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Effects of medium composition and nutrient limitation on loss of the recombinant plasmid pLG669-z and β-galactosidase expression by *Saccharomyces cerevisiae*

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The effects of medium composition, nutrient limitation and dilution rate on the loss of the recombinant plasmid pLG669-z and plasmid-borne β-galactosidase expression were studied in batch and chemostat cultures of *Saccharomyces cerevisiae* strain CGpLG. The difference in growth rates between plasmid-free and plasmid-containing cells ($\Delta\mu$) and the rate of segregation (R) were determined and some common factors resulting from the effect of medium composition on plasmid loss were identified. Glucose-limited chemostat cultures of CGpLG grown on defined medium were more stable at higher dilution rates and exhibited $\Delta\mu$-dominated plasmid loss kinetics. Similar cultures grown on complex medium were more stable at lower dilution rates and exhibited R-dominated plasmid loss kinetics. Overall plasmid stability was greatest in phosphate-limited chemostat cultures grown on defined medium and was least stable in magnesium-limited cultures grown on defined medium. $\Delta\mu$ decreased and R increased with increased dilution rate, irrespective of medium composition. Increased plasmid loss rates at high or low dilution rates would appear to be characteristic of loss kinetics dominated by R or $\Delta\mu$, respectively. Growth of glucose-limited chemostat cultures on complex medium decreased $\Delta\mu$ values but increased R values, in comparison to those cultures grown on defined medium. Any increased stability that a complex medium-induced reduction of $\Delta\mu$ may have conferred was counteracted by an increased R value. Increased β-galactosidase productivity was correlated with increased plasmid stability only in glucose-limited chemostat cultures grown on defined medium and not in those grown on complex medium. Previous studies have yielded contrasting responses with regard to the effect of dilution rate on recombinant plasmid loss from *S. cerevisiae*. Our findings can account for these differences and may be generally valid for the stability of similar yeast plasmid constructs. This information would facilitate the design of bioprocesses, where recombinant plasmid instability results in reduced culture productivity.

Keywords: plasmid instability; chemostat; yeast

Introduction

Fermentation processes based on recombinant organisms depend on the retention of the DNA encoding a recombinant gene of interest. If this gene is located on a extrachromosomal element such as a plasmid then production will depend on plasmid maintenance. Plasmid maintenance will generally place a burden on a cell, reducing its growth rate relative to the plasmid-free host. This can result in a rapid decrease in the proportion of plasmid-containing cells within a growing population. Using a simple homogenous unsegregated model [28], plasmid loss will be a function of the rate at which plasmids are incorrectly partitioned (segregation rate: **R**) and the growth rate difference between plasmid-free and plasmid-containing clones ($\Delta\mu$). Both the environment and the nature of the plasmid and host are known to influence $\Delta\mu$ and **R** [6,34].

Most strains of *Saccharomyces cerevisiae* carry a cryptic plasmid, known as the 2-μm circle plasmid, which is stably maintained in the nucleus at copy numbers between 60 and 100. Four plasmid-encoded *trans*-acting gene products (FLP, REP1, REP2 and RAF) and two *cis*-acting sites (ORI and STB) are required for 2-μm plasmid segregation, copy number amplification and control [13,43]. The replication and segregational mechanisms of the 2-μm plasmid are similar to those used for chromosomes [43]. This plasmid provides the basis for many recombinant yeast vectors [36] which fall into two basic groups: full 2-μm sequence and 2-μm fragment-based vectors. A full 2-μm sequence vector consists of the 2-μm plasmid into which a recombinant cassette has been introduced. The cassette consists of a yeast-selective marker, usually linked to a bacterial plasmid origin of replication and antibiotic marker, allowing propagation and selection in both *S. cerevisiae* and *Escherichia coli*. These vectors can be maintained relatively well, at high copy numbers and independently of other sequences, but they are large in size and possess only a few useful unique restriction enzyme sites. To reduce these problems, 2-μm fragment-based vectors have been constructed by introducing the minimal *cis*-acting 2-μm plasmid replicon into a bacterial vector. Although these vectors are smaller and have a better choice of restriction enzyme sites than full 2-μm sequence vectors, they require the presence of native 2-μm plasmid-encoded *trans*-acting gene products for stable maintenance [24].

Impoolsup *et al* [21] and Bugeja *et al* [5] showed that the instability kinetics of two 2-μm-based plasmids, pJDB248 and pLG669-z, were completely different. Bugeja *et al* [5] and Kleinman *et al* [25] reported increased plasmid loss rates from glucose-limited chemostat cultures of *S. cer-*

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evisiae S150-2B (pJDB248) grown at low dilution rates on defined medium. Similar patterns of plasmid loss were observed from bacterial host-vector systems [8,14,32,40,46]. The plasmid pJDB248 is a full 2-μm sequence vector which consists of a recombinant marker cassette inserted into the RAF gene of the native 2-μm plasmid [1]. Although the exact role of RAF is unclear, it is implicated in the control of FLP expression which is essential for copy number amplification [30]. Bugeja *et al* [5] concluded that insertional inactivation of the 2-μm plasmid RAF gene had led to disruption of the segregational mechanism at low dilution rates.

The instability of the recombinant 2-μm fragment-based plasmid, pLG669-z, has been studied extensively in fed-batch and chemostat culture systems [7,18,19,21,22,41]. This plasmid consists of a bacterial cloning vector into which a yeast selection marker, the 2-μm minimal replicon (the ORI-STB region) and an *E. coli* β-galactosidase expression cassette have been cloned. Contrary to the loss kinetics of pJDB248, Impoolsup *et al* [21] observed that plasmid loss rates increased as the dilution rate was increased in glucose-limited chemostat cultures of *S. cerevisiae* YNN24 pLG669-z grown on complex medium. They concluded that increased instability at high dilution rates resulted primarily through increased rates of segregation (**R**) [21].

Our previous results [34] suggested that the contrasting responses of plasmid loss rates to changes in dilution rate, observed by Bugeja *et al* [5], Kleinman *et al* [25] and Impoolsup *et al* [21], may be explained by differences in medium composition rather than differences in plasmid construction. We reported that patterns of plasmid loss from glucose-limited chemostat cultures of the 2-μm fragment-based plasmid, pYEαa4, in *S. cerevisiae* AH22 in response to changes in dilution rate, were dependent on the composition of the medium. The plasmid, pYEαa4 is similar in construction to pLG669-z but encodes for a constitutively expressed α-amylase gene instead of the β-galactosidase gene in pLG669-z. While the instability of pYEαa4 from chemostat cultures grown on glucose-limited defined medium was $\Delta\mu$-dominated and was greater at lower diluton rates, instability from similar cultures grown on glucose-limited complex medium was greater at the higher dilution rate and instability kinetics were **R**-dominated [34]. This suggested that two underlying principles may govern the effect of medium composition on overall plasmid instability through opposing effects on $\Delta\mu$ and **R** [34]. Rates of segregation (**R**), derived from recombinant yeast cultures, consistently increase with increased dilution rate while $\Delta\mu$ decreases. Growth of recombinant cultures on complex media reduces $\Delta\mu$ but increases **R**. In this report we provide evidence that this hypothesis is generally applicable to other recombinant plasmid vectors in *S. cerevisiae*.

The instability of similar bacterial plasmids showed varied responses to growth conditions in continuous cultures of *E. coli* [6,32] and *B. subtilis* [12]. From these studies, no general rule could be applied to predicting plasmid loss from cultures grown under different limitations. Few studies have been carried out on the effect of nutrient limitation on the rate of plasmid loss from recombinant yeast. Caunt *et al* [7] reported that continuous cultures of *S. cerevisiae* YNN24 pLG669-z, exposed to stepped decreases in dissolved oxygen tension are more prone to increased plasmid loss rates. O'Kennedy *et al* [34] reported only minor differences between plasmid loss rates derived from glucose-, magnesium- and phosphate-limited nonselective chemostat cultures of *S. cerevisiae* AH22 pYEαa4. The apparent instability appears to be determined by an association of factors affecting the host/plasmid combination.

We set out to compare the instability of the 2-μm fragment-based vector, pLG669-z with that of the pYEαa4 [34]. We show that the pattern of loss in response to dilution rate was affected by medium composition, in a fashion similar to that observed in our previous study on the vector pYEαa4 [34] but the effects of nutrient limitation, however, were distinctly different.

Materials and methods

Strain and plasmid

The plasmid pLG669-z [16] was maintained in *S. cerevisiae* strain CG379 (Mat *a* [cir$^+$] *ura*3 *trp*1 *ade*1 *leu*2 *his*3; YGSC, Berkeley, California). The plasmid contained the yeast URA3 gene and a section of the *E. coli lac*Z gene, under the control of the *Saccharomyces* CYC1 promoter. This promoter is derepressed under conditions of glucose limitation or growth on a non-fermentable carbon source [16].

Media and culture conditions

Batch and continuous cultures were grown at 30°C on CCM (complex) medium [26] or one half-strength Wickerham's defined medium (DCM) [45] maintained at pH 5.2 ± 0.05 with 0.1 M succinic acid/NaOH buffer (BDH, Poole, UK). The nonselective complex medium, CCM, consisted of D-glucose (20 g L^{-1}), bacteriological peptone (5 g L^{-1}; Oxoid, Unipath, Basingstoke, UK) and yeast extract (5 g L^{-1}; Oxoid). DCM consisted of D-glucose (20 g L^{-1}), $(NH_4)_2SO_4$ (3.5 g L^{-1}), KH_2PO_4 (0.5 g L^{-1}), $MgSO_47H_2O$ (0.25 g L^{-1}), $CaCl_2$ $2H_2O$ (0.25 g L^{-1}), NaCl (0.25 g L^{-1}), myo-inositol (5 mg L^{-1}), Ca-pantothenate (1 mg L^{-1}), nicotinic acid (200 μg L^{-1}), pyridoxin-HCl (200 μg L^{-1}), thiamine-HCl (200 μg L^{-1}), *p*-amino benzoic acid (100 μg L^{-1}), biotin (10 μg L^{-1}), folic acid (1 μg L^{-1}), riboflavin (100 μg L^{-1}), H_3BO_3 (0.25 mg L^{-1}), $MnCl_3$ (0.2 mg L^{-1}), $ZnSO_4 \cdot 7H_2O$ (0.2 mg L^{-1}), $FeCl_3 \cdot 6H_2O$ (0.1 mg L^{-1}), $Na_2MoO_4 \cdot 2H_2O$ (0.1 mg L^{-1}), KI (0.05 mg L^{-1}), $CuSO_4$ (0.02 mg L^{-1}). DCM-nonselective was supplemented with L-histidine, uracil, adenine, tryptophan (20 mg L^{-1}; Sigma) and L-leucine (30 mg L^{-1}; Sigma). When selective conditions were required (DCM-selective), uracil was omitted. DCM plates were prepared with agar bacteriological No. 1 (20 g L^{-1}; Oxoid, UK). YPD plate medium consisted of D-glucose (20 g L^{-1}), bacteriological peptone (20 g L^{-1}), yeast extract (10 g L^{-1}) and agar technical (20 g L^{-1}; Oxoid). YMP-X-gal plate medium [35] consisted of glycerol (4% v/v), mycological peptone (20 g L^{-1}; Oxoid), yeast extract (10 g L^{-1}) and agar technical (20 g L^{-1}). X-gal (40 μg ml^{-1}; Sigma) was dissolved in dimethylformylamide and added after autoclaving.

Batch cultures

Maximum specific growth rates (μ_{max}) were determined in 500-ml baffled flasks containing 100 ml of medium with 20 g L^{-1} filter-sterilized D-glucose as a carbon source. The flasks were incubated in a rotary shaker waterbath (Infors AG, Basel, Switzerland) operating at 250 rpm. Optical density was monitored at 600 nm over the initial 8 h of growth and converted to dry cell weight (mg ml^{-1}) by use of a standard curve. Maximum specific growth rates (μ_{max}) were calculated by non-linear regression. The proportions of the population which were Ura^+ βgal^+ at the beginning and the end of the 8-h growth period (about 0.85 and 0.83 respectively) were not significantly different.

Plasmid stability in batch cultures was determined in triplicate serial cultures (10 ml) grown on nonselective media (DCM or CCM) and shaken at 200 rpm. Every 24 h, about 2×10^5 cells were subcultured into fresh medium. After 96 h, samples were removed and the proportion of plasmid-containing cells was determined.

Chemostat cultures

Chemostat cultures (500 ml) were carried out using the apparatus described by Fleming *et al* [11]. Cultures were aerated at 750 ml min^{-1} and mixed by means of a magnetic stirrer. When CCM was used, vegetable oil (Dunnes Stores, Galway, Ireland) was added at regular time intervals (75 μl every 6 h) to suppress foaming. Adjustments were applied to CCM and DCM to ensure nutrient limitation, which was confirmed by the method of Goldberg and Er-el [15]. Chemostat cultures were limited by glucose (0.5 g L^{-1}), magnesium (as $MgSO_4 \cdot 7H_2O$, 50 mg L^{-1}; D-glucose, 5 g L^{-1}) or phosphate (as KH_2PO_4, 2 mg L^{-1}; D-glucose, 5 g L^{-1}) [34].

Determination of the proportion of recombinant plasmid-containing colonies

Suitably diluted samples from batch and chemostat cultures were plated onto YPD and YMP-X-gal media. The proportion of plasmid-containing cells in a sample was the number of blue colonies divided by the total colonies grown on YMP-X-gal plates after 72 h incubation at 30°C. Colonies grown on YPD plates were also replica-plated onto DCM-selective, DCM-nonselective and YMP-X-gal plates to verify the proportion of plasmid-containing cells. No evidence of segregation of β-gal and Ura phenotypes was noted and the plasmid was deemed structurally stable.

β-galactosidase enzyme assay

β-galactosidase activity in the cultures was determined in triplicate by the method of Guarente and Ptashne [16]. One international unit (IU) of β-galactosidase is defined as the amount of enzyme which hydrolyzes 1 nmol of *o*-nitrophenyl-β-D-galactoside (ONPG; Sigma) per min at 28°C. Final results were expressed as cell specific activity (IU mg^{-1} cell biomass).

Data analysis

Transient productivity of β-galactosidase in chemostat cultures was calculated by the integration method suggested by Pirt [37]. Plasmid loss parameters (**R** and $\Delta\mu$) were estimated by non-linear regression as outlined by O'Kennedy *et al* [33]. This method was essentially that proposed by Davidson *et al* [9], where the parameters of Equation 1, defined by Lenski and Bouma [28], were optimised such that experimental data were fitted.

$$\overline{p_t^+} = \frac{p_0^+(\Delta\mu + \mathbf{R})}{([1 - p_0^+]\Delta\mu + \mathbf{R})e^{(\Delta\mu + \mathbf{R})t} + \Delta\mu p_0^+} \quad (1)$$

$\overline{p_t^+}$ is the predicted proportion of plasmid-containing cells at time = t, where p_0^+ is the proportion of plasmid-containing cells at time = 0. Optimum values for $\Delta\mu$ and **R** were obtained by minimizing the error between experimental data and the trajectory described by the above equation. Errors on the parameters, $\Delta\mu$ and **R**, were determined using the general jackknife method, reviewed by Robinson [38]. Modified data series were constructed from the original time *vs* proportion of plasmid-containing cells, where single data pairs were omitted. Non-linear regression was then repeated on each modified data series, providing N 'pseudovalues' for $\Delta\mu$, **R** and p_0^+. The standard error on each of these parameters was then derived from the pseudovalues.

The overall instability of plasmids from batch cultures (S_L; Eqn 2) was defined as the exponential rate of plasmid loss over a given time interval = t [29].

$$S_L = 2\left(1 - \exp\left\{\frac{\ln(p_t^+/p_0^+)}{t}\right\}\right) \quad (2)$$

Rather than reporting solely the final proportion of plasmid-containing cells, Equation 2 takes into account both the initial and final proportions of plasmid-containing cells. Dunn *et al* [10] noted, however, that calculation of overall plasmid instability using this method could define plasmids as equally stable though their plasmid loss trajectories were completely different. The area under the plasmid loss curve was suggested as a more appropriate measure of overall plasmid stability ($\sigma(N_+)$) in chemostat cultures. This is obtained by integration of Equation 1 yielding

$$\sigma(N_+) = \frac{1}{\Delta\mu}\ln\left(\frac{\Delta\mu + \mathbf{R}}{\Delta\mu + \mathbf{R} - p_0^+(1 - e^{-(\Delta\mu + \mathbf{R})N_+})}\right) \quad (3)$$

where $\sigma(N_+)$ is the overall stability over N_+ = 60 generations [10].

Results

Effect of plasmid carriage on batch cultures

The introduction of pLG669-z into the host CG379 reduced μ_{max} by around 10% in non-selective batch cultures grown on defined (DCM) and complex (CCM) media (Table 1). Selective batch cultures of CGpLG used as inocula contained a significant percentage of plasmid-free cells (*ca* 15%) so it would be expected that growth rate differences would have a significant effect on plasmid loss. Loss rates (S_L) after 96 h ($\approx$ 37 generations) demonstrated that pLG669-z was unstable in DCM-nonselective but was, however, stable in CCM.

Table 1 Growth rates and plasmid loss rates of batch cultures of *S. cerevisiae* CGpLG grown on nonselective media

Medium	CG379 (no plasmid)	CGpLG		
	$\mu_{max} \pm$ s.e.m. (h^{-1})	$\mu_{max} \pm$ s.e.m. (h^{-1})	$\Delta\mu_{max}$[a] $\times 10^{-2}$ (h^{-1})	Segregational loss[b] (S_L gen^{-1})
DCM	0.287 ± 0.005	0.260 ± 0.001	2.7 (>95%)	0.0354 ± 0.0023
CCM	0.301 ± 0.001	0.266 ± 0.005	3.5 (>95%)	–0.0015 ± 0.0020

[a]Levels of significance (in brackets) were determined by Student's *t*-test.
[b]Plasmid loss rates in batch cultures were calculated using Equation 2 described in Materials and Methods.

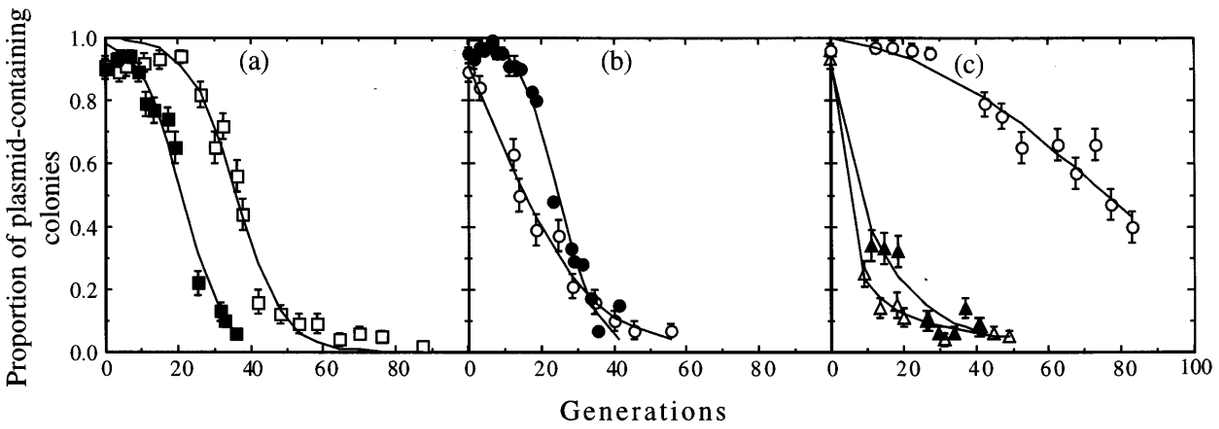

Figure 1 Plasmid loss from *S. cerevisiae* CGpLG grown in nonselective chemostat culture. Error bars represent the standard error on the proportion of plasmid-containing colonies. D = dilution rate. Best fit lines are derived from $\Delta\mu$, **R** and p_0^+ determined by jackknifed non-linear regression (Table 2). (a) Glucose-limited DCM, D = 0.16 h^{-1} (□); D = 0.06 h^{-1} (■). (b) Glucose-limited CCM, D = 0.15 h^{-1} (○); D = 0.05 h^{-1}) (●). (c) Magnesium-limited DCM, D = 0.13 h^{-1} (△); D = 0.11 h^{-1} (▲). Phosphate-limited DCM, D = 0.14 h^{-1} (○).

Effect of medium composition on the pattern of plasmid loss from chemostat cultures at different dilution rates

Plasmid loss from chemostat cultures grown under various conditions is shown in Figure 1. Overall plasmid loss ($\sigma(N_+)$) was dependent on dilution rate, medium type and limitation (Table 2). The pattern of plasmid loss, in response to altered dilution rate, was markedly different between cultures grown on glucose-limited DCM-nonselective or in glucose-limited CCM. In cultures grown on glucose-limited DCM-nonselective (Figure 1a), the overall stability was greater in cultures grown at the higher dilution rate (0.16 h^{-1}) than in cultures grown at the lower dilution rate (0.06 h^{-1}). Since decreased stability correlated with increased $\Delta\mu$, we concluded that $\Delta\mu$ was the dominant factor governing plasmid loss from chemostat cultures grown on glucose-limited DCM (Table 2).

In cultures grown on glucose-limited CCM (Figure 1b), the overall stability was greater in cultures grown at the lower dilution rate (0.05 h^{-1}) than in cultures grown at the higher dilution rate (0.15 h^{-1}). Even though $\Delta\mu$ was greater than **R** at the lower dilution rate, a four-fold reduction of $\Delta\mu$ and a 40-fold increase in **R** decreased stability at the higher dilution rate, thus reversing the pattern observed in

Table 2 Plasmid loss kinetics from chemostat cultures of *S. cerevisiae* CGpLG grown on nonselective media

Medium and limitation	D (h^{-1})	Overall stability[a] $\sigma(N_+)$	$\Delta\mu$[b] (10^{-2} gen^{-1})	**R**[b] (10^{-2} gen^{-1})	β-gal productivity[a] (IU mg^{-1} h^{-1})
DCM, glucose	0.16	36	15.4 ± 0.15	0.06 ± 0.00	2728
DCM, glucose	0.06	21	18.6 ± 0.14	0.00 ± 0.00	182
CCM, glucose	0.15	19	4.93 ± 0.20	2.40 ± 0.10	422
CCM, glucose	0.05	25	18.4 ± 0.01	0.06 ± 0.02	185
DCM, magnesium	0.13	8	–34.1 ± 1.17	34.8 ± 0.99	N/A
DCM, magnesium	0.11	13	–2.85 ± 1.16	8.67 ± 1.18	N/A
DCM, phosphate	0.14	52	3.50 ± 0.06	0.23 ± 0.01	N/A

[a]Overall stability ($\sigma(N_+)$; $N_+ = 60$ gen) was calculated using Equation 3, defined in Materials and Methods. β-galactosidase productivity was calculated as described in the Materials and Methods. N/A: not applicable due to glucose repression.
[b]Values of $\Delta\mu$ and **R** are expressed as the parameter ± s.e.m. which was calculated from jackknifed values (see Materials and Methods).

similar cultures grown on DCM-nonselective. Since decreased stability correlated with increased **R**, we concluded that **R** was the dominant factor responsible for plasmid loss from chemostat cultures grown on glucose-limited CCM.

Effect of nutrient limitation on plasmid loss from chemostat cultures of CGpLG

Studies with recombinant *E. coli* [23,32,46] and *B. subtilis* [12] have shown that limitations other than glucose can have a profound effect on plasmid loss. Phosphate- and magnesium-limited chemostat cultures of CGpLG were grown on DCM-nonselective with glucose ($5\ g\ L^{-1}$) as a non-limiting carbon source, such that plasmid-borne β-galactosidase expression was repressed. Even under these repressed conditions, rapid plasmid loss was observed in magnesium-limited cultures. Loss kinetics and the response of plasmid loss to slight changes in dilution rate showed **R**-dominated instability. Parameter values determined from plasmid loss in magnesium-limited cultures were, however, very variable between cultures exhibiting similar loss patterns (Figure 1c and Table 2). It is possible that the values obtained from the higher dilution rate are spurious due to the small number of data points obtained during the loss phase. Nevertheless, a greater than 100-fold increase in **R** over glucose-limited DCM cultures demonstrates that the segregation rate of pLG669-z was particularly sensitive to magnesium-limitation. CGpLG was most stable when grown in phosphate-limited cultures (Figure 1c). This stabilization, in comparison to plasmid loss from cultures grown on glucose-limited DCM-nonselective, resulted from a five-fold reduction of $\Delta\mu$.

β-galactosidase expression in batch and chemostat culture

β-galactosidase expression in batch cultures grown on 2% glucose exhibited low levels of residual activity which was consistent with repression of the CYC1 promoter. Under conditions of glucose-limited chemostat culture, the CYC1 promoter was derepressed and β-galactosidase expression was induced. Medium composition affected the productivity of β-galactosidase (Table 2). The highest productivities were observed in cultures grown on DCM-nonselective at the high dilution rate. Increased overall stability resulted in higher productivity in cultures grown in glucose-limited DCM-nonselective but not in those grown in CCM.

Effect of plasmid loss on β-galactosidase expression

In studies where β-galactosidase expression was considered to be completely derepressed, a simple correlation was observed between the loss rates of the proportion of plasmid-bearing cells and plasmid-borne gene expression [21,42]. No simple correlation was apparent between the proportion of plasmid-containing cells and normalised cell-specific β-galactosidase expression in glucose-limited chemostat cultures (Figure 2) but it was evident that at least two phases existed.

Discussion

The study of plasmid loss from *E. coli* has yielded many conflicting conclusions for similar host-plasmid systems. Plasmid vectors for *E. coli*, based on the Col E1 replicon, exhibit varied responses to changes in dilution rate when grown under different environmental conditions [8,14,32,40,46]. Similar studies carried out on recombinant *S. cerevisiae* have also yielded conflicting results and no attempt has been made to account for these differences [5,21,25,26]. It is apparent from this that it is not always possible to provide general principles which govern plasmid loss in cultures grown under different nutrient conditions.

Our previous studies have focused on the loss of the 2-μm fragment-based vector, pYEαa4, from *S. cerevisiae* AH22 in chemostat cultures [34]. This plasmid is a 2-μm fragment-based vector, similar in construction to the plasmid used in this study, pLG669-z. The plasmid, pYEαa4, encodes for a constitutively expressed α-amylase gene while pLG669-z encodes for regulated β-galactosidase which is repressed in the presence of a fermentable carbon source or under glucose-sufficient conditions. Our findings indicated that the lack of continuity between previous studies on the effect of dilution rates on plasmid loss [5,21,25] may have been due to large changes in the relative contributions of **R** and $\Delta\mu$ to overall plasmid loss, brought about by different medium composition. We suggest that few general principles could be applied to predict the effect of medium composition on plasmid loss.

The effect of nutrient composition and dilution rate on plasmid stability and recombinant β-galactosidase expression was examined by comparing plasmid loss from glucose-limited chemostat cultures grown on DCM-nonselective or CCM (Figure 1a, b). The overall plasmid stability ($\sigma(N_+)$) of chemostat cultures grown on DCM-nonselective was greater at higher dilution rates while being greater at the lower dilution rate in cultures grown on CCM (Table 2). Plasmid loss kinetics derived from chemostat cultures grown on DCM-nonselective were dominated by the effects of $\Delta\mu$. This pattern of plasmid loss from cultures grown on DCM-nonselective was similar to that observed for many bacterial plasmids [4,6,39,46] and for the full 2-μm sequence vector, pJDB248 [5,25]. As dilution rate is reduced, competition between subpopulations is expected to increase [17] leading to increased $\Delta\mu$ and instability.

Overall plasmid stability in chemostat cultures grown on CCM was greater at the lower dilution rate ($D = 0.05\ h^{-1}$; Table 2) even though there was a four-fold reduction of $\Delta\mu$ at the higher dilution rate ($D = 0.15\ h^{-1}$). This increased plasmid stability was associated with a reduced rate of plasmid segregation (**R**), which increased as the dilution rate was increased. It was concluded that **R** was the dominant factor which determined plasmid loss in cultures grown on CCM, which concurs with previous studies [21,34]. In comparison to chemostat cultures grown on DCM-nonselective, values of $\Delta\mu$ derived from cultures grown on CCM, were reduced in cultures grown at the high dilution rate (Table 2). This indicated that grown on complex medium may alleviate the metabolic burden attributable to plasmid carriage. Plasmid loss parameters derived from similar chemostat cultures of AH22 pYEαa4 also exhibited this behaviour [34]. In both cases, any increased stability that this complex-medium-induced reduction of $\Delta\mu$ may have conferred was counteracted by increased **R**. Values of $\Delta\mu$ derived

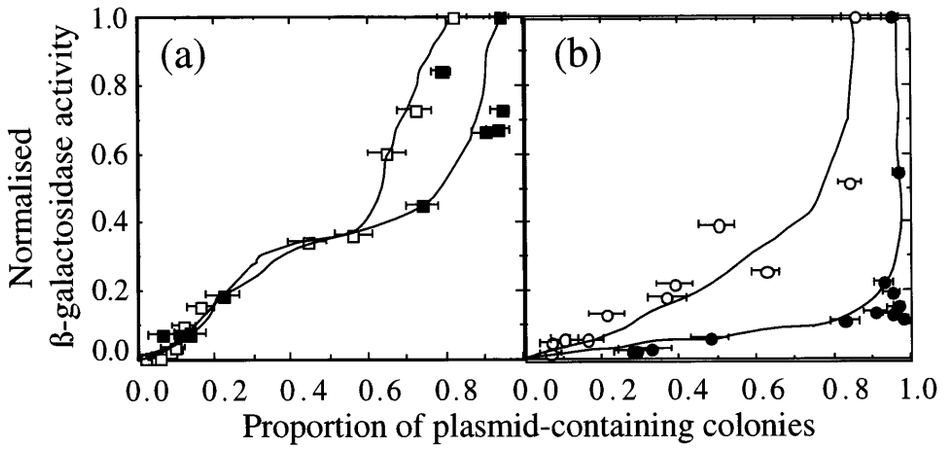

Figure 2 Relationship between the proportion of plasmid-containing cells and cell-specific β-galactosidase activity. Cell-specific activity was normalised against the maximum activity observed during cultures. D = dilution rate. (a) Glucose-limited DCM; D = 0.16 h^{-1}, Max activity = 983 IU mg^{-1} (□); D = 0.06 h^{-1}, Max activity = 165 IU mg^{-1} (■). (b) Glucose-limited CCM, D = 0.15 h^{-1}, Max activity = 144 IU mg^{-1} (○); D = 0.05 h^{-1}, Max activity = 578 IU mg^{-1} (●).

from cultures grown on CCM were generally greater than those obtained by Impoolsup *et al* [21]. Since the dilution rates used in this study were lower than those used by Impoolsup *et al* [21], competition would be expected to increase at the lower dilution rates and it would be expected that the $\Delta\mu$ values derived from these cultures would be greater than those found by Impoolsup *et al* [21].

This study also concurs with our previous observations that values of **R**, derived from recombinant cultures grown on complex medium, are generally greater than those derived from cultures grown on defined medium. Continuous cultures of recombinant *B. subtilis* [31] and *S. cerevisiae* [20] grown on complex media have a reduced plasmid copy number in comparison to those grown on defined media. This reduced copy number provides the simplest explanation for the increased values of **R** derived from cultures grown on CCM. Although no determinations of plasmid copy number were carried out, the reduced β-galactosidase productivity which chemostat cultures grown on CCM exhibited (Table 2) would agree with this hypothesis.

As with other studies [21,34], **R** was observed to increase with increasing dilution rate, irrespective of medium composition. This may also be due to the effect of reduced plasmid copy number at higher dilution rates [2,3,27]. This is not, however, reflected in the β-galactosidase productivity (Table 2).

Previously, only minor differences were observed in plasmid loss rates derived from glucose-, magnesium- and phosphate-limited cultures of *S. cerevisiae* AH22 pYEαa4 grown at similar dilution rates [34]. A comparison between glucose-limited and magnesium-limited chemostat cultures of CGpLG grown on DCM-nonselective (Figure 1a, c) shows that plasmid loss from magnesium-limited cultures was much more rapid and displayed **R**-dominated loss kinetics (Figure 1c; Table 2). Since β-galactosidase expression was repressed by the presence of non-limiting concentrations of glucose, this increased instability could not be attributed to plasmid-borne expression. The elevated values of **R**, derived from magnesium-limited cultures indicated that a deficiency in either the replication or segregational mechanisms may have been responsible for the increased plasmid loss rates. This would be supported by a recent study which suggests that magnesium limitation primarily exerts its influence on the cell division cycle of *S. cerevisiae* [44]. Chemostat cultures of CGpLG grown on phosphate-limited DCM-nonselective were significantly more stable than those grown on glucose-limited DCM-nonselective (Figure 1c; Table 2) and exhibited a five-fold reduction of $\Delta\mu$. An attempt to establish a phosphate-limited culture at a similar dilution rate, where β-galactosidase was induced by growth on raffinose (2.5 g L^{-1}), resulted in washout of the culture. This suggested that repression of the *LacZ* gene was primarily responsible for the increased stability in non-induced phosphate-limited cultures due to the reduction of plasmid-derived metabolic load. Caulcott *et al* [6] have suggested that plasmids which exhibit $\Delta\mu$-dominated kinetics may be subject to certain nutrient limitations which lead to high plasmid loss rates, whereas plasmids which exhibit **R**-dominated kinetics could be subject to an alternative group of limitations.

It appears that few general rules can be derived from previous studies on the effect of growth conditions on the stability of recombinant microorganisms [5,21,25,34]. There are some common factors between these and our present study findings. Glucose-limited chemostat cultures grown on defined medium were typically more stable at higher dilution rates and exhibited $\Delta\mu$-dominated plasmid loss kinetics while similar cultures grown on complex medium were typically less stable at higher dilution rates and exhibited **R**-dominated plasmid loss kinetics. Competition between plasmid-free and plasmid-containing cells tended to be lower while **R** tended to be greater in glucose-limited cultures grown on complex medium in comparison to similar cultures grown on defined medium. These common factors may facilitate the design of conditions which maximize recombinant plasmid stability, thereby enhancing product yields. The analysis of plasmid loss under a given set of culture conditions should be carried out at least at two dilution rates since increased loss rates at high or low

dilution rates would appear to be diagnostic for loss kinetics dominated by **R** or $\Delta\mu$, respectively.

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