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SLOW GROWTH PHENOTYPE - A POSSIBLE APPROACH TO IMPROVED PLASMID MAINTENANCE IN *SACCHAROMYCES CEREVISIAE*.

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SUMMARY
Transformation-induced slow growth phenotype (SGP) in yeast is repressed in the presence of 2μm plasmids. A full 2μm-sequence-based recombinant plasmid (pJB502) was found to be more stable in a 2μm-free- [cir°] strain of *Saccharomyces cerevisiae* than in a cir$^+$ strain. This could not be attributed to differences in growth rate calculated from kinetic analysis of plasmid loss, but transformed [cir°] isolates, which had lost the recombinant plasmid, exhibited varying degrees of SGP in batch culture. One of these isolates was outcompeted in chemostat culture by the recombinant-plasmid-containing strain, suggesting that improved plasmid maintenance can result from SGP in cir° hosts.

INTRODUCTION

In *Saccharomyces cerevisiae*, physical methods of DNA transformation can induce non-specific chromosomal mutations resulting in a slow growth phenotype (SGP). This phenotype is only expressed in 2μm-plasmid-free [cir°] strains and reintroduction of the 2μm-plasmid suppresses SGP (Danhash *et al.*, 1991; Mead *et al.*, 1987), though the nature of this suppression is unknown. It is possible that a host with the potential to exhibit SGP could produce improvements in recombinant plasmid stability.

Changes in the proportion of plasmid-containing cells in a growing population are dependent on the rate of incorrect plasmid partitioning during daughter cell formation (Segregation Rate: **R**) and growth rate differences between plasmid-free and plasmid-containing clones (Δμ). Both the environment and the nature of the plasmid and host will influence Δμ and **R** (O'Kennedy *et al.*, 1995). Plasmid maintenance generally places 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. If SGP is induced upon recombinant plasmid loss, an improvement in plasmid retention could result due to the increased competitive ability of the plasmid-containing population which should be expressed by an improvement in Δμ.

This study set out to investigate this proposition, using a full 2μm-based-plasmid (pJB502) which was introduced into two hosts by a physical method (lithium acetate transformation). One host (DBY746) contained the endogenous 2μm-plasmid. The other (DBB1) did not, but was otherwise isogenic.

MATERIALS AND METHODS

Strains and plasmid

Plasmid pJB502 (Bijvoet *et al.*, 1991) was a full-2μm-sequence-based *Escherichia coli/Saccharomyces cerevisiae* shuttle vector with a cassette containing bacterial origin and selection markers inserted in a silent region of the yeast 2μm-plasmid. Selection in yeast hosts was via URA3 auxotrophy. The plasmid, pJB502 was introduced into *S. cerevisiae* DBY746 (Mat α his3-Δ1 trp1-289_a leu2-3 leu2-112 ura3-52 [cir$^+$]) and its derivative, DBB1, by the lithium acetate method (Gietz and Schiestl, 1991). DBB1 was a spontaneous 2μm-free segregant [cir°-s] of DBY746 which was isolated from chemostat culture.

Media and culture conditions

Yeast extract-Peptone-Dextrose (YPD) medium with 20 g glucose/l as a carbon source was as described by Guthrie and Fink (1991). Defined chemostat medium consisted of one half-strength Wickerham's defined medium (Wickerham, 1946) buffered to pH 5.2 with 0.025M citrate-phosphate buffer. Non-selective defined chemostat medium (DCM non-selective) was supplemented with all auxotrophic requirements, including uracil, according to Guthrie and Fink (1991). When selective conditions were required (DCM selective), uracil was omitted. Plates were prepared with Agar Bacteriological No.1 (20g/l ; Oxoid). All cultures were incubated at 30°C.

Batch cultures

Plasmid stability in batch cultures was determined in triplicate 10ml serial cultures grown on DCM non-selective medium and shaken at 200 r.p.m. Every 24 h, about 2 x 10^5 cells were subcultured into fresh medium. At intervals, samples were removed and the proportions of plasmid-containing isolates (p^+) were determined.
Growth rates were determined from cultures in 500ml baffled flasks containing 100ml DCM non-selective medium which were incubated in a shaking water bath at 250 r.p.m. Absorbance at 600nm was monitored over the initial 8 h of culture and converted to dry cell weight (mg/ml) by use of a standard curve. The maximum specific growth rate (μ_{max}) was calculated by non-linear regression. The significance of differences in growth rates between strains was determined by Student's *t*-test. Levels of significance below 95% were considered insignificant.

Chemostat cultures

Chemostat cultures were carried out in the apparatus described by Fleming *et al.* (1988). Cultures were aerated at 1.5 v.v.m and mixed by means of a magnetic stirrer. Glucose was added to DCM non-selective medium at a concentration of 0.5g/l so as to ensure limitation. At intervals, samples were removed and the proportions of plasmid-containing isolates (p^+) were determined. The kinetics of plasmid loss in chemostat cultures were analysed by the method of Davidson *et al.* (1990) and model parameters optimised as described by O'Kennedy *et al.* (1994).

Determination of the proportion of recombinant-plasmid-containing colonies

Appropriate dilutions of samples were spread-plated onto YPD medium. After 48h incubation, colonies were replica plated onto DCM selective and DCM non-selective media. After 96h incubation the numbers of colonies growing on each plate were used to calculate the proportion of plasmid containing colonies (p^+)

RESULTS

Growth rates of original strains.

No significant difference was observed between the growth rates (μ_{max}) of the original hosts (DBY746 and DBB1) or between transformants (DBY746 pJB502 and DBB1 pJB502), indicating that the presence of the endogenous 2μm-plasmid had no discernible effect on growth rates (Table 1). A slight but significant difference in μ_{max} between DBY746 pJB502

and the original host (DBY746) showed that the recombinant plasmid imposed a small burden.

Host	Plasmid Phenotype	$\mu_{max} \pm$ s.e.m.	$\%\Delta\mu_{max}$
DBY746	[cir$^+$]	0.253±0.006 (7)	--
DBB1	[cir$^\circ$]	0.245±0.005 (15)	--
DBY746	[cir$^+$; pJB502]	0.237±0.006 (8)	**-6.3**
DBB1	[cir$^\circ$; pJB502]	0.237±0.003 (26)	-3.3

Table 1. Growth rates of original strains. The number of independent determinations is shown in parentheses. For pJB502-containing strains, p^+ at inoculation was between 0.87 and 0.94 and did not decrease significantly over the course of the determination. $\%\Delta\mu_{max}$ is the growth rate difference between pJB502-containing strains and their original hosts expressed as a percentage. Significant differences are marked in **bold**.

Stability of pJB502-containing strains in batch and chemostat culture

The recombinant plasmid was more stable in the 2μm-free strain (DBB1 pJB502) than in the 2μm-containing strain (DBY746 pJB502) in both batch and chemostat cultures (Fig. 1). Kinetic analysis of plasmid loss in chemostat cultures demonstrated that instability in both strains was dominated by Δμ (the difference in growth rates: Table 2). If SGP was the direct cause of improved plasmid retention by the cir° strain, a corresponding decrease in Δμ might be expected. No significant decrease was observed, but the rate of plasmid segregation (**R**) was significantly reduced in the 2μm-free transformant (DBB1 pJB502).

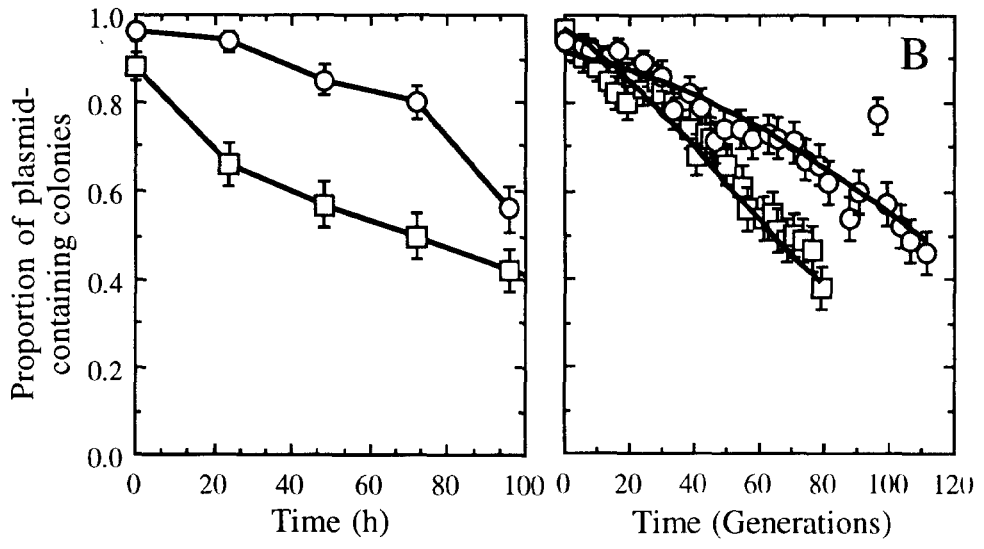

Figure 1. Plasmid loss in batch cultures (**A**) and glucose-limited ($D=0.15h^{-1}$) chemostat cultures (**B**) of DBY746 pJB502 (□) and DBB1 pJB502 (○).

Host	Plasmid Phenotype	Overall Plasmid stability*	$\Delta\mu \pm$ s.e.m. (10^{-2} gen^{-1})	**R** $\pm$ s.e.m. (10^{-2} gen^{-1})
DBY746	[cir$^+$; pJB502]	63	2.18±0.24	0.48±0.01
DBB1	[cir° ; pJB502]	93	2.15±0.01	0.00±0.00

Table 2. Kinetic analysis of plasmid loss from glucose-limited chemostat cultures of DBY746 pJB502 and DBB1 pJB502 ($D=0.15h^{-1}$). $\Delta\mu$ and **R** are as defined in the introduction. *Overall plasmid stability at 100 generations was calculated from kinetic parameters by the integration method of Dunn *et al.* (1995).

Growth rates of chemostat isolates spontaneously cured of pJB502

Since the origin of SGP is in the transformation process, the growth rates of the original untransformed hosts may be different from those of recombinant-plasmid-free clones which appear as a result of instability. In order to investigate this, the growth rates of recombinant-plasmid-free clones isolated from 96h serial batch cultures were determined (Table 3). Seven out of eight pJB502-free isolates derived from DBB1 pJB502 had significantly reduced growth rates in comparison to the original host of which five displayed a significant growth rate disadvantage over their plasmid-containing parent. Seven out of eight recombinant-plasmid-free isolates derived from DBY746 pJB502 exhibited growth rates which were not significantly different from that of their original host, of which six showed a significant growth rate advantage over their plasmid-containing parent. These results show that recombinant plasmid loss in cir° (but not cir$^+$) cultures resulted in the formation of plasmid-free clones exhibiting SGP. It may be postulated that these would be unable to compete with their plasmid-containing parent.

Cured isolates derived from DBB1 [cir°; pJB502].			Cured isolates derived from DBY746 [cir$^+$; pJB502].		
Isolate	$\Delta\mu_{max}$ Original host	$\Delta\mu_{max}$ p$^+$ Parent	Isolate	$\Delta\mu_{max}$ Original host	$\Delta\mu_{max}$ p$^+$ Parent
BB 2	**-26.0%**	**-23.6%**	BY 4	*-7.1%*	*-0.8%*
BB 8	**-24.9%**	**-22.4%**	BY 8	*-1.3%*	*5.4%*
BB 6	**-14.8%**	**-11.9%**	BY 5	*2.3%*	**9.2%**
BB 4	**-13.7%**	**-10.8%**	BY 1	*2.4%*	**9.2%**
BB 1	**-11.6%**	**-8.7%**	BY 6	*3.0%*	**9.9%**
BB 3	**-8.8%**	*-5.7%*	BY 7	*4.1%*	**11.1%**
BB 7	**-7.2%**	*-4.1%*	BY 2	*5.8%*	**13.0%**
BB 5	*-2.0%*	*1.3%*	**BY 3**	**8.5%**	**15.8%**

Table 3. Differences in μ_{max} between cured isolates and original (untransformed) hosts or p$^+$ (transformed) parents as a % of original host or p$^+$ parent. At least three independent determinations were made for each isolate. Significant differences (>95%) are marked in **bold.** Significant negative differences between an isolate and the original host denote the SGP$^+$ phenotype.

Competition of DBB1 pJB502 and a derived pJB502-cured isolate

Isolate BB 2, which was derived from DBB1 pJB502 as described in the previous section and which had been identified as exhibiting SGP, was competed against DBB1 pJB502 in a chemostat culture. The culture was initially inoculated with DBB1 pJB502 and showed plasmid loss due to instability equivalent to $p^+ = 0.85$ over 11 generations. A mid-exponential phase batch culture of BB2 was then added so as to reduce p^+ to around 0.55. Within 10 generations p^+ had increased to over 0.80 and remained relatively constant for a further 40 generations (Fig. 2). It was concluded that DBB1 pJB502 was capable of outcompeting one of its plasmid-free derivatives.

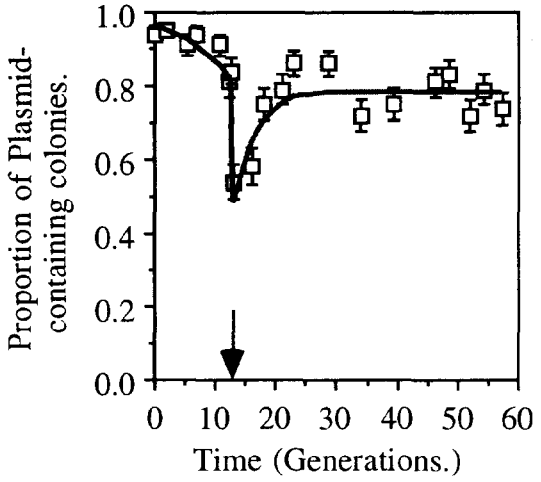

Figure 2. Competition of DBB1 pJB502 against a cured DBB1 isolate (BB2) in glucose-limited chemostat culture at D = 0.15 h^{-1}. The arrow denotes addition of isolate BB 2 (see text).

DISCUSSION

Danhash *et al.* (1991) have suggested that SGP could be used to improve plasmid maintenance by modulating the growth rate difference ($\Delta\mu$) between plasmid-free and plasmid-containing cells. In the present investigation, otherwise isogenic cir$^+$ and cir$^\circ$ hosts were used since it was hypothesised that they would be either SGP$^-$ or SGP$^+$ respectively upon recombinant plasmid loss. The hypothesis was shown to be correct, with spontaneously cured cir$^\circ$ isolates typically exhibiting SGP and possessing growth rates significantly less than that of their parent transformant (Table 3). By contrast, spontaneously cured cir$^+$ isolates typically possessed growth rates which did not differ significantly from that of their original host and therefore did not exhibit SGP. A competition study between the cir$^\circ$ transformant and a spontaneously cured isolate confirmed that SGP had rendered the plasmid-free clone less fit than its recombinant precursor (Fig. 2)

The improved plasmid stability observed in both batch and continuous cultures of the cir° transformant might be taken as confirmation of the role of SGP in promoting plasmid stability, but kinetic analysis of chemostat cultures failed to provide proof of its influence. Though plasmid loss kinetics were dominated by $\Delta\mu$, only **R** varied significantly between the cir° and cir$^+$ recombinant strains. The method of analysis employed in this study (Davidson *et al.*, 1990) assumes constant values of $\Delta\mu$ and **R.** Since a significant variation was noted in the degree of SGP expression by plasmid-free cir° clones, it is possible that such a form of analysis is over-simplistic.

Other studies have also shown that full-2μm-sequence vectors, such as pJB502, tend to be more stable in a cir° host (Bijvoet *et al.*, 1991; Walmsley *et al.*, 1983). This was believed to be the result of a maximum limit on the copy number of 2μm sequences (both endogenous 2μm and recombinant plasmids) within a given host. The absence of endogenous 2μm-plasmids from the host would thus allow the recombinant plasmid to attain a higher copy number, and consequently reduce the rate of segregation (**R**). It should be possible to differentiate between the relative influences of copy number and SGP on the differences in plasmid stability observed in this study by the use of strains produced by the introduction of pJB502 into the cir° host by cytoduction. Such a strain should exhibit the same copy number for the recombinant plasmid as the present cir° construct, but would not manifest SGP on plasmid loss.

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