

# Temperature and Growth Rate Effects on the *hok/sok* Killer Locus for Enhanced Plasmid Stability

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The *hok/sok* locus, isolated from the multiple-resistance plasmid R1 of *Escherichia coli*, is very efficient at ensuring the stable maintenance of plasmids in Gram-negative systems by killing plasmid-free cells as they arise. To investigate independently the influence of temperature and growth rate on the effectiveness of *hok/sok*, continuous fermentations have been conducted with the pUC-based, IPTG-induced,  $\beta$ -galactosidase expression vector pTKW106. At fixed temperature (37 °C), decreasing the dilution rate decreased plasmid stability, and at a fixed, low dilution rate ( $D = 0.15/h$ ), decreasing the temperature resulted in an increase in plasmid stability. These trends are explained by the specific  $\beta$ -galactosidase activity of each continuous fermentation: higher, specific, recombinant protein expression led to decreased plasmid stability (due to either segregational or structural instability, as determined by plasmid DNA isolation). A representative fed-batch medium produced more  $\beta$ -galactosidase on a volumetric basis than M9C in the chemostat, and addition of the *hok/sok* locus increased segregational stability by 8–22-fold in continuous fermentations that lacked antibiotic selection pressure and in which  $\beta$ -galactosidase was constantly expressed at 12% of total cell protein for 60 h (43–47 generations).

## Introduction

Cloned genes must be maintained stably for productive fermentations (Kapralek and Jecmen, 1992). Plasmids are common expression vectors that may lose the cloned gene due to segregational instability (loss of the whole plasmid from the bacterium) or structural instability (mutations such as deletions, insertions, and rearrangements of the plasmid DNA) (Ensley, 1985). To enhance plasmid segregational stability, physical methods [such as two-stage reactor operations (Siegel and Ryu, 1985), whole-cell immobilization (Sayadi et al., 1989), and dilution rate cycling (Weber and San, 1988)], chemical methods [antibiotic selection pressure (Ensley, 1985)], and genetic methods [auxotrophic complementation (Porter and Black, 1991), bacteriocins (Lauffenburger, 1987), and phage  $\lambda$  (Padukone et al., 1992)] have been used, although these methods are often expensive (e.g., antibiotics), cumbersome (e.g., two-stage operations), or non-general (e.g., auxotrophic complementation and phage techniques).

The enhancement of plasmid segregational stability using the *hok/sok* (formerly *parB*) locus offers many advantages, in that it is a genetic approach that stabilizes plasmids by killing plasmid-free cells as they form, independent of the mechanism that generates the plasmid-free daughter cell (Gerdes, 1988). Transcription of both mRNA from the cell-killing gene (*hok*) and antisense RNA from the suppressor gene (*sok*) is constitutive; however, plasmid-bearing cells are not killed since the suppressor is made in excess. Upon plasmid loss, the suppressor RNA decays more rapidly than the *hok* mRNA, with the result that a 52 amino acid Hok protein is expressed, binds to the inner membrane, and collapses the transmembrane potential. This prevents cell respiration and leads to cell death (Gerdes et al., 1986).

Because of the small size of *hok/sok* (580 bp), expression vectors may be rapidly constructed. It is an extremely general stabilization technique in that *hok/sok*

is effective in all of the Gram-negative bacteria that have been tried (including *Escherichia coli*, *Xanthomonas campestris*, *Pseudomonas putida*, and *Serratia marcescens*) (Gerdes, 1988; Pimenta et al., 1992; Wood et al., 1990; Wu and Wood, 1994), and no modifications are required for the chromosome of the host bacterium in which the plasmid is placed; thus, the *hok/sok*-containing vector may be used in a wide variety of hosts. Furthermore, it is a general technique, in that cells that are stabilized with *hok/sok* have no special medium requirements.

The effectiveness of the *hok/sok* system has been illustrated by many authors through batch and continuous fermentations (Moerloose et al., 1992; Nesvera et al., 1991; Pimenta et al., 1992; Schweder et al., 1992; Wood et al., 1990). However, none of these studies have examined the general aspects of this system since *hok/sok* usually is used to stabilize plasmids for specific applications without regard for the effects of medium, extent of cloned-gene expression, temperature, or growth rate.

Although the growth rate affects plasmid segregational stability (Brownlie et al., 1990; Nancib and Boudrant, 1992), its influence is complicated and difficult to predict since the factors that influence growth rate also directly or indirectly affect plasmid stability. Hence, contradictory results have been observed regarding the influence of growth rate on plasmid segregational stability (Brownlie et al., 1990; Nancib and Boudrant, 1992).

Copy number is recognized as an important factor for maintaining plasmids in high copy number systems since most of these plasmid systems are randomly partitioned to daughter cells (Lauffenburger, 1987). Many authors have reported the inverse relationship between copy number and growth rate (Betenbaugh et al., 1989; Ryan et al., 1989; Sayadi et al., 1989; Siegel and Ryu, 1985). Because of this close relationship between growth rate and copy number, copy number was analyzed in this

study while evaluating the effect of growth rate on plasmid stability. In addition, since the extra metabolic burden from cloned-gene expression has a significant effect on plasmid structural stability (Kapralek and Jecmen, 1992), the extent of cloned-gene expression was investigated while evaluating the *hok/sok* system.

Temperature also affects plasmid segregational stability (Aiba and Koizumi, 1984); however, there are no obvious temperature trends in recombinant *E. coli* systems in the literature. Therefore, temperature has been considered while evaluating the *hok/sok* system. Since one purpose of this study is to examine the practicality of the *hok/sok* system, temperatures were chosen to mimic normal fed-batch and batch conditions.

Batch studies conducted by this lab have shown that *hok/sok* stabilizes the highly unstable, pUC derivative pMJR1750 (Stark, 1987) and have provided useful information regarding medium and cloned-gene expression effects (Wu and Wood, 1994). Since temperature and growth rate affect plasmid stability and are important controlled parameters in large-scale fermentations, continuous fermentation studies have been conducted in this work to probe the impact of these parameters on the effectiveness of *hok/sok*. In addition, the continuous studies allow the enhancement in segregational stability provided by the *hok/sok* locus to be rigorously evaluated relative to the unstabilized plasmid during fermentations in which plasmid stability is challenged severely by constantly expressing the cloned gene. The mechanism of plasmid loss was also determined by isolating the plasmid DNA during the course of the fermentations.

## Materials and Methods

**Bacterial Strain and Plasmids.** *E. coli* strain BK6 (Wood et al., 1990) was chosen as the host for this continuous study because it was used to evaluate the *hok/sok* system in earlier batch studies (Wu and Wood, 1994). This host is convenient since it completely lacks the *lacZ* gene (to avoid homologous recombination), contains *lacY*<sup>+</sup>, and is a *recA* mutant. Its genotype is  $\Delta(lacIPOZ)-C29, lacY^+, hsdR, galU, galK, strA^R, leuB6, trpC9830, \Delta(srl-recA)306::Tn10$ , with the result that BK6 cannot produce  $\beta$ -galactosidase due to a genetically stable deletion while it retains the ability to produce  $\beta$ -galactosidase permease (LacY protein). Hence, this strain can be used conveniently with MacConkey agar (Mac) plates to indicate cells harboring plasmids that produce  $\beta$ -galactosidase. Plasmid-containing cells form red colonies, whereas plasmid-free cells form white colonies.

The  $\beta$ -galactosidase expression vector pTKW106 (Wood et al., 1990; Wood and Peretti, 1991) (*hok/sok*<sup>+</sup>, Kan<sup>R</sup>, *lacI*<sup>Q</sup>+, *ptac::lacZ*<sup>+</sup>, 9176 bp) was constructed previously by cloning the *hok/sok* stability locus into the ampicillin-resistance gene of the unstable expression vector pMJR1750 (Stark, 1987) (Amp<sup>R</sup>, *lacI*<sup>Q</sup>+, *ptac::lacZ*<sup>+</sup>, 7504 bp). Both plasmids produce  $\beta$ -galactosidase upon the addition of the noncleavable lactose analog isopropyl  $\beta$ -D-thiogalactopyranoside (IPTG) and are tightly regulated due to overexpression of the LacI repressor from the *lacI*<sup>Q</sup> allele present on the plasmid.

**Continuous Fermentations.** The influence of temperature and growth rate was checked through continuous fermentations performed in a 2-L New Brunswick Scientific Bioflo III fermentor filled with 1 L of medium. The fermentor automatically controlled the temperature and pH through PID control. The dilution rate was determined by checking the flow rate through a burette with a stopwatch every 4–8 h. The fermentor was monitored by Advanced Fermentation Software (New Brunswick Scientific) using an IBM-type PC; this program acquired data on dissolved oxygen, pH, tempera-

ture, and agitation speed every 5 min. Air was supplied to the reactor at the rate of 1 L/min, and a dissolved oxygen probe (Ingold) was used to monitor oxygen levels in the fermentor to ensure that the cells were growing aerobically (the dissolved oxygen level was kept greater than 25% of saturation). pH was controlled at 7.0 by adding NH<sub>4</sub>OH, and an external pH meter (Fisher Model 910) was used to correct any drift that occurred with the fermentor pH probe (Ingold). Agitation was kept constant at 500 rpm (although 1000 rpm was used during the fermentation with fed-batch medium to maintain the DO<sub>2</sub> level greater than 25%).

BK6/pTKW106 was grown at two different dilution rates (0.15 and 0.5/h) and at two different temperatures (37 and 30 °C) in the chemostat without antibiotics. A –84 °C frozen glycerol (20%) culture was streaked on 37 °C Mac–kanamycin (50 µg/mL, U.S. Biochemical) plates to obtain single colonies for the inoculum. After 14–18 h of growth at 37 °C, a single red colony from the Mac–kanamycin plate was aseptically transferred to a 250-mL flask filled with 20 mL of M9C Trp medium (Rodriguez and Tait, 1983) or a representative fed-batch medium (M9C with yeast extract, vitamins, and trace metals) (Fieschko and Ritch, 1986; Wu and Wood, 1994) supplemented with 50 µg/mL kanamycin. These starter cultures were grown for 12 (or 24) h at 37 °C (or 30 °C) and 250 rpm in a rotary shaker. Ten milliliters (1 vol %) of the starter culture was transferred to the fermentor as the inoculum. The antibiotic-free continuous fermentation media used in this study were M9C Trp and fed-batch medium supplemented with 0.5 mM IPTG (dioxane-free, U.S. Biochemical). BK6/pMJR1750 was cultivated in an analogous fashion, with 100 µg/mL ampicillin (Sigma; instead of kanamycin) used for its starter cultures.

During the continuous fermentations, a 0.5-mL sample was taken from the fermentor every 3–6 h for OD(600 nm) measurement. A 25-mL sample was taken every 5–10 h to determine the  $\beta$ -galactosidase activity (Wood and Peretti, 1991) (20 mL), isolate plasmid DNA (pDNA) (Rodriguez and Tait, 1983) (2 mL), obtain the OD (0.5 mL), and determine the cell number along with the fraction of  $\beta$ -galactosidase-producing cells (5–500 µL).

Plasmid stability was determined by diluting the fermentation sample (10<sup>–3</sup>–10<sup>–7</sup>), culturing it on MacConkey plates that lacked antibiotic (each plate averaged 100–300 colonies), and incubating the plates at 37 °C in order to determine the fraction of  $\beta$ -galactosidase-producing cells (red colonies). White colonies on Mac plates indicated cells that no longer produced  $\beta$ -galactosidase due to plasmid structure change or wholesale plasmid loss. These results were corroborated by analyzing the pDNA content as a function of time, as will be described later.

The number of stable generations of growth until 90% of the cell population possessed plasmids (*gen*<sub>90% point</sub>) was determined by counting the hours of exponential growth during the batch growth phase at the beginning of the continuous fermentation (*t*<sub>fermentor batch phase</sub>), as well as the hours of continuous feed to the reactor (*t*<sub>chemostat 90% plasmid-bearing</sub>):

$$\text{gen}_{90\% \text{ point}} = \frac{t_{\text{fermentor batch phase}}}{(\ln 2)/\mu_{\text{max}}} + \frac{t_{\text{chemostat 90\% plasmid-bearing}}}{(\ln 2)/\text{dilution rate}}$$

The maximum specific growth rates ( $\mu_{\text{max}}$ ) of BK6/pTKW106 in various media and at various temperatures were determined as described previously (Wu and Wood,

1994). The maximum specific growth rates of BK6/pMJR1750 were estimated using those of BK6/pTKW106 (since pMJR1750 was too unstable in BK6 to determine the growth rate directly).

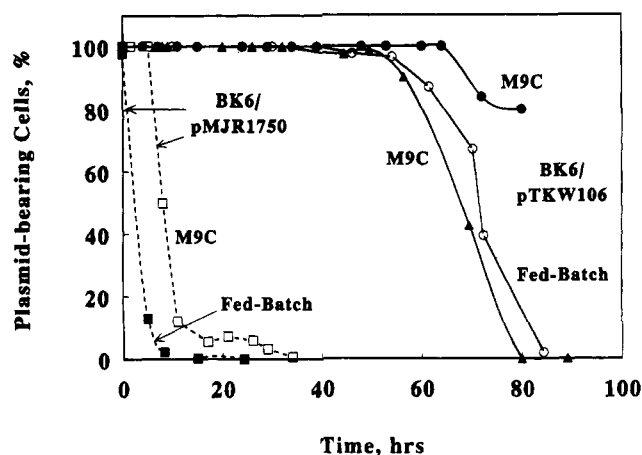
**Plasmid Structure and Copy Number.** Plasmid instability was further characterized as segregational or structural instability by examining the size of pTKW106 during the course of the fermentation using agarose gel electrophoresis (this data also verified the Mac plate plasmid stability results by indicating the extent of the cell population that was plasmid-bearing). To check the size of the plasmid, a 2-mL aliquot was taken from the 25-mL fermentation broth sample and stored at  $-20^{\circ}\text{C}$  for later analysis. Thawed samples were adjusted to contain the same number of cells by using the equivalent of 0.5 mL of cells with  $\text{OD} = 5.0$  for all of the continuous fermentations. Plasmid minipreparations were conducted using the protocol of Rodriguez and Tait (1983), with the modification that an internal reference plasmid (pBR322) was used to gauge slight differences in plasmid isolation efficiency. *E. coli* GM33/pBR322 cells were added (0.5 or 0.3 mL) to each fermentation pDNA sample and mixed thoroughly before initiating the plasmid isolation protocol. Isolated pTKW106 and pBR322 pDNA were digested with *Eco*RI (NE Biolabs) for 1 h at  $37^{\circ}\text{C}$ . The digested samples were analyzed using a 0.6 wt % agarose gel, and horizontal electrophoresis was conducted at 260 V·h.

The relative copy number provided information regarding the plasmid content of the cells under different growth conditions and was used to help interpret the SDS-PAGE and specific enzyme activity results. Relative copy number was determined using horizontal agarose gel electrophoresis with the internal pBR322 standard as described earlier, using a fermentation sample obtained when the population was 100% plasmid-containing. The pDNA was digested with *Hind*III enzyme (U.S. Biochemical) since both pBR322 and pTKW106 contain unique *Hind*III site. The gel band intensities were compared quantitatively on a single gel using a scanning densitometer (Bio-Rad GS-670 with Molecular Analyst software).

**$\beta$ -Galactosidase Activity, SDS-PAGE, and OD Measurements.**  $\beta$ -Galactosidase enzyme activity was measured using D. M. Miller's method (Wood and Peretti, 1991). A 20-mL sample was placed on ice immediately after removing the sample from the fermentor (to avoid degradation of the enzyme), and  $\beta$ -galactosidase activity (nmol/min·mL) was calculated on the basis of the conversion rate of uncolored *o*-nitrophenyl  $\beta$ -D-galactopyranoside (ONPG, Sigma) to yellow *o*-nitrophenol.

Specific  $\beta$ -galactosidase activity (nmol of ONPG cleaved/min·AU) was calculated by dividing the enzyme concentration (nmol/min·mL) by the spectrophotometrically determined cell density (AU/mL, where AU is the  $\text{OD}_{600\text{ nm}}$  value). Since  $\beta$ -galactosidase activities were obtained shortly after sampling the fermentor, these values were used as an indicator of the fraction of cells harboring the plasmid during the course of the fermentation; as plasmid-free cells took over the fermentation, the  $\beta$ -galactosidase activity decreased. Hence,  $\beta$ -galactosidase activity was used to discern the end of the fermentation. Furthermore,  $\beta$ -galactosidase specific enzyme activity was used as a check on the size of the  $\beta$ -galactosidase band observed using SDS-PAGE. In addition, the specific enzyme activity provided quantitative information regarding cloned-gene expression.

SDS-PAGE was used to discern the fraction of  $\beta$ -galactosidase as a percentage of total cell protein by using stacking (3.5%) and separating (12%) polyacrylamide gels and the discontinuous buffer system of Laemmli (1970).



**Figure 1.** BK6/pTKW106 (*hok/sok*<sup>+</sup>) and BK6/pMJR1750 (*hok/sok*<sup>-</sup>) continuous fermentation stability data at  $D = 0.5/\text{h}$  and  $37^{\circ}\text{C}$  in M9C-Trp medium and fed-batch medium supplemented with 0.5 mM IPTG and no antibiotics (five separate fermentations shown).

After the fermentation reached steady state (the dissolved oxygen level remained constant), a 20-mL sample was taken and immediately stored at  $-84^{\circ}\text{C}$  for later SDS-PAGE analysis. Total cellular protein was isolated from the 20-mL thawed fermentation samples as described previously (Wu and Wood, 1994). The individual bands of  $\beta$ -galactosidase in the continuous runs were quantified with a laser scanning densitometer (Molecular Dynamics Personal Densitometer).

The optical density of the fermentation broth sample was used to monitor the fermentation process, as well as to discern the onset of plasmid instability. As the plasmid was lost by the cells, the OD usually increased by 10–30%. A 0.5-mL sample was diluted by 1/10 with 4.5 mL of  $37^{\circ}\text{C}$  LB medium (Rodriguez and Tait, 1983), and the diluted sample was measured at a wavelength of 600 nm to obtain the optical density of the sample.

## Results

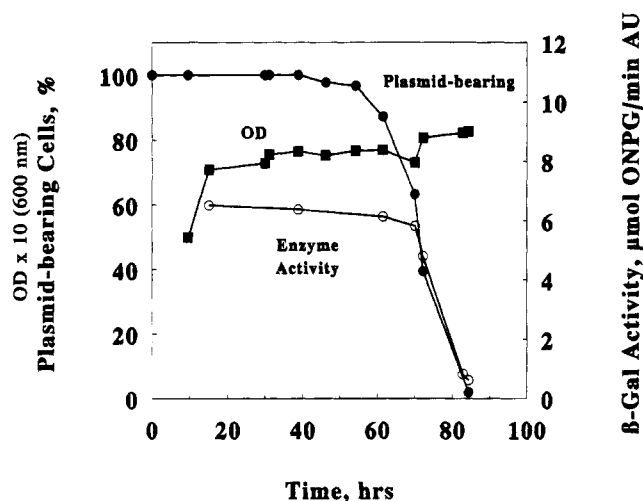
The effects of temperature and growth rate on plasmid stability were examined by continuous fermentations with high cloned-gene expression (0.5 mM IPTG) at different dilution rates (0.15 and 0.5/h) and temperatures ( $37$  and  $30^{\circ}\text{C}$ ). Figure 1 shows the enhancement in plasmid stability that is obtained by adding the *hok/sok* locus to the unstable plasmid pMJR1750, in terms of the percentage of the population of cells that harbors the plasmid as a function of time in the chemostat. Representative analyses of cellular OD, specific  $\beta$ -galactosidase activity, and the fraction of cells bearing the plasmid as a function of time for three of the nine continuous fermentations are shown in Figures 2–4. In order to compare data from fermentations with bacteria doubling at different rates, the absolute time of plasmid stability during the continuous fermentations was converted to the number of generations of growth before plasmid instability occurred (generations until 10% of the cells has lost the plasmid).

**Addition of the *hok/sok* Stability Locus.** Addition of the *hok/sok* locus to plasmid pMJR1750 led to a dramatic increase in plasmid segregational stability for all of the continuous fermentations conducted since pMJR1750 was extremely unstable with high, constant cloned-gene expression (Figure 1 and Table 1). In M9C-Trp medium ( $D = 0.5/\text{h}$ ,  $37^{\circ}\text{C}$ ), *hok/sok* addition led to an 8-fold increase in stability (47 vs 6 generations). In fed-batch medium ( $D = 0.5/\text{h}$ ,  $37^{\circ}\text{C}$ ), pMJR1750 was lost in just two generations in the chemostat; although, due to its extreme instability in this medium, we were unable to inoculate the chemostat with a 100% plasmid-bearing

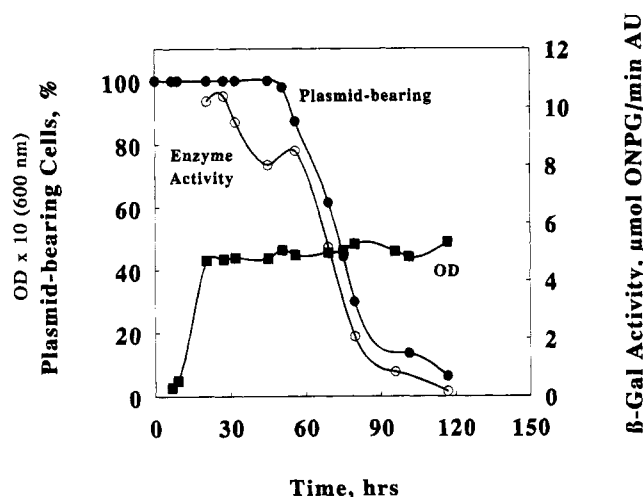
**Table 1. Plasmid Stability with Continuous Fermentations of BK6/pTKW106 (*hok/sok*<sup>+</sup>) and BK6/pMJR1750 (*hok/sok*<sup>-</sup>) with Constant Induction (0.5 mM IPTG) and Various Media, Dilution Rates (*D*), and Temperatures (No Antibiotics)<sup>a</sup>**

strain	medium/0.5 mM IPTG	dilution rate (h <sup>-1</sup> )/temp (°C)	time (h) (90% plasmid-bearing)	no. of generations (90% plasmid-bearing)	β-gal activity <sup>b</sup> (100% pb) (nmol of ONPG/min·AU)	plasmid loss mechanism
BK6/pTKW106 ( <i>hok/sok</i> <sup>+</sup> )	fed-batch medium	0.5/37	60	43	6450	structural
	M9C-Trp	0.5/37	67	50	5500	segregational
		0.5/37	57	43	6630	segregational
	M9C-Trp	0.15/37	34	12	11934	structural
		0.15/37	54	17	10306	structural
	M9C-Trp	0.15/30	146	34	5500	structural
BK6/pMJR1750 ( <i>hok/sok</i> <sup>-</sup> )	M9C-Trp	0.5/37	6	6		segregational
	fed-batch medium	0.5/37	2	2		segregational
		0.5/37	2	2		segregational

<sup>a</sup> Dual entries indicate that the fermentation was performed twice. <sup>b</sup> pb, plasmid-bearing cells.



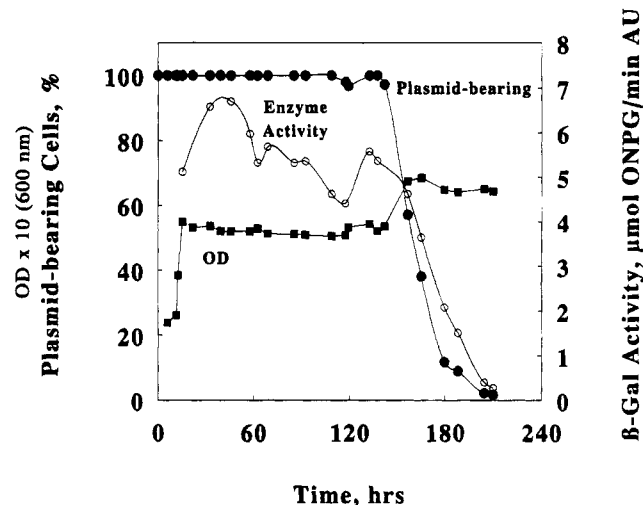
**Figure 2.** BK6/pTKW106 continuous fermentation stability, OD, and β-galactosidase specific enzyme activity in fed-batch medium supplemented with 0.5 mM IPTG and no antibiotics at *D* = 0.5/h and 37 °C (run 8).



**Figure 3.** BK6/pTKW106 continuous fermentation stability, OD, and β-galactosidase specific enzyme activity in M9C-Trp medium supplemented with 0.5 mM IPTG and no antibiotics at *D* = 0.15/h and 37 °C (run 14).

culture of BK6/pMJR1750 (for both chemostat experiments with fed-batch medium, the starter culture was found to be 98% and 99% plasmid-bearing after 12 h of growth). In contrast, pTKW106 (*hok/sok*<sup>+</sup>) was maintained stably for 43 generations of continuous, high specific β-galactosidase expression in fed-batch medium (22-fold increase). Hence, addition of the *hok/sok* locus dramatically and consistently improved plasmid stability.

For all of the fermentations, there was a precipitous drop in the number of plasmid-bearing cells once some plasmid-free cells were formed (Figures 1–4). This rapid

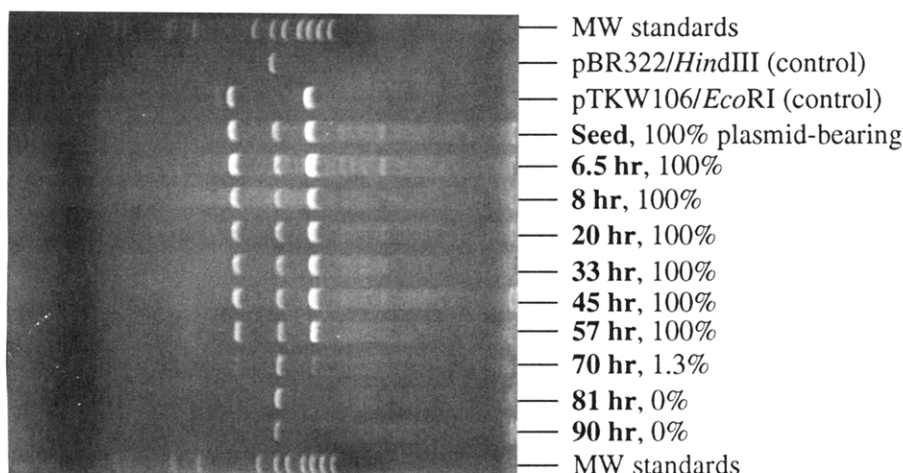


**Figure 4.** BK6/pTKW106 continuous fermentation stability, OD, and β-galactosidase specific enzyme activity in M9C-Trp medium supplemented with 0.5 mM IPTG and no antibiotics at *D* = 0.15/h and 30 °C (run 11).

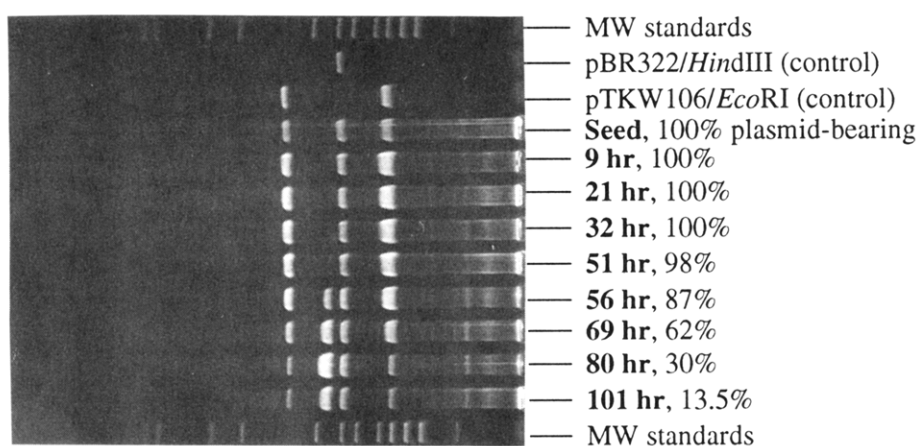
decrease is a direct result of the faster growth rate of the plasmid-free cells relative to plasmid-bearing cells (which produce β-galactosidase at high levels). The maximum specific growth rates ( $\mu_{\max}$ ) of BK6/pTKW106 in M9C-Trp/0.5 mM IPTG at 37 and 30 °C are 0.69 and 0.32/h, respectively, and 0.64/h in fed-batch/0.5 mM IPTG medium at 37 °C (Wu and Wood, 1994). In contrast,  $\mu_{\max}$  for BK6 is 1.05/h and 1.12/h in M9C-Trp/0.5 mM IPTG and fed-batch media at 37 °C, respectively. Therefore, plasmid-free cells grow 52% faster at 37 °C in M9C-Trp/0.5 mM IPTG medium.

The *hok/sok* locus stabilizes plasmids by killing efficiently daughter cells that lack plasmids. In order to gauge the extent and impact of this killing (along with the metabolic burden of making β-galactosidase and maintaining the plasmid), the continuous fermentations were conducted to the point at which nearly 0% of the cellular population contained the original pTKW106 plasmid (representative chemostat data shown in Figures 2–4). Table 2 lists the changes in cell number and OD as the plasmid was lost or severely structurally modified. In all of the fermentations, both the cell number and the OD increased. The most significant result was obtained for the M9C continuous fermentation conducted at 37 °C and *D* = 0.5/h, where the plasmid was lost completely due to pure segregational instability. In this case, the OD increased by 50% and the cell number increased by 150% upon complete loss of the plasmid (and termination of the expression of β-galactosidase).

As for the rapid drop in the number of plasmid-bearing cells once plasmid instability occurred, these increases in OD and cell number upon plasmid loss occur because the cloned-gene product (β-galactosidase) and other plas-



**Figure 5.** Examination of plasmids isolated from a BK6/pTKW106 continuous fermentation in M9C-Trp medium supplemented with 0.5 mM IPTG and no antibiotics at  $D = 0.5/h$  and  $37^\circ\text{C}$  (run 13). DNA molecular weight standards from NE Biolabs ( $\lambda$  BstEII digest, from right): 14 140, 8454, 7242, 6369, 5686, 4822, 4324, and 3675 bp. The percent plasmid stability shown was determined from red/white colony counts of MacConkey plates.



**Figure 6.** Examination of plasmids isolated from a BK6/pTKW106 continuous fermentation in M9C-Trp medium supplemented with 0.5 mM IPTG and no antibiotics at  $D = 0.15/h$  and  $37^\circ\text{C}$  (run 14). DNA standards are as in the legend to Figure 5. The percent plasmid stability shown was determined from red/white colony counts of MacConkey plates.

**Table 2. Cell Number and Optical Density for BK6/pTKW106 (*hok/sok*<sup>+</sup>) Fermentations (0.5 mM IPTG) at the Onset of Plasmid Instability (90% Plasmid-Containing Point) and for Plasmid-Free Cells (0% Plasmid-Bearing)**

temp ( $^\circ\text{C}$ )/ dilution rate ( $\text{h}^{-1}$ )	medium (0.5 mM IPTG)	plasmid-free cells and OD (near 0% point)	plasmid-bearing cells and OD (at 90% point)	cell no. w/o plasmid/ cell no. with plasmid
37/0.5	fed-batch medium	$50 \times 10^7$	$2 \times 10^7$	25.0
		7.7	7.4	
37/0.5	M9C-Trp	$5 \times 10^7$	$2 \times 10^7$	2.5
		4.3	2.9	
37/0.15		$28 \times 10^7$	$4 \times 10^7$	7.0
		5.0	4.3	
30/0.15		$50 \times 10^7$	$30 \times 10^7$	1.7
		6.5	5.2	

mid-mediated genes are no longer expressed; therefore, plasmid-free cells have a higher specific growth rate. Hence, an increase in cell density is expected since, at a fixed dilution rate, the Monod model predicts that cell density should increase with increasing  $\mu_{\text{max}}$  (Bailey and Ollis, 1986).

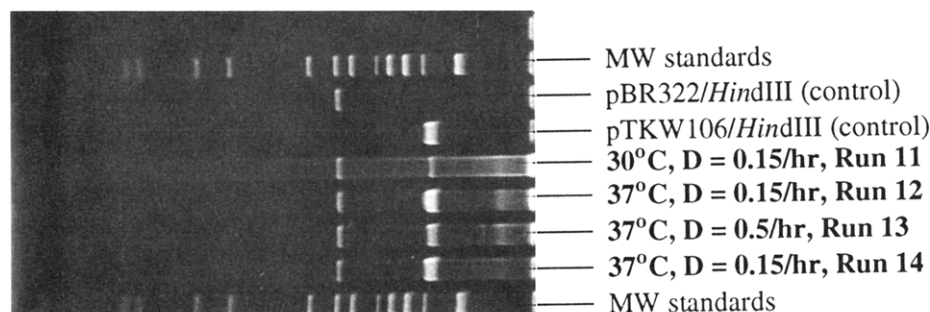
**Plasmid Structure Examination.** In order to determine the mode of plasmid instability (segregational vs structural), plasmid DNA was isolated from the fermentation broth during the course of the fermentation. By loading pDNA from the same amount of cells (constant OD), adding a constant amount of an internal standard (pBR322), and restricting the pDNA with *EcoRI*, pTKW106 band intensity could be used to gauge the relative amount of the original plasmid pTKW106 present at any time, and the formation of structural deletions in this plasmid could be observed (if any

occurred). Table 1 summarizes the results of this analysis in terms of the plasmid-loss mechanism for each of the continuous fermentations.

Figure 5 is a representative horizontal electrophoresis gel that shows the *segregational* loss of plasmid pTKW106 for a fermentation conducted at  $37^\circ\text{C}$  and  $D = 0.5/h$  (run 13). As the gel shows, the two pTKW106 bands (from *EcoRI* digestion) decrease over 70–90 h; this indicates that the plasmid was completely lost during the fermentation and a plasmid-free cell overtook the chemostat. These results also corroborate the independent determinations of the fraction of plasmid-bearing cells obtained from red/white colony formation on Mac plates.

Figure 6 is a representative gel that shows that, at  $37^\circ\text{C}$  and  $D = 0.15/h$ , the reduction in specific  $\beta$ -galactosidase activity shown in Figure 3 is a result of *structural* instability. In this fermentation, a new 3.8-kb pDNA





**Figure 7.** Comparison of the relative copy number of BK6/pTKW106 continuous fermentations in M9C-Trp/0.5 mM IPTG and no antibiotics at different temperatures and dilution rates. DNA standards are as in the legend to Figure 5.

**Table 3.** Fraction of  $\beta$ -Galactosidase from SDS-PAGE, Specific  $\beta$ -Galactosidase Activity, and Relative Copy Number of BK6/pTKW106 Continuous Cultures in M9C-Trp Medium Supplemented with 0.5 mM IPTG

temp ( $^{\circ}$ C)/ dilution rate ( $\text{h}^{-1}$ ) % plasmid-bearing	$\beta$ -galactosidase (% total cell protein)	$\beta$ -gal specific activity (nmol of ONPG/min·AU)	relative copy number
37/0.5, 100% (run 13)	12	6850	1.0
37/0.15, 100% (run 14)	17	10300	1.5
30/0.15, 100% (run 11)	not measured	5500	0.15

band (just below the internal standard band) forms and becomes the dominant plasmid as the fermentation continues. This new band is a deletion derivative of pTKW106 and was dominant at the end of fermentations at lower dilution rates (0.15/h) at both 37 and 30  $^{\circ}$ C. In addition, all deletion-derivative plasmids were found to be very close in size. After analysis through restriction enzyme digestions (*Bam*HI, *Eco*RI, *Pst*I, *Hind*III, *Sma*I, and *Nde*I) and horizontal gel electrophoresis, the deletion derivatives of pTKW106 were found to have very similar structures. They contained both the *hok/sok* locus and the kanamycin-resistance gene, but probably no *lacZ* and only part of the *lacI<sup>Q</sup>* gene. Hence, it appears that *lacZ* and *lacI<sup>Q</sup>* are deleted from pTKW106 (on the basis of the disappearance of the *Bam*HI and *Eco*RI sites in this region).

**Growth Rate.** The growth rate affected plasmid stability (in terms of the number of generations until the plasmid is lost) since the generations at the 90% plasmid-containing point were decreased from an average of 47 to 15 when growth rate was decreased from 0.5 to 0.15/h at 37  $^{\circ}$ C in M9C-Trp/0.5 mM IPTG (Table 1). This implies that an increasing dilution rate increases plasmid stability. This result is opposite that of Nancib and Boudrant (1992), but agrees with the observations of Sayadi et al. (1989). After an examination of the plasmid structural results with agarose gel electrophoresis (Figure 6, as a representative result for the two  $D = 0.15/\text{h}$  fermentations), it is obvious that the original plasmid (pTKW106) was gradually replaced by a structurally modified plasmid having a smaller size (3800 bp) in both fermentations at a 0.15/h dilution rate and 37  $^{\circ}$ C. This result indicates that plasmid structural instability, not plasmid segregational instability, made the plasmid more unstable at the lower dilution rate.

**Temperature.** No clear trends developed regarding the effect of temperature on plasmid *segregational* instability (Table 1). However, for *structural* instability, the number of generations at the 90% plasmid-containing point were decreased from 34 generations at 30  $^{\circ}$ C ( $D = 0.15/\text{h}$ ) to 15 generations at 37  $^{\circ}$ C ( $D = 0.15/\text{h}$ ). This implies that lowering the cultivation temperature can increase the plasmid *structural* stability by 2.3-fold; however, this increase in plasmid stability came at the cost of a 2-fold decrease in  $\beta$ -galactosidase specific activity.

**Specific Enzyme Activity and SDS-PAGE.** Specific  $\beta$ -galactosidase activity (Table 1) indicated the

degree of cloned-gene expression during the continuous fermentations. For example, the specific enzyme activity at 37  $^{\circ}$ C and  $D = 0.15/\text{h}$  was high (av: 11 120 nmol of ONPG/min·AU) compared to 6100 nmol of ONPG/min·AU at  $D = 0.5/\text{h}$  and 37  $^{\circ}$ C. Therefore, a decreasing growth rate resulted in a 1.8-fold increase in the specific enzyme activity.  $\beta$ -Galactosidase activity is not reported for the three fermentations with the unstable plasmid pMJR1750 because it was nearly completely lost during the batch growth phase (before continuous operation).

$\beta$ -Galactosidase activity also indirectly indicated the number of plasmid-containing cells (Figures 2–4). The specific enzyme activity trends match the % plasmid-containing curves very well (Figures 2–4). This suggests that specific enzyme activity is a useful indicator of the fraction of cells harboring the original plasmid, as well as the onset and degree of plasmid instability.

$\beta$ -Galactosidase as a fraction of the total cell protein was determined for the continuous fermentations using SDS-PAGE and a scanning densitometer. Table 3 shows that 0.5 mM IPTG resulted in a significant metabolic burden ( $\beta$ -galactosidase at 12–17% of the total cell protein) on BK6/pTKW106, regardless of the growth conditions. This suggests that the *hok/sok* system can efficiently improve plasmid segregational stability, even with constant, high-level cloned-gene expression. Furthermore, the fraction of  $\beta$ -galactosidase data observed with SDS-PAGE (Table 3) is consistent with the  $\beta$ -galactosidase enzyme activity data (Table 3), in that the 41% increase in  $\beta$ -galactosidase as a fraction of total cell protein seen upon decreasing  $D$  from 0.5 to 0.15/h at 37  $^{\circ}$ C is reflected in the 55% increase in specific  $\beta$ -galactosidase enzyme activity for the same change in continuous fermentation conditions.

**Copy Number.** To determine the relative copy number in the continuous fermentations at various temperatures and dilution rates, a horizontal electrophoresis gel was run with pDNA samples from representative fermentations (Figure 7). By loading each gel well with pDNA from the same amount of cells (constant OD) and by using the internal pBR322 standard band to confirm that there were no significant differences in the preparation of each sample, scanning densitometry could be used to compare the pTKW106 relative band intensities for each fermentation to gauge relative copy number. These results are listed in Table 3.

At 37  $^{\circ}$ C, lowering the dilution rate (from 0.5 to 0.15/h) results in a higher relative copy number (about a 50%

increase). This observation agrees with many authors' results (Betenbaugh et al., 1989; Ryan et al., 1989). In general, a higher copy number ensures higher plasmid segregational stability in multiple copy number systems. However, although a decreasing growth rate increased the copy number, plasmid stability decreased. This is due to structural changes that occurred in the plasmid (unique fourth band in Figure 6 at 50–101 h), causing instability (rather than segregational instability). Therefore, the copy number data cannot be related directly to plasmid segregational stability due to the structural instability that arose, primarily at lower growth rates (Table 1).

It was also interesting to note that as the temperature was decreased from 37 to 30 °C at  $D = 0.15/h$ , the relative copy number decreased by roughly 10-fold (Table 3). This phenomenon has been noted with other pUC plasmids by these authors (unpublished results).

### Discussion

Two dilution rates, 0.5 and 0.15/h, were used to examine growth rate effects at a fixed temperature (37 °C). A dilution rate of 0.5/h was used to compare these continuous results to earlier batch fermentation data (Wu and Wood, 1994); this dilution rate is close to the maximum specific growth rate of BK6/pTKW106 in minimal medium with high cloned-gene (*lacZ*) expression ( $\mu = 0.69/h$  in 0.5 mM IPTG) (Wu and Wood, 1994). A dilution rate of 0.15/h was chosen since the difference between 0.15 and 0.5/h is large enough to investigate the influence of growth rate on cell metabolism and plasmid stability. In addition, Fieschko and Ritch (1986) have shown that growth rates near 0.15/h are good for high cell density, fed-batch *E. coli* fermentations, since these conditions help to avoid both oxygen limitation and the accumulation of partially oxidized products (which may be toxic to the cells).

To investigate temperature effects on plasmid stability, the dilution rate was fixed at 0.15/h and growth temperatures were set at 30 or 37 °C. The temperature 37 °C was chosen because it is the optimal temperature for growing *E. coli* strains, and 30 °C was chosen because the difference between 30 and 37 °C is large enough to examine the influence of temperature on cell metabolism and plasmid stability. In addition, Fieschko and Ritch (1986) used 30 °C as the fermentation temperature for high cell density, fed-batch fermentations. Since recombinant *E. coli* cultures often are grown using fed-batch or batch fermentations to reach high cell density, it seemed reasonable to choose 30 °C as the other cultivation temperature.

The *hok/sok* locus proved efficient in improving plasmid segregational stability during continuous fermentations without antibiotic selection pressure. Stability was increased from 6 generations at the 90% plasmid-containing point (BK6/pMJR1750, 37 °C and  $D = 0.5/h$ ) to 47 generations (BK6/pTKW106, 37 °C and  $D = 0.5/h$ ). Therefore, the addition of the *hok/sok* locus increased stability by 8-fold (Table 1), while  $\beta$ -galactosidase was expressed continuously at 12% of total cell protein. In a similar manner, the *hok/sok* locus increased plasmid stability by 22-fold in the fed-batch medium. These continuous fermentation results agree well with shake-flask studies using *hok/sok*, which indicated that a 17–29-fold improvement in segregational stability was obtained by adding *hok/sok* to pMJR1750 for growth in LB and fed-batch media supplemented with 2 mM IPTG and  $\beta$ -galactosidase expression at 15% and 6% of total cell protein, respectively (Wu and Wood, 1994).

The influence of growth rate on plasmid segregational stability was also investigated. However, no segrega-

tional stability conclusions can be made because plasmid structural instability occurred. Therefore, for elucidating the relation between growth rate and plasmid segregational stability using the *hok/sok* system, a more structurally stable plasmid or vector/host system should be chosen (although the structural changes were surprising, in that *lacZ* is deleted from the chromosome of host BK6). The relatively large size (9176 bp) and high copy number of pTKW106 are not advantageous for avoiding structural deletions.

By examining the relative copy number and specific  $\beta$ -galactosidase activity at 37 °C in M9C-Trp/0.5 mM IPTG (Figure 7 and Table 3), it was found that the relative copy number at the lower dilution rate (0.15/h) was roughly 1.5-fold higher than that at dilution rate 0.5/h, and the average specific enzyme activity at  $D = 0.15/h$  (11 120 nmol of ONPG/min·AU) was about 1.8-fold that at 0.5/h (6100 nmol of ONPG/min·AU) with identical inducer concentrations (0.5 mM IPTG). The higher enzyme activity is probably due to the higher copy number, because the relative increases in activity and copy number are relatively well-matched (1.8- vs 1.5-fold). Hence, cloned-gene expression is closely related to gene dosage at 37 °C for this strain. The observation that a lower dilution rate yields a higher copy number agrees with many other authors' observations (Betenbaugh et al., 1989; Ryan et al., 1989).

In addition, the higher plasmid content may also contribute to the structural instability seen for both of the continuous fermentations conducted at 0.15/h and 37 °C. Higher plasmid content provides more sites for plasmid–plasmid recombination. In addition, the idea that a higher expression level results in higher plasmid structural instability agrees with the results of Kapralek and Jecmen (1992), who used a pUC9 derivative to produce calf prochymosin. In the current study, decreasing the temperature from 37 to 30 °C (at  $D = 0.15/h$ ) resulted in a 230% increase in plasmid structural stability. The increase in structural stability was probably the result of both the 10-fold reduction in the relative copy number (less plasmid–plasmid homologous recombination) and the 50% decrease in specific  $\beta$ -galactosidase activity. It appears that a higher copy number is advantageous for increasing segregational stability as long as structural instability is not a major concern.

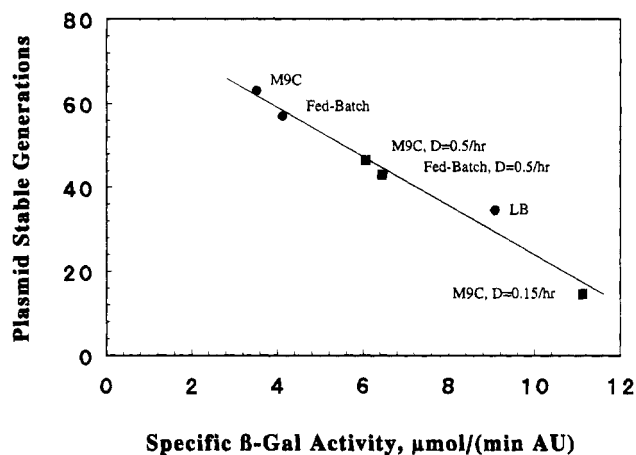
Interestingly,  $\beta$ -galactosidase production *per gene* was 5-fold greater at 30 °C than at 37 °C ( $D = 0.15/h$ ) since one-tenth the gene dosage resulted in only a 50% reduction in  $\beta$ -galactosidase specific activity. This must be due to greater translational efficiency, if one assumes that the promoter strength was fixed at constant induction (0.5 mM IPTG).

The results obtained from these continuous fermentations indicate that productivity is different when growth conditions are changed. With productivity expressed volumetrically as the amount of enzyme (expressed as activity, nmol/min) synthesized per hour of fermentation, it is clear that, for M9C-Trp medium, a dilution rate of 0.5/h and a temperature of 37 °C were more productive than either a lower dilution rate or both a lower temperature and a lower dilution rate (Table 4). Decreasing the dilution rate enhances the specific enzyme activity by 2-fold and the cell density by 1.5-fold; however, volumetric flow is decreased by 3.3-fold. Although decreasing the temperature from 37 to 30 °C at the 0.15/h dilution rate can prolong stability by 2-fold (34 vs 15 generations), the productivity is only 60% that at the 0.15/h dilution rate and 37 °C. Furthermore, the increase in stability is less than that at  $D = 0.5/h$  and 37 °C.

In comparing the different media at  $D = 0.5/h$  and 37 °C, the fed-batch medium was found to be more produc-

**Table 4.  $\beta$ -Galactosidase Specific Enzyme Activity and Productivity (at 0.5 mM IPTG) for Different Specific Growth Rates and Temperatures**

medium (0.5 mM IPTG)	dilution rate ( $\text{h}^{-1}$ )/ temp ( $^{\circ}\text{C}$ )	av stability (90%) time (h), no. of generations	specific activity $\beta$ -gal (nmol of ONPG/min·AU)	volumetric $\beta$ -gal activity (nmol of ONPG/min·mL)	productivity (nmol of ONPG/min·h)
M9C-Trp	0.5/37	62, 47	6630	18560	$9.28 \times 10^6$
	0.15/37	44, 15	11120	48370	$7.25 \times 10^6$
	0.15/30	146, 34	5500	28600	$4.29 \times 10^6$
fed-batch	0.5/37	60, 43	6450	45150	$22.6 \times 10^6$



**Figure 8.** Number of generations the plasmid maintained stably (90% point) vs specific  $\beta$ -galactosidase activity at  $37^{\circ}\text{C}$ . Data are from both shake-flask (exponential growth in LB, M9C-Trp, and fed-batch supplemented with 2 mM IPTG media, shown by circles) (Wu and Wood, 1994) and continuous fermentations (fed-batch and M9C-Trp media supplemented with 0.5 mM IPTG,  $D = 0.15$  and  $0.5/\text{h}$ , shown by squares). Line is linear regression of the data.

tive (Table 4) by 240%. This increase in productivity is due to a 2.6-fold higher cell density (Table 2). This result also verifies the use of this kind of medium to reach high cell density in fed-batch fermentation (Fieschko and Ritch, 1986).

Plasmid stability for the M9C-Trp fermentation was slightly better than that in fed-batch medium (47 vs 43 generations), even though the plasmid instability mechanism was different (segregational vs structural instability). This slight increase in stability in M9C-Trp is reasonable compared to the slightly smaller specific  $\beta$ -galactosidase activity (6065 in M9C-Trp vs 6450 nmol/min·AU in fed-batch medium). This and the other stability results presented in Table 1 indicate that plasmid stability is correlated with cloned-gene expression: as  $\beta$ -galactosidase specific activity increases, plasmid stability decreases. Using batch fermentation results obtained with BK6/pTKW106 in LB, fed-batch, and LB supplemented with 2 mM IPTG media at  $37^{\circ}\text{C}$  (Wu and Wood, 1994), along with the  $37^{\circ}\text{C}$  continuous-culture results of Table 1 ( $D = 0.15$  and  $0.5/\text{h}$ ), the number of stable generations of growth before 10% of the population loses the plasmid is found to be dictated linearly by the specific  $\beta$ -galactosidase activity (Figure 8). It should be noted that this relationship is based on batch and continuous fermentations conducted with various media (LB, fed-batch, and M9C with either 0.5 or 2.0 mM IPTG) and at different specific growth rates ( $0.15$ – $0.75/\text{h}$ ). Hence, plasmid stability is determined by the extent of cloned-gene expression.

### Conclusions

We have shown that the addition of the *hok/sok* stability locus dramatically improves the segregational stability of the pUC derivative pMJR1750 in host BK6 when cells are cultivated continuously in the absence of antibiotics and under the constant stress of high  $\beta$ -ga-

lactosidase expression. In terms of the number of generations in which the plasmid was maintained stably, in M9C medium at  $37^{\circ}\text{C}$  and  $D = 0.5/\text{h}$ , the addition of *hok/sok* resulted in an 8-fold improvement in plasmid retention (47 vs 6 generations) while the cell produced  $\beta$ -galactosidase at 12% of total cell protein. A 22-fold increase in stability was observed in fed-batch medium with similar  $\beta$ -galactosidase expression. Therefore, it is possible to cultivate cells continuously for significant periods of time without antibiotic selection pressure using the *hok/sok* stability locus.

In addition, the representative fed-batch medium maximized the continuous expression of  $\beta$ -galactosidase by 2.4-fold on a volumetric productivity basis compared to M9C. Decreasing the growth rate from  $0.5$  to  $0.15/\text{h}$  at a fixed temperature ( $37^{\circ}\text{C}$ ) resulted in 2-fold higher specific  $\beta$ -galactosidase activity; however, plasmid stability was decreased by 3-fold. It was also found that this and other trends may be explained by correlating the number of generations the plasmid is stably maintained with the amount of expression of the recombinant protein ( $\beta$ -galactosidase). Cultivation at  $30^{\circ}\text{C}$  served to reduce the copy number by 10-fold.

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