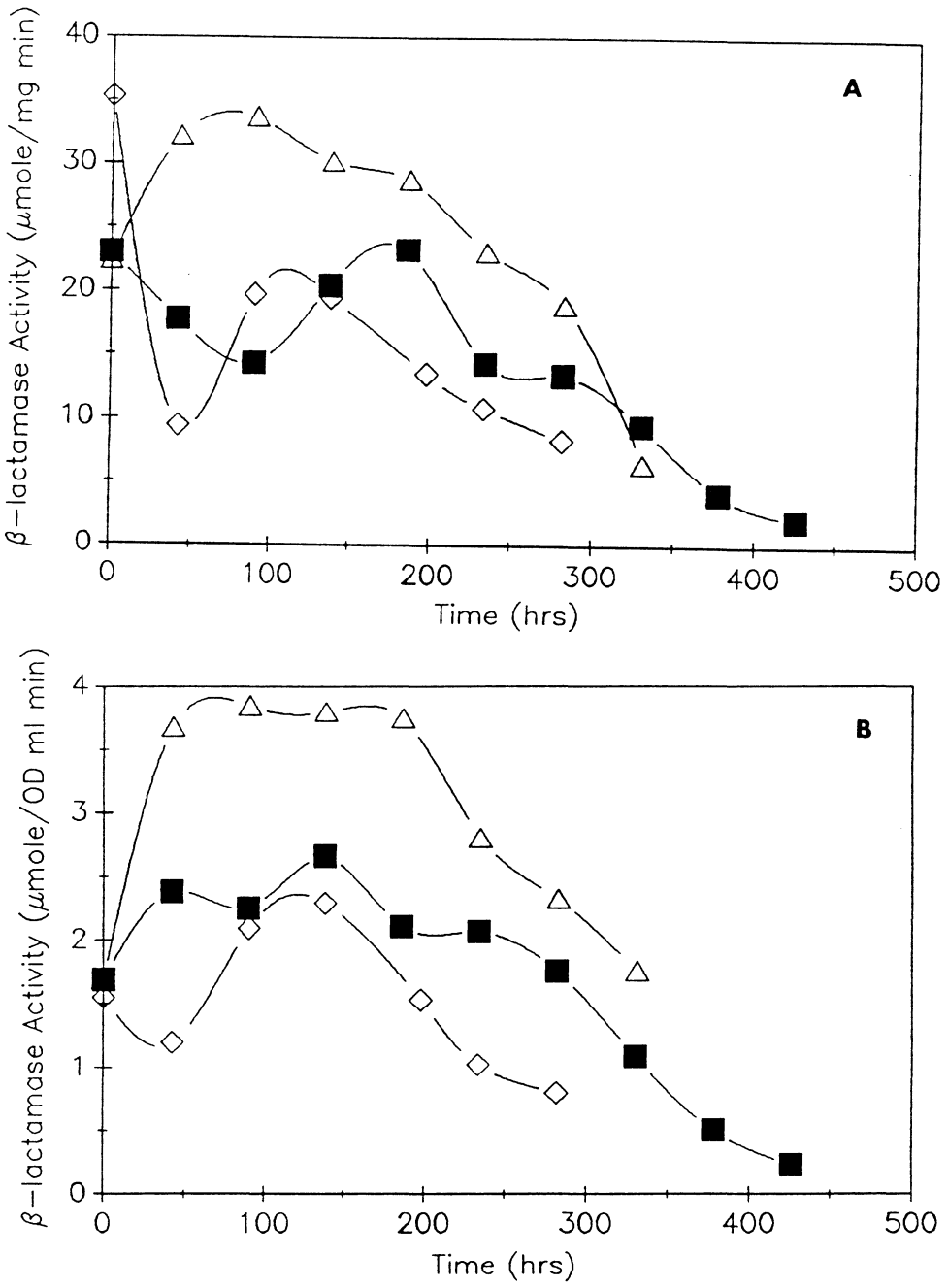


Figure 4-3. (a) Specific β-lactamase activity, based on total protein, for 2/2 frequency cycling (■) and control experiments, D = 0.31 (△) and 0.45 (◇) hr^{-1}. (b) Expression of β-lactamase activity/ml culture/OD for 2/2 frequency cycling (■) and control experiments, D = 0.31 (△) and 0.45 (◇) hr^{-1}.

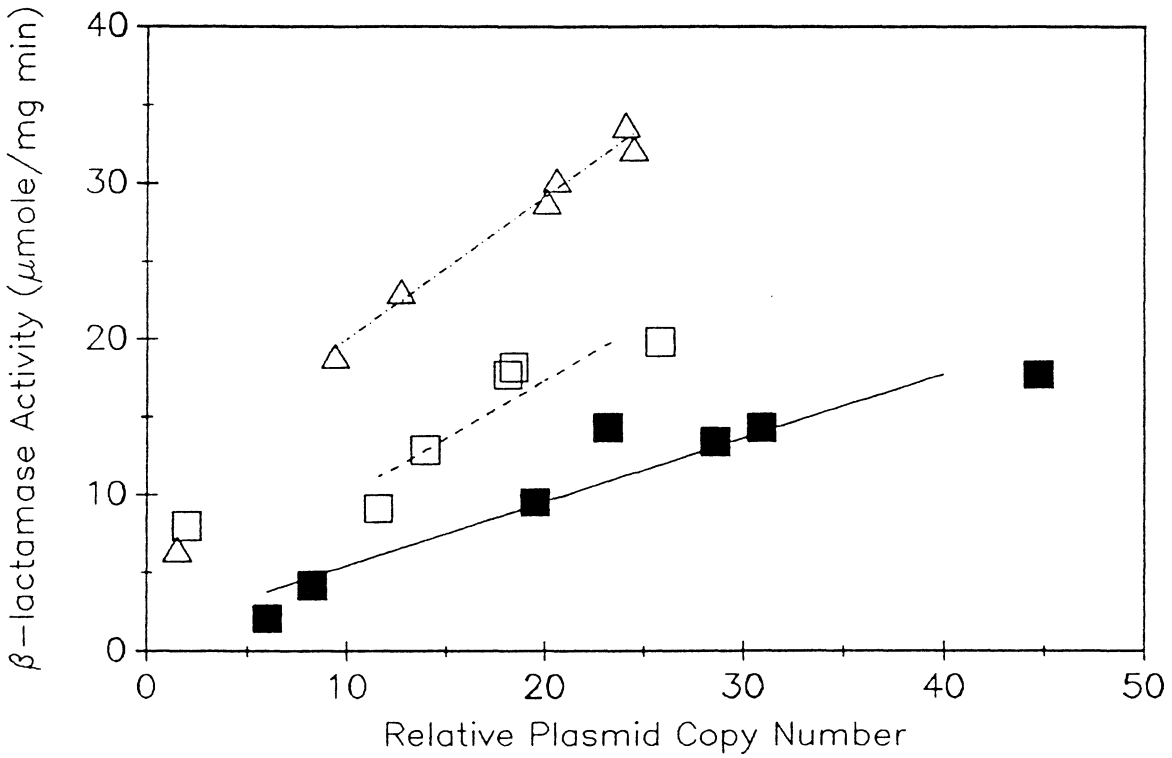


Figure 4-4. Relationship between the cloned gene product, β-lactamase activity, and the relative plasmid copy number for 2/2 frequency cycling (■) and control experiments, D = 0.31 (△) and 0.61 (□) hr^{-1}.

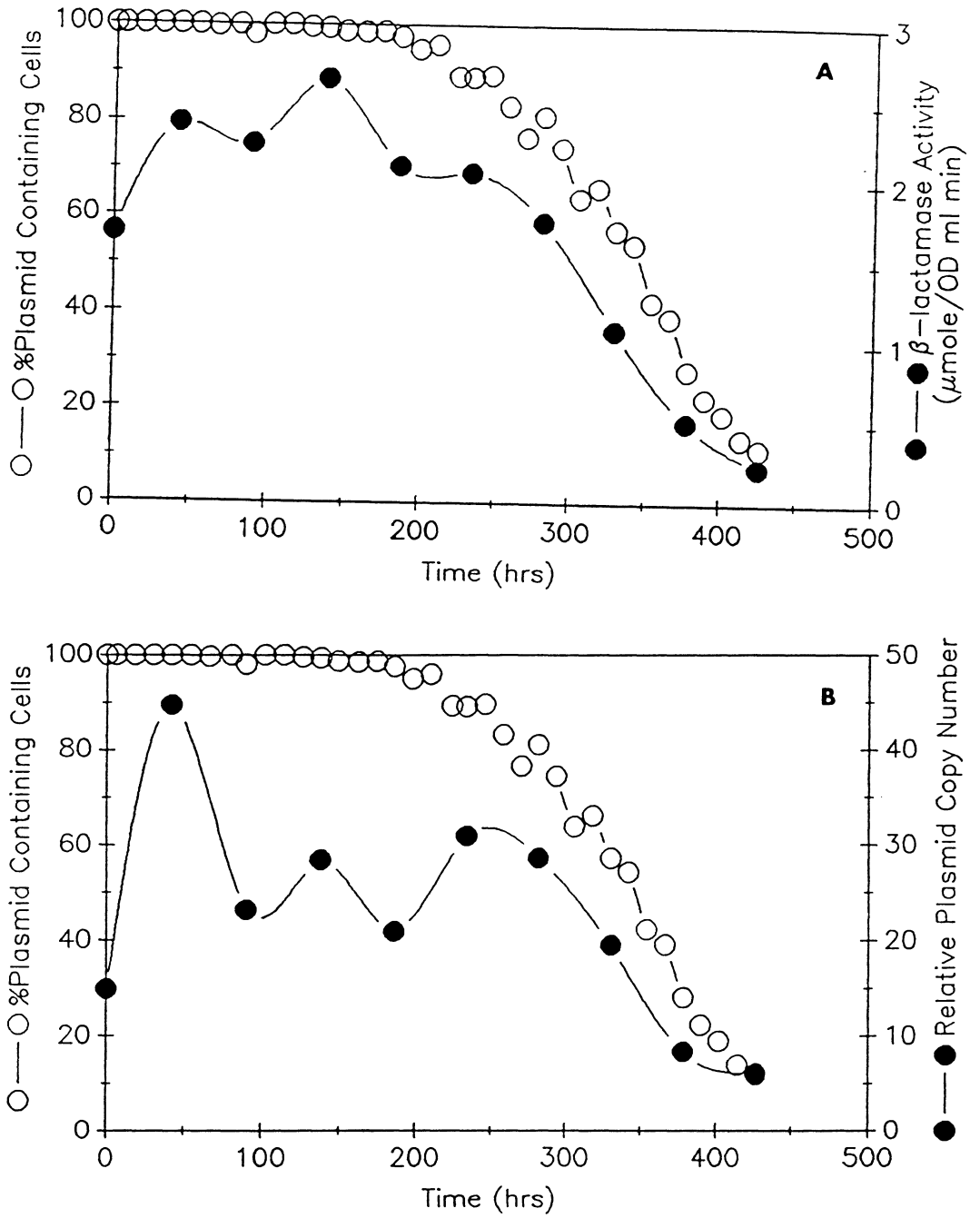


Figure 4-5. Comparison of the percentage of plasmid-containing cells and the (a) β-lactamase activity and (b) relative plasmid copy number, plasmid DNA per chromosome DNA for the 2/2 cycling frequency experiment.

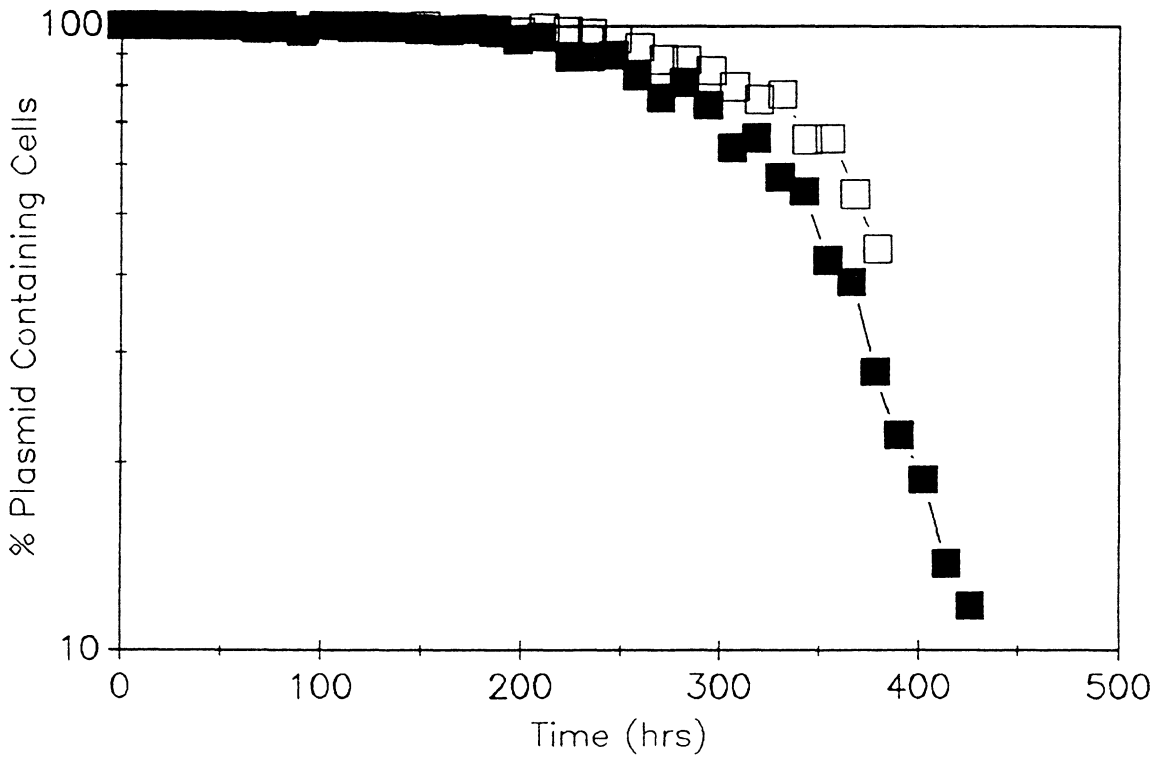


Figure 4-6. Effect of cycling frequency on reactor stability. Comparison of 2/1 frequency, dilution rate was switched between 0.46 and 0.31 hr^{-1} at a frequency of two and one hour, respectively, (□) and 2/2 frequency (■) experiment.

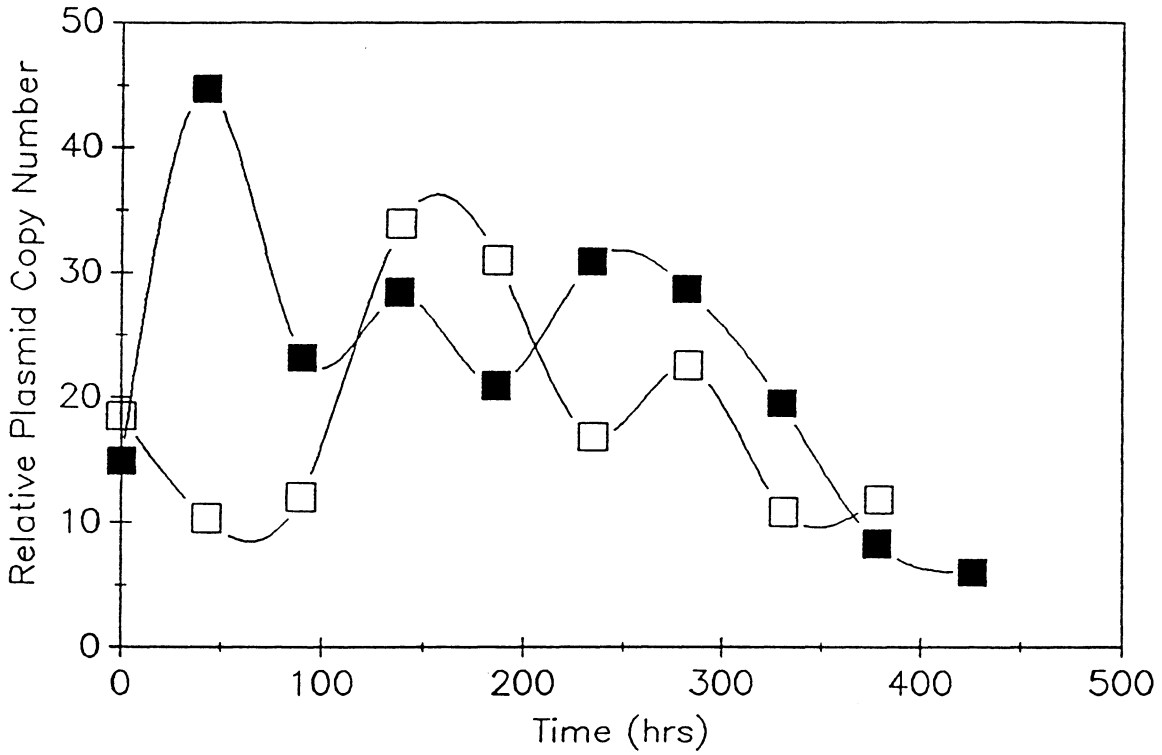


Figure 4-7. Comparison of relative plasmid copy number, plasmid DNA per chromosome DNA, for 2/1 frequency cycling (□) and 2/2 frequency (■) experiments.

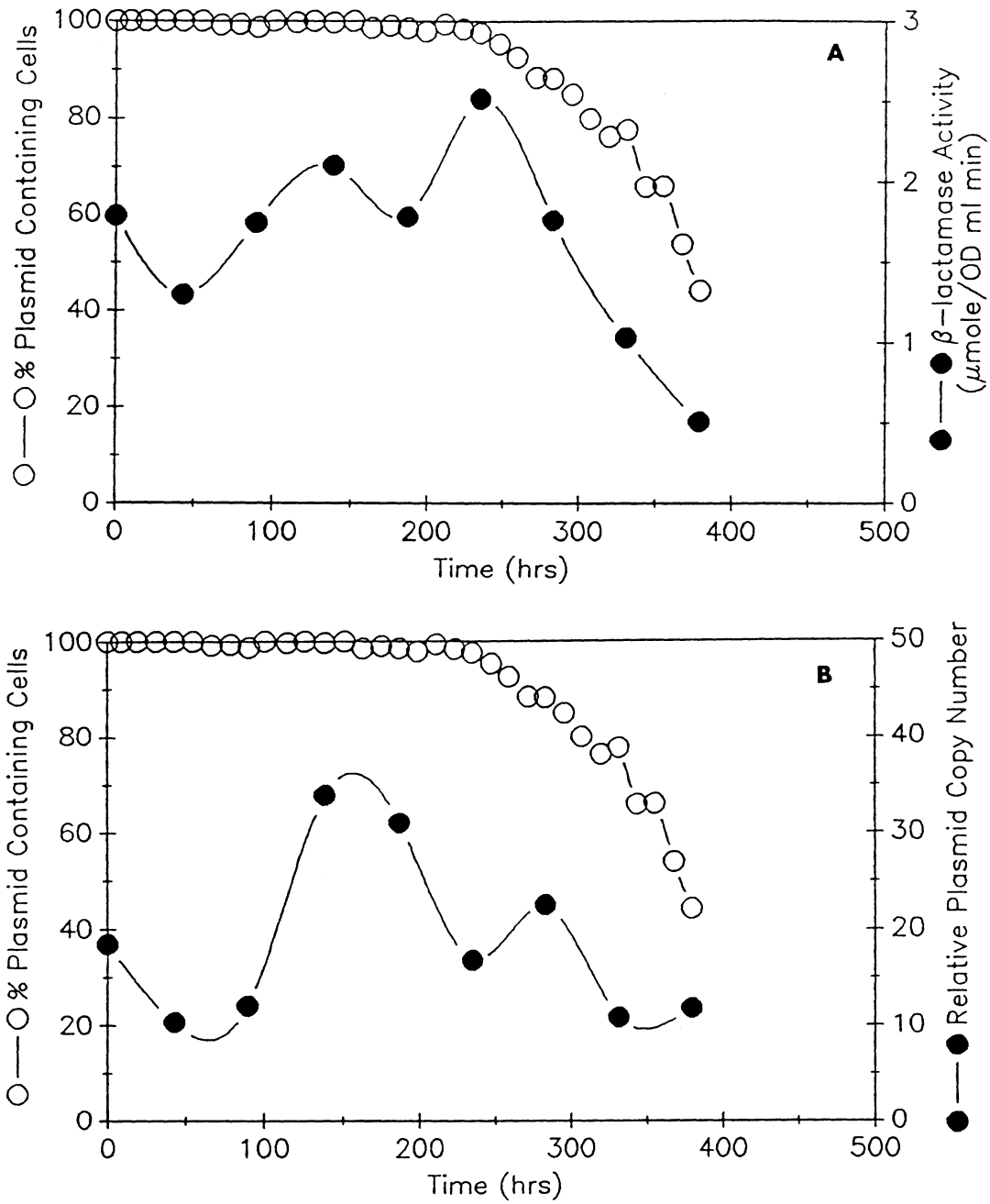


Figure 4-8. Comparison of the percentage of plasmid-containing cells and the (a) β-lactamase activity and (b) relative plasmid copy number, plasmid DNA per chromosome DNA, for the 2/1 cycling frequency experiment.

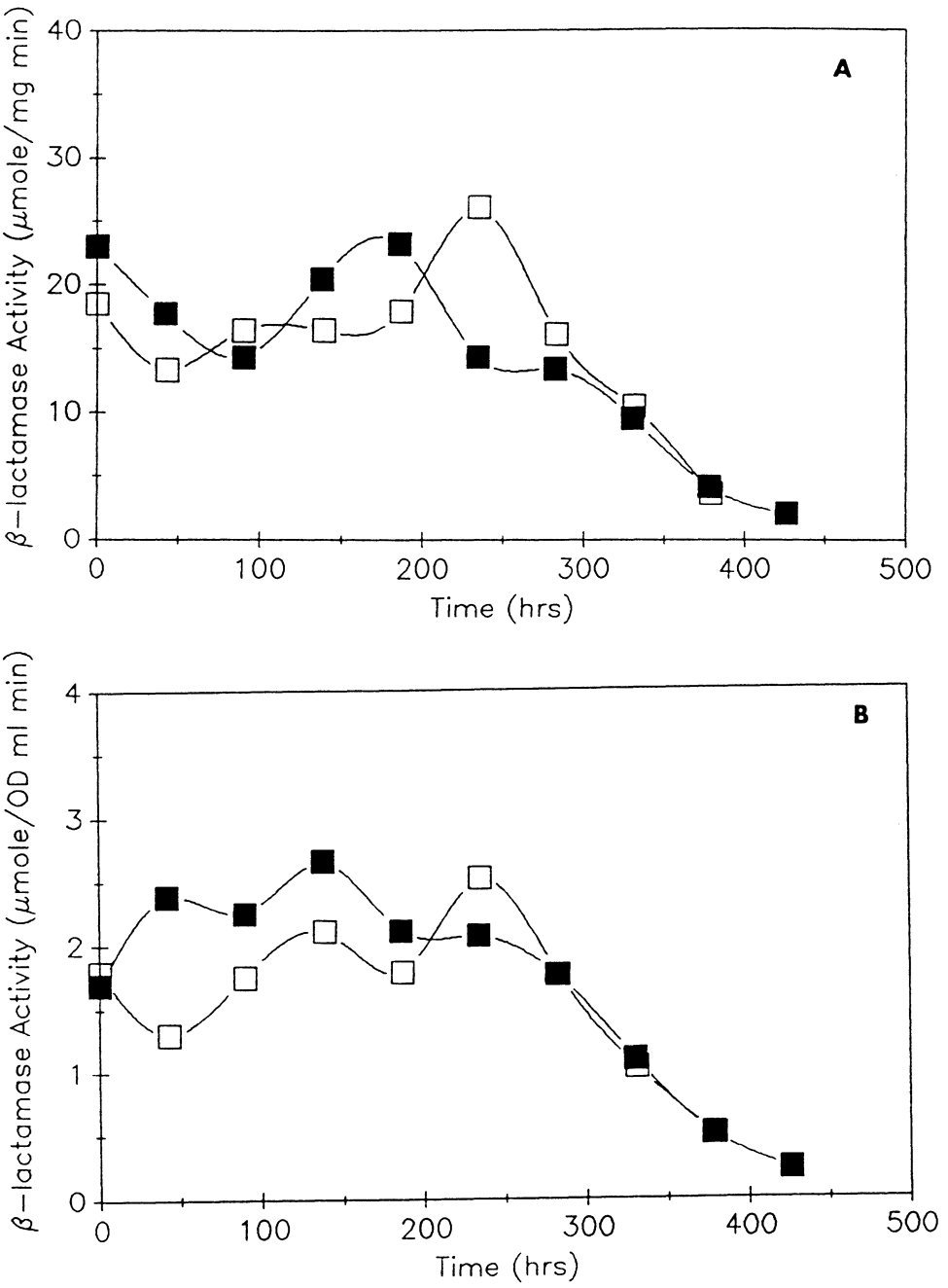


Figure 4-9. (a) Specific ß-lactamase activity, based on total protein, for 2/1 frequency cycling (□) and 2/2 frequency cycling experiment (■). (b) Expression of ß-lactamase activity/ml culture/ OD for 2/1 frequency cycling (□) and 2/2 frequency cycling experiment (■).

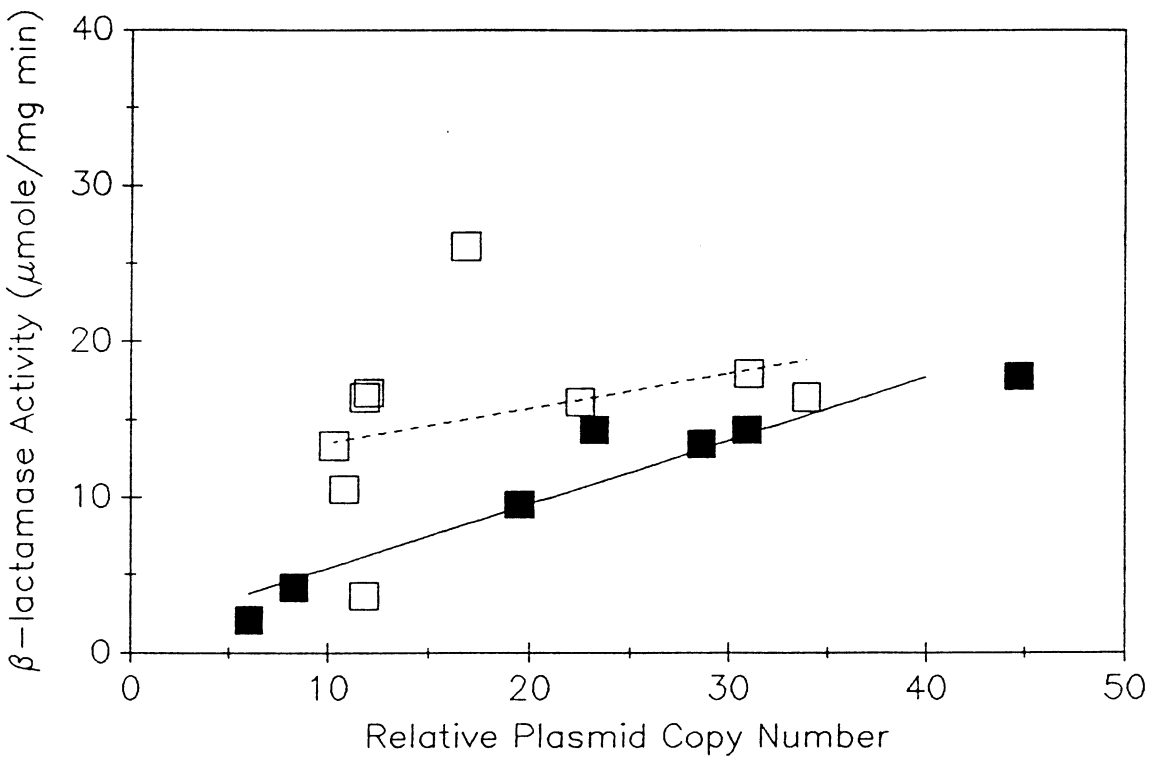


Figure 4-10. Relationship between the cloned gene product, β-lactamase activity, and the relative plasmid copy number for 2/1 frequency cycling (□) and 2/2 frequency cycling (■) experiment.

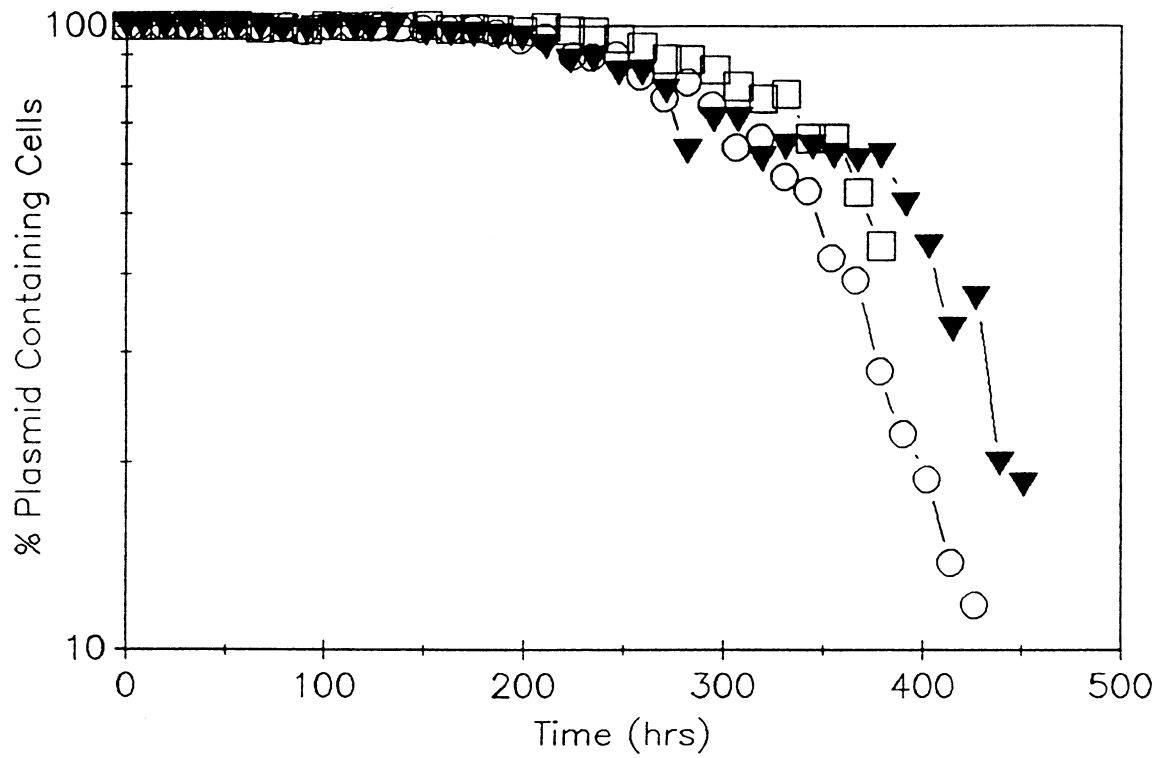


Figure 4-11. Effect of cycling frequency on reactor stability. Comparison of random cycling frequency, dilution rate was switched between 0.31 and 0.46 hr^{-1} with the overall frequency kept between 2/2 and 2/1, (▼), 2/2 frequency (○), and 2/1 cycling frequency (□) experiments.

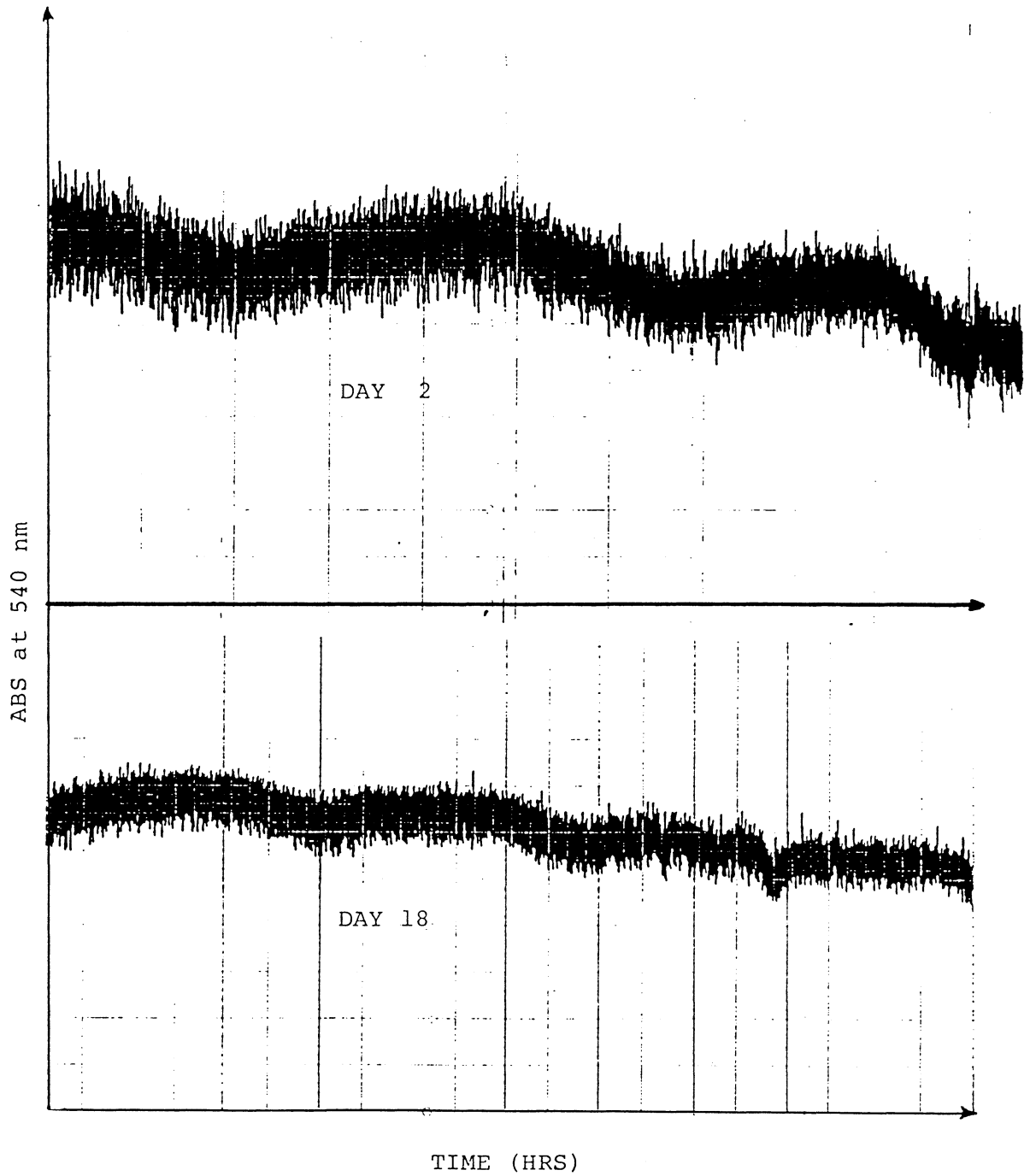


Figure 4-12. Adaptation of the fermentation culture to the perturbations in the dilution rate under random cycling.

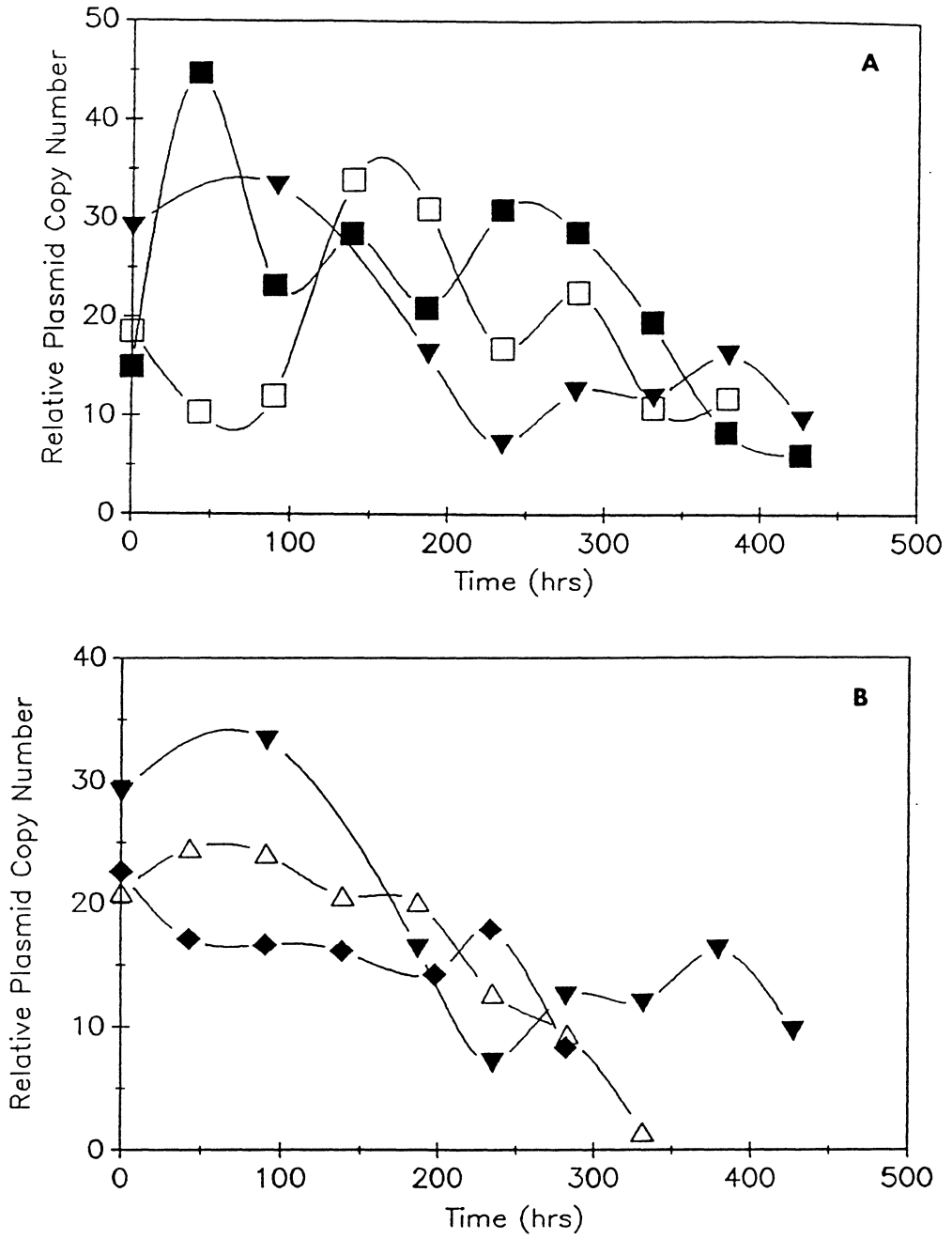


Figure 4-13. Comparison of relative plasmid copy number, plasmid DNA per chromosome DNA, for (a) random cycling (▼), 2/1 frequency cycling (□), and 2/2 frequency (■) experiments, and (b) random cycling (▼), and D = 0.31 (△) and 0.45 (◆) hr^{-1}.

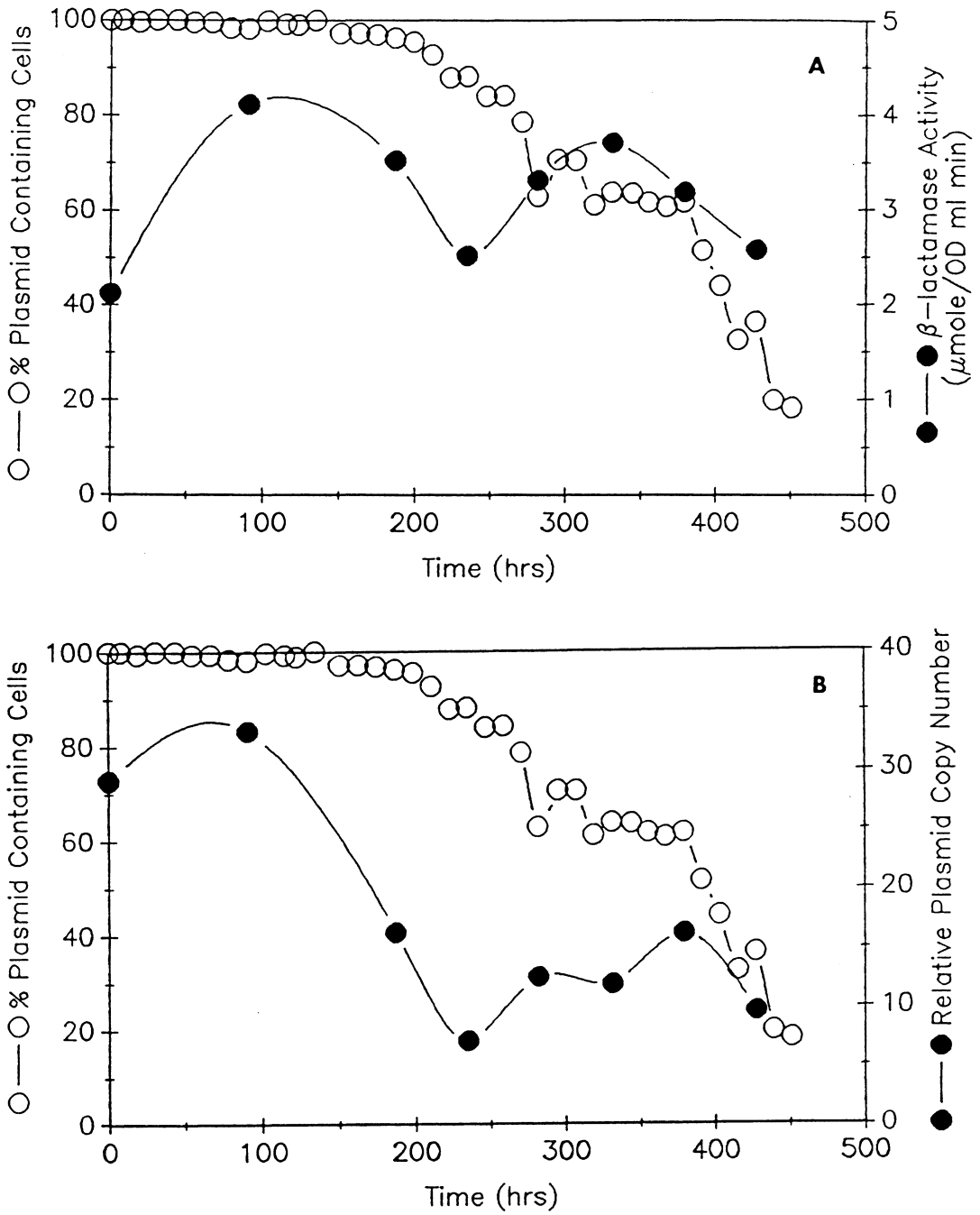


Figure 4-14. Comparison of the percentage of plasmid-containing cells and the (a) β-lactamase activity and (b) relative plasmid copy number, plasmid DNA per chromosome DNA for the random cycling frequency experiment.

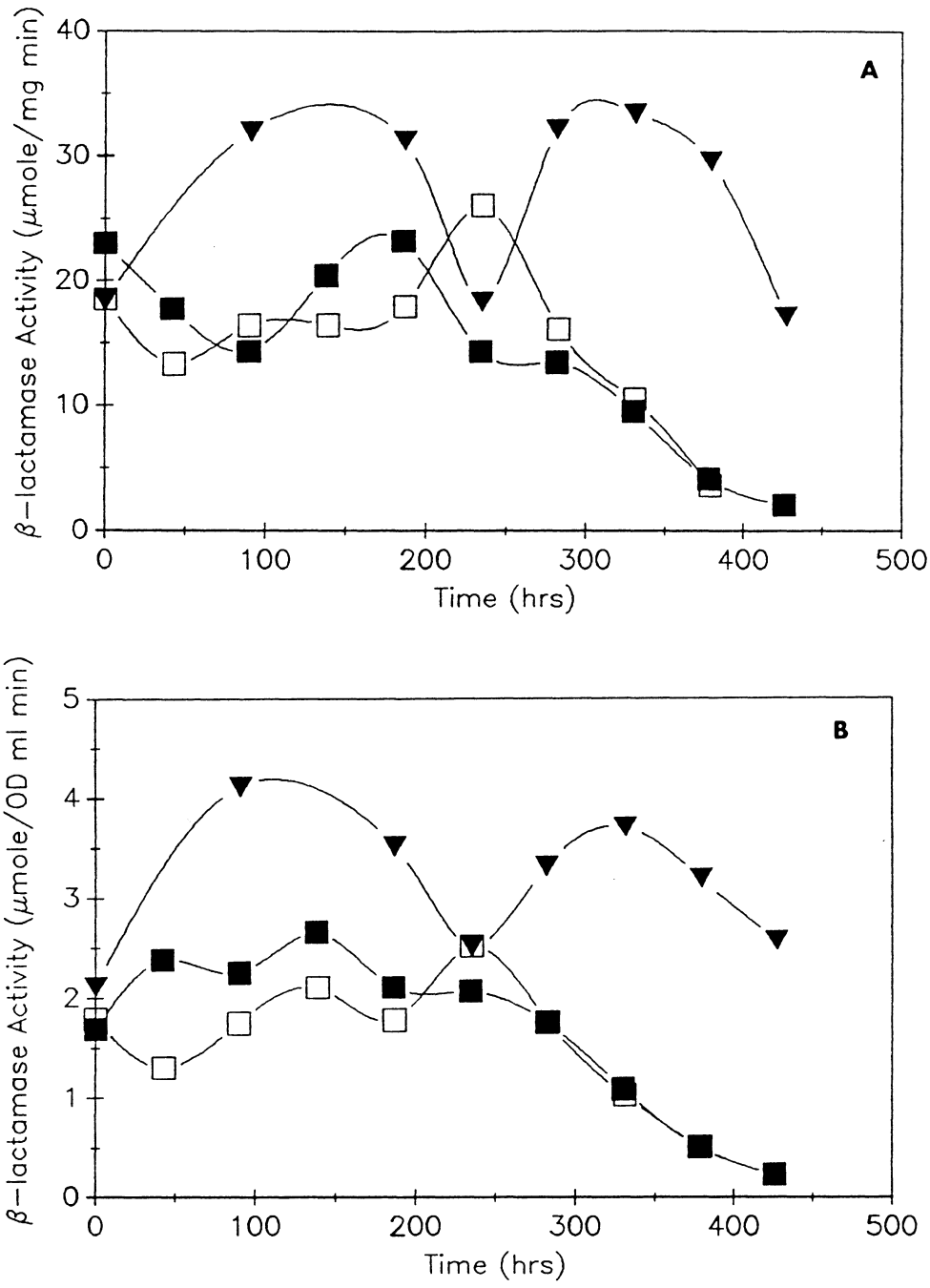


Figure 4-15. (a) Specific β-lactamase activity, based on total protein, for random frequency (▼), 2/1 frequency cycling (□), and 2/2 frequency cycling experiment (■). (b) Expression of β-lactamase activity/ml culture/OD for random frequency (▼), 2/1 frequency cycling (□), and 2/2 frequency cycling experiment (■).

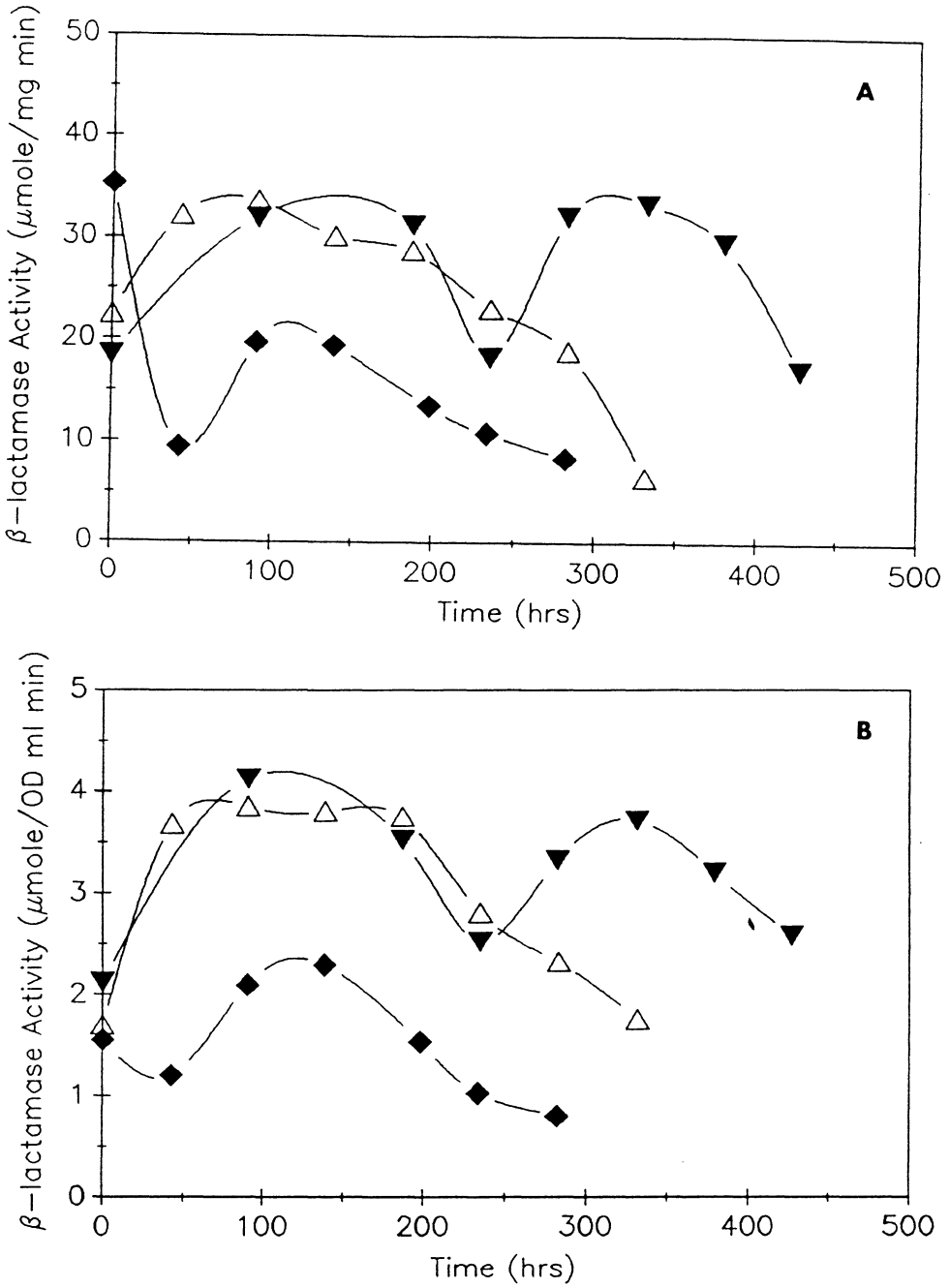


Figure 4-16. (a) Specific β-lactamase activity, based on total protein, for random frequency (▼) and D = 0.31 (△) and 0.45 (◆) hr^{-1}. (b) Expression of β-lactamase activity/ml culture/OD for random frequency (▼) and D = 0.31 (△) and 0.45 (◆) hr^{-1}.

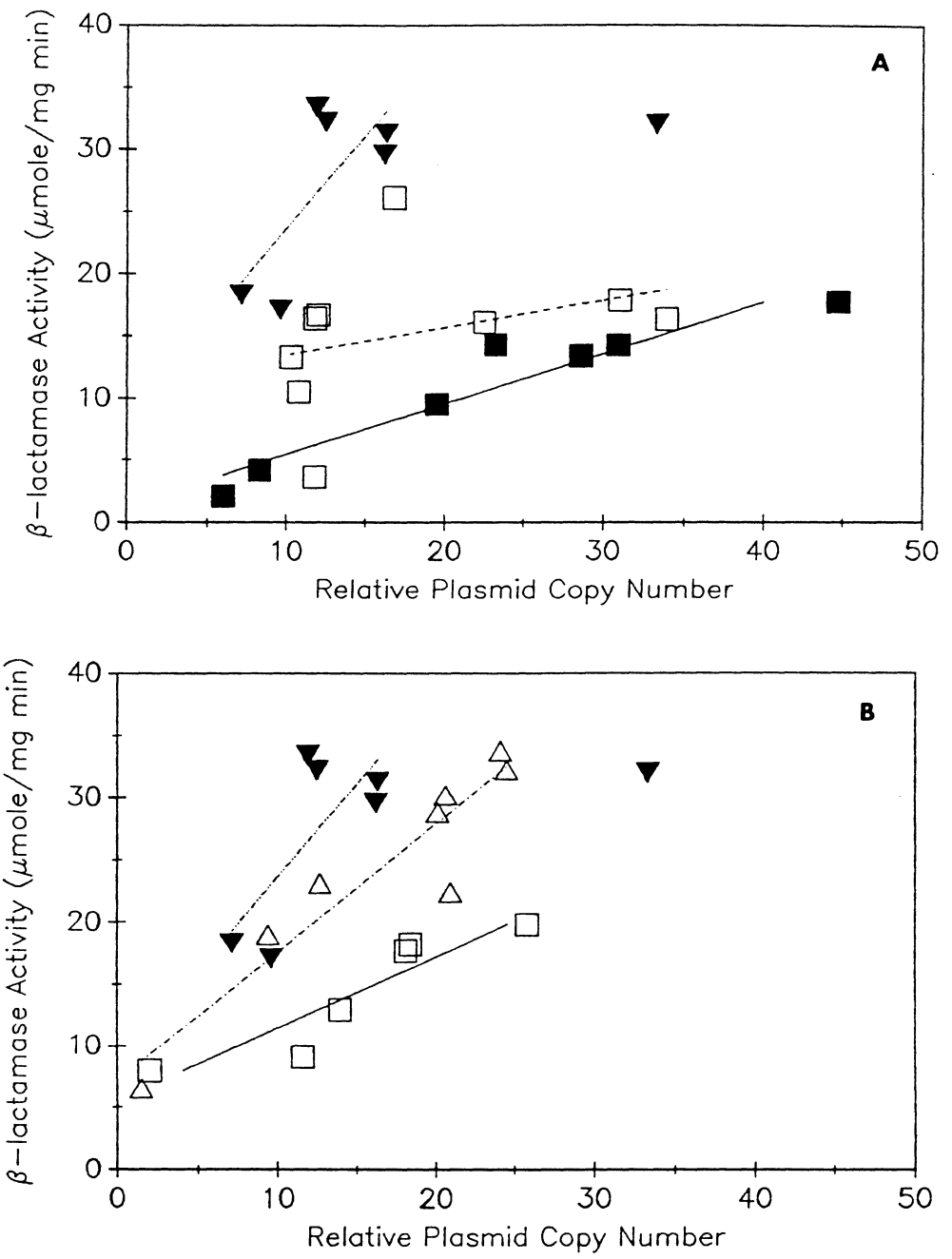


Figure 4-17. Relationship between the cloned gene product, β-lactamase activity, and the relative plasmid copy number for (a) random frequency (▼), 2/1 frequency cycling (□), and 2/2 frequency cycling experiment (■) and (b) random frequency (▼) and control experiments, D = 0.31 (△) and 0.61 (□) hr^{-1}.

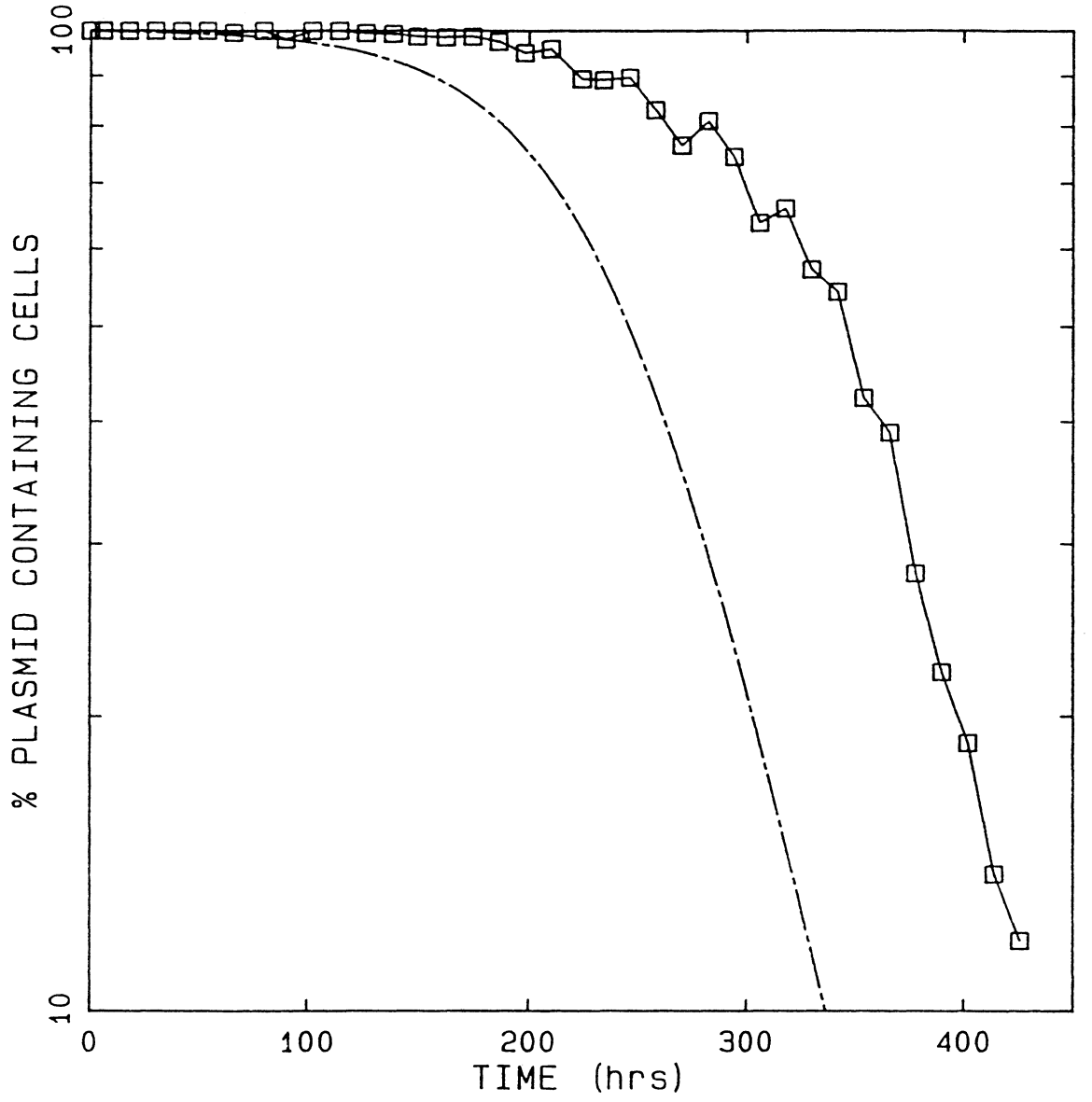


Figure 4-18. Comparison of model prediction (—·—·—) for parameters $\delta\mu_H$ = -0.025, p_H= 1.0e^{-4} and $\delta\mu_L$ = -0.023, p_L= 3.0e^{-5}, Chapter 3, against experimental data (□) for 2/2 frequency cycling at D = 0.31 and 0.46 hr^{-1}.

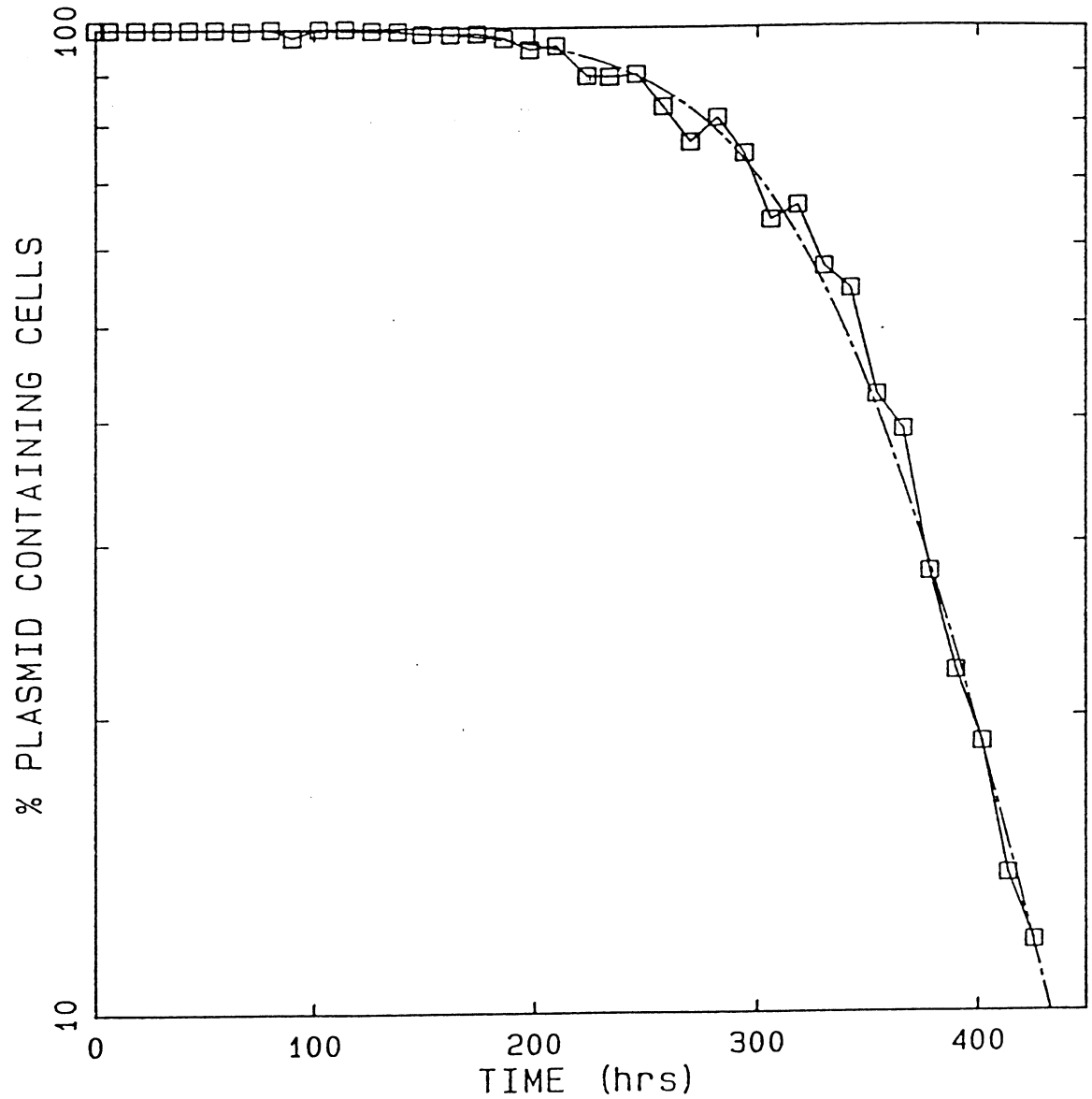


Figure 4-19. Model prediction with parameters based on time-averaged values ($\delta\mu$ = -0.023 and p = $9.6e^{-6}$) for the 2/2 frequency cycling experiment.

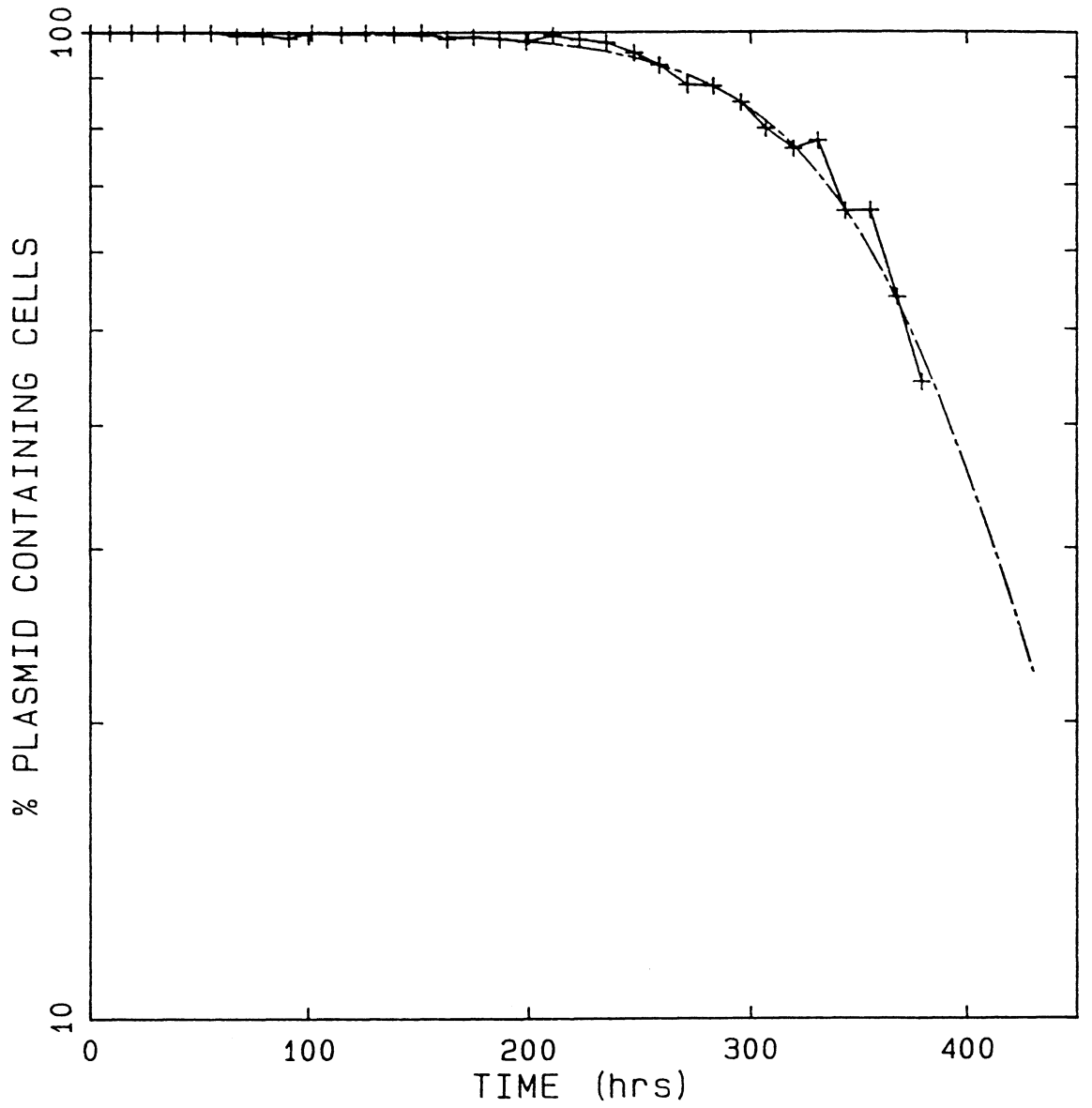


Figure 4-20. Model prediction with parameters based on time-averaged values ($\delta\mu$ = -0.022 and p = $5.9e^{-6}$) for the 2/1 frequency cycling experiment.

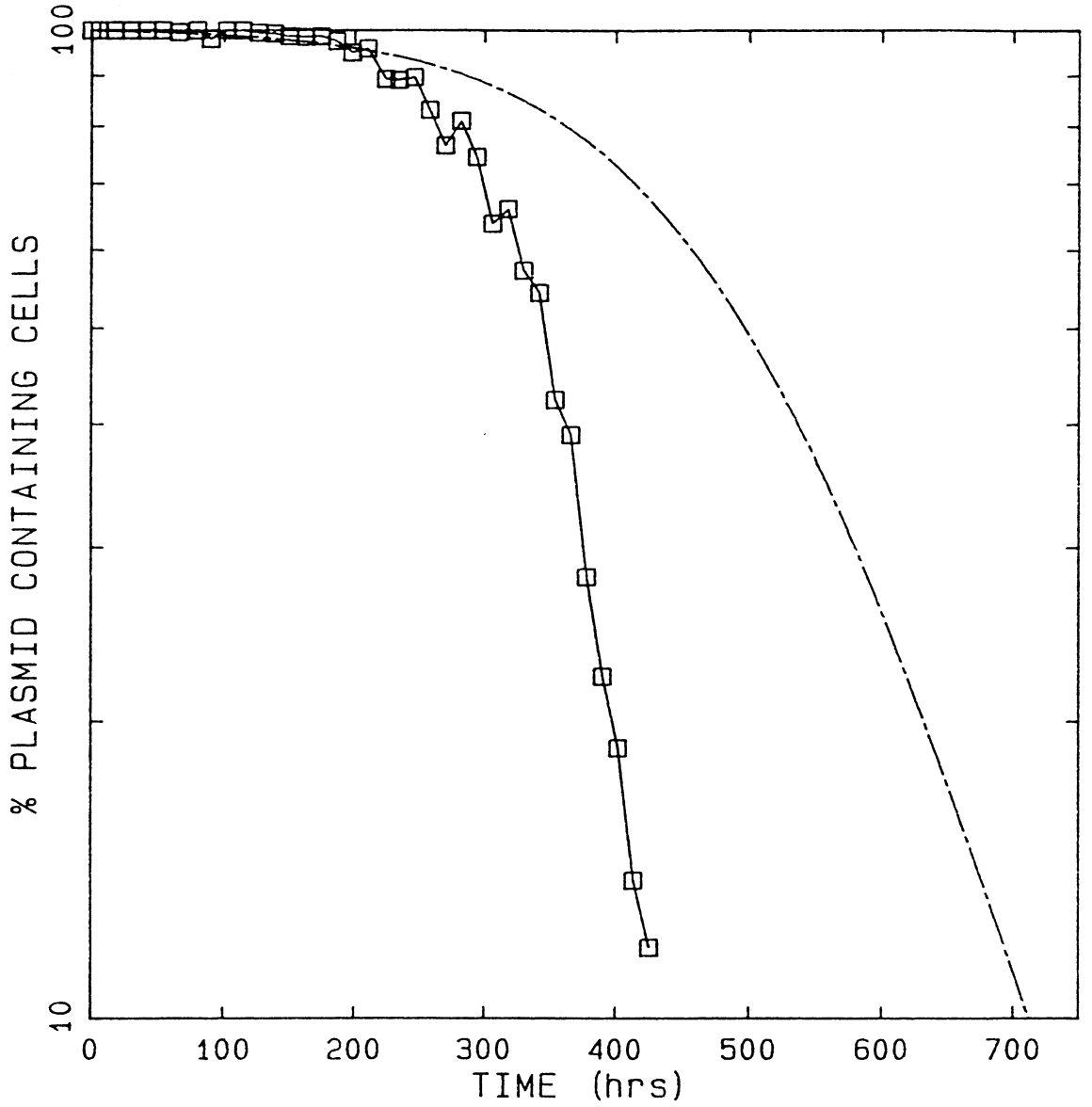


Figure 4-21. Model prediction (—-—) based on measured parameters $\delta\mu_H$ = -0.0082, p_H= 1.0e^{-4} and $\delta\mu_L$ = -0.012, p_L= 3.0e^{-5} for 2/2 frequency cycling experimental data (□) at D = 0.31 and 0.46 hr^{-1}.

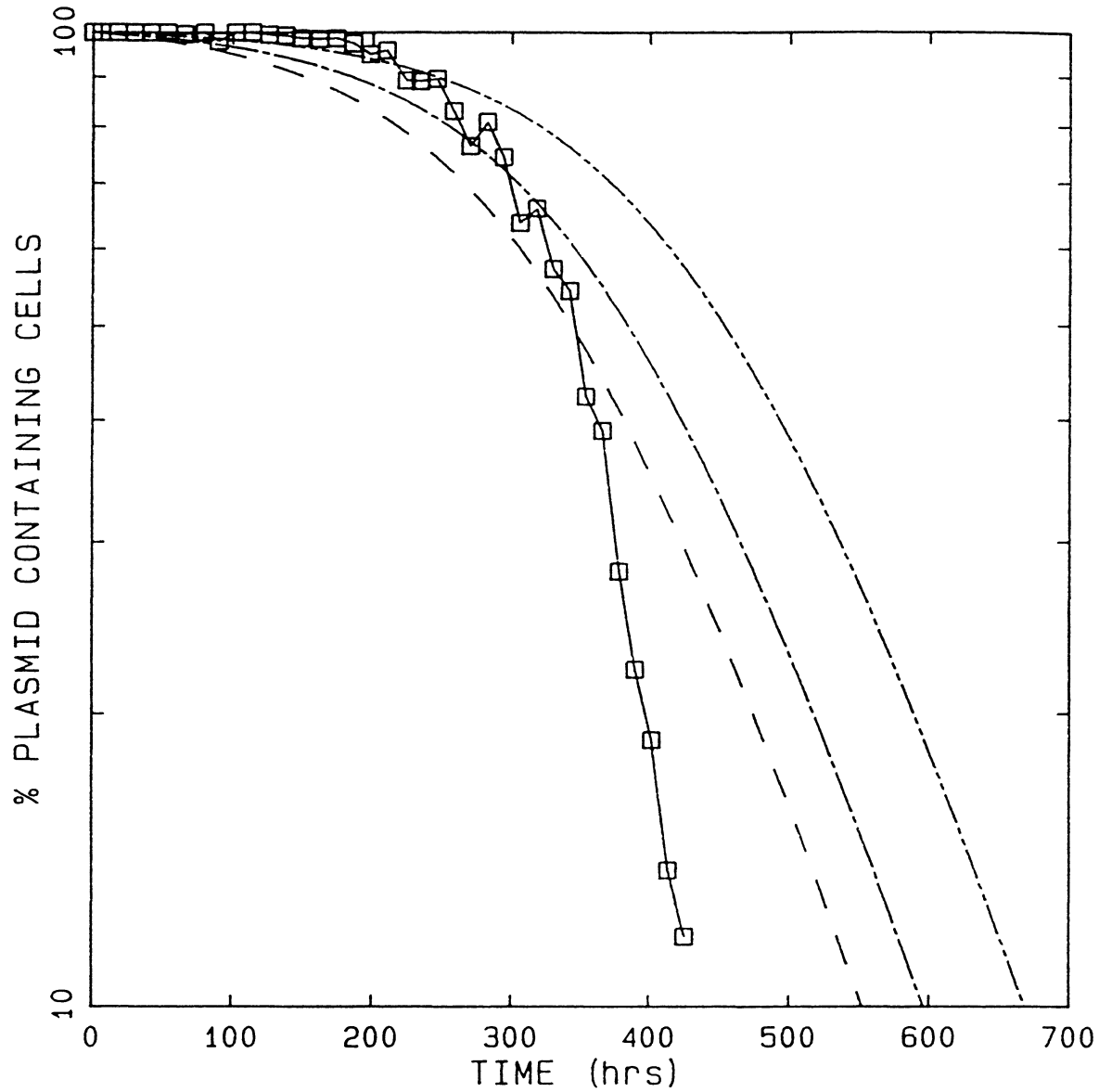


Figure 4-22. Model prediction for 2/2 frequency cycling experiment with different values of p and constant $\delta\mu$ ($\delta\mu_H$ = -0.0082 and $\delta\mu_L$ = -0.012 and p = $1.0e^{-4}$ (— —), p = $2.0e^{-4}$ (— — —), and p = $3.0e^{-4}$ (— — —).

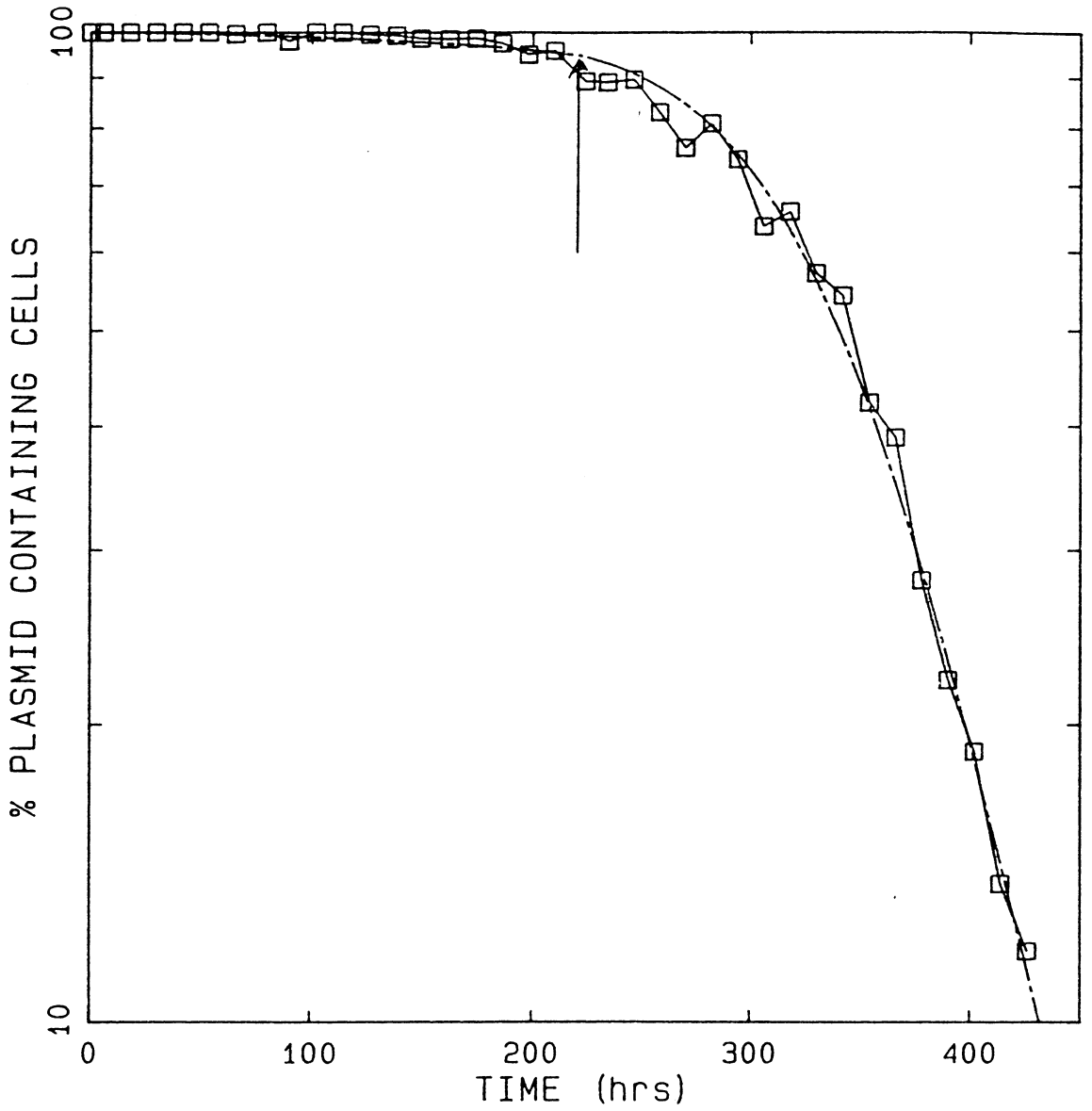


Figure 4-23. Model prediction for 2/2 frequency cycling experiment with different sets of $\delta\mu$.
0 - 220 hrs: $\delta\mu_H = -0.0082$; $p_H = 1.0e^{-4}$
$\delta\mu_L = -0.012$; $p_L = 3.0e^{-5}$
220hrs - end: $\delta\mu_H = -0.025$; $p_H = 1.0e^{-4}$
$\delta\mu_L = -0.023$; $p_L = 3.0e^{-5}$

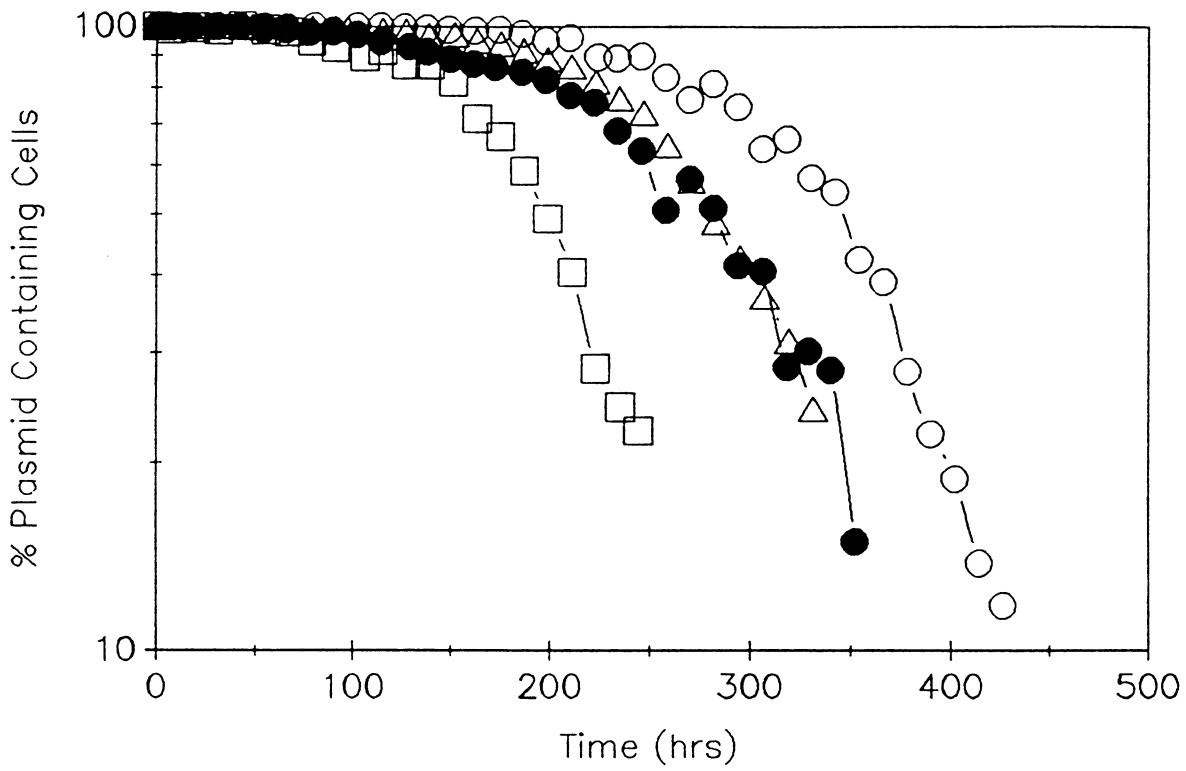


Figure 4-24. Effect of cycling amplitude on reactor stability. Comparison of 2/2 amplitude cycling, dilution rate was switched between 0.31 and 0.61 hr^{-1} at a frequency of two hours, (●) and 2/2 frequency, dilution rate was switched between 0.31 and 0.46 hr^{-1} at a frequency two hours, (O) and control experiments, D = 0.31 (△) and 0.61 (□) hr^{-1}.

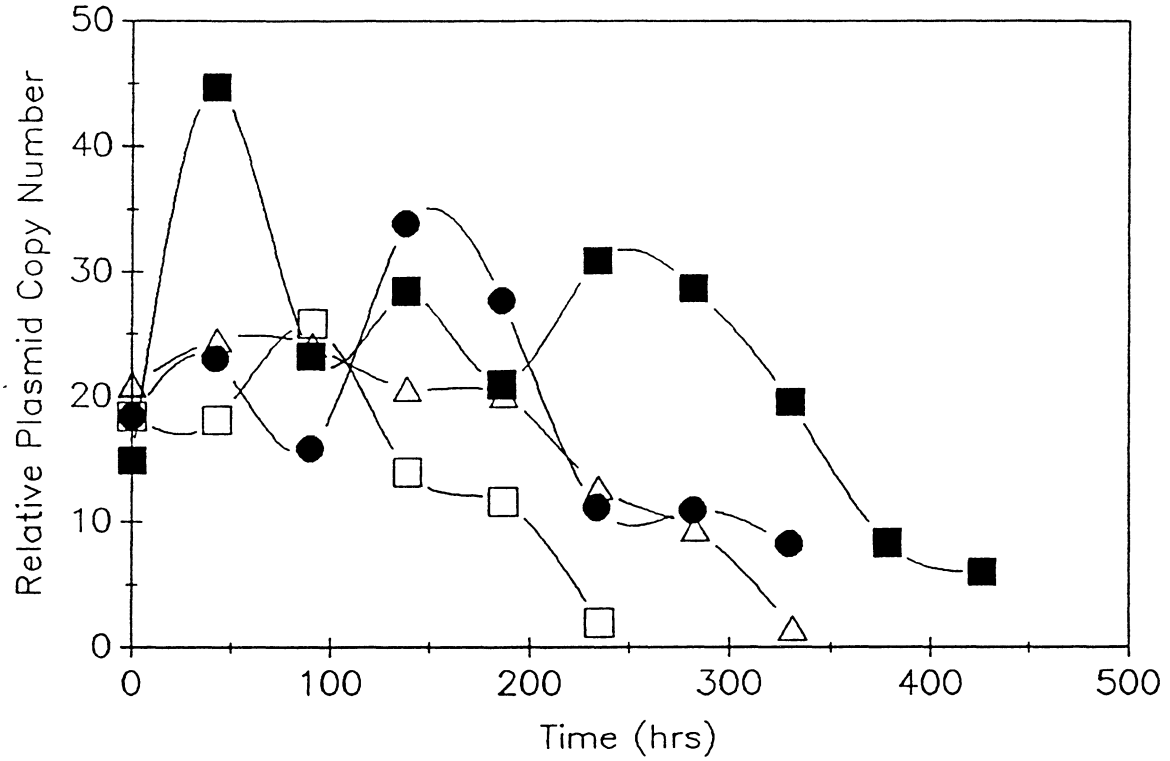


Figure 4-25. Comparison of relative plasmid copy number, plasmid DNA per chromosome DNA, for 2/2 amplitude cycling, dilution rate was switched between 0.31 and 0.61 hr^{-1} at a frequency of two hours, (●) and 2/2 frequency, dilution rate wasswitched between 0.31 and 0.46 hr^{-1} at a frequency two hours, (■) and control experiments, D = 0.31 (△) and 0.61 (□) hr^{-1}.

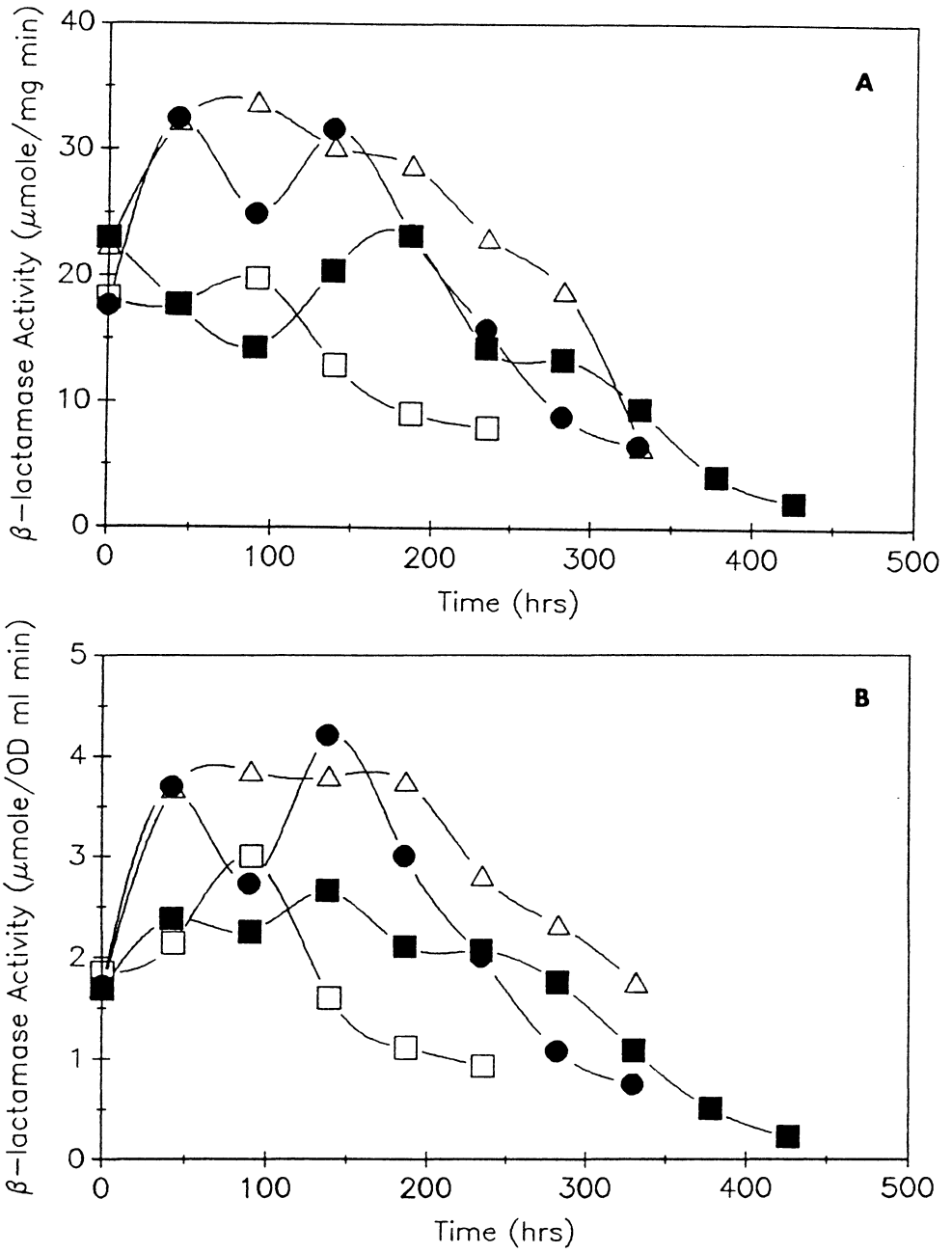


Figure 4-26. (a) Specific β-lactamase activity, based on total protein, and (b) expression of β-lactamase activity/ml culture/OD for 2/2 amplitude cycling, dilution rate was switched between 0.31 and 0.61 hr^{-1} at a frequency of two hours,(●) and 2/2 frequency, dilution rate was switched between 0.31 and 0.46 hr^{-1} at a frequency two hours, (■) and control experiments, D = 0.31 (△) and 0.61 (□) hr^{-1}.

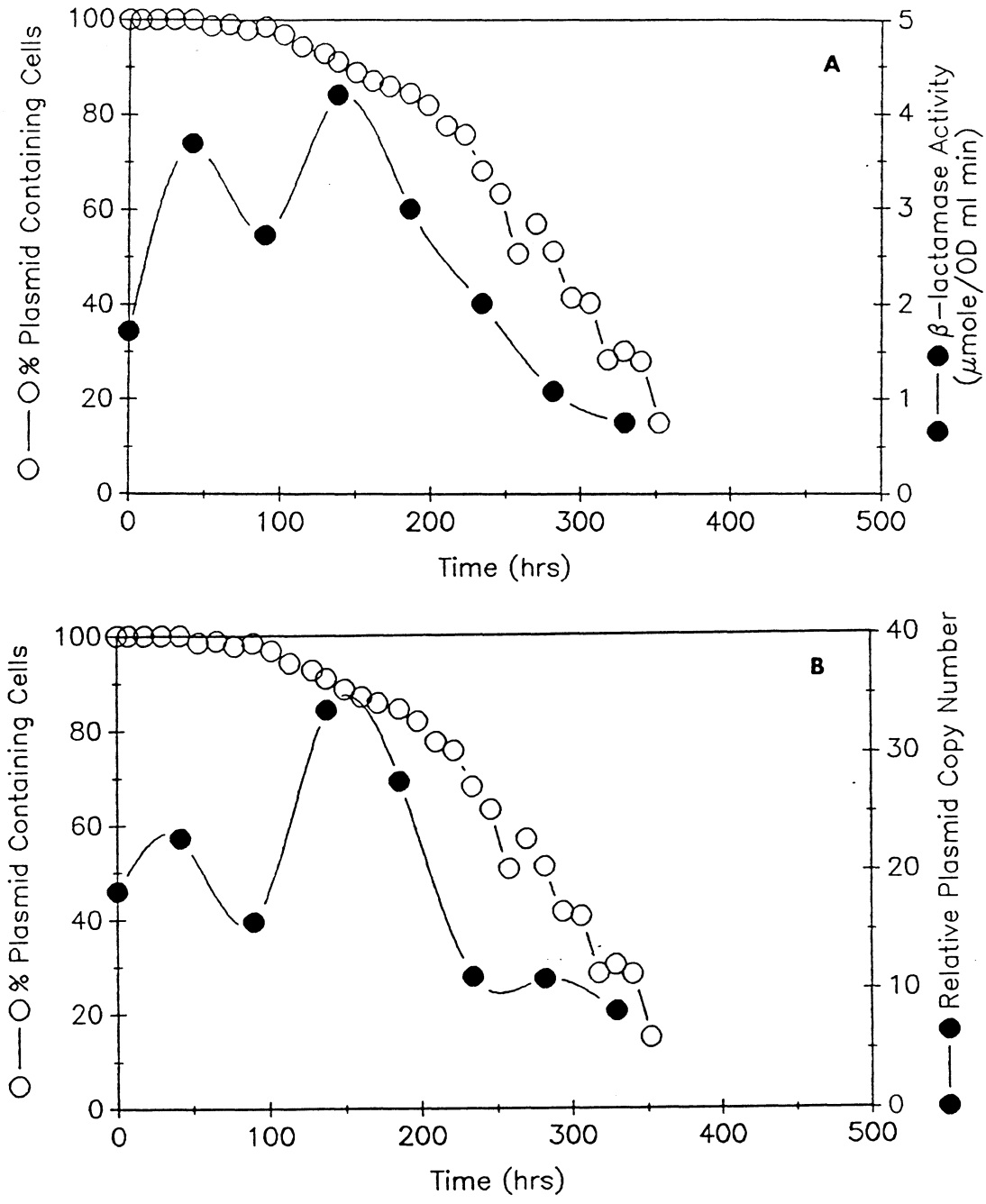


Figure 4-27. Comparison of the percentage of plasmid-containing cells and the (a) β-lactamase activity and (b) relative plasmid copy number, plasmid DNA per chromosome DNA, for the 2/2 amplitude, D = 0.31 and 0.61 hr^{-1}, cycling experiment.

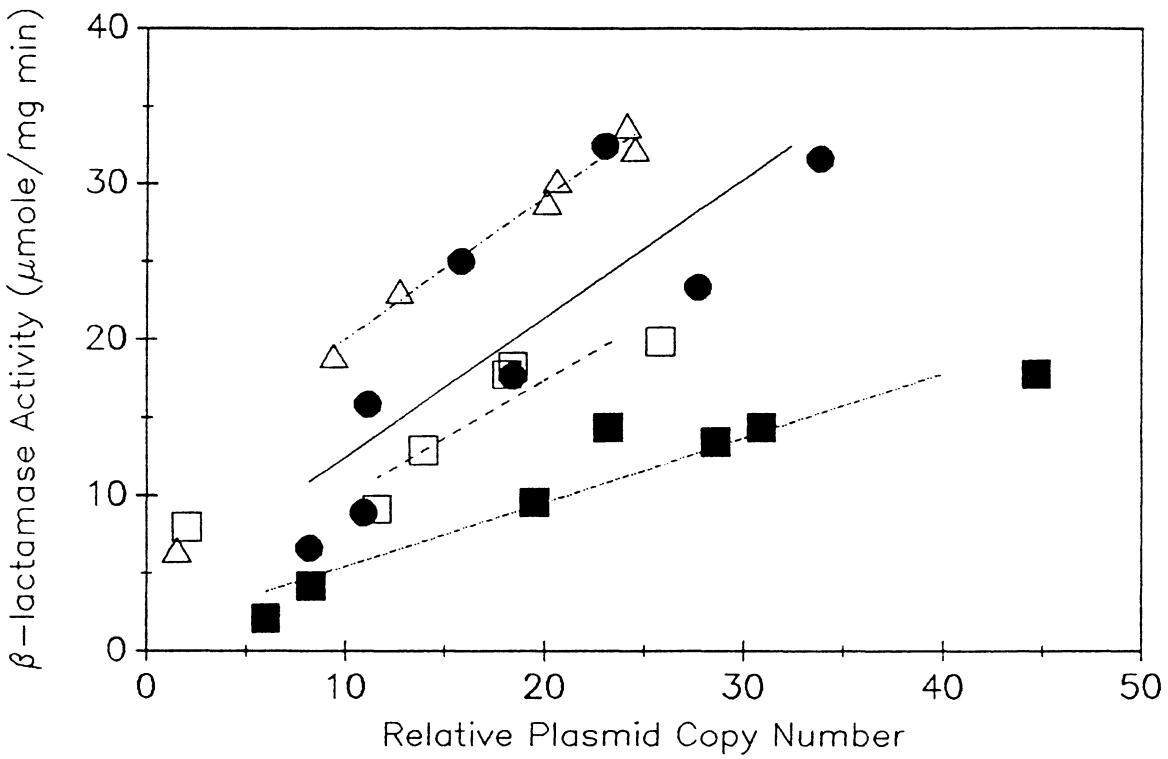


Figure 4-28. Relationship between the cloned gene product, β-lactamase activity, and the relative plasmid copy number for 2/2 amplitude cycling, dilution rate was switched between 0.31 and 0.61 hr^{-1} at afrequency of two hours, (●) and 2/2 frequency, dilution rate was switched between 0.31 and 0.46 hr^{-1} at a frequency two hours, (■) and control experiments, D = 0.31 (△) and 0.61 (□) hr^{-1}.

CHAPTER 5

POPULATION DYNAMICS OF A RECOMBINANT CULTURE IN A CHEMOSTAT UNDER PROLONGED CULTIVATION

5.1 Introduction

The dynamics of a recombinant culture in a chemostat with non-selective media have been discussed in Chapter 3. In general, for an unstable plasmid system, it was observed that the culture goes through a stable lag phase followed by a rapid increase in the plasmid-free population. Two parameters have been found to have significant effects on the overall dynamics of the chemostat culture. These two parameters are the probability of plasmid loss due to faulty segregation during cell division and the difference in the specific growth rate between the recombinant and reverted cells. Several lumped parameter models have been proposed which predict the experimental results fairly well [99,129,195]. However, these models fail to predict two observations: 1) a decrease in β-lactamase activity which usually precedes a decrease in the population of ampicillin resistant cells [254] and 2) the gradual decline in the minimum inhibitory concentration and plasmid content under prolonged chemostat cultivation [110]. More recently, a consistent discrepancy has been observed to exist between the spread and replica plating methods used to determine the percentage of plasmid-containing cells[248].

The difference is more pronounced during the transient period when the plasmid-free cells begin to emerge. It is speculated that the discrepancy is due to the detection of different sub-populations by the two methods. These sub-populations could have a significant effect on the stability and productivity of a plasmid-containing culture.

The focus of this chapter is to examine the dynamics of a recombinant culture in a chemostat by monitoring the ability of the culture to form colonies on plates supplemented with different ampicillin concentrations. A previously derived model is extended to describe the results obtained.

5.2 Experimental Evidence of the Heterogeneity of a Plasmid-Containing Population

Many techniques have been used in the past to study plasmid stability. Commonly used methods include enzymatic assays of the cloned gene product, determination of the plasmid copy number, and cell plating [72,92,213]. Although all of these techniques were used in this work, the focus of this section is to compare the results from two different cell plating techniques, described in Chapter 2, used to estimate the segregational stability of *E. coli* RR1 harboring the plasmid pBR322 in a chemostat under prolonged cultivation. The data obtained from the two plating methods are an indication of the existence of plasmid-containing sub-populations.

The results from two chemostat experiments operating at dilution rates of 0.31 and 0.61 hr^{-1} are given in Figure 5-1. Despite the use of multiple plates in the spread method, the data obtained are still noisy. Nevertheless, there is no apparent inconsistency between the two plating techniques during the first 100 hours. However, after approximately 150 hours into the experiments, a difference in the estimate of the percentage of plasmid-containing cells, as reflected by the two methods, can be observed. The replica method usually yields a higher estimate. This period corresponds to the time when the portion of plasmid-free cells began to increase rapidly. The differences between the two procedures can be shown to be within a 95% confidence level using the null hypothesis testing method. Toward the end of the experiments, when the culture is dominated by the plasmid-free cells, the data from the two methods converge again and the statistical significance of the deviations diminishes. Data from other experiments also exhibit similar behavior.

Included in Figure 5-1 are the normalized results for the plasmid copy number and the β-lactamase activity for the two experiments. The enzyme activity and copy number were normalized with respect to the highest values. The normalized plasmid copy number follows more closely to the curve determined by the spread plate method. The β-lactamase activity is more scattered but follows a similar trend of

gradual decline. As previously shown in Figures 3-13 and 3-15, the β-lactamase activity and plasmid copy number drop before a decrease in the population of plasmid-containing cells is observed.

Since the only major difference between the spread and replica methods is the origination of the colony: in the spread method an individual colony is formed from a single cell while in the replica method a colony is formed from a cluster of cells transferred onto the antibiotic supplemented plate. The discrepancy between the methods might be due to synergistic effects. It is postulated the recombinant population, under prolonged cultivation, becomes heterogeneous with respect to the plasmid copy number as time progresses: a "weaker" sub-population carrying the plasmid, but not strong enough to confer sufficient antibiotic resistance capability, emerges. The higher estimate of the percentage of plasmid-containing cells by the replica method may be due to the inclusion of the weaker sub-populations. This speculation is consistent with the observation of a decrease in the minimum inhibitory concentration of a chemostat culture, even for a stable plasmid system, with time [110] and the decrease in the plasmid copy number and β-lactamase activity before the appearance of plasmid-free cells. Culture heterogeneity with respect to the copy number has also been observed using a flow cytometer [48].

A series of chemostat experiments were conducted in an

attempt to monitor the sub-population within the recombinant culture. The percentage of cells that were capable of forming colonies on plates supplemented with 25 mg/l tetracycline (Tc) or 25, 50, 100, 500, 2000 mg/l ampicillin (Ap). The time profiles for the fraction of cells that grew on the antibiotic supplemented plates from two chemostat experiments are shown in Figures 5-2 and 5-3. The dilution rates were kept constant at 0.31 and 0.61 hr^{-1}, respectively. The results from two cyclic experiments, described in Chapter 4, are included in Figures 5-4 and 5-5. The general trend is similar among the different antibiotic plates. The culture exhibited a stable lag phase followed by a gradual decline in the fraction of ampicillin resistant cells. No significant differences among the plates were observed during the lag phase. However, as previously seen in Figure 5-1, substantial deviations appeared during the transient period when the plasmid-free cells begin to emerge. The statistical significance of these differences diminishes when the culture is dominated by the plasmid-free cells. One additional point should be made concerning the ability of the plasmid-containing culture to grow on the 25 mg/l ampicillin plates. Initially, it was believed that this concentration was too low to differentiate between the plasmid-carrying and plasmid-free cells. This speculation was based on experiments, Figures 5-4 and 5-5, in which 100% of the cells were able to form colonies on these plates

continually throughout the experiment. Further experiments, Figures 5-2 and 5-3, however, proved contradictory. The reason for the discrepancy between these results could be due to the reactor conditions in which the cells were cultured. In the transient experiments, unlike the control runs, the cells are capable of forming colonies on these plates. More detailed experiments, such as studying the effects of the transient environment on a plasmid-free culture, are necessary to determine if this is due to the plasmid copy number or some other mechanism caused by the dilution rate oscillations.

Another representation of the same data, obtained by determining the differences between the results from two plates with successive increasing ampicillin concentration, is given in Figures 5-6 - 5-8. The histograms represent the percentage of cells that form colonies within a certain ampicillin concentration range. It should be noted that almost all cells sampled can grow on the 2000 mg/l Ap plates at the beginning of the experiment. However, before the appearance of the plasmid-free cells, a sub-population which can form colonies on the 100-500 mg/l, but not on the 2000 mg/l Ap plates, is detected. As time progresses, the percentage of cells that form colonies on the 2000 mg/l Ap decreases while it increases on the lower ampicillin concentration plates. Toward the end of the experiment, the percentage of population within the 100-500 mg/l range drops and the population below the 100 mg/l Ap plates increases

rapidly.

It has been previously demonstrated [247,249] that plasmid maintenance of a chemostat culture under randomly oscillating dilution rates is greatly enhanced. The results, of the random cycling experiment are shown in Figure 5-9. The behavior of the population distribution is similar to that shown in Figures 5-6 - 5-8. However, it is interesting to note that the intermediate sub-population, within the 100-500 mg/l Ap range, remained in the reactor for a much longer period before another sub-population, not able to grow on or above the 100 mg/l Ap plate, increases rapidly. This result suggests the prolonged existence of the intermediate sub-population may play an important role in the increased stability of the plasmid-containing culture.

In summary, these results indicate the culture is heterogeneous with respect to its ability to form colonies on plates supplemented with different ampicillin concentrations in a chemostat culture under prolonged cultivation. The degree of heterogeneity increased as time progressed. If it is assumed that the ability of the culture to form colonies in a particular ampicillin concentration is related to the plasmid copy number [239], the results can be interpreted as an indication that the culture is heterogeneous with respect to the plasmid copy number. This is further supported by theoretical models [95,204] and heterogeneity experiments using a flow cytometer [47,87,218].

The actual cause of the low copy number population to harbor a lesser plasmid content is not known. It is believed, however, that it is due mainly to physiological effects. This speculation is based on experimental results in which the low copy number isolates were observed to be able to revert back to the normal copy number after growing in enriched conditions. This observation was seen in previous experiments [110].

If the shift to a lower copy number is due to physiological effects one question that arises is the inability of the cell, harboring a lower than normal plasmid copy number, to reach its full plasmid complement during continuous culture. Initially, it was speculated that the cells would have enough time, especially when comparing the time needed for plasmid replication versus the doubling time of the cell, to recover. However, recent results have shown that after a nutritional shift-up plasmid replication was inhibited for approximately half a mass doubling time [21]. In addition, the theoretical recovery time from faulty segregation of a steady-state plasmid concentration at balanced cell growth at dilution rates of 0.69 and 0.35 hr^{-1} were ten and eight generations or ten and sixteen hours, respectively [198]. The recovery time, therefore, is much larger than the doubling time of the cell. It is speculated under continuous cultures the cells do not have the

appropriate amount of time for recovery. These cells will have a growth advantage when compared to cells with a higher plasmid copy number. This results in favorable growth for the lower copy number cells.

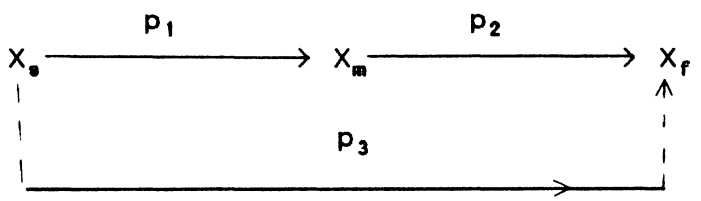
Finally, it should be pointed out that due to the small sample size involved, a portion of the data collected between the 500 and 2000 mg/l ampicillin supplemented plates cannot be shown to be statistically significant. However, if only three sub-populations are considered, that is, when the cells are grouped according to their ability to form colonies on 100 mg/l, between 100 and 2000 mg/l, or above 2000 mg/l Ap, most of the data obtained can be shown to be within a 95% confidence level using chi-square tests. The data interpreted in this manner will be used in the following parameter estimation and model comparison study.

5.3 Model Development

The experimental results presented in the previous section indicate that the recombinant culture is heterogeneous with respect to the plasmid copy number. As such, a population balance model with the plasmid copy number as a parameter would be more appropriate to describe the culture behavior.

Population balance models have been used to describe heterogeneity in cell age and size or mass [1,87], cell length [39], and cell volume [88]. Recently, a population balance

model has been developed to describe the dynamics of a plasmid-containing culture [253]. A similar model with the plasmid copy number as a parameter for the above system can be constructed by following the same framework. However, in view of the lack of sufficient data, a simplified three sub-population model, which provides a qualitative agreement of the observed reactor behavior, is proposed. A previously derived lumped parameter two population model [99,129,195] is extended by the addition of a relatively lower copy number sub-population. In this model, the recombinant culture is grouped into three major classes according to their plasmid copy number. The interactions among these three classes are schematically shown in the following diagram:



In the above scheme, X_s stands for the sub-population which harbors a high plasmid copy number while X_m refers to the sub-population which carries a relatively lower plasmid copy number. As the division of X_s and X_m is qualitative in nature, the boundary between the two sub-populations does not have a definitive copy number. In the model, it is assumed

that both sub-populations will lose their plasmid due to imperfect segregation during cell division. The complete shedding of the plasmid will result in a plasmid-free sub-population, X_f. In addition, the high copy number population will migrate into the lower sub-population. This shifting of copy number, as discussed previously, is believed to be mainly due to physiological effects.

The dynamics of the system can be described by the following set of ordinary differential equations:

$$dX_s/dt = (\mu_s - D)X_s - p_1\mu_s X_s - p_3\mu_s X_s \qquad (5\text{-}1a)$$

$$dX_m/dt = (\mu_m - D)X_m - p_2\mu_m X_m + p_1\mu_s X_s \qquad (5\text{-}1b)$$

$$dX_f/dt = (\mu_f - D)X_f + p_2\mu_m X_m + p_3\mu_s X_s \qquad (5\text{-}1c)$$

where X_s, X_m, and X_f denote the high and low copy number plasmid-containing and plasmid-free species, respectively. μ_s, μ_m, and μ_f are the corresponding specific growth rates. p_1 represents the rate of population shift from the high copy number population to the lower sub-population. The probability of plasmid loss due to imperfect segregation during cell division are denoted by p_2 and p_3, respectively. If plasmid partitioning during cell division is purely a random event, as is the case for ColE1-type plasmid, p_2 should be larger than p_3. According to this scheme, the dynamics of

the fraction of plasmid-carrying cells with high copy number, y_s, defined as

$$y_s = X_s/X_T \tag{5-2}$$

is given below

$$dy_s/dt = y_s[\mu_s - p_1' - p_3'] - y_s[\mu_s y_s + \mu_m y_m + \mu_f y_f] \tag{5-3a}$$

similarly, y_m and y_f can be found to be

$$dy_m/dt = y_m[\mu_m - p_2'] + y_s p_1' - y_m[\mu_s y_s + \mu_m y_m + \mu_f y_f] \tag{5-3b}$$

and

$$dy_f/dt = y_f\mu_f + y_m p_2' + y_s p_3' - y_f[\mu_s y_s + \mu_m y_m + \mu_f y_f] \tag{5-3c}$$

where $p_1' = p_1\mu_s$, $p_2' = p_2\mu_m$, and $p_3' = p_3\mu_s$. Expressed in terms of relative growth rate and by using the fact that the sum of the population fractions should add up to unity, equation 5-3 can be rewritten as

$$dy_s/dt = y_s[\delta\mu_1 - p_1' - p_3'] - y_s[\delta\mu_1 y_s + \delta\mu_2 y_m] \tag{5-4a}$$

$$dy_m/dt = y_m[\delta\mu_2 - p_2'] + y_s p_1' - y_m[\delta\mu_1 y_s + \delta\mu_2 y_m] \tag{5-4b}$$

and

$$dy_f/dt = y_m p_2' + y_s p_3' - y_f[\delta\mu_1 y_s + \delta\mu_2 y_m] \tag{5-4c}$$

where $\delta\mu_1$ and $\delta\mu_2$ are defined as

$$\delta\mu_1 = \mu_s - \mu_f \text{ and } \delta\mu_2 = \mu_m - \mu_f$$

It should be pointed out that $\delta\mu_1$ and $\delta\mu_2$ represent the growth rate differences between the high and low copy number sub-population relative to the revertant. It is commonly accepted that the presence of plasmids will pose an additional burden to the host cells resulting in a slower growth rate for the recombinant cells than the plasmid-free cells. This implies that both μ_s and μ_m are smaller than μ_f. Moreover, $\delta\mu_2$ is expected to be smaller than $\delta\mu_1$ due to gene dosage effects.

5.4 Estimation of Model Parameters

The five parameters in the previous model cannot be determined directly from the existing data. Nevertheless, instead of fitting the model to the experimental results by blindly adjusting the parameters, an attempt is made to best estimate these parameters.

As shown in Appendix I, the slope and intercept from a semi-log plot of y_m/y_s as a function of time provide a rough estimate for $\delta\mu_1-\delta\mu_2$ and p_1'. The other parameters are more difficult to obtain. However, it is possible to obtain an order of magnitude estimate by using a reduced model which combines the high and low copy number sub-populations. The estimation scheme previously developed [195] can be directly applied to the reduced model to obtain an 'averaged' estimate of the sum of $\delta\mu_1$ and $\delta\mu_2$, and p_2' and p_3', respectively.

Finally, p_2' and p_3' are assumed to follow an inverse relationship with respect to the plasmid copy number. Based on experimental data, p_2' is estimated to be three to four times that of p_3' in the following computer simulation studies.

5.5 Comparison of Model Prediction with Experimental Data

The parameters determined by the procedures outlined in the previous section for the two control runs with the dilution rate of 0.61 and 0.31 hr^{-1} are listed in Table 5-1. It should be pointed out that the parameters used have not been optimized to best fit the experimental data.

The time profiles of the three sub-populations for the dilution rate of 0.61 hr^{-1} are shown in Figure 5-10. The model agrees qualitatively with the experimental data. However, a faster drop in the higher copy number population is predicted by the model. This results in an earlier appearance of the plasmid-free population. The model also correctly predicts the behavior of the low copy number population which reaches a maximum and then declines, but at a slower rate.

The fraction of cells which form colonies on the 100 mg/l ampicillin plates is compared to the β-lactamase activity, Figure 5-11. The β-lactamase activity is calculated by

assuming the productivity is related to the copy number. Specifically, the model assumed a 50% expression level by the low copy number sub-population as compared to the higher copy number sub-population. The model correctly predicts a lag of the decrease in the fraction of ampicillin resistant cells behind the decrease of the β-lactamase activity of the culture. The lag is due to the appearance of a lower plasmid copy number sub-population before a plasmid-free population is detected.

The results from an experiment with a dilution rate of 0.31 hr^{-1} are given in Figures 5-12 and 5-13. Similar behavior is obtained. It can be seen that the model agrees with the experimental data.

5.6 Conclusions

The most important conclusions and observations from these experiments are:

1. A statistically significant discrepancy in the percent of plasmid-containing data existed between the spread and replica plating techniques. This difference appeared only after approximately 150 hours into the chemostat experiments. Based on these results it is postulated that the recombinant population, under prolonged cultivation becomes heterogeneous with respect to the plasmid copy number resulting in different plasmid-

containing sub-populations.

2. A series of chemostat experiments were conducted in an attempt to monitor the sub-populations within the recombinant culture. The percentage of cells that were capable of forming colonies on plates supplemented with different ampicillin concentrations ranging from 25 to 2000 mg/l during the chemostat experiments were measured. It was observed that almost all cells sampled were able to grow on the high concentration of ampicillin supplemented plates at the beginning of the experiment. However, a sub-population which formed colonies on an intermediate ampicillin concentration, but not on the high concentration plate, was detected before the appearance of the plasmid-free population. As time progressed, the percentage of this sub-population increased, reached a maximum, then decreased toward the end of the experiment. At this time the culture was dominated by a sub-population which was not able to form colonies on the 100 mg/l ampicillin plates.
3. The results indicate that three major processes may occur in the chemostat: 1) a gradual shift of the higher plasmid copy number population toward a relatively lower population, 2) the complete shedding of the plasmid due to faulty plasmid segregation during cell division, and 3) growth competition among the sub-populations. The exact cause for the shift from a high copy number to a

relatively low copy number population is not known. Current results indicate it is mainly due to physiological effects. This speculation is based on experimental results in which the low copy number isolates were observed to be able to revert back to the normal copy number after growing in enriched conditions.

4. To account for all the sub-populations an extension of a previously derived model is performed. The model agrees qualitatively with the experimental results. The model also correctly predicts a lag of the decrease in the fraction of ampicillin resistant cells behind the decrease of the β-lactamase activity of the culture.

5. The existence of the sub-populations of the recombinant culture will have significant implications in the long term productivity of continuous cultures. However, it is not clear if the phenomenon is common to other plasmids with different replication mechanisms or structures.

Table 5-1. Parameters used in the extended model to describe the behavior of the culture sub-populations.

Dilution Rate (hr^{-1})	0.31	0.61
p_1'	1.5×10^{-4}	1.5×10^{-4}
p_2'	7.5×10^{-4}	1.1×10^{-4}
p_3'	2.5×10^{-4}	2.8×10^{-4}
$\delta\mu_1$	-0.033	-0.039
$\delta\mu_2$	-0.020	-0.014

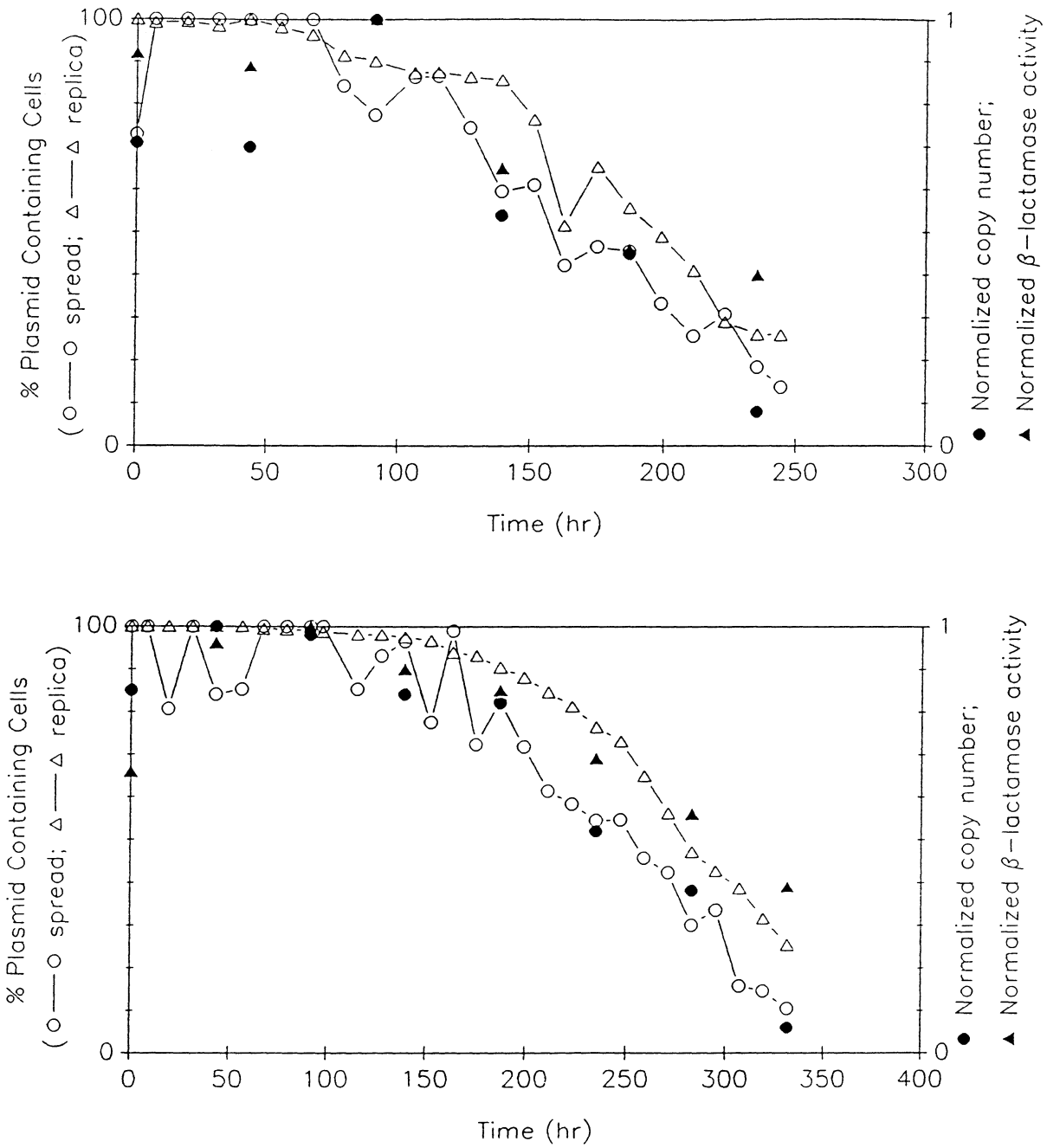


Figure 5-1. Comparison of spread and replica plating methods for dilution rates of a) 0.61 hr^{-1} and b) 0.31 hr^{-1}. Spread data (○), replica data (△), normalized copy number (●), and normalized β-lactamase activity (▲).

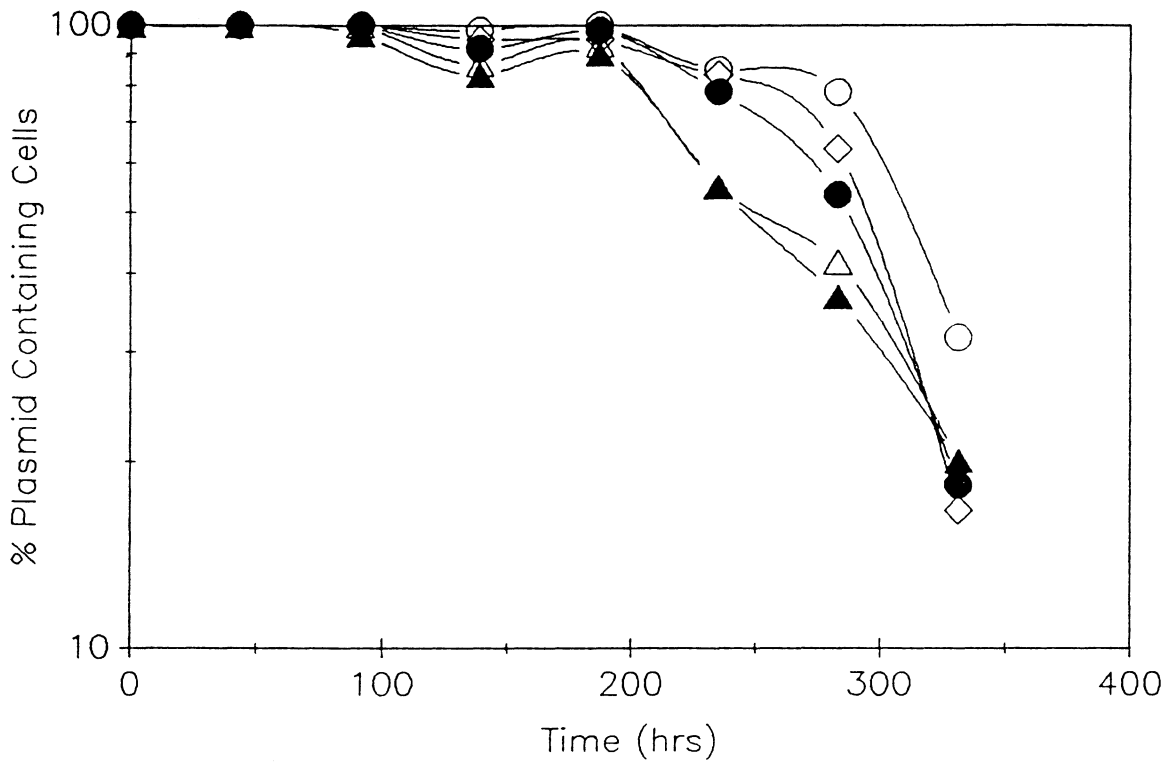


Figure 5-2. Percentage of cells that formed colonies on plates supplemented with 25 (○), 50 (◇), 100 (●), 500 (△), and 2000 (▲) mg/l ampicillin for D = 0.31 hr^{-1}.

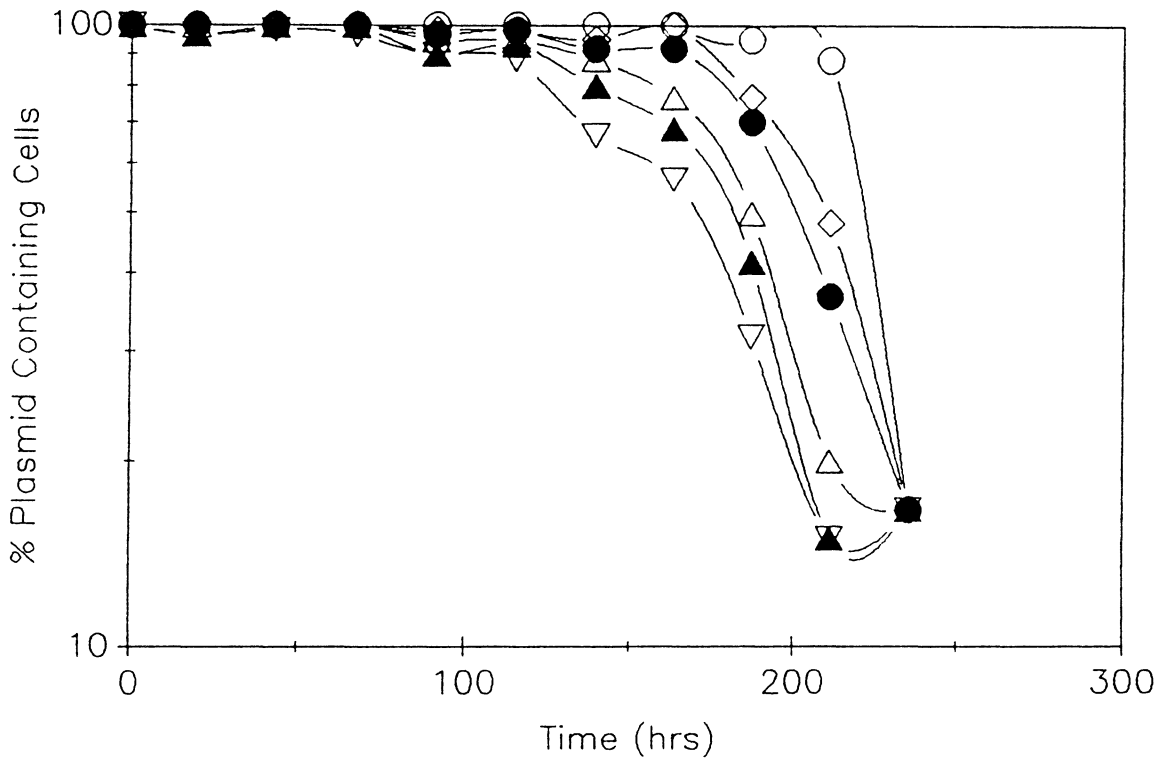


Figure 5-3. Percentage of cells that formed colonies on plates supplemented with 25 (○), 50 (◇), 100 (●), 500 (△), and 2000 (▲) mg/l ampicillin and 25 mg/l tetracycline (▽) for $D = 0.61\ hr^{-1}$.

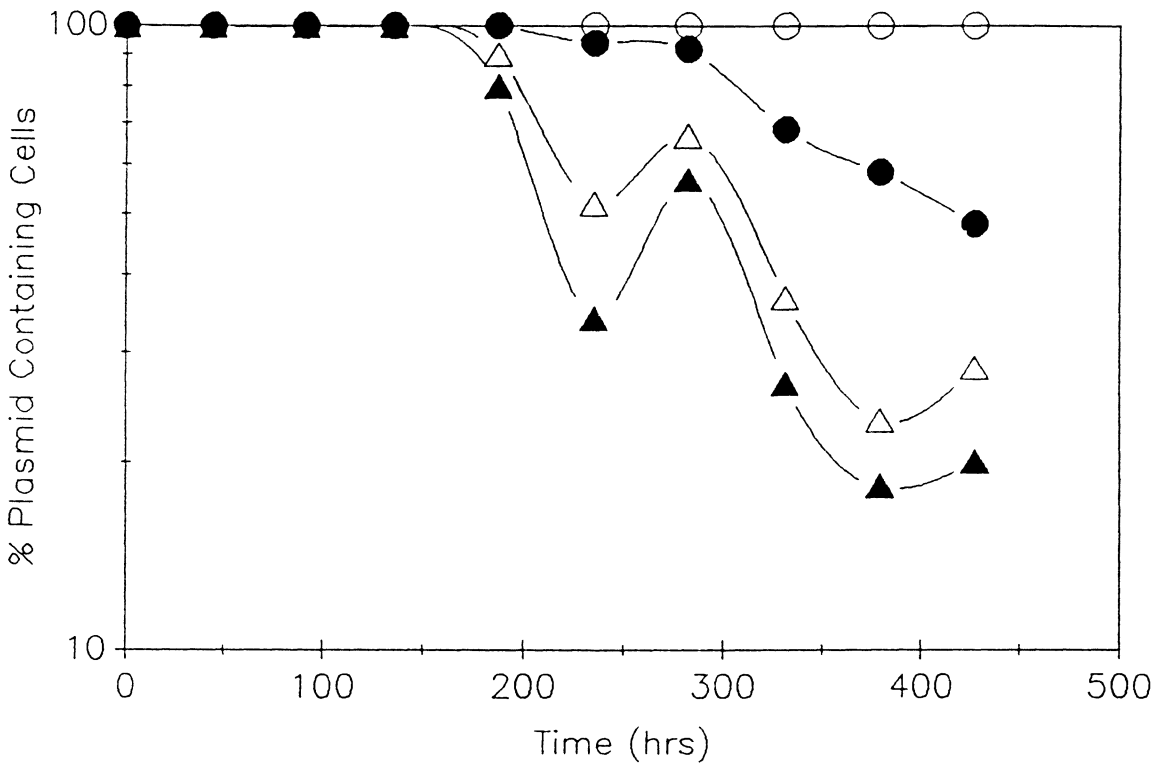


Figure 5-4. Percentage of cells that formed colonies on plates supplemented with 25 (○), 100 (●), 500 (△), and 2000 (▲) mg/l ampicillin for a cyclic experiment with the dilution rate oscillating randomly between D = 0.31 and 0.46 hr^{-1}.

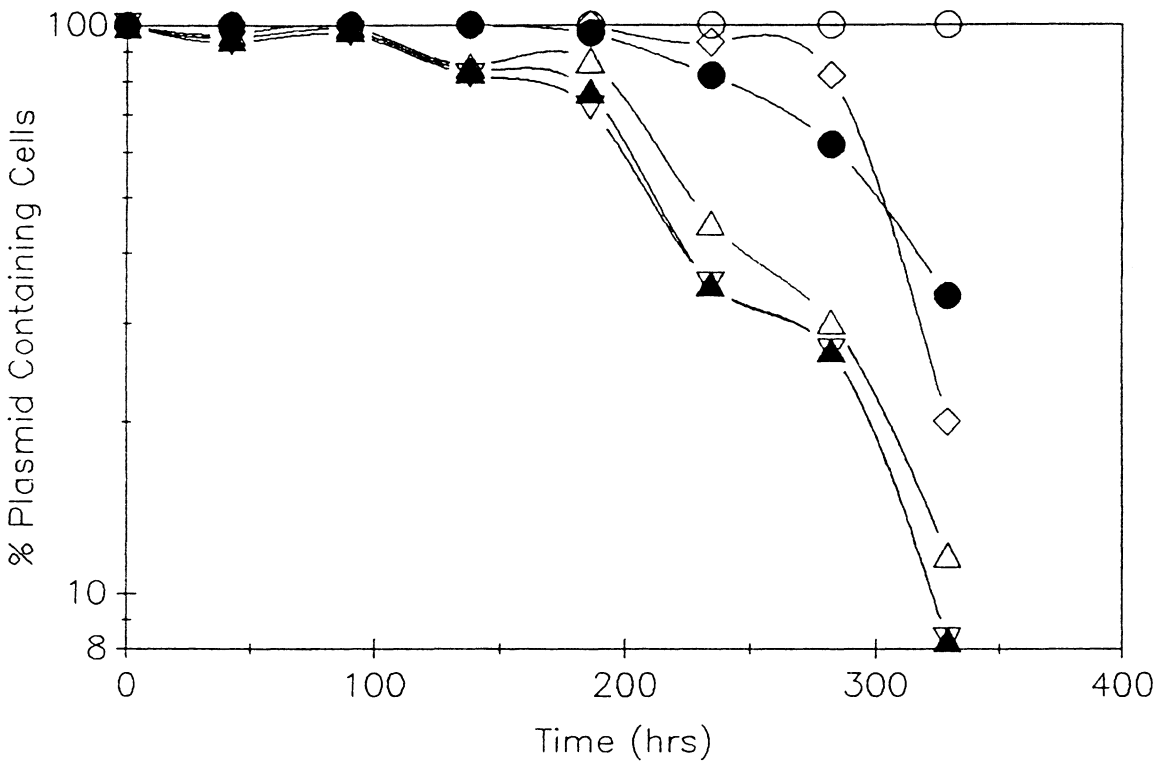


Figure 5-5. Percentage of cells that formed colonies on plates supplemented with 25 (○), 50 (◇), 100 (●), 500 (△), and 2000 (▲) mg/l ampicillin and 25 mg/l tetracycline (▽) for a cyclic experiment with the dilution rate oscillating every two hours between D = 0.31 and 0.60 hr^{-1}.

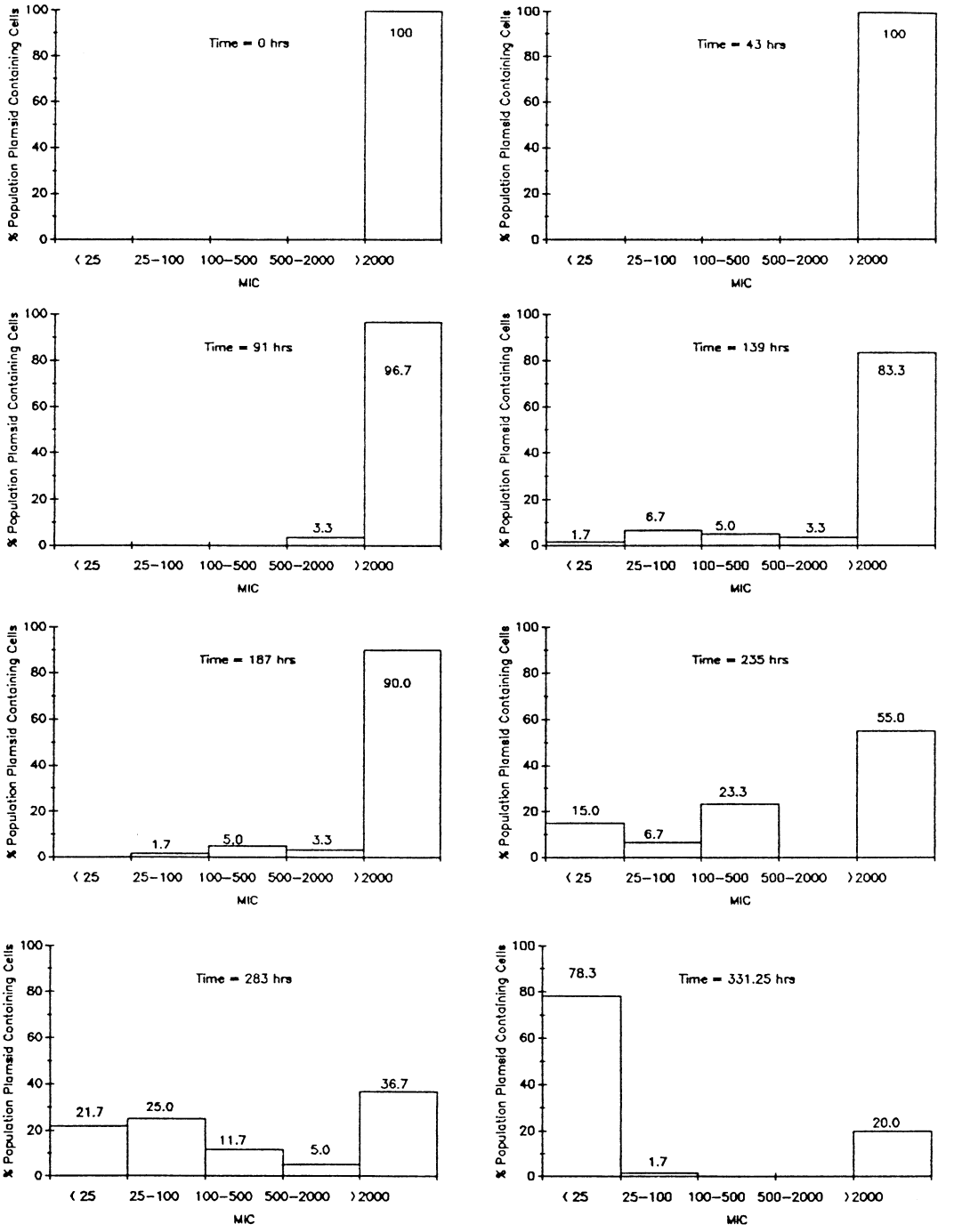


Figure 5-6. Histogram showing the percentage of cells that formed colonies between a range of ampicillin supplemented plates for D = 0.31 hr^{-1}.

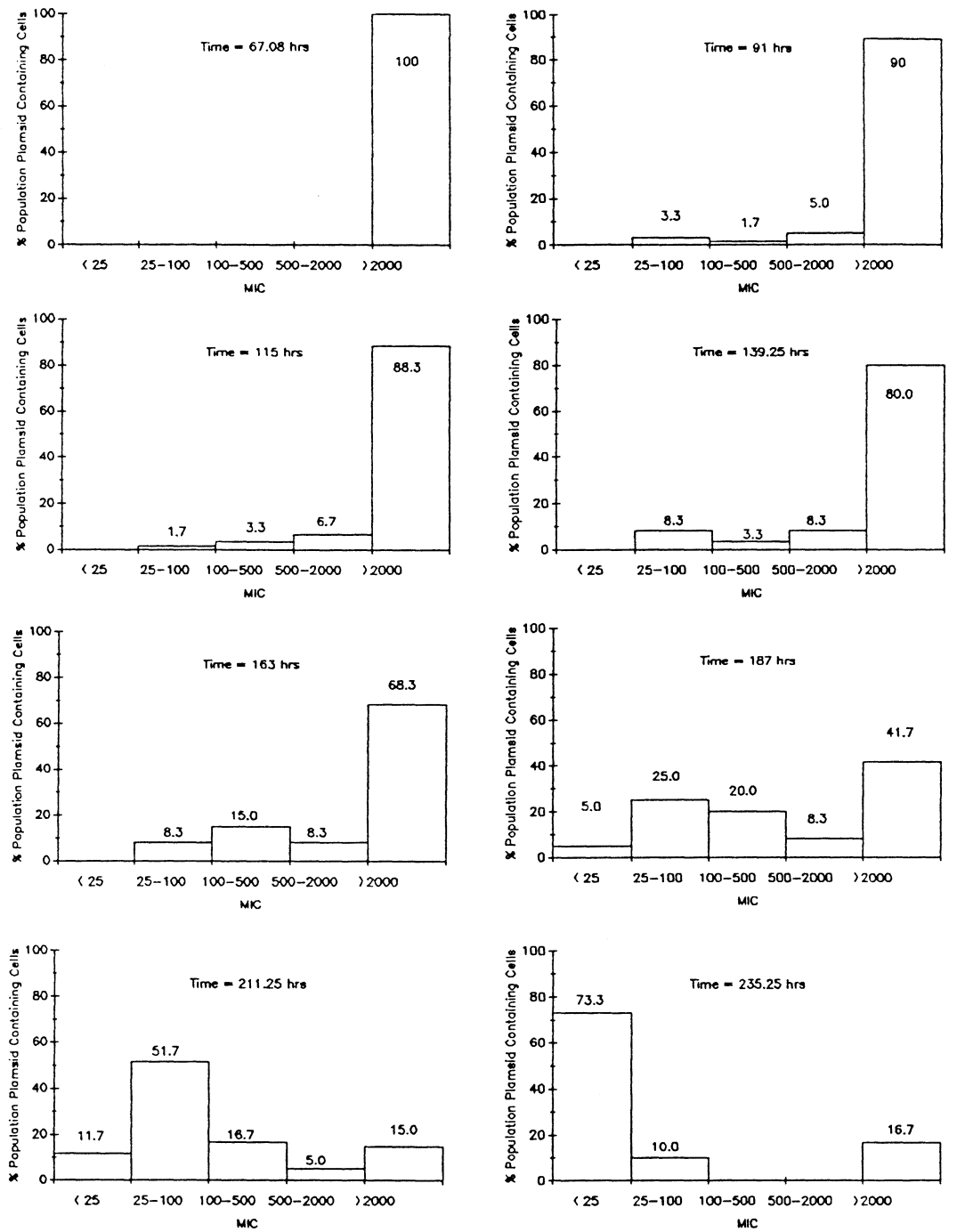


Figure 5-7. Histogram showing the percentage of cells that formed colonies between a range of ampicillin supplemented plates for D = 0.61 hr^{-1}.

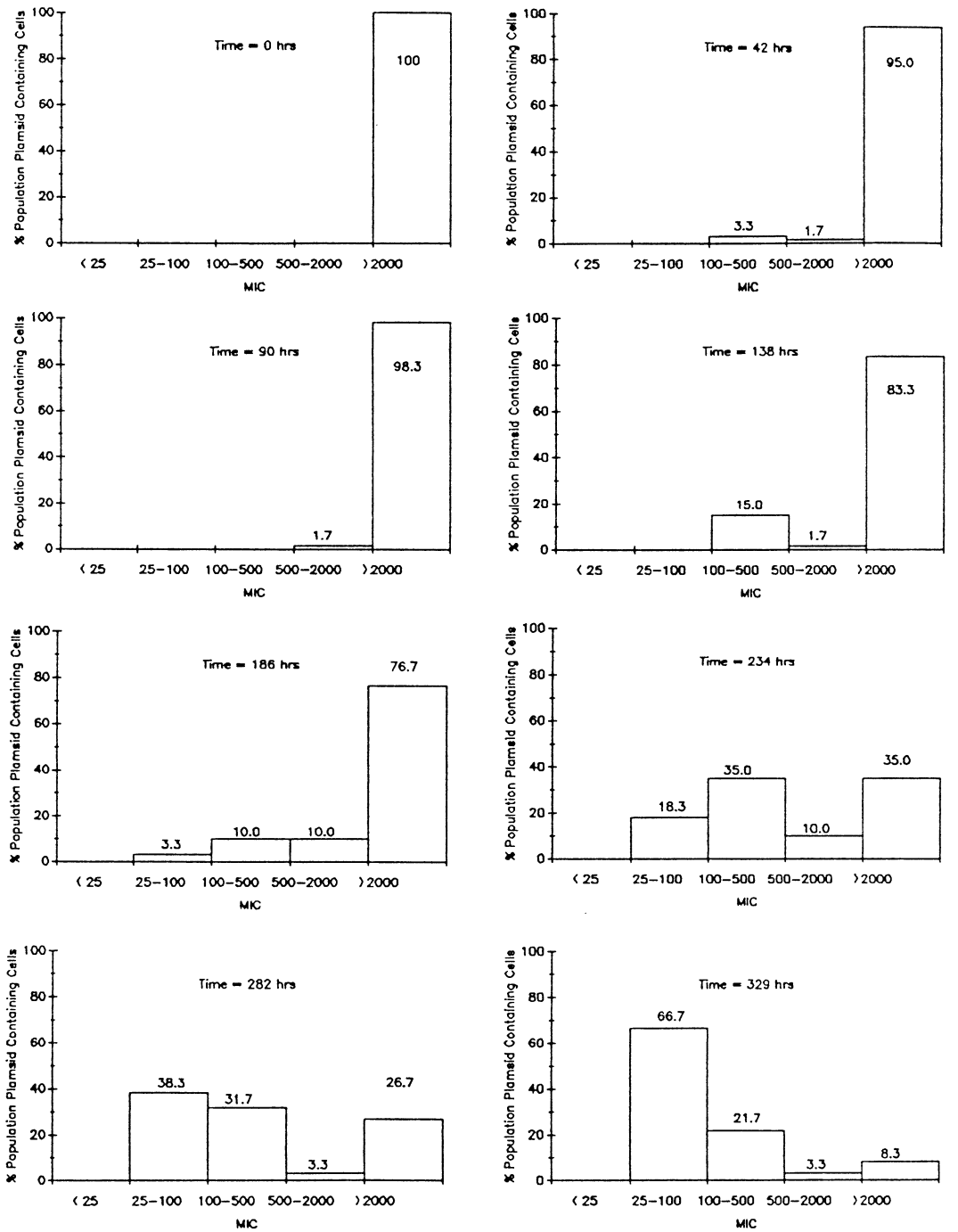


Figure 5-8. Histogram showing the percentage of cells that formed colonies between a range of ampicillin supplemented plates for a cyclic experiment with the dilution rate oscillating every two hours between D = 0.31 and 0.60 hr^{-1}.

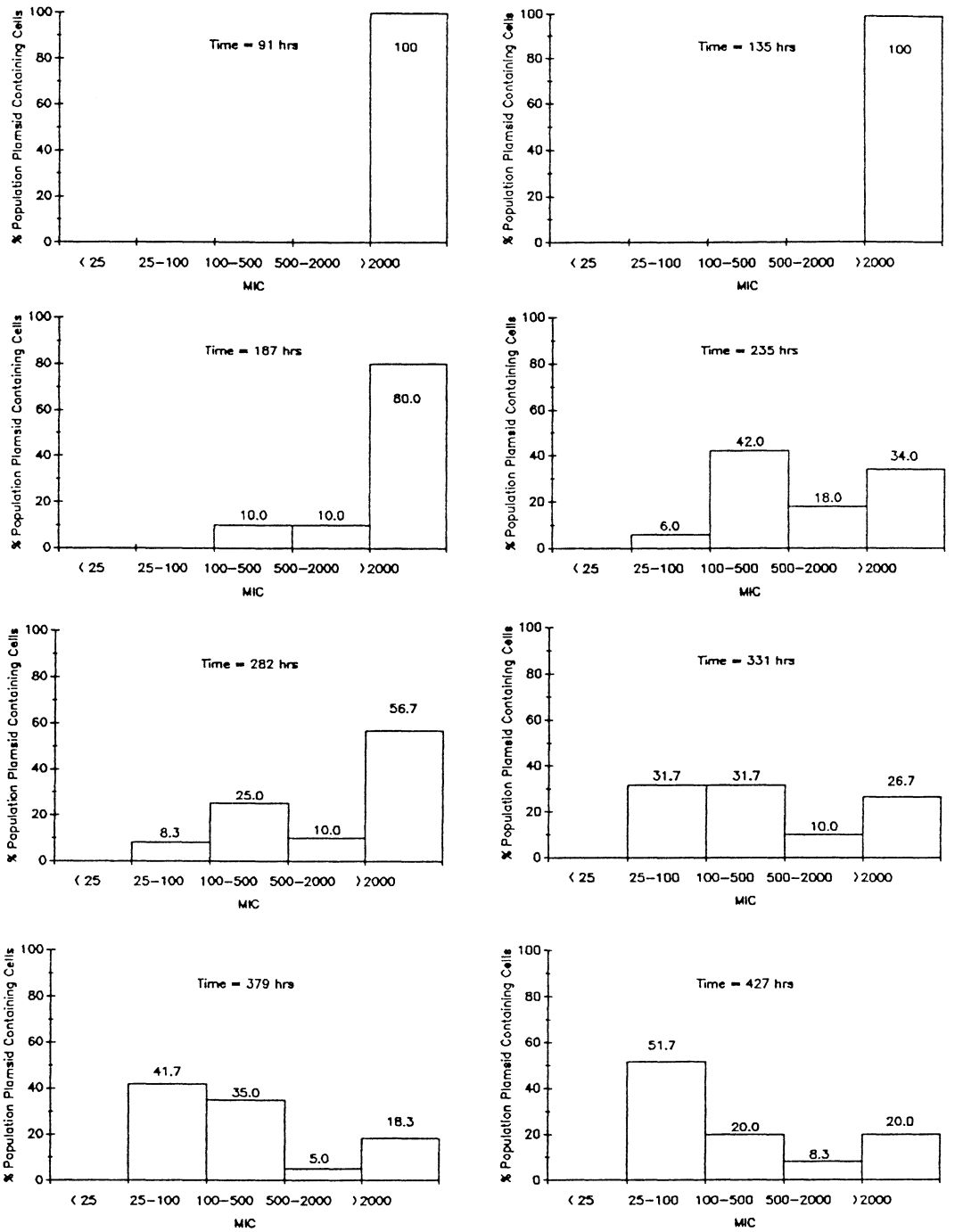


Figure 5-9. Histogram showing the percentage of cells that formed colonies between a range of ampicillin supplemented plates for a cyclic experiment with the dilution rate oscillating randomly between D = 0.31 and 0.46 hr^{-1}.

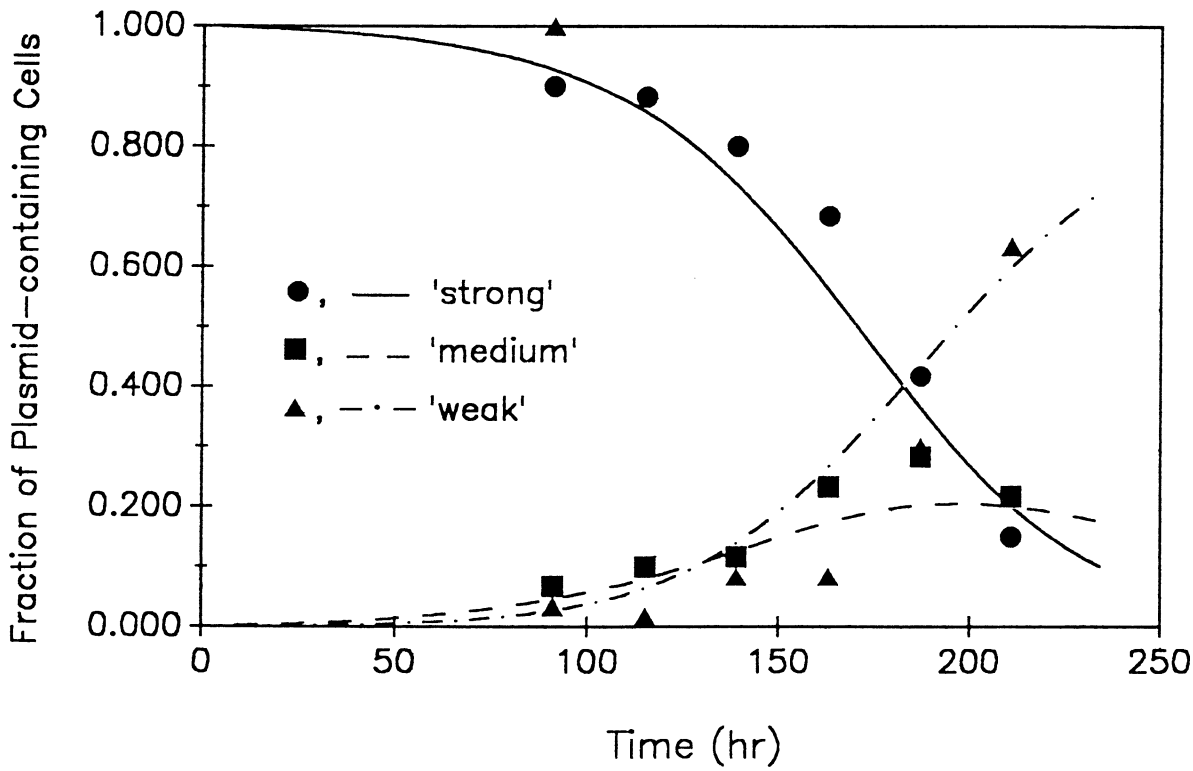


Figure 5-10. Comparison of the model prediction with the experimental results for D = 0.61 hr^{-1}. High copy number sub-population: experimental data (●) and model prediction (——), low copy number sub-population: experimental data (■) and model prediction (– –), and plasmid-free population: experimental data (▲) and model prediction (– - –).

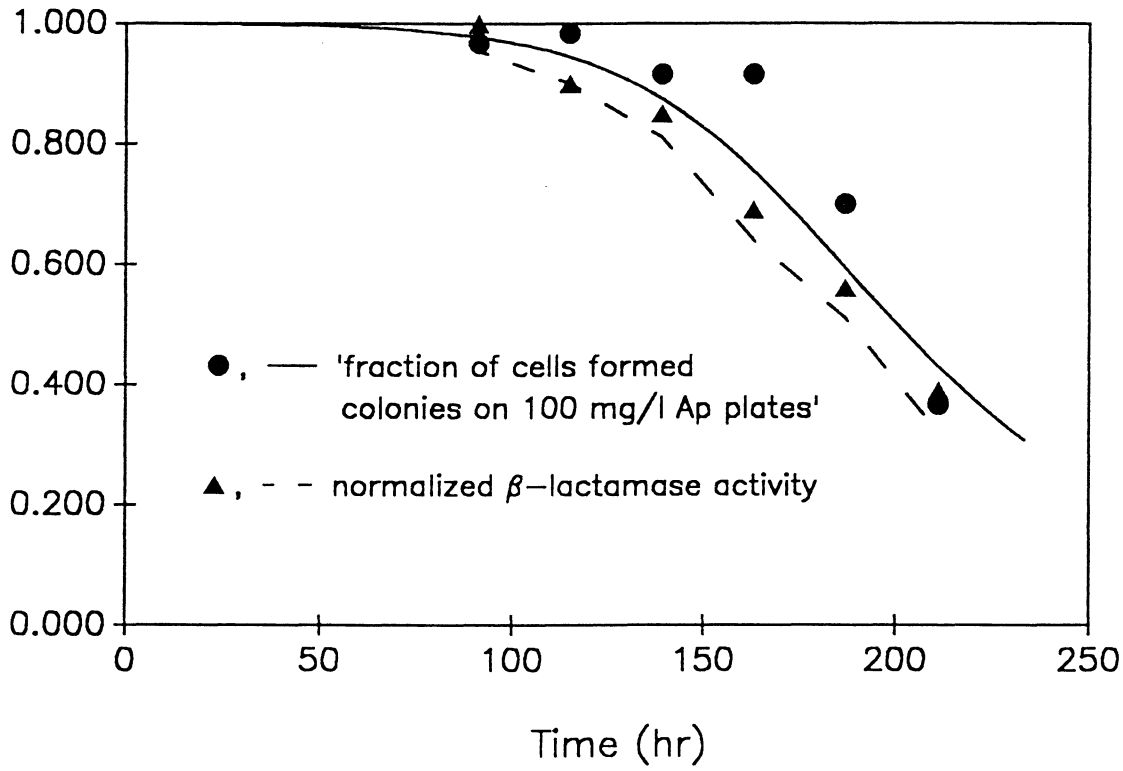


Figure 5-11. Comparison of the model prediction with the experimental results for D = 0.61 hr^{-1}. Fraction of cells that formed colonies on 100 mg/l ampicillin plates: experimental data (●) and model prediction (——) and the normalized β-lactamase activity: experimental data (▲) and model prediction (— —).

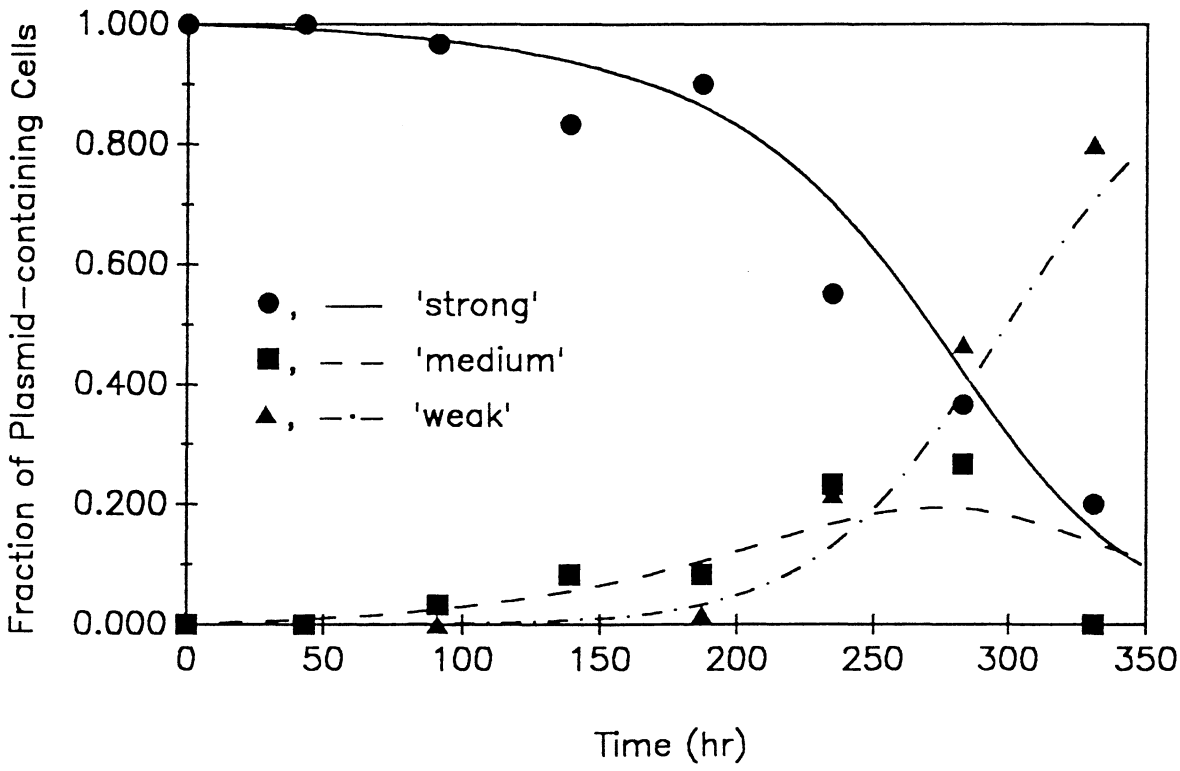


Figure 5-12. Comparison of the model prediction with the experimental results for D = 0.31 hr^{-1}. High copy number sub-population: experimental data (●) and model prediction (——), low copy number sub-population: experimental data (■) and model prediction (– –), and plasmid-free population: experimental data (▲) and model prediction (–·–).

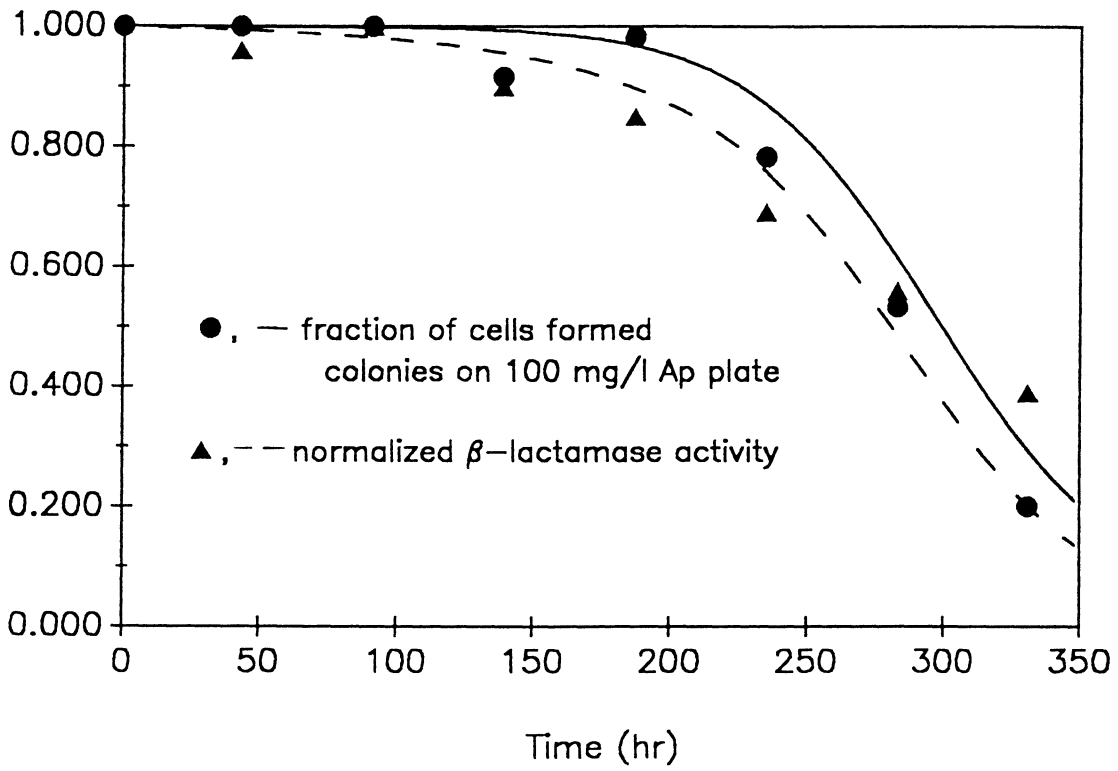


Figure 5-13. Comparison of the model prediction with the experimental results for D = 0.31 hr^{-1}. Fraction of cells that formed colonies on 100 mg/l ampicillin plates: experimental data (●) and model prediction (——) and the normalized β-lactamase activity: experimental data (▲) and model prediction (– –).

CHAPTER 6

THE USE OF A PARTITION LOCUS TO INCREASE THE STABILITY OF pBR322 PLASMID IN *ESCHERICHIA COLI*

6.1 Introduction

The stable maintenance of the plasmid in a growing culture is dependent on two primary factors. The first factor, is the faithful replication of the plasmid. The rate of replication of each specific plasmid is regulated in order to ensure a constant plasmid copy number per cell under different growth conditions. The regulation mechanism is dependent on the type of plasmid [201]. The second factor is the accurate segregation of the plasmids during cell division so the daughter cell receives at least one copy of each plasmid from the parent cell. As shownn in Chapter 3, if a mechanism governing the accurate partitioning of the plasmids is not present the distribution of the plasmids in the daughter cells will occur randomly. Eventually, this will produce a plasmid-free culture. There, is evidence, however, that some mechanism exists which guarantees a more accurate partitioning of the plasmid set during cell growth [151,164]. This is more apparent in cultures where low copy number plasmids are stably inherited [106,163,165].

The presence of a specific DNA sequence that assures stable plasmid inheritance in a growing cell population has

been observed in many laboratories. The region affecting the partitioning of the plasmid has been identified in the Clo DF13 [83,84,85], Ti [64], and ColE1 plasmid [223]. Systems involved in plasmid partition have also been found in low copy number plasmids P1 [8], F [170], and the *incFII* plasmid, NR1 [151]. Two of the more studied partitioning systems are those on the R1 [164] and pSC101 plasmid [145].

In spite of its low copy number [163,165] the R1 plasmid is stably maintained in growing cultures. This stability is not only due to an efficient replication mechanism, but also to the additional stability systems that are located on the plasmid [164]. Three different stability systems have been identified and are commonly referred to as ParA, ParB, and ParD [19,66]. Although the exact function of the ParA system is not known, it is believed to be required for the even partitioning of the plasmids during cell division [67]. The ParB system is responsible for counterselecting plasmid-free cells [68]. While the ParA and ParB systems are located away from the replication origin [164] the third system, ParD, is located near the origin [19]. The ParD codes for two proteins, P10 and P12 [20]. The genes of the P10 and P12 proteins are denoted as *kis* (suppressor of killing) and *kid* (killing determinant), respectively [20]. As in the cases of the Ccd system of the F plasmid [107,170] and the ParB system of the R1 plasmid [68,184], the ParD system also has been shown to stabilize a plasmid-containing culture by interfering

with the growth or viability of the plasmid-free cells resulting from faulty segregation during cell division. This is due to the activity of the P12 protein and implied instability of the P10 protein. It should be remembered that although three of the stability systems of the R1 have been identified the exact mechanism governing plasmid segregation has yet to be directly demonstrated.

Since Meacock and Cohen [145] identified and characterized the genetic function, designated *par*, responsible for the stable maintenance of the pSC101 plasmid numerous studies have been performed to elucidate the structure and mechanism of this gene. The *par* locus has been found to be a 375 bp Eco RI-Ava I DNA fragment adjacent to the origin of replication [152]. The nucleotide sequence of the *par* gene has also been reported [35,152]. No obvious proteins or segments with the structural characteristics of transcriptional or translational start signals were found in the *par* region. However, a hairpin-loop structure, formed by one of two lateral segments with an middle section, has been found to be involved in the even distribution of the plasmids to the daughter cells [38,236]. Deletion of either end segment and the middle segment resulted in behavior resembling a *par*$^-$ phenotype. However, deletion of only an end segment produced a stable plasmid. The resulting plasmid, though, demonstrated an increased incompatibility with the wild-type

plasmid. Deletion of all three segments produced a faster rate of plasmid loss than expected. This plasmid was termed a super-*par* mutant. Based on these results a model is proposed in which the *par* system allows nonreplicating plasmids of the intracellular pool to be treated as individual molecules resulting in the even distribution of the plasmids into the dividing cells [236]. In the super-*par* phenotype the plasmids are possibly lumped as one molecule and not counted as individual plasmids. However, this model is proposed solely based on experimental observations.

It is also known that the *par* function is independent of copy number control and does not determine plasmid incompatibility. In addition, it is not directly connected with the plasmid replication functions [145]. It is suggested that the *par* locus functions similarly to the centromere of eukaryotic cells [145], which is responsible for the accurate division of the DNA molecules during mitosis. There is some evidence that the *par* region directs the binding of the plasmid DNA to the outer membrane of the host bacteria [81]. A host protein, identified as DNA gyrase, was also found to bind specifically to the *par* locus [243]. The actual stabilization mechanism conferred by the *par* locus, however, is still not clear.

Many studies have been performed in which the ParA and ParB genes, from the R1 plasmid, *ccd* and sop stability genes, from the F plasmid, and the *par* locus, from the pSC101, was

cloned to plasmids lacking a stability factor [18,128,215,260]. In all cases, the stability of the plasmid had been increased. However, three points concerning these experiments should be made. First, an increase in stability was a function of the stability factor added to the plasmid [18]. Second, the experiments generally did not proceed longer than 160 generations. From previous experiments [246,247] it is believed this length of time is not adequate for long-term plasmid stability analysis. Third, in some cases [215] the stability of some of the plasmids, after the addition of the *par* locus, was not complete. The stability function of the *par* gene appeared to be dependent on the vector and the growth medium. In fact, the *par* locus does not stabilize all plasmid molecules under all conditions and does not stabilize Escherichia coli minichromosomes [94].

This chapter will focus on the effect of the *par* function on the stability and productivity of the pBR322 plasmid under prolonged conditions. With this in mind, an experiment was performed in which the stability of the pBR322 plasmid containing the *par* locus was studied. The dilution rate was kept constant at 0.61 hr^{-1}. The results were then compared to previous experiments and are presented in the following sections.

6.2 Construction of the pBR322-*par* Plasmid

Plasmid pBR322-*par* was constructed by first digesting both the pBR322 and pΔ37-*par* with EcoRI. The digested DNA fragments were then allowed to self-ligate in the presence of T4 ligase. After transformation and screening of the ampicillin resistant colonies, clones containing the *par* locus were isolated, Figure 2-2. Construction and isolation of the plasmid pBR322-*par* was performed by Peng Yu.

An important consideration in constructing the plasmid was the location of the *par* gene on the pBR322 plasmid. As seen in Figure 2-2a, the *par* locus is located between the Ap^R and Tc^R genes. A region localized in the region of the *Hind* III site, within the Tc^R gene, was found to cause a destabilization of the plasmid [121,128]. If this region is altered by the construction of the *par* plasmid an increase in plasmid stability may not be solely due to the *par* locus. The β-lactamase activity might also be affected if the position of the *par* locus somehow interferes with the transcription of this gene. Fortunately, the results from the continuous experiment indicate that the position of the *par* locus does not interfere with either the Tc resistance or the β-lactamase activity of the cell. In addition, the unique behavior of the culture, as seen in Figure 6-1, signifies that any change in the plasmid stability is probably due only to the *par* locus and not its position on the plasmid. Generally, it has been

observed that the *par* locus is effective regardless of location and orientation on the plasmid [145].

6.3 Effect of the *par* Locus on the Stability of the pBR322 Plasmid

The stability of the plasmid pBR322-*par* is compared to a control experiment, described in Chapter 3, using the pBR322 plasmid. The dilution rates for both experiments were maintained at 0.61 hr^{-1}. The results are given in Figure 6-1a. Initially, the addition of the *par* locus does not appear to have a significant effect on the stability of the plasmid. The lag time is slightly increased by approximately 50 hours. At this point, as previously observed, the fraction of plasmid-free cells are detectable in the culture. For the next 100 hours the behavior of the culture then resembles that of the par^- plasmid, in which random plasmid partitioning occurs. It should be pointed out that literature data are seldom reported beyond this time [215,260]. This is unfortunate, as the behavior of the par^+ plasmids in the later part of the experiment, as seen in Figure 6-1a, is the most interesting and unexpected. Instead of showing a continual decline of the plasmid-containing population, the cells reach a pseudo-steady-state where a mixed culture of plasmid-containing and plasmid-free cells is stably maintained. During the period of 200 and 375 hours the par^+ culture

demonstrates a very gradual decrease of the plasmid-containing cells. Within this time portion the percentage of plasmid-containing cells decreases from 63 to 38%. This is rather unusual. In most cases (Chapter 3) when the percentage of plasmid-containing cells reaches this level, approximately 40 to 50%, the culture cannot be stably maintained. The 'plateau' is not maintained indefinitely and after 400 hours into the experiment the plasmid-carrying population drops in a manner similar to previous experiments, Chapter 3 and 4.

Three points should be made concerning this result. First, is the behavior demonstrated by the *par*$^{+}$ plasmid. As shown in Figure 6-1b, this trend resembles that of a plasmid-containing culture exposed to random oscillations in the dilution rate, D = 0.31 and 0.46 hr^{-1}. However, there are some differences. The population in the random experiment is maintained at a higher plateau, reflecting a higher percentage, approximately 60 to 70%, of plasmid-containing cells. This plateau, however, is only 100 hours, half the time period of the plateau generated by the *par*$^{+}$ plasmid.

The second point is the cause of the stable region in this experiment. In the random experiment the stability was believed to be generated by random perturbations of the dilution rate, resulting in an environment advantageous to the plasmid-containing cells. As the culture in the *par* experiment was not exposed to any known oscillations a

different mechanism is responsible for the stability of the culture. Two possible explanations are explored. In the first, the stability could be the result of plasmid conjugation. Similar results were predicted by a model describing a plasmid capable of conjugation [58]. As, the plasmid pBR322 is not conjugative this is probably not the case. In addition, there was no evidence of conjugation by the *par*$^+$ plasmid in subsequent experiments. The presence of the *par* locus is a more probable explanation. If it is assumed that a decrease in the plasmid-containing population is accompanied by a decrease in the plasmid copy number, Chapter 5, a population with a lower plasmid copy number exists during the stable plateau. In addition, it has been speculated that the decrease of the plasmid copy number and expression preceding the decrease in the plasmid-containing cells, Figure 6-2, is caused by a gradual loss of plasmids from the cells producing a sub-population characterized by a lower plasmid copy number. The *par* locus, in turn, might be more efficient in evenly dividing the plasmids between the daughter cells at a lower copy number. This could result in the observed stable population. This agrees with the proposed mechanism in which the *par* site on the plasmids must compete with each other for binding sites on the cell membrane [81, 243]. In addition, the copy number of the vector pSC101, in which the *par* locus was originally identified, is

approximately two [252]. The *par* locus is reasonably effective at this copy number. The final decrease in the percentage of plasmid-containing cells is likely due to the 'take-over' of the plasmid-free cells and not a failure of the *par* locus.

The last point to be made concerns the existence of a heterogeneous population. The advantage of a *par* locus is the increased accuracy of plasmid partitioning between two daughter cells. Based on this, it is assumed that the par^+ culture would be able to completely stabilize the culture resulting in a homogeneous copy number population. As previously demonstrated, Figure 6-1, the addition of the *par* locus to a plasmid does not necessarily ensure full stability. In addition, as seen in Figures 6-3 and 6-4, the culture composition is not homogeneous, but heterogeneous, similar to the par^- systems. Sub-populations, based on the MIC study, evolve after 100 hours. This corresponds to the transient period when the plasmid-free cells begin to emerge.

The behavior of the population distribution is similar to the previous experiments, Figure 6-4a and 6-4b. In the par^+ culture, though, the sub-populations are more evenly distributed during the stable plateau. A gradual shift of one sub-population to another is also observed. It was suggested, Chapter 5, that the sub-population reflects a heterogeneity of the plasmid copy number. The stable period

then consists of a large percentage of low copy number cells. This would agree with one of the assumptions proposed to explain the increase in stability of the *par*$^+$ culture.

6.4 Effect of the *par* Locus on the Plasmid Copy Number and Expression of the pBR322 Plasmid

The *par* locus has been shown to increase the stability of many plasmids [128,215,260]. However, there have been no reports of its effect on plasmid copy number and expression. The purpose of this section is to examine this effect in comparison to a *par*$^-$ culture. The β-lactamase activity of the *par*$^+$ and *par*$^-$ cells is shown in Figure 6-5a. As expected, in the first 250 hours there is no noticeable difference between the two cultures. After this time the activity of the *par*$^+$ culture increases slightly then gradually decreases. This trend corresponds to the period, up to 375 hours, when the plasmids are stably maintained.

A more interesting comparison is the activity of the *par*$^+$ culture with that of a culture grown under random oscillations, Figure 6-6b. The unique behavior of the random cycling culture, in which a drop in the activity occurred followed by an increase, was mentioned in Chapter 4. The activity of the culture reached a level corresponding to the activity of a culture with a full complement of plasmids.

This does not occur in the *par*$^+$ culture. The activity of this culture shows the same decrease, interesting enough it occurs at the same time period as the decrease in the random culture. However, the *par*$^+$ culture is not able to reach its original activity level.

It would appear as though the addition of the *par* locus, except for prolonging the period of productivity, has no other effect on the plasmid expression. This is not the case with respect to the plasmid copy number. As shown in Figure 6-6a, the *par* locus has a significant effect on the relative plasmid copy number, defined as the plasmid DNA per chromosome DNA. Although the behavior is similar between the *par*$^+$ and *par*$^-$ cultures, the population containing the *par* locus has a higher plasmid copy number. This could be due to the effective partitioning of the plasmids in the *par*$^+$ cells resulting in a decrease in the rate of plasmid loss. The increase in the lag time, Figure 6-1a, supports this assumptions. The rapid decrease, from 100 to 200 hours, in the plasmid content may be due to the appearance of the plasmid-free cells and a gradual loss of plasmid copies per cell.

The resemblance between the behavior of the plasmid content in the *par*$^+$ culture and the random cycling culture should be pointed out, Figure 6-6b. Both cultures show a drop in the plasmid content before a stable level is attained.

There is a slight shift between the two cultures. This corresponds to the time difference in the decrease in the plasmid-containing cells. The *par*$^+$ culture has a shorter lag time than the culture exposed to random perturbations.

In general, the addition of the *par* locus to the pBR322 plasmid proved beneficial. Both the stability and the plasmid copy number of the culture increased. Although there was no immediate effect on the β-lactamase activity, the long term productivity also increased. Until the mechanism of the *par* function is better understood, however, the exact relationship between stability, plasmid copy number, and expression and the *par* locus is not clear.

6.5 Conclusions

To summarize, the more important observations from this experiment are:

1. The presence of the *par* locus to the pBR322 plasmid affected the stability of the culture. Not only was the lag time increased, but a mixed population of plasmid-containing and plasmid-free cells was stably maintained for approximately 200 hours. In addition, the culture was maintained at a very low percentage of plasmid-carrying population. This could have been due to the effectiveness of the *par* locus at a low copy number.

2. The *par*$^+$ culture, similar to experiments without the *par* locus, under prolonged cultivation became heterogeneous with respect to the plasmid copy number. This resulted in sub-populations with different plasmid copy numbers.
3. The β-lactamase activity was not significantly affected by the *par* locus. Due to an increase in stability, though, the *par* gene had an effect on the long term productivity.
4. The addition of the *par* locus also affected the plasmid copy number. An increase, compared to the *par*$^-$ culture, in the copy number was observed in the initial stage of the experiment. After 100 hours the copy number decreased, reached a minimum, then stabilized. Although the cause of this behavior is not clear, it is speculated that it is due to the effectiveness of the *par* function.
5. Based on all these results, the effect of the *par* locus on stability, plasmid copy number, and expression, random oscillations of the dilution rate appears to be the optimum operating mode. It is possible, however, that exposing a *par*$^+$ culture to random perturbations might result in a greater increase in the plasmid stability, copy number, and expression.

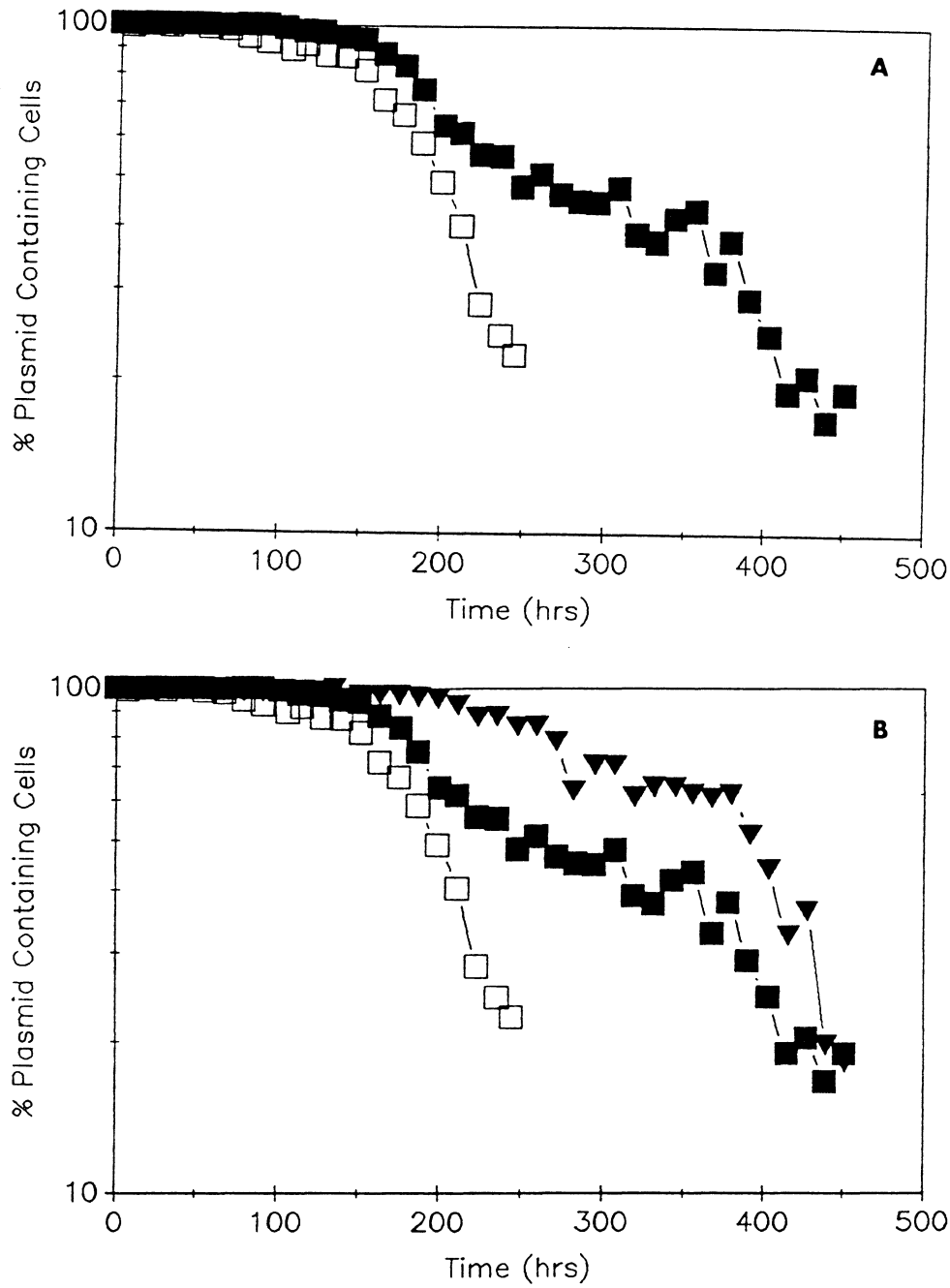


Figure 6-1. Comparison of the stability of a plasmid-containing culture for (a) the plasmid pBR322 (□) and plasmid pBR322-*par* (■) at D = 0.61 hr^{-1} and (b) the plasmid pBR322 (□) and plasmid pBR322-*par* (■) at D = 0.61 hr^{-1} and the plasmid pBR322 under random cycling, D = 0.31 and 0.46 hr^{-1} (▼).

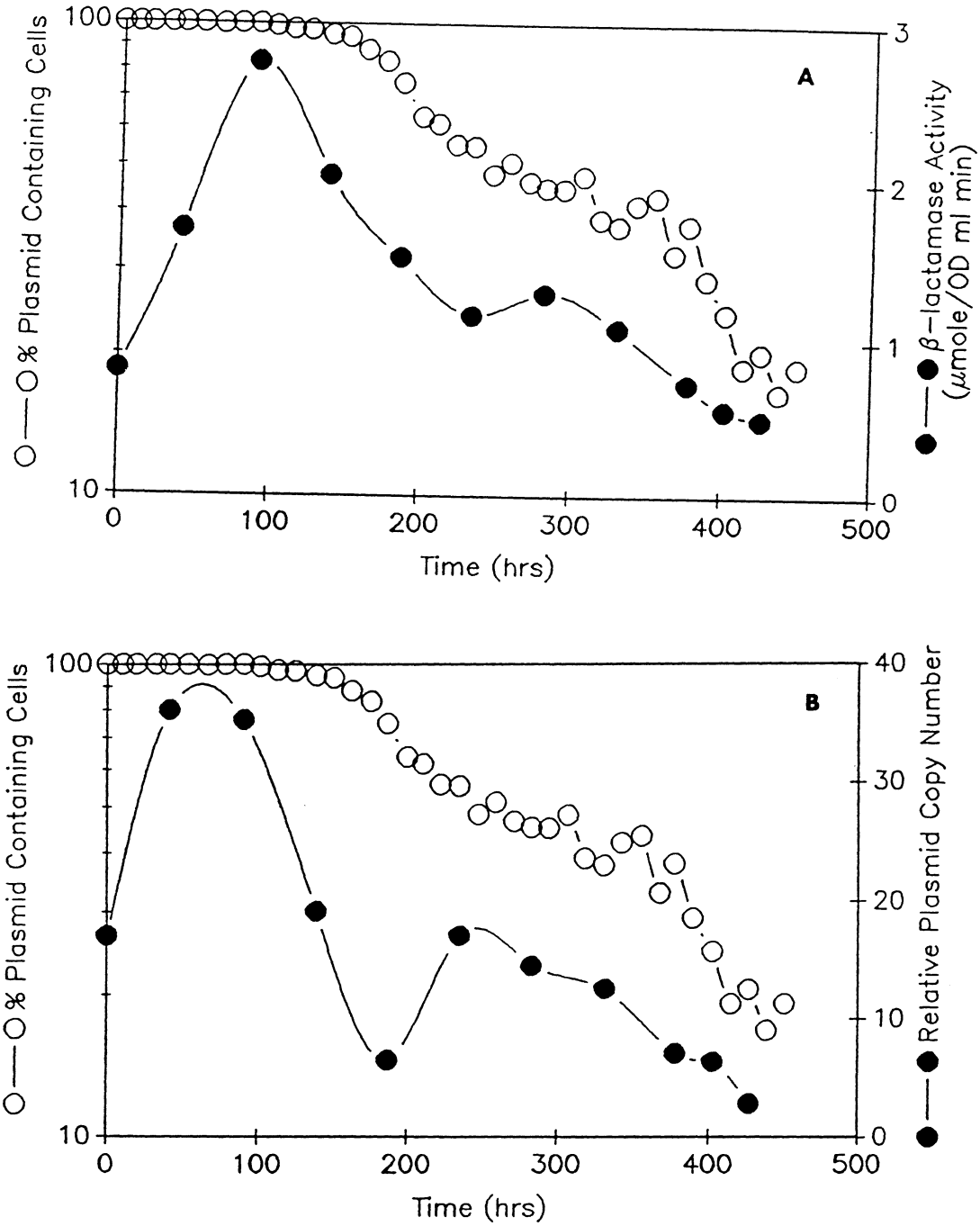


Figure 6-2. Comparison of the percentage of plasmid-containing cells pBR322-*par* and (a) the specific β-lactamase activity and (b) the relative plasmid copy number, plasmid DNA per chromosome DNA.

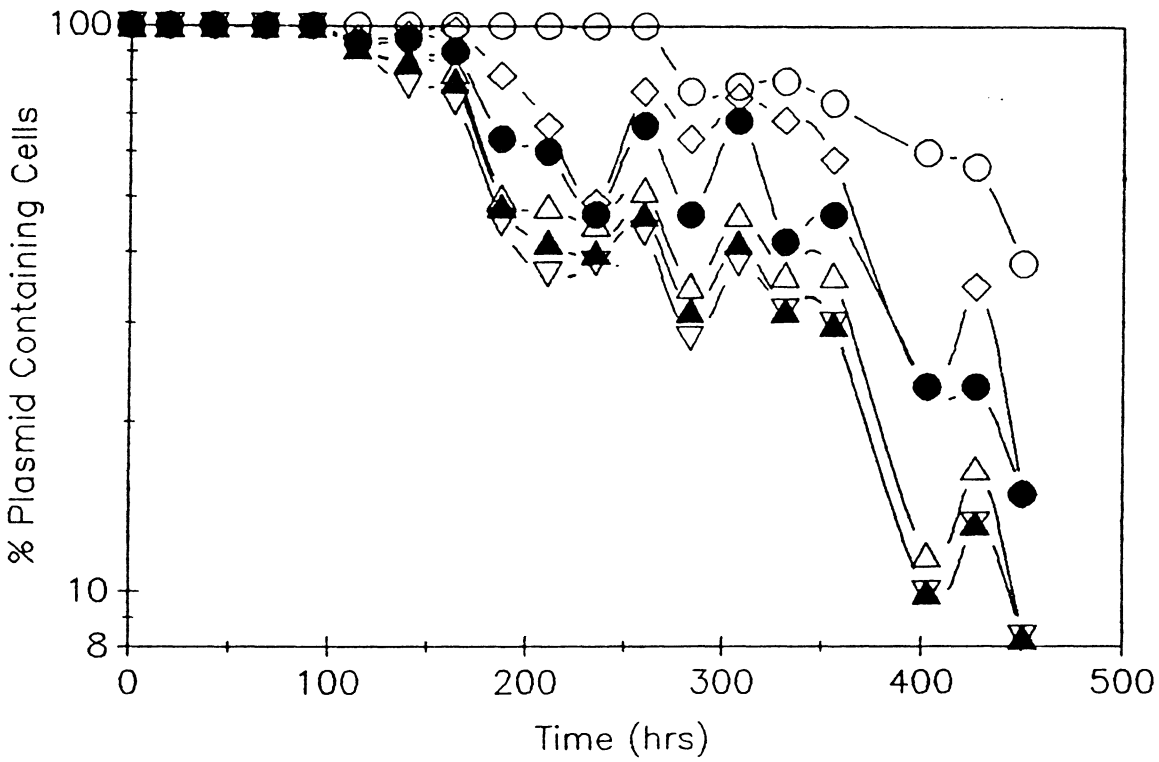


Figure 6-3. Percentage of cells that formed colonies on plates supplemented with 25 (○), 50 (◇), 100 (●), 500 (△), and 2000 (▲) mg/l ampicillin and 25 mg/l tetracycline (▽) for plasmid pBR322-par at D = 0.61 hr^{-1}.

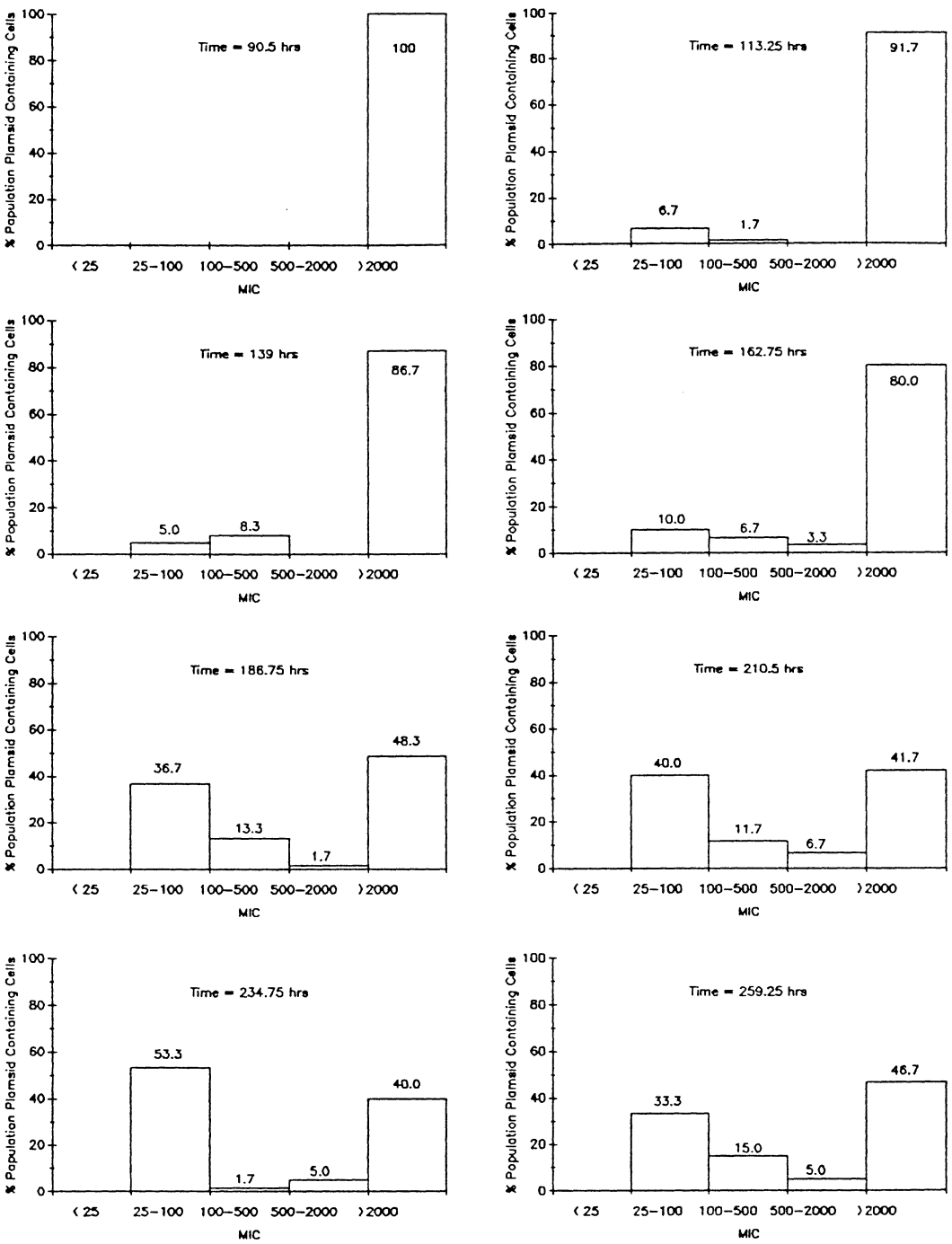


Figure 6-4a. Histogram showing the percentage of cells that formed colonies between a range of ampicillin supplemented plates for pBR322-*par* at D = 0.61 hr^{-1} from 90.5 to 259.25 hours.

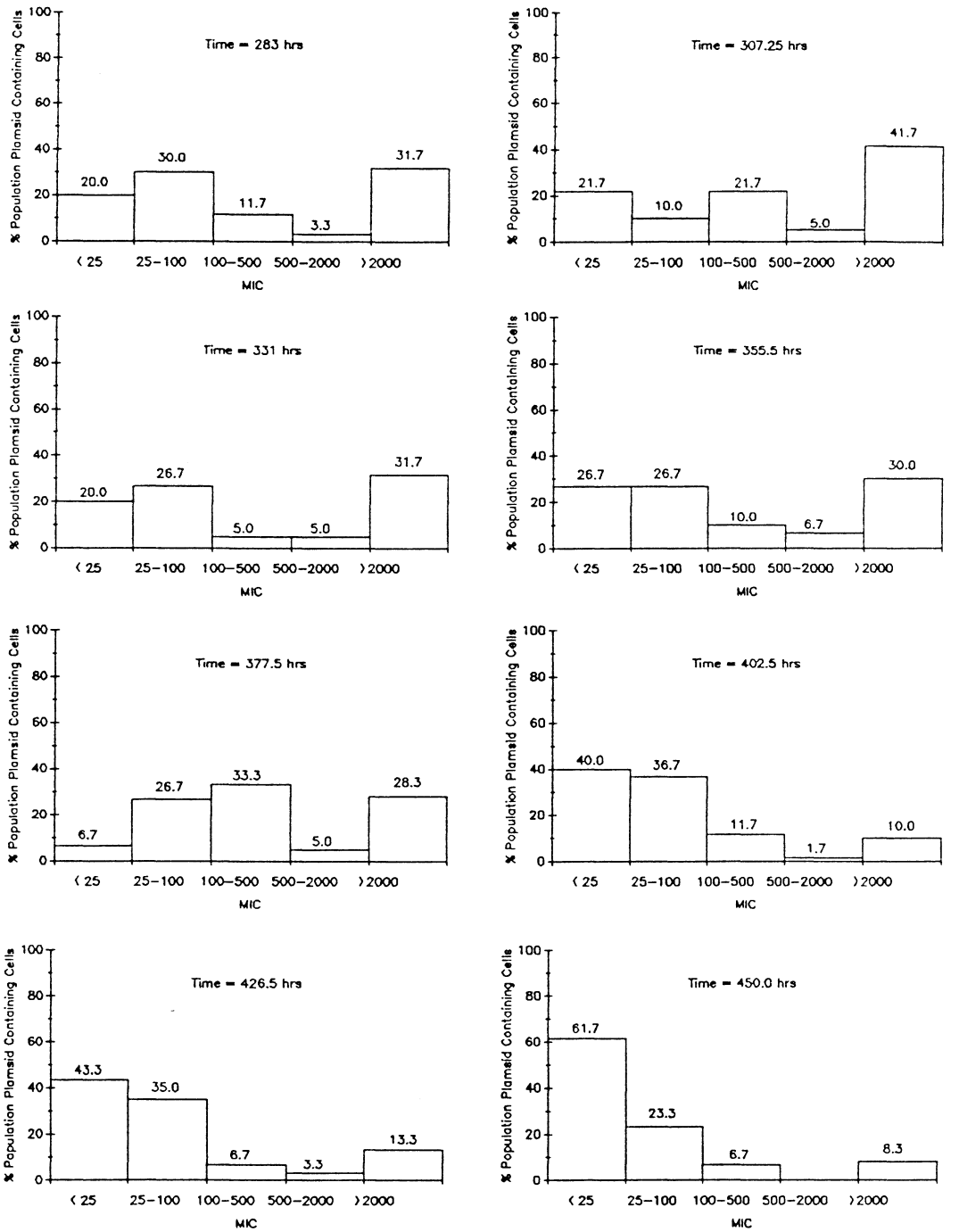


Figure 6-4b. Histogram showing the percentage of cells that formed colonies between a range of ampicillin supplemented plates for pBR322-par at $D = 0.61\ hr^{-1}$ from 283 to 450 hours.

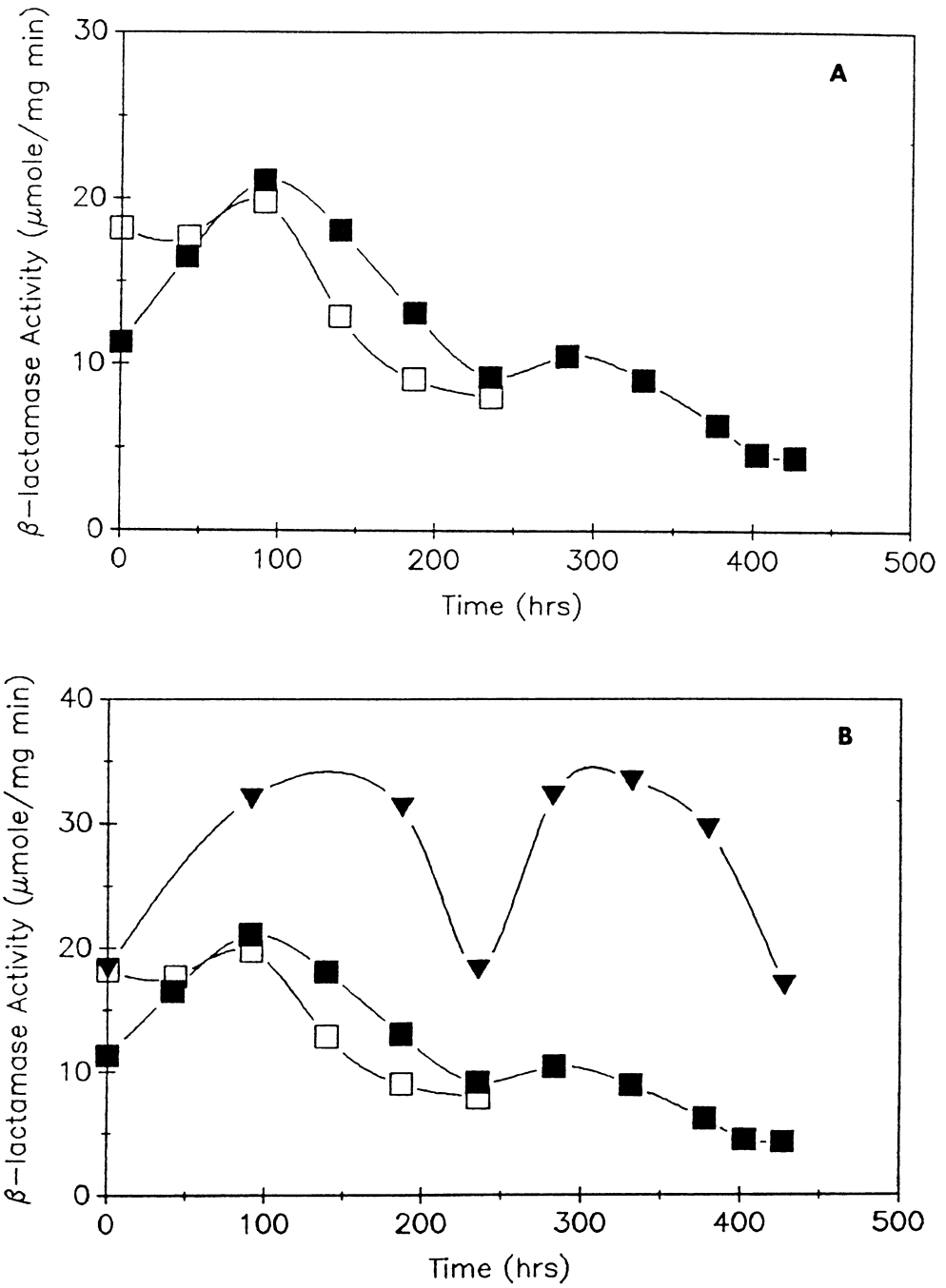


Figure 6-5. Specific β-lactamase activity, based on the total protein present, for (a) plasmid pBR322-*par* (■) and pBR322 (□) at D = 0.61 hr^{-1} and (b) plasmid pBR322-*par* (■) and pBR322 (□) at D = 0.61 hr^{-1} and plasmid pBR322 under random cycling, D = 0.31 and 0.46 hr^{-1} (▼).

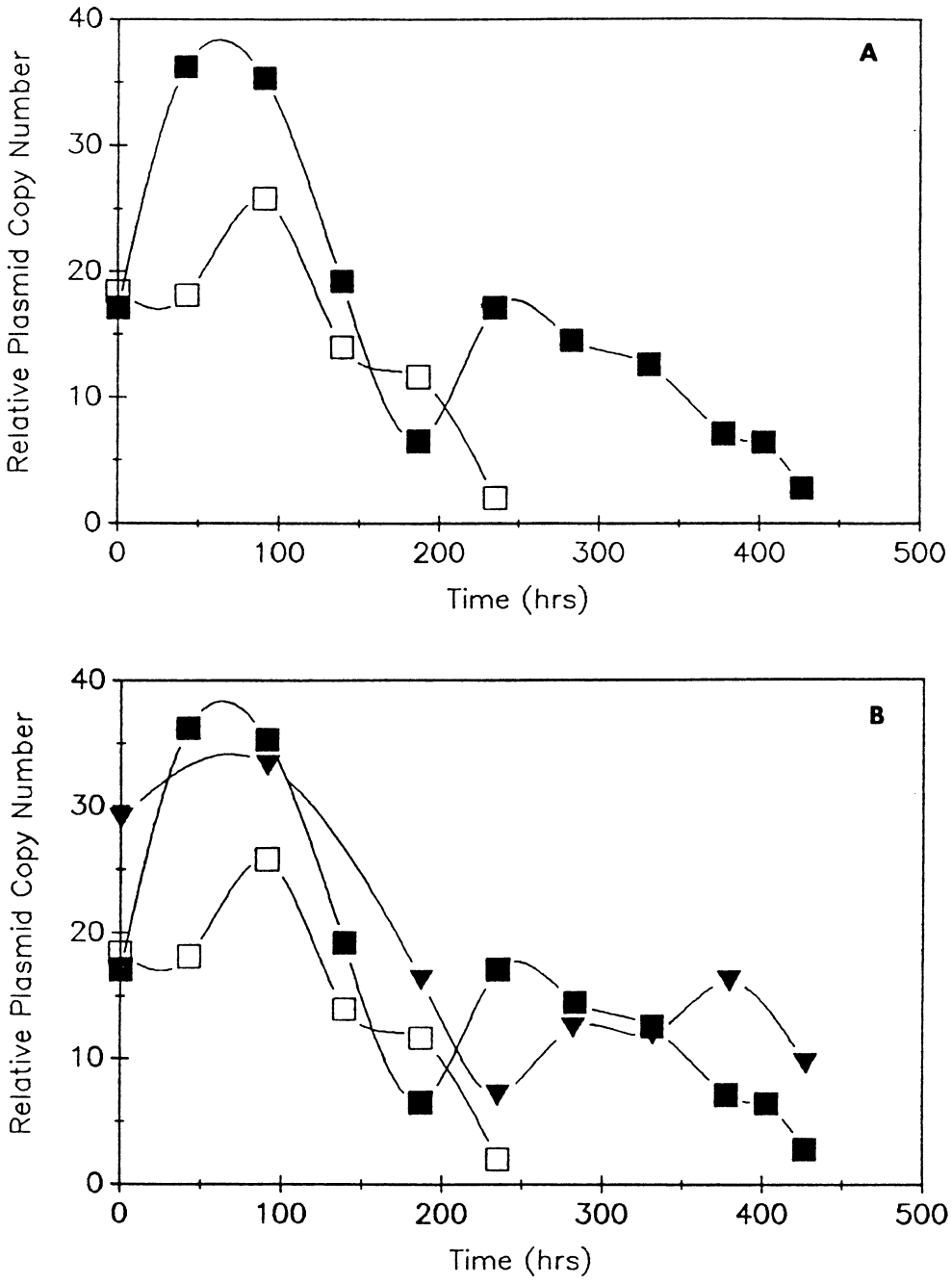


Figure 6-6. Relative plasmid copy number, plasmid DNA per chromosome DNA, for (a) plasmid pBR322 (□) and pBR322-*par* (■) at D = 0.61 hr^{-1} and (b) plasmid pBR322 (□) and pBR322-*par* (■)at D = 0.61 hr^{-1} and plasmid pBR322 under random cycling, D = 0.31 and 0.46 hr^{-1} (▼).

CHAPTER 7

SUMMARY AND RECOMMENDATIONS

7.1 Summary

Plasmid stability is one of the potential factors that determines the productivity of recombinant populations. Therefore, the understanding of various factors affecting plasmid maintenance is of both fundamental and practical importance. In this work the effect of reactor parameters such as medium composition, genetic make-up of the plasmid, and dilution rate, on plasmid maintenance and expression were investigated.

In the first set of experiments the stability and gene expression of plasmid pBR322 in a chemostat with complex non-selective medium at different dilution rate were studied. It was observed that pBR322 was eventually lost from the population after a long lag period. The rate of plasmid loss decreased as the dilution rate increased. This result was different from those obtained with cells grown in defined medium, where plasmid loss was observed to decrease with increasing dilution rate. In addition, it was observed that the cloned gene expression, β-lactamase, and plasmid copy number were also affected by the growth rate.

Plasmid stability and expression were also influenced by copy number and plasmid structure. In two separate

experiments plasmids pDM247 and pBR322*par* were compared to the results obtained using plasmid pBR322. Plasmid pDM247, a low copy number plasmid, was found to increase stability. However, because the plasmid is present in low copy number, approximately one half the pBR322 copy number, the gene expression was much lower than previously observed with plasmid pBR322. In the second experiment the plasmid pBR322 was stabilized by the addition of the *par* gene. The *par* locus has been previously shown to decrease plasmid loss due to faulty segregation. In this work it was found that not only did the lag time increase, but a mixed population of plasmid-containing and plasmid-free cells was maintained for approximately 200 hours, a fairly substantial time. The β-lactamase activity was not significantly effected by the *par* locus. It did, however, have an affect on the plasmid copy number.

The most significant results were obtained from the transient experiments in which a population of Escherichia coli carrying the plasmid pBR322 were exposed to square-wave perturbatios in the dilution rate. Under these conditions the culture was capable of maintaining a mixed population of cells for a longer period of time than a culture grown under a constant dilution rate. The loss of the plasmid-containing cells appears to be due to the adaptation of the culture to the oscillations. It is speculted at this point any advantage created by the perturbations in the dilution rate has been

eliminated. The copy number and expression were also affected by th transient environment. The results were dependent on the amplitude and frequency of the dilution rate oscillations.

Based on experimental and model results two mechanisms were proposed to describe the observed enhanced stability. First, under transient conditions, the recombinant cells respond quicker to the environmental changes, thereby diminishing the relative growth advantage of the plasmid-free cells. Second, under these conditions, the cells are induced to maintain a higher copy number. The increase in copy number might lead to a lowering of the probability of plasmid loss due to random partitioning.

One common phenomenon observed in all experiments was that the plasmid-containing cells existed as a heterogeneous population. The existence of the sub-populations of the recombinant culture could have significant effects and implications in the long term productivity of continuous cultures.

In summary, the results obtained proved to be both interesting and promising. This work demonstrates how the stability and production of a plasmid-containing culture can be optimized by the manipulation of reactor paramters. It is essential, however, to understand the interrelationships between the growth rate, plasmid stability, cloned gene expression, and culture conditions. This should be approached at both the engineering and molecular levels.

7.2 Engineering Approach

This research has shown that steady-state operation of continuous fermenters is not necessarily the optimum operation strategy for recombinant cultures. Based on this speculation, further transient condition experiments should be studied. Besides additional investigations of the oscillation amplitude and frequency, the effects of perturbations in other parameters such as dissolved oxygen, temperature, and pH, on plasmid stability, copy number, and expression should be examined. One promising experiment would be to study the effect of temperature oscillations on temperature-dependent replication plasmids. Perturbations in the pH have also appeared to affect plasmid stability. However, it is not known whether this was due to a change in the pH or some other artifact of the system. A reactor strategy in which the recombinant cells are cultured under perturbations arising from repeated fed batch operation could also be beneficial.

Transient environments might also have different effects on different plasmids. One plasmid worth studying is the plasmid pBR322*par* in which plasmid stability was enhanced by the addition of the *par* gene. Oscillations of reactor conditions, i.e., dilution rate, might result in an even greater stability. In addition, the gene expression, as seen in the random cycling experiment, might also increase.

Another experiment, where the cells are continuously cultured in a selective medium, might prove useful in understanding the phenomena observed in the first stages of all the experiments. At this point it is not known why the gene expression increases in the first 100 hours of the experiment. In addition, an experiment in which a heterogeneous population is inoculated into a reactor might be beneficial in studying the effect and recovery of the plasmid-containing sub-populations.

These experiments would provide useful information in determining the optimal operation of a bioreactor. However, without the ability to understand or predict the behavior of a plasmid-containing population under transient operation it would be a "hit or miss" experimentation process. In addition, the optimal operating strategy might not be limited by conventional parameters, but by the plasmid and host cell being used. Therefore, it is also necessary to study this system at a molecular level.

7.3 Molecular Approach

This section focuses on the elucidation of the underlying mechanisms governing plasmid stability, replication, segregation, and expression under a transient environment.

The effects of perturbations on the macromolecular composition, including RNA I and RNA II, of a plasmid-containing culture should be examined. This would give an

understanding of plasmid replication under these conditions. Pulse labeling and analysis of the mRNA [177] would also aid in determining the effect of a transient environment on protein translation and the cloned gene expression.

The investigation of the plasmid-containing sub-population might prove useful in understanding plasmid segregation. The use of flow cytometry would provide a useful tool as it allows a rapid sampling of a large sample size. In addition, plasmids in which replication could be turned on and off would also be helpful. Work is currently underway in constructing a plasmid where replication could be increased or decreased by the addition of an inducer, such as IPTG for the lac operon.

Regulation of plasmid replication might prove beneficial in reducing if not eliminating plasmid instability due to plasmid loss. Through the use of minimum inhibitory concentration plates which detect the gradual loss of plasmids it is possible to predict the onset of the plasmid-free cells. If at this point plasmid replication could be induced by the addition of IPTG it would be possible to prevent the formation of the plasmid-free cells. A better system is the regulation of the plasmid replication through a pH or temperature shift. On an industrial scale, regulation by these two parameters is less costly and easier than chemical induction.

In general, further insight into the relationship between the growth environment and plasmid maintenance and expression

and ultimately the development of optimum bioreactor operation will result from work at both the engineering and molecular level.

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APPENDIX I

DETERMINATION OF MODEL PARAMETERS USED IN CHAPTER 5

A new variable w, defined as the ratio between the high copy number sub-population and the lower number sub-population, is given by

$$dw/dt = d(y_m/y_s)/dt$$

$$= w[\mu_m - \mu_s - p_2' + P_1' + P_3'] + p_1'$$

with $w(t=0) = 0$ for a culture which starts with only a high copy number population. If the parameters are assumed to be constant, the analytical solution to the above equation is given by

$$\ln[\ \alpha w/p_1' + 1] = \alpha t$$

where

$$\alpha = \mu_m - \mu_s - p_2' + p_1' + p_3'$$

As α/p_1' is usually much larger than 1, the approximation

$$\ln[\ \alpha w/p_1'] = \alpha t$$

will hold when w is not too small. The parameters α and p_1' can be determined from the slope and the intercept of a semi-log plot with w plotted as a function of time. A typical plot for $D = 0.61\ hr^{-1}$ is shown in Figure A-1. Finally, if $|\mu_m - \mu_s| >> |p_2' - p_1' - p_3'|$, then $\alpha \sim \mu_m - \mu_s$.

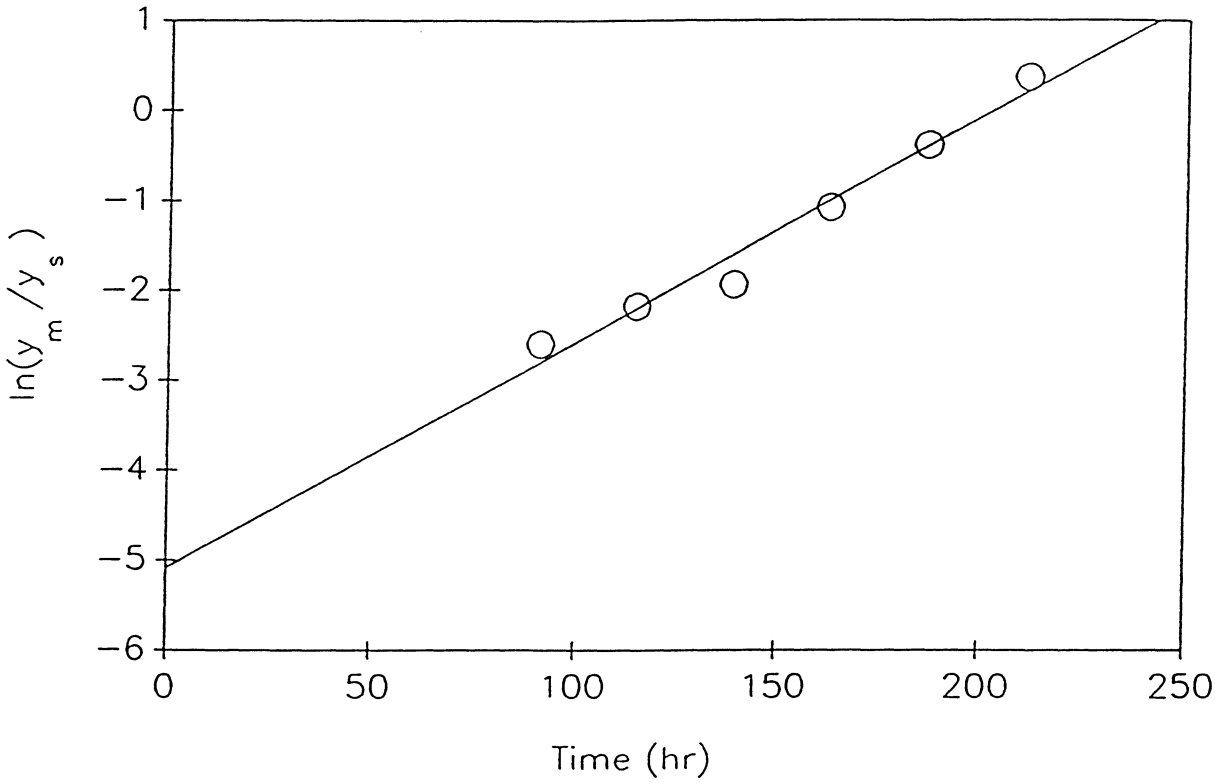

Figure A-1. Estimation of $\mu_m - \mu_s$ and p_1' from a plot of $\ln(y_m/y_s)$ as a function of time.

APPENDIX II

The null hypothesis testing method was used to determine whether the difference between the data obtained from the spread and replica plating techniques was statistically significant. The difference of the means of two normal distributions having equal but unknown variances was examined [82].

Briefly, x_1 is the average of a sample of size n_1 from a normal population $N(\mu_1,\sigma_1^2)$ and x_2 is the average of an independent sample of size n_2 from a normal population $N(\mu_2,\sigma_2^2)$ where σ_1^2 and σ_2^2 are unknown. It is desired to test, at a certain significance level, the hypothesis

$$H_o: \mu_1 - \mu_2 = 0 \qquad \text{(A-1)}$$

against the alternatives

$$H_1: \mu_1 - \mu_2 = \delta > 0 \qquad \text{(A-2)}$$

of the two samples. The sizes, averages, and variances of the two samples are represented by n, x, and s, respectively. The method used is a right-sided test with the critical region defined as the set of values (x_1,x_2,s_1,s_2) for which

$$\left| \frac{x_1 - x_2}{s_w\sqrt{1/n_1 + 1/n_2}} \right| > t_{n1 + n2 - 2;\alpha} \qquad \text{(A-3)}$$

where

$$s_w^2 = \frac{(n_1 - 1)s_1^2 + (n_2 - 1)s_2^2}{n_1 + n_2 - 2} \qquad \text{(A-4)}$$

In general, if x_1, x_2, and s_w satisfy equation A-4 then x_1 and x_2 differ significantly at the 100α level of significance [82]. In comparing the discrepancy between the spread and replica plating techniques, at a 5% level of significance

$$n_1 = n_2 = 200$$

$$s_1 = s_2 = 5$$

and

$$t_{398;.05} = 1.645$$

Based on these parameters the differences between the two procedures must be greater than 0.82% to be statistically significant.