Physiological aspects of growth and recombinant DNA stability in *Saccharomyces cerevisiae*

C. Anthony Mason

Swiss Federal Institute for Water Resources and Water Pollution Control (EAWAG),

Swiss Federal Institutes of Technology (ETH) Zürich, Ueberlandstrasse 133, CH-8600 Dübendorf, Switzerland

Received 20 September 1990; accepted 14 February 1991

Key words: growth physiology, plasmid stability, recombinant cell culture, *Saccharomyces cerevisiae*

Abstract

Despite the fact that plasmid stability in the yeast *Saccharomyces cerevisiae* is influenced by both genetical and physiological parameters most attention has been focussed on the former. Physiological factors affecting the stability of plasmids have been poorly characterized despite the need for such information in order to optimize the use of *S. cerevisiae* as a host for recombinant protein production processes. The physiology of wild type *S. cerevisiae* differs considerably when grown using different cultivation techniques. A limited amount of phenomenological data has been reported concerning plasmid instability effects under these different conditions and in this article these have been collected together with the intention of providing an overview to instability effects and to try and propose reasons as to how the physiological response to different growth conditions can be manifested as stability/instability effects.

1. Introduction

In recent years, there has been significant interest in the use of microorganisms for the production of heterologous polypeptides and proteins. The main advantages associated with the use of yeasts as a host for the production of eukaryotic heterologous proteins are well known and systems have been developed for the synthesis of various pharmaceutical and fine chemical products including hepatitis B surface antigen, interferons and calf chymosin. Optimization of production systems and large scale industrial application of recombinant DNA mediated systems requires evaluation and maximization of both the genetic and physiological potentials available. This sets as a prerequisite the detailed understanding of the behaviour of all elements of the system, their interactions and the consequences

of both internally and externally mediated perturbations and of long term physical, chemical and biochemical change. Genetical and physiological optimization are closely linked and both have to be considered together in the evaluation of system performance. There has been a great deal of good qualitative research on the genetic aspects of stability in recombinant systems, especially in the case of the bacteria. Despite the tremendous potential that recombinant yeast systems offer, the level of understanding still lags markedly behind that of the bacteria. While many phenomenological effects have been described, their genetical and physiological basis remain largely unresolved.

In the absence of additional DNA in the cell, the growth physiology of yeasts is already highly complicated. When additional DNA is present, this might interfere either directly or indirectly with the

degradative, energetic, biosynthetic and secretory pathways and mechanisms that occur in the cell and thus could have an adverse effect on the physiology. In this review the relationship between physiology and stability will be investigated based on information available in the literature. When looked at together, there is very little known concerning the interaction between physiology and genetics and this is somewhat unexpected given the importance of this question and the need for maximization of stability and thereby expression in industrial processes. This review will focus mainly on *Saccharomyces cerevisiae*, partly because there is a great deal of information available concerning the physiology of this organism and partly because of the importance of *S. cerevisiae* in industrial processes. Whilst the use of *S. cerevisiae* in the brewing and baking industries has historically been the major application, other industries and technologies are now making use of the properties of yeasts (Sturley & Young 1988). For example, in waste treatment *Candida utilis* is used in the treatment of sulphide wastes and *Kluyveromyces fragilis* is used in the treatment of whey. For the production of fine chemicals, including pharmaceuticals, much attention has been focussed on *S. cerevisiae*, but other yeasts like *Pichia pastoris* are also important and it might well prove that such methylotrophic yeasts offer significant economic advantages in the long term and will receive greater attention accordingly (Egli & Mason 1991). Genetic manipulation of *S. cerevisiae* is being proposed with two objectives: strain construction and strain improvement. Much of the current effort in the application of molecular biological manipulation to industrial yeast strains is designed to produce strains which are more productive, more competitive or more resistant to adverse influences in a particular industrial system. *S. cerevisiae* is being used much more effectively for the production of eukaryotic proteins, since both correct conformation and secretion from the production cells can be more readily achieved, compared with expression systems developed for *Escherichia coli* where eukaryotic proteins are frequently not produced with the correct tertiary structure (Zabriskie & Acuri 1986).

2. Yeast plasmids

There are very few naturally occurring plasmids in yeasts (Toh-e et al. 1982) as compared with the bacteria where a range of different naturally occurring plasmids are known to exist. The natural yeast plasmids are surprisingly stable, for example, the mitotic stability of the 2 μ m circular plasmid found in the yeast *S. cerevisiae* is almost identical to that of the chromosome, despite a slight but reproducible effect on growth rate (2 μ m containing strains have a 1.5–3% longer generation time than plasmid free cells (Walmsley et al. 1983; Mead et al. 1986a). The high stability of the 2 μ m plasmid has been attributed to the tight regulation of copy number. Normally, the 2 μ m plasmid is present in the nucleus at a copy number between 60 and 100 per cell, and the plasmid has evolved a system whereby this copy number can be maintained (Futcher 1986). Should the copy number decrease during division, it can undergo multiple rounds of replication to bring its copy number back up to the original level (Sigurdson et al. 1981, Som et al. 1988). The 2 μ m plasmid is very widespread and has been found in virtually all strains of *S. cerevisiae* and *S. carlsbergensis*. The open reading frames in the 2 μ m plasmid all encode functions associated with plasmid maintenance and amplification. There is no other known function. Recently, Volkert et al. (1989) described the 7 different plasmids that have been found in yeast species (in *Zygosaccharomyces rouxii*, *Z. balii*, *Z. fermentati* and in *Kluyveromyces drosophilae*). Several features of these plasmids are similar to the 2 μ m plasmid, e.g., they all have the unique sequences bordered by a pair of inverted repeats and lack any known functions. Linear plasmids have also been found in several yeast species including the three which were recently detected in the yeast *Pichia inositolovora* (Ligon et al. 1989). These linear plasmids are located in the cytoplasm and have physical characteristics reminiscent of the yeasts *K. lactis* and *Saccharomycopsis crataegensis*. However, they lack the killer toxin production capability present on that of *K. lactis* (Ligon et al. 1989).

Fig. 1. The cell cycle of *Saccharomyces cerevisiae*.

3. Cell cycle

It is possible that the nature of the yeast cell cycle might have an influence on host plasmid interactions thereby affecting plasmid stability. In the yeast cell cycle, replication occurs by production of a daughter cell as a bud on the mother cell. As shown in Fig. 1, the bud grows and eventually separates from the mother cell at division. Division in *S. cerevisiae* is asymmetrical, the daughter cell being smaller at birth than the mother cell (Hartwell & Unger 1977). Since there is a size requirement before initiation of division in *S. cerevisiae* (Johnston et al. 1977) the daughter cell must first grow to attain this size, whilst the mother cell is already at this critical size, so that the cell cycle time for the daughter cell is longer than that for the mother cell (Lord & Wheals 1981). The degree of asymmetry at division, i.e., the size of the daughter cell at birth relative to the mother cell, is growth rate dependent (Lord & Wheals 1980). The faster the cells are growing, the lower the degree of asymmetry (Lord & Wheals 1980, 1981; Thompson & Wheals 1980). Nutrient depletion causes the cells

to stop progress through the cell division cycle before passage through 'start' i.e., the cells accumulate as uninucleated unbudded cells in the G1 phase (Piñon 1978, 1979; Piñon & Pratt 1979). During the S-phase, the DNA is replicated. This phase occupies 25 to 50% of the cell cycle time of glucose grown cells. When other carbon sources are used the G1 phase occupies a longer period of time, however, the time spent in the S-phase appears not to be drastically altered (Barford & Hall 1976). Cells grown under nitrogen limitation have also an extended G1-phase. However, under these conditions the other phases are also affected with the S and G2 periods requiring more time for completion, resulting in much longer cell cycle times and thereby lower growth rates (Rivin & Fangman 1980 a,b; Johnston et al. 1980). The replication of plasmid DNA also takes place during the S-phase and usually there is only one round of replication (Zakain et al. 1979; Fangman et al. 1983). The exception to this is the specific amplification system in low copy number cells of the 2 μ m plasmid (Futcher 1986). However, even in this case there is only a single initiation followed by rolling circle replica-

tion producing a multimer which is eventually cut to produce single copies of the plasmid. 'Start' (Fig. 1) is the first point in the cell cycle under genetic control, as indicated by the ability to isolate temperature sensitive mutants which halt at this point in the cell cycle (Wheals 1989). Once the cells have passed start they are committed to a mitotic division even in the absence of further growth. Once the cells have reached start additional growth occurs only in the daughter cell, there is little change in the size of the mother cell following attainment of the critical size necessary for division.

4. Recombinant system considerations

There are a number of options available for strain construction and these have been described in detail by Parent et al. (1985) and Bitter et al. (1987). Essentially, genes on integrating plasmids usually show low transformation efficiencies (Hinnen et al. 1978), but can attain relatively high gene stability in *S. cerevisiae* (Zhu et al. 1986). Recently there have been reports of using the rDNA sites as sites for integration for the integrative plasmids, and this has been shown to enable copy numbers to be as high as 200 (Lopes et al. 1989). Growth experiments with one such construct suggest that optimum expression and stability of recombinant proteins occurs when the copy number is limited to 20–25 (Van der Aar et al. 1990). Episomal plasmids based on the *S. cerevisiae* 2 μ m plasmid, require the original stability and amplification functions to be encoded in trans either on accompanying native 2 μ m plasmids coexisting in the cell or by incorporation of the appropriate functions on the chromosome. The copy number of episomal plasmids is typically about 20–40 per genome but the stability is less than that of the native 2 μ m plasmid (Futcher & Cox 1984; Bugeja et al. 1989).

Replicating plasmids, based on the presence of a selectable yeast gene, contain an autonomously replicating sequence (ARS) from the yeast chromosome apparently enabling autonomous replication. It has been suggested that these autonomously replicating sequences might be functionally linked to nuclear compartmentalization, such that the

very high instability of replication plasmids could be accounted for by the bias of the plasmid to its mother cell during cell division (Newlon 1989; Murray & Szostak 1983). Plasmids which contain a chromosomal centromere sequence have also been used for cloning. These plasmids behave like a chromosome at division, pairing up on the spindle body at mitosis. This ensures very stable division of mother and daughter plasmids at cell division, but limits the plasmid copy number to 1 (Parent et al. 1985).

Irrespective of the nature of the recombinant system adopted it is important to characterize the stability of the host-plasmid construct and ensure that the plasmid is both stably maintained in the population and stably passed on to the progeny. In order to comprehensively understand this issue it is necessary to understand how host cell physiology can influence both stability and expression of the heterologous gene. Physiological stability is considered to be slightly different from genetic stability which is more concerned with the ability for the DNA to replicate under standardized laboratory conditions. Physiological stability takes into account how the recombinant organisms respond to their growth environment and to dynamic conditions. Plasmid behaviour is governed by the conditions prevalent in the host cell i.e., plasmid encoded functions are dependent in the first instance on the host cell for the provision of such factors as energy and biosynthetic precursors. Anything that affects the physiology of the cell could therefore have an influence on the stability and expression of the plasmid should the altered conditions result in a change in availability of host-mediated functions. Correspondingly, it is important to consider the opposite effect, i.e., the effect of heterologous gene maintenance and expression on the physiology of the host cell. Expression of plasmid genes and heterologous protein production are using resources such as nutrients, precursors and energy normally available to the host cell for other purposes and they can compete or interfere with various other functions in the host cell namely in the secretory pathways or in energy generation. This effect results in internal perturbation of the physiology of the host cell. There is relatively little known about

Fig. 2. Fate of plasmid containing *S. cerevisiae* in a culture growing under selective conditions. (A) Normal segregation of mother and daughter cells where daughter cells contain the plasmid. (B) Instability event resulting in the production of plasmid-free daughter cells which, due to cytoplasmic transfer of complimentary product, can grow even in a selective environment. Eventually the complimentary product will be diluted out thereby producing cells incapable of continued survival. (C) Lysis of mother cell resulting in release of cell contents into culture fluid making available a supply of complimentary product for uptake by plasmid-free cells to enable enhanced survival especially for those cells where the level of inherited complimentary product has become inadequate.

this aspect despite the importance with respect to plasmid stability and to function of both the plasmid and the host cell (Mason & Bailey 1989).

Figure 2 shows schematically the types of cells that can be found in a growing culture of plasmid containing *S. cerevisiae* in selective medium. Plasmid loss results in a plasmid free daughter cell (P⁻), which is only capable of growth when it contains the complementary product required to overcome the selective conditions imposed. However, since the complementary product is frequently cytoplasmic in nature (e.g., an amino acid) and that a certain fraction of the cytoplasmic contents of the mother cell are passed into the daughter cell at birth it is possible plasmid free daughter cells will contain a sufficient amount of the complementary product to overcome the inhibition and be able to

grow in the selective environment. This replication/cytoplasmic transfer process is able to repeat itself over several generations and instances where as many as 5 generations have been described before the complementary product is eventually diluted out of the cytoplasm to such an extent that growth is no longer possible in the selective environment (Murray & Szostak 1983; Srienc et al. 1986; Bailey et al. 1986; Wittrup & Bailey 1988).

Cellular lysis is another fate possible for plasmid containing cells whereby the cellular contents are released into the culture fluid. Cells devoid of both plasmid and of complementary product can then take up the complementary product from the culture fluid and grow, a process known as cryptic growth (Mason & Hamer 1987). For efficient production of heterologous proteins both the extent to

which plasmid free cells arise and the lysis rate of the plasmid containing cells must be minimized. In many industrial production systems, the use of specialized selective media is neither time nor cost effective. Therefore most attention is focussed on growth and stable expression of product using non-selective media. In such systems since the plasmid can impose a metabolic burden on the host cell (Bentley et al. 1990) it is possible that the plasmid-free cells will have a growth advantage in the culture and might therefore become dominant. This requires that the growth of plasmid-free strains must be restricted in some way since under non-selective growth conditions, all of the cell types shown in Fig. 2 will be able to grow.

In order to analyse the culture to determine the relevant fractions of the different types of cell which are present one has to rely on the accuracy and work within the limitations of the particular techniques that are being used. Many of the techniques involved in physiological studies of recombinant microorganisms are derived from the genetical literature where such techniques were designed for purposes other than growth studies or physiological characterization. A large number of standard techniques are now used for this latter purpose under conditions where the results can be ambiguous, for example, by using media lacking certain trace elements. Similarly, complex defined media can result in methodology derived errors especially in the case of growth of slow growing cells taken from minimal media.

5. Instability

There are various ways by which instability in a plasmid and host construct can arise. Futcher & Cox (1984) investigated stability of a wide range of host plasmid constructs and concluded that considerable variation exists in both stability and copy number from plasmid to plasmid and from host to host. These authors also discussed the various ways in which instability could arise, including incompatibility, loss of function, size, selection, segregation, copy number and host ploidy effects. For example, incompatibility results in instability in

such cases as where the coexistence of a 2 μ m-based episomal plasmid and the endogenous 2 μ m plasmid is impossible in the same cell due to complications in the copy number amplification and/or control system (Gerbaud & Guérineau 1980; Jayaram et al. 1983). Loss of function causes instability if deletions or insertions occur in the replicative functions of the plasmid (Broach & Hicks 1980). Furthermore, integration of the selection markers into the chromosome will result in loss of stability under selective conditions. Plasmid size has also been shown to be a factor involved in instability, in that the larger the plasmid the lower is its apparent stability. Physical interactions during mitosis has been suggested as a possible reason (Volkert et al. 1989). According to this theory, the nucleus at mitosis is assumed to have a very complex 3-dimensional structure full of tubules and spindles. These represent physical barriers to the smooth passage of a plasmid from a mother to a daughter cell, the extent of passage inhibition being a function of plasmid size. Selection is also another factor where there is a requirement for a certain number of copies of a gene for it to be effective. For example the use of a defective LEU2 gene on the plasmid pJDB219 requires that many copies must be present for complementation, enabling only the high copy number plasmid-containing strains to grow in a selective environment (Erhart & Hollenberg 1983; Futcher & Cox 1984). Segregational instability is the most frequently discussed form of instability and results when a low frequency transfer of plasmid occurs from mother to daughter. The physiological or genetical basis for these phenomena are less clear, but in the case of the ARS plasmids, inherent segregational instability could be caused by the preferential mother-cell bias due to affinity of the ARS sequence to specific nuclear components in the mother cell (Newlon 1989; Murray & Szostak 1983). Copy number regulation is also a feature where the higher the copy number the more stable the plasmid will be. This is based mostly on a probability of transfer function (Jayaram et al. 1983; Futcher & Cox 1984). Host ploidy has been shown to have an influence on stability (Mead et al. 1986b). Some suggestions have been made that this might be associated with chromoso-

mally located stability function (Kikuchi & Tohe 1986), enabling cells with higher ploidy to be more stable than those with lower ploidy (Spalding & Tuite 1989).

6. Growth physiology

Physiological characterization of a cell during growth under different conditions has to be correlated with phenomenologically observed instability effects reported under these growth conditions. In the following sections, the physiology of *S. cerevisiae* under various culture conditions is discussed with the aim of showing how the physiology of growth might provide insight to understand the nature of some of the instability patterns observed during culture of recombinant yeast cells under the different conditions. Growth of *S. cerevisiae* in aerobic batch culture, using glucose as carbon and energy substrate, results in a diauxic growth pattern in which glucose is used in the first phase of growth with the concomitant production of cell biomass. As a result of fermentative pathways ethanol is produced as a by-product (or end-product) of metabolism. When glucose is exhausted, a diauxic lag follows during which time metabolism is altered to enable utilization of ethanol oxidatively as substrate for the second phase of growth. Schepers et al. (1987) studied various cellular parameters such as cell size, RNA content and protein content using flow cytometry to determine how these vary during growth of *S. cerevisiae* in batch culture. The results they obtained indicated that during exponential growth on glucose there is a distribution in cell sizes made up of unbudded cells in the G1 phase with a characteristic peak at 5 μm , and a broader peak containing cells with sizes varying from 6 μm –12 μm and therefore at different phases of budding. Following glucose exhaustion, the population of cells larger than 6 μm decreased quite rapidly such that by the diauxic lag phase there remained only a single major peak at 6 μm . During the subsequent phase of growth on ethanol, Schepers et al. found that the unbudded daughter cells constituted a new size cluster less than 6 μm , the budding cell population reaching a maximum cell

size of 6 μm . This latter population slowly disappeared so that at the end of the second growth phase the population was composed almost exclusively of the smaller 2–5 μm cells.

Both RNA content and protein content of the *S. cerevisiae* also varied during batch growth (Schepers et al. 1987). Protein content increased slightly during growth on glucose, but as soon as glucose became exhausted, the protein content, measured on a per cell basis, decreased. The RNA content decreased gradually during growth on glucose, however, as soon as this was exhausted there was a rapid drop in the level of RNA which then further continued to decline during subsequent growth on ethanol.

Correlation of plasmid stability under batch growth conditions with physiology

Data available correlating plasmid stability to physiology during batch cultivation is somewhat limited. One example was reported by Hjortsu et al. (1985) who found using flow-cytometry that plasmid stability was highest during exponential growth on glucose where ca. 12% of the cells at birth were plasmid-free. Instability increased upon exhaustion of the glucose in the culture up to 25–28%. However, correlation with physiological state beyond this was not given. In contrast, Copella & Dhurjati (1989) using an episomal plasmid in a *cir*^o host, found that plasmid stability decreased during the active growth phase, but that it stabilized at a level of ca. 80% in the stationary phase following glucose exhaustion. This indicates that there is a strong dependence for plasmid stability on the nature of the host-plasmid construction, a feature which has also been observed by others (Walmsley et al. 1983; Futcher & Cox 1984). The nature of the coordinated interactions between physiological state and plasmid (in)stability during batch culture is unclear. There have been many mechanisms proposed to explain the phenomena, many of which are based upon the fact that the daughter cell size is smaller following exhaustion of glucose in the culture fluid. This has led to the conclusion that more difficulties are encountered in getting the plasmid into the daughter cells at a sufficient copy number (Coppella & Dhurjati

1989). However, one should be a little careful in simply relating the increased probability of producing a cell without plasmid to the fact that the cells are smaller and therefore 'packaging' is more difficult. What is necessary is to correlate the fact that the onset of increased plasmid instability is associated with major energetic and physiological changes in the growth cycle of the cells and this has not really been looked into to any great extent, despite its possible relevance. Ultimately one must try to associate both the physical factors and the physiological to understand the true nature of the interactions involved.

Fed batch

Optimization of industrial fermentation processes requires high product concentrations and high volumetric productivities whilst minimizing raw material costs. When a recombinant system is involved, high gene stability and high level gene expression are also necessary. Frequently, in a production system, the cells can become inhibited either by by-products (e.g., ethanol) or even the recombinant product might be inhibitory. Therefore the higher the cell concentration the greater the potential for inhibition. One rationalization is to use cells grown under such conditions whereby growth is optimized in the exponential phase with a continuous feed of fresh substrate at a rate to the medium such that the residual substrate concentration is zero. In this fed-batch process, high cell densities can be reached prior to product biosynthesis in the absence of by-product formation (Tsai et al. 1987). Moreover, maintaining growth in the exponential phase also ensures that plasmid stability is maintained at an operational high level (Hjortsu et al. 1985). In *S. cerevisiae*, growth is maintained in exponential phase by using metabolic feedback to control the rate at which glucose is added such that the culture grows under glucose limitation and glucose is oxidized completely to produce biomass and CO₂ only. For fed-batch grown cultures of *S. cerevisiae* the respiratory quotient is frequently used as the control indicator.

Plasmid stability during fed batch culture

Results of fed batch experiments with recombinant *S. cerevisiae* have been shown by a number of groups. For example, Fieschko et al. (1987) in a series of experiments with *S. cerevisiae* RH218 containing a plasmid with a *trp1* selection marker, were able to achieve cell biomass concentrations up to 200 grams dry weight per litre. Although they reported some difficulties with oxygen transfer above a density of 90 g/l and the plasmid was not expressing any particular product. However, these data serve to illustrate that it is potentially possible to achieve very high biomass levels under appropriate conditions. In order to examine the response of their system when a heterologous product was expressed from *S. cerevisiae* during fed-batch cultivation, they used a strain of *S. cerevisiae* containing plasmid pPG96y4AYC which contained the gene for human immune interferon (IFN- γ). With this system a final cell density of only ca. 15 g/l was possible and the stability was severely affected. Only 11% of the culture contained plasmid at the end of the experiment, despite the use of selective growth conditions. This result was found to be due to the inhibition of growth and of plasmid stability by the product of the recombinant plasmid. In order to overcome this growth inhibitory effect an alternative strategy was used whereby the cells were first grown up to very high cell densities and by using a variety of different promoter systems, the expression of the recombinant gene was only induced following attainment of high biomass concentration. In this manner, very high expression levels (2.2×10^{10} units/l representing 3–5% of total cell protein) could be obtained together with high levels of plasmid stability. However, it should be noted that to increase stability of the plasmid in this system, a diploid host cell was used (see section 5).

Continuous culture

The yeast *Saccharomyces cerevisiae* has been very extensively studied in continuous culture, and this technique has proved to be a very important tool both for physiologists and for geneticists. Unfortu-

nately, the aim is often to study the long term behaviour of a particular cell under steady state conditions based on the assumption that steady state culture dynamics are established. However, especially for the case of continuous cultures of recombinant microorganisms, the concept of a 'steady state' must be subject to question. Amongst the frequently used definitions of steady state is that of attainment of a constant amount of a particular parameter, the most commonly used being constant biomass. Whilst this might be a pertinent indicator and 'steady-states' might occur readily for this lumped parameter, it is not necessarily a reflection of the physiological state of the culture. Using this technique, minor fluctuations in biomass are probably a reflection of massive changes in physiology. One only needs to compare the times required for a steady state to be reached as judged from, for example, residual substrate concentrations (Senn 1989) to understand the dynamic behaviour of the microorganisms. When plasmid containing populations are cultivated under continuous culture conditions, the time constant of the process of copy number adjustment after a change in physiological conditions is longer than the time constant of biomass concentration adjustment. This, together with instability effects, means that it is unlikely that a steady state can be established with respect to either the genetic composition of the culture or cellular composition.

Physiology of Saccharomyces cerevisiae in continuous culture

The growth of *S. cerevisiae* in continuous culture can be divided into three regimes. The first is at low growth (dilution) rates where glucose is degraded oxidatively to produce only biomass and CO₂ and a relatively high cell yield results. At high growth (dilution) rates, glucose is also metabolized by fermentative metabolism to ethanol and since ethanol is an energy rich compound the yield of biomass is reduced. High growth rate situations are therefore analogous to the situation as found in batch culture except that the system is now a flow through continuous culture. Between these two growth regimes is a range of growth (dilution) rates at which glucose

is both oxidatively and fermentatively metabolized (respiro-fermentative metabolism). Cells growing under these conditions are very sensitive to changes in their external environment and react with sudden and long-lasting changes in their metabolism. For example, with *S. cerevisiae* CBS 8066 growing in continuous culture at a dilution rate between 0.30 h⁻¹ and 0.38 h⁻¹ any change in the dilution rate by more than 0.02 h⁻¹ results in quite drastic changes in the culture including a change of metabolism to alcohol fermentation after which an extensive period of time requiring many residence times is required before steady-state conditions are re-established (Postma et al. 1989).

From both flow cytometry and direct microscopic observation, one can associate very closely changes in the proportions of different cell sizes with changes in the physiology of the culture (Scheper et al. 1987). Cells with a diameter between 6 and 12 µm constitute only a very low percentage of the total culture at very low growth rates but their proportion increases with increasing growth rate (decreasing doubling time) becoming much more numerous during the respiro-fermentative metabolism phase. At the higher growth rates this fraction of cells dominates the culture. As the dilution rate (growth rate) is increased, the generation time decreases and the size of the daughter cells at birth increases (Lord & Wheals 1980, 1981; Thompson & Wheals 1980). Also, at the lower growth rates there are a larger proportion of unbudded daughter cells in the population whilst at higher growth rates there are very few unbudded daughter cells such that the majority of cells present exist as budding mother cells. This fact also implies that the cell cycle time in the G1 phase is very much shorter, as would be expected, since the degree of asynchrony between the daughter and mother cells is either considerably reduced or even non-existent. Data concerning the RNA and protein contents of the cell also indicate that with increasing dilution rate, marked changes occur around the transition phase from oxidative to respiro-fermentative metabolism (Scheper et al. 1987). Whilst these parameters serve as indicators of physiology and that major changes in these compo-

nents occur in *S. cerevisiae* as a function of dilution rate, plasmid stability in continuous culture should also reflect these differences in physiology. Curiously, despite the central nature of this question, there has been remarkably little effort in this direction in the literature.

Where results have been made available there exists a certain degree of controversy and confusion. Generalizations are clearly impossible since one has two superimposed effects namely differences in plasmid structure/function and differences in host physiology. Considerable differences in response have been observed by different groups with respect to stability using both different plasmids, hosts, and different growth conditions.

Plasmid stability during continuous culture

In selective media, DiBiasio & Sardonini (1986) have shown that their host-plasmid construct was more stable at higher than at lower growth rates, i.e., the stability in selective media increased with increasing growth rate. In contrast, in non-selective environments, growth of recombinant *S. cerevisiae* resulted in a slightly different response in that the stability of the 2 μ m-based pLG69Z plasmid studied for example by Impoolsup et al. (1989) in *S. cerevisiae* YN124 was found to decrease with increasing dilution rate. In the latter case, there was no indication during the reported experiments of the physiological status of the culture although on examination of the reported curves showing the rate of plasmid loss at various dilution rates there appeared to be a dichotomy between a dilution rate of 0.23 h⁻¹ where the rate of generation of plasmid free cells was 0.0039 h⁻¹ and a dilution rate of 0.27 h⁻¹ where a rate of 0.011 h⁻¹ was found. It is within this range that one would expect the transition from oxidative to oxido-reductive metabolism to occur. Also using non-selective medium, the results of Kleinman et al. (1986) indicate somewhat different effects. Unfortunately, although these results are frequently discussed, there has been some confusion in the literature concerning the growth conditions used and therefore erroneous interpretation of the results by other researchers has occurred. The conditions used were clearly defined in the original paper as being non-selective. The data

of Kleinman et al. (1986) indicated the opposite effect to that found by Impoolsup et al. (1989), in that the stability of the plasmid pJDB248, in *S. cerevisiae* strain S150-2B, increased with increasing dilution (growth) rate. However, the dilution (growth) rates investigated by Kleinman ranged from 0.025 h⁻¹–0.227 h⁻¹, whilst Impoolsup et al. investigated a range from 0.15 h⁻¹ to 0.42 h⁻¹ with only one measurement taken below 0.22 h⁻¹ such that different physiological regimes were encountered in the two pieces of work. Recently, Bugeja et al. (1989) have also reported higher stability at higher growth rates using the same host and plasmid as used by Kleinman et al. (1986). However, these experiments were also carried out over a relatively narrow range of low growth rates, i.e., between 0.05 h⁻¹ and 0.12 h⁻¹.

Discussion of plasmid stability phenomena in continuous culture

The reasons for these differences between high and low dilution rates and between selective and non-selective growth conditions are difficult to explain. It is evident from the various reports discussed here that plasmid stability in non-selective media varies considerably with growth (dilution) rate. The apparent conclusion is that at very high dilution rates, the plasmid stability decreases with increasing dilution rate. At intermediate growth rates plasmid stability is at a maximum and at lower growth rates plasmid stability decreases with decreasing dilution rate. It is therefore important to be able to understand the mechanisms and interactions behind this phenomenological response. One possible explanation is based on the cell cycle of *S. cerevisiae*. At lower growth rates, the size of the daughter cell relative to the mother cell is much smaller. Moreover, the relative numbers of daughter cells compared to those of mother cells is much higher at low growth rates than at faster growth rates (Thompson & Wheals 1980) since the G1 phase requires longer for completion at slower growth rates as a consequence of the smaller size of the daughter cells at birth. Therefore, at lower growth rates, in a completely mixed system, more unbudded daughter cells than mother cells will be eliminated from the bioreactor in direct proportion to the relative

amounts of the two cell types in the culture fluid. Combining this with the fact that plasmid instability results almost exclusively from a mother cell biased segregational inefficiency means that the probability of washing out a plasmid-free cell at lower dilution (growth) rates is much higher than the probability of washing out a plasmid containing mother cell and therefore the stability is high. With increasing growth rate, the relative numbers of daughter cells to mother cells decreases and therefore the higher proportion of plasmid-free daughter cells washed out diminishes and therefore plasmid stability would be predicted to decrease with increasing dilution rate. How then can the fact that plasmid stability decreases with decreasing dilution rate below a particular threshold maximum be explained? One possibility for this is that when one considers very slow growing continuous cultures at decreasing dilution rates the cells are exposed to increasing levels of starvation. When *S. cerevisiae* cells are subject to starvation conditions, transfer out of their normal cell-cycle into a so-called G_0 phase which represents a resting stage for the yeast and is detectable as phase light cells. During this time the metabolic activity is maintained at an absolute minimum for maximum survival (Bugeja et al. 1982). What causes the transition into G_0 is unknown as is the trigger out of G_0 but what is known is that such cells are not growing. At the very low dilution rate used in some of the experiments reported for example by Kleinman et al. (1986) and by Bugeja et al. (1989), with decreasing dilution rate the number of cells moving into the G_0 phase increased (Britton & Wheals 1987). Moreover, one can speculate that since the transition into G_0 is the response to a stressed environmental effect and those cells containing plasmids are the ones with the greatest metabolic burden, they are therefore the ones most likely to succumb under starvation conditions, and may thus be more likely to enter into the G_0 phase than the plasmid-free cells. This would then explain why plasmid-containing cells are more likely to be lost from the reactor at lower growth rates since they will not replicate from the G_0 phase. Thus with decreasing dilution (growth) rate the stability of the plasmid containing population would be expected to de-

crease as is in fact the case reported. However, such a hypothesis remains to be confirmed.

In selective media, to provide an explanation of the phenomenon described by DiBiasio & Sardoni (1986) is more difficult since there is insufficient data available to confirm the trends predicted by these authors, i.e., that plasmid stability in selective medium increases with increasing dilution rate. Clearly this conforms with what is known for the conditions in non-selective media but only at low growth rates. One possible mechanism which would explain the results for the trends seen in selective media is that since under such conditions only those cells which contain the plasmid can grow, and if at very low dilution (growth) rates the rate of cellular lysis is high, then one would expect that a larger number of plasmid-free cells might be maintained in the selective environment by cryptic growth on the complementary product released from the lysed plasmid containing cells. Thus decreasing the dilution rate results in an increased rate of lysis and a higher number of plasmid-free cells, maintained in the culture via cryptic growth and thereby a decreasing plasmid stability with decreasing growth rate. This hypothesis also needs to be substantiated by experiment but could provide an insight into reasons for the observed phenomena of plasmid instability variability in continuous culture.

The mechanisms and effects of metabolic status on plasmid stability has been shown to be significant, but the mechanism of action is a long way from being understood. One example is the effect of dissolved oxygen concentration on the stability of plasmids at different dilution rates. Caunt et al. (1989) carried out a series of experiments growing *S. cerevisiae* YN124 containing the plasmid pLG669Z at various dilution rates under conditions of high dissolved oxygen and then followed the rate of plasmid loss from such cultures. After a few generation times the dissolved oxygen level was changed by the investigators such that oxygen became limiting and they again monitored the rate of plasmid loss. Their results for the high dissolved oxygen region corresponded to those of Impoolsup et al. (1989) with the highest rate of plasmid loss at higher dilution rates. However under conditions of

low dissolved oxygen, the results were reversed, i.e., the highest rate of plasmid loss under conditions of low dissolved oxygen was found to occur at lower dilution rates. Growth rate dependent enhanced stability effects as a result of the use of dissolved oxygen cycling to stabilize plasmids in *S. cerevisiae* has been shown to occur by Caunt et al. (1990) based on a theory described by Stephens and Lyberatos (1988).

As a generalisation, it is reasonable to say that the descriptions of the relationship between physiology and instability in continuous cultures of *S. cerevisiae* have really only started to uncover the reasons for the observed effects and it is still very necessary to understand further the mechanisms involved.

Immobilized cell culture

Advantages of immobilized cell culture techniques include the possibilities to achieve a high specific rate of product synthesis and also a high rate of substrate consumption. As one would expect, the physiology of immobilized cells is considerably different from cells under suspended culture conditions. This was originally assumed to be purely due to difference in environmental conditions (e.g., pH, temperature, dissolved oxygen, ion and nutrient concentration) but it is also important to consider the consequences of physical contact with the surface which can result in physical effects on the immobilized cell. In an elegant series of experiments, Doran & Bailey (1986) have been able to show using a flow cytometer to examine the cell size, DNA content, RNA content, and protein content in individual cells which were carefully removed from the gelatin coated glass bead attachment support, that considerable changes in the macromolecular composition occurs in *S. cerevisiae* upon immobilization. They showed that the level of DNA in immobilized cells on a per cell basis was on average 3.8 X higher than in corresponding suspended cell systems ranging as high as 8 X that of suspended cells. Also, the amount of RNA was reduced by about 4 times and the amount of protein was only about 2/3 of that in suspended

cells. However, since there was a corresponding reduction in cell size, the amount of protein per unit volume cell was effectively unchanged between immobilized and suspended cells despite quite considerable changes in the DNA. Given the higher quantity of DNA it is then potentially possible that amplification of gene dosage can also be achieved by immobilization of cells of *S. cerevisiae*, thereby enhancing both gene stability and product formation. There are unfortunately very few published reports which actually deal with this question but those that have appeared have tended to agree with the prediction that in immobilized recombinant cell systems a high level of stability can be found (Walls & Gainer 1989).

It is also important to consider the fact that in practically all bioreactors a biofilm occurs during cultivation on the walls and other surfaces in the reactor (Topiwala and Hamer 1971). These films are often unnoticed but can contribute significantly to the persistence of plasmid containing strains by serving as a more stable reservoir for the replenishment of the less stable suspended population particularly in continuous cultures. Failure to account for the contribution of wall growth to the overall culture dynamics can result in major misconceptions concerning the stability of particular constructs.

7. Concluding remarks

In summary it is clear that the answers to the question of plasmid stability in yeast in relation to physiology remains very unclear and incomplete. Certainly there is a link between growth physiology and plasmid stability but how this is manifested is unknown, although it is clearly a response to many factors. Various factors have been discussed in this review which play a role in the physiological effects on plasmid stability. Clearly those discussed are not exclusively responsible for instability effects and a great deal more work is necessary to examine the relevance of other physiological parameters, particularly growth rate differences between plasmid-containing and plasmid free cells, cell cycle related phenomena such as daughter cell size, and

metabolic and energetic effects, for example oxidative or fermentative metabolism. As has been discussed here, the effects at different growth rates are consequences not only of how fast the cells are growing but also concern extensive changes in the cells physiological state.

It is important to realize that plasmid stability requires definition with respect to the physiology of the host microorganism. Once redefined then optimization of a process culture with specific production objectives might be achieved. The ultimate objective of metabolic manipulation of the yeast cell is to direct metabolism for the production of a useful product. Provided that the physiological factors during cell cultivation are comprehensively considered, only then can optimization of product formation be efficiently achieved.

Acknowledgement

I would like to thank G. Hamer and T. Egli for their valuable comments.

References

- Aar PC van der, Lopes TS, Klootwijk J, Groeneveld Ph, Versveld HW van & Stouthamer AH (1990) Consequences of phosphoglycerate kinase overproduction for the growth and physiology of *Saccharomyces cerevisiae*. *Appl. Microbiol. Biotechnol.* 32: 577–587
- Bailey JE, Da Silva NA, Peretti SW, Seo J-H & Srienc F (1986) Studies of host plasmid interactions in recombinant microorganisms. *Ann. NY. Acad. Sci.* 469: 194–211
- Barford JP & Hall RJ (1976) Estimation of the length of cell cycle phases from asynchronous cultures of *Saccharomyces cerevisiae*. *Exp. Cell Res.* 102: 276–284
- Bentley WE, Mirajalili N, Andersen DA, Davis RH & Kompa-la DS (1990) Plasmid-encoded protein: The principal factor in the 'metabolic burden' associated with recombinant bacteria. *Biotechnol. Bioeng.* 35: 668–681
- Bitter GA, Egan KM, Koski RA, Jones MO, Elliott SG & Giffin JC (1987) Expression and secretion vectors for yeast. *Meth. Enzymol.* 153: 516–544
- Britton NF & Wheals AE (1987) Mathematical models for a G_0 phase in *Saccharomyces cerevisiae*. *J. Theor. Biol.* 125: 269–281
- Broach JR & Hicks JB (1980) Replication and recombination functions associated with the yeast plasmid 2 μ m circle. *Cell* 21: 501–508
- Bugeja VC, Piggott JR & Carter BLA (1982) Differentiation of *Saccharomyces cerevisiae* at slow growth rates in glucose limited continuous culture. *J. Gen. Microbiol.* 128: 2707–2714
- Bugeja VC, Kleinman MJ, Stanbury PF & Gingold EB (1989) The segregation of the 2 μ m-based yeast plasmid pJDB248 breaks down under conditions of glucose-limited growth. *J. Gen. Microbiol.* 135: 2891–2897
- Caunt P, Impoolsup A & Greenfield PF (1989) The effect of oxygen limitation on stability of a recombinant plasmid in *Saccharomyces cerevisiae*. *Biotechnol. Lett.* 11: 5–10
- Caunt P, Impoolsup A & Greenfield PF (1990) A method for the stabilization of recombinant plasmids in yeast. *J. Biotechnol.* 14: 311–320
- Copella SJ & Dhurjati P (1989) α -factor directed expression of the human epidermal growth factor in *Saccharomyces cerevisiae*. *Biotechnol. Bioeng.* 33: 976–983
- DiBiasio D & Sardonini C (1986) Stability of continuous culture with recombinant organisms. *Ann. N.Y. Acad. Sci.* 469: 111–117
- Doran PM & Bailey JE (1986) Effects of immobilization on growth, fermentation properties and macromolecular composition of *Saccharomyces cerevisiae* attached to gelatin. *Biotechnol. Bioeng.* 28: 73–87
- Egli T & Mason CA (1991) Mixed Cultures and Mixed Substrates. In: Goldberg I & Rokem S (Eds) *The Biology of the Methylophiles* (pp 00–00) Butterworth Press
- Erhart E & Hollenberg CP (1983) The presence of a defective LEU2 gene on 2 μ m DNA recombinant plasmids is responsible for curing and high copy number. *J. Bacteriol.* 156: 625–635
- Fieschko JC, Egan KM, Ritch T, Koski RA, Jones M & Bitter G (1987) Controlled expression and purification of human immune interferon from high cell density fermentations of *Saccharomyces cerevisiae*. *Biotechnol. Bioeng.* 29: 1113–1121
- Futcher AB (1986) Copy number amplification of the 2 micron plasmid of *Saccharomyces cerevisiae*. *J. Theor. Biol.* 119: 197–204
- Futcher AB & Cox BS (1984) Copy number and the stability of 2 μ m circle-based artificial plasmids of *Saccharomyces cerevisiae*. *J. Bacteriol.* 157: 283–290
- Gerbaud C & Guérineau M (1980) 2 μ m plasmid copy number in different yeast strains and repartition of endogenous and 2 μ m chimeric plasmids in transformed strains. *Current Genetics* 1: 219–228
- Golpal CV, Broad D & Lloyd D (1989) Bioenergetic consequences of protein overexpression in *Saccharomyces cerevisiae*. *Appl. Microbiol. Biotechnol.* 30: 160–165
- Hartwell LH & Unger MW (1977) Unequal division in *Saccharomyces cerevisiae* and its implication for the control of cell division. *J. Cell Biol.* 75: 422–435
- Hinnen A, Hicks JB & Fink GR (1978) Transformation of yeast. *Proc. Natl. Acad. Sci. USA* 75: 1929–1933
- Hjortsu MA, Dennis KE & Bailey JE (1985) Quantitative characterization of plasmid instability in *Saccharomyces cerevisiae* using flow cytometry cell sorting. *Biotechnol. Lett.* 7: 21–24
- Impoolsup A, Caunt P & Greenfield PF (1989) Effect of growth rates on stability of a recombinant plasmid during continuous

- culture of *Saccharomyces cerevisiae* in non-selective medium. *J. Biotechnol.* 10: 171–180
- Jayaram M, Li YY & Broach JR (1983) The yeast plasmid 2 μ circle encodes components required for its high copy propagation. *Cell* 34: 95–101
- Johnston GC, Pringle JR & Hartwell LH (1977) Coordination of growth with cell division in the yeast *Saccharomyces cerevisiae*. *Exp. Cell Res.* 105: 79–98
- Johnston GC, Singer RA, Sharrow SO & Slater ML (1980) Cell division in the yeast *Saccharomyces cerevisiae* growing at different growth rates. *J. Gen. Microbiol.* 118: 479–484
- Kikuchi Y & Toh-e A (1986) A nuclear gene of *Saccharomyces cerevisiae* needed for stable maintenance of plasmids. *Mol. Cell Biol.* 6: 4053–4059
- Kleinman MJ, Gingold EB & Stanbury PF (1986) The stability of the yeast plasmid pJDB248 depends on the growth rate of the culture. *Biotechnol. Lett.* 8: 225–230
- Ligan JM, Bolen PL, Hill DS & Bothast RJ (1989) Physiological and biochemical characterization of linear DNA plasmids of the yeast *Pichia inositolovora*. *Plasmid* 21: 185–194
- Lopes TS, Klootwijk J, van der Aar PC, van Heerikhuizen H, Raué HA & Planta RJ (1989) High copy-number integration into the ribosomal DNA of *Saccharomyces cerevisiae* a new vector for high-level expression. *Gene* 79: 199–206
- Lord PG & Wheals AE (1980) Asymmetrical division of *Saccharomyces cerevisiae*. *J. Bacteriol.* 142: 808–818
- Lord PG & Wheals AE (1981) Variability in individual cell cycles of *Saccharomyces cerevisiae*. *J. Cell Sci.* 50: 361–376
- Marquet M, Alouani S, Haas ML, Loison G & Brown SW (1987) Double mutants of *Saccharomyces cerevisiae* harbour stable plasmids. Stable expression of a eukaryotic gene and the influence of host physiology during continuous culture. *J. Biotechnol.* 6: 135–145
- Mason CA & Hamer G (1987) Cryptic growth in *Klebsiella pneumoniae*. *Appl. Microbiol. Biotechnol.* 25: 577–584
- Mason CA & Bailey JE (1989) Effects of plasmid presence on growth and enzyme activity of *Escherichia coli* DH5 α . *Appl. Microbiol. Biotechnol.* 32: 54–60
- Mead DJ, Gardner DCJ & Oliver SG (1986a) The yeast 2 micron plasmid.: Strategies for the survival of a selfish DNA. *Mol. Gen. Genet.* 205: 417–421
- Mead DJ, Gardner DCJ & Oliver SG (1986b) Enhanced stability of a 2 μ m-based recombinant plasmid in diploid yeast. *Biotechnol. Lett.* 8: 391–396
- Murray AW & Szostak JW (1983) Pedigree analysis of plasmid segregation in yeast. *Cell* 34: 961–970
- Newlon CS (1989) Deoxyribonucleic acid organization and replication. In: Rose AH & Harrison JS (Eds) *The Yeasts*, Vol 3 (pp 57–116) Academic Press London
- Parent SA, Fenimore CM & Bostian KA (1985) Vector systems for the expression analysis and cloning of DNA sequences in *Saccharomyces cerevisiae*. *Yeast* 1: 83–138
- Piñón R (1978) Folded chromosome in non-cycling yeast cells. Evidence for a characteristic G₀ phase. *Chromosoma* 67: 263–274
- Piñón R (1979) A probe into nuclear events during the cell cycle of *Saccharomyces cerevisiae* in *cdc* mutants with arrest in G₁. *Chromosoma* 70: 337–352
- Piñón R & Pratt D (1979) Folded chromosomes of mating factor arrested cells: comparison with G₀ arrest. *Chromosoma* 73: 117–129
- Postma E, Verduyn C, Scheffers WA & van Dijken JP (1989) Enzymic analysis of the crabtree effect in glucose-limited chemostat cultures of *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol.* 55: 468–477
- Rivin CJ & Fangman WL (1980a) Cell cycle phase expansion in nitrogen limited cultures of *Saccharomyces cerevisiae*. *J. Cell Biol.* 85: 96–107
- Rivin CJ & Fangman WL (1980b) Replication fork rate and origin activation during the S phase of *Saccharomyces cerevisiae*. *J. Cell Biol.* 85: 108–115
- Scheper Th, Hoffman H & Schügerl K (1987) Flow cytometric studies during culture of *Saccharomyces cerevisiae*. *Enz. Microb. Technol.* 9: 399–405
- Senn H (1989) Kinetik und regulation des Zuckerabbaus von *Escherichia coli* ML 30 bei tiefen Zuckerkonzentrationen. Dissertation ETH Nr. 8831
- Sigurdson DC, Gaarder ME & Livingston DM (1981) Characterization of the transmission during cytoductant formation of the 2 μ m DNA plasmid from *Saccharomyces*. *Mol. Gen. Genet.* 183: 59–65
- Som T, Armstrong KA, Volkert FC & Broach JR (1988) Auto-regulation of 2 μ m circle gene expression provides a model for maintenance of stable plasmid copy levels. *Cell* 52: 27–37
- Spalding A & Tuite MF (1989) Host-plasmid interactions in *Saccharomyces cerevisiae*: Effect of host ploidy on plasmid stability and copy number. *J. Gen. Microbiol.* 135: 1037–1045
- Srienc F, Campbell JL & Bailey JE (1986) Analysis of unstable recombinant *Saccharomyces cerevisiae* population growth in selection medium. *Biotechnol. Bioeng.* 28: 996–1006
- Stephens ML & Lyberatos G (1988) Effect of cycling on the stability of plasmid-bearing microorganisms in continuous culture. *Biotechnol. Bioeng.* 31: 464–469
- Sturley SL & Young TW (1988) Genetic Manipulation of commercial yeast strains. In: Russell GE (Ed) *Yeast Biotechnology* (pp 1–38) Intercept, Wimbourne Dorset
- Thompson PW & Wheals AE (1980) Asymmetrical division of *Saccharomyces cerevisiae* in glucose limited chemostat culture. *J. Gen. Microbiol.* 121: 401–409
- Toh-e A, Tada S & Oshima Y (1982) 2 μ m DNA-like plasmids in the osmophilic haploid yeast *Saccharomyces cerevisiae*. *J. Bacteriol.* 151: 1380–1390
- Topiwala HH & Hamer G (1971) Effect of wall growth in steady state continuous cultures. *Biotechnol. Bioeng.* 13: 919–922
- Tsai LB, Mann M, Morris F, Rotgers C & Fenton D (1987) The effect of organic nitrogen and glucose on the production of recombinant human insulin-like growth factor in high cell density *Escherichia coli* fermentations. *J. Ind. Microbiol.* 2: 181–187
- Volkert FC, Wilson DW & Broach JR (1989) Deoxyribonucleic acid plasmids in yeasts. *Microbiol. Rev.* 53: 299–317

- Walls EL & Gainer JL (1989) Retention of plasmid bearing cells by immobilization. *Biotechnol. Bioeng.* 34: 717–724
- Walmsley RM, Gardner DCJ & Oliver SG (1983) Stability of a cloned gene in yeast grown in chemostat culture. *Mol. Gen. Genet.* 192: 361–365
- Wheals AE (1989) Biology of the cell cycle in yeast. In: Rose AH & Harrison JS (Eds) *The Yeasts*, Vol 1 (pp 283–377) Academic Press, London
- Wittrup KD & Bailey JE (1988) A segregated model of recombinant multicopy plasmid propagation. *Biotechnol. Bioeng.* 31: 304–310
- Zabriskie DW & Acuri EJ (1986) Factors influencing productivity of fermentation employing recombinant microorganisms. *Enz. Micro. Technol.* 8: 706–717
- Zakain VA, Brewer BJ & Fangman WL (1979) Replication of each copy of the yeast 2 micron DNA plasmid occurs during the S-phase. *Cell* 17: 923–934
- Zhu J, Contreras R & Fiers W (1986) Construction of stable laboratory and industrial yeast strains expressing a foreign gene by integrative transformation using a dominant selection system. *Gene* 50: 225–237