Evaluation of the *hok/sok* Killer Locus for Enhanced Plasmid Stability

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The effectiveness of the *hok/sok* plasmid stability locus and mechanism of cloned-gene loss was evaluated in shake-flask cultures. Addition of the *hok/sok* locus dramatically increased apparent plasmid segregational stability relative to the *hok/sok*$^-$ control. In terms of the number of generations before 10% of the population became plasmid-free, segregational stability was increased by 11- to 20-fold in different media in the absence of induction of the cloned-gene (*hok/sok*$^+$ plasmid stable for over 200 generations in all media tested). With constant expression of β-galactosidase in the absence of antibiotic, the segregational stability of the plasmid containing *hok/sok* was increased more than 17- to 30-fold when β-galactosidase was expressed at 7–15 wt % of total cell protein. Although the *hok/sok* system stabilized the plasmid well in four different media (Luria-Bertani (LB), LB glucose, M9C Trp, and a representative fed-batch medium), the ability of *hok/sok* to maintain the plasmid with induction of the cloned gene decreased as the complexity of the media increased. This result is better interpreted in terms of the influence of cloned-gene expression on plasmid maintenance; plasmid segregational stability decreased linearly as specific β-galactosidase activity increased.
Key words: plasmid stability • cloned gene • *hok/sok* locus

INTRODUCTION

Plasmids have become common as carriers of genes whose products are of industrial importance.[5] However, large-scale, commercial production of foreign proteins using recombinant plasmid technology is often hampered by plasmid instability (segregational or structural),[8] and much work has been conducted in *E. coli* to combat plasmid segregational instability, including both physical means (novel reactor strategies)[3,18,20,23] as well as genetic means (phage techniques and complementation of host auxotrophic markers).[14,16]

Another exciting and more general technique to combat segregational instability in *E. coli* and other Gram-negative hosts is to clone the 580-bp *hok/sok* stability locus (formerly *parB*) of the stable resistance plasmid R1 into an unstable plasmid. This stability locus prevents faster-growing, plasmid-free cells from overtaking the plasmid-bearing cells by killing the plasmid-free cells as they arise.[5,22] The *hok/sok* locus stabilizes plasmids by encoding a cell-killing gene (*hok*) whose mRNA is translated only when the cell loses the plasmid. Upon plasmid loss, the 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.[6] The advantages of using this locus include ease of use (rapid addition of *hok/sok* to the expression vector),[5] no need for modification of the bacterial host (so the modified plasmid may be used in many different hosts), broad-host-range effectiveness (*hok/sok* is active in all Gram-negative strains tried, including *Escherichia coli, Xanthomonas campestris, Pseudomonas putida,* and *Serratia marcescens*),[5,15,25] and no medium constraints.

Short-term, continuous-reactor experiments showing the effectiveness of the *hok/sok* stability locus (in conjunction with antibiotics and the *recA* mutation in the host chromosome) for stabilizing the β-galactosidase-expression vector pMJR1750 were conducted previously by this group.[24] Other researchers[12,13,15,19] have also shown the effectiveness of the *hok/sok* locus for plasmid maintenance; however, none of these studies include a detailed investigation into the general factors which affect the ability of the *hok/sok* locus to maintain plasmids.

Based on our prior encouraging results and the need to investigate further the suitability of stabilizing plasmids with the *hok/sok* locus in the absence of antibiotic-selection pressure, this shake-flask study was conducted. Since different nutritional limitations (e.g., carbon, nitrogen, phosphate, and magnesium) influence plasmid stability,[18] four different media (without antibiotics) were used to study the impact of medium on *hok/sok*. Along with LB complex medium, LB medium supplemented with glucose, and M9C Trp minimal medium, a semidefined medium used for high-cell-density, recombinant, fed-batch fermentations[4] was evaluated. In addition, plasmid-based, cloned-gene expression was varied to see its impact on *hok/sok*-enhanced, segregational stability.

MATERIALS AND METHODS

Bacterial Strain and Plasmids

E. coli strain BK6[24] was used for all the shake-flask experiments and has genotype Δ(*lacIPOZ*)*C29*, *lacY*$^+$, *hsdR*,

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galU, *galK*, *strA*^{R}, *leuB6*, *trpC9830*, Δ(*srl-recA*)306::*Tn* 10, with the result that BK6 cannot produce β-galactosidase due to the genetically-stable *lacZ* deletion while it still produces β-galactosidase permease (Lac Y protein). Therefore, this strain can be used conveniently with MacConkey agar (Mac) plates to indicate cells harboring plasmids that produce β-galactosidase. Plasmid-containing cells form red colonies, whereas plasmid-free cells form white colonies. The *recA* deletion of BK6 greatly decreases the possibility of homologous recombination between the plasmid and chromosome and between plasmids.[2,9]

The β-galactosidase-expression vector pTKW106[24,26] (*hok/sok*$^+$, Kan^R, *lacI*$^{Q+}$, *ptac::lacZ*$^+$, 9176 bp) was constructed previously by cloning the *hok/sok* stability locus into the ampicillin-resistance gene of the unstable expression vector pMJR1750[21] (Amp^R, *lacI*$^{Q+}$, *ptac::lacZ*$^+$, 7504 bp). Both plasmids produce β-galactosidase upon addition of the noncleavable, lactose analog isopropyl-β-D-thiogalactopyranoside (IPTG), and are tightly regulated due to overexpression of the LacI repressor from the *lacI*^{Q} allele present on the plasmid.

Sequential-Dilution Shake-Flask Studies

Two sets of batch experiments were conducted using sequential dilution of 20-mL, shake-flask cultures cultivated in 250-mL Erlenmeyer flasks. In the first set of experiments, the cloned gene (*lacZ*) was not induced (0.0 m*M* IPTG); and in the second set, β-galactosidase was fully expressed[26] by adding 2.0 m*M* IPTG to evaluate the *hok/sok* locus under the heavy metabolic burden of constant, cloned-gene expression.

The four media used in this study were M9C Trp,[17] LB medium,[17] semidefined medium,[4] and modified complex medium. The modified complex medium consisted of LB plus 60 g/L glucose, and the semidefined medium contained 5 g/L glucose, 7 g/L K_2HPO_4, 8 g/L KH_2PO_4, 1 g/L $MgSO_4 \cdot 7H_2O$, 5 g/L $(NH_4)_2SO_4$, 5 g/L yeast extract, and 1 mL/L each of a concentrated stock solution of vitamins and trace metals. The stock vitamin solution[4] contained 0.42 g/L riboflavin, 5.4 g/L pantothenic acid, 6.1 g/L niacin, 1.4 g/L pyridoxine, 0.06 g/L biotin, and 0.04 g/L folic acid. The stock trace metals solution[4] contained 27 g/L $FeCl_3 \cdot 6H_2O$, 2 g/L $ZnCl_2 \cdot 4H_2O$, 2 g/L $CaCl_2 \cdot 6H_2O$, 2 g/L $NaMoO_4 \cdot 2H_2O$, 1.9 g/L $CuSO_4 \cdot 5H_2O$, 0.5 g/L H_3BO_3, and 100 mL/L concentrated HCl.

To initiate the BK6/pTKW106 shake-flask, plasmid-stability studies, a −84°C frozen glycerol (20%) culture was streaked onto 37°C Mac-kanamycin (50 μg/mL, US Biochemical) plates to obtain a single red colony for each inoculation (100 μg/mL ampicillin plates were used for BK6/pMJR1750). To evaluate the *hok/sok* system in the absence of induction of *lacZ*, after 14 to 18 h at 37°C, a single red colony of BK6/pTKW106 from the Mac-kanamycin plate was aseptically transferred to a 250-mL flask filled with 20 mL M9C Trp media (also LB, modified complex, or semidefined media). To evaluate the *hok/sok* locus under the constant stress of expression of β-galactosidase, the media were supplemented with 2 m*M* IPTG (dioxane-free, US Biochemical).

Zero time for all the batch fermentations in this study was the moment the red colony was transferred to the 250-mL shake-flask. Cultures were grown 12 h at 37°C and 250 rpm in a New Brunswick Series 25 rotary shaker. Every 12 h, 10 μL were taken from the culture and aseptically transferred to another flask filled with 20 mL of the same medium for the next 12-h growing cycle. Fermentations were terminated as the fraction of plasmid-containing cells approached 0%. A 0.5 mL broth sample was taken and diluted by 1/10 to obtain the optical density (600 nm) of the cells after each 12-h growth cycle. Multiple fermentations were performed to confirm the results.

Plasmid Stability and Specific Growth Rates

To ascertain plasmid stability during the shake-flask fermentations, the fraction of plasmid-containing cells (as a function of time) was determined by obtaining a 5–500 μL broth sample (taken at the end of each shake-flask cycle), diluting, and spreading on Mac agar plates. The fraction of β-galactosidase-expressing colonies was found by averaging the fraction of red colonies from two plates (with each plate containing roughly 100–300 colonies). Plasmid segregational or structural instability (e.g., loss of *lacZ*) was indicated by the appearance of white colonies on the Mac plates (and corroborated by analyzing the pDNA content as a function of time as described below).

To discern whether there were any plating efficiency differences between BK6 (Lac$^-$) and BK6/pTKW106 (Lac$^+$), LB cultures of these two strains were sampled at OD_{600} = 0.5, 1.1, and 2.1, diluted (10^{-5} – 10^{-7}), and grown on six Mac plates for each OD. The number of colonies formed for each culture were then counted.

To compare cultures with different specific growth rates, the absolute time at which 10% of the cell population had lost the plasmid (as determined by Mac plates) was converted into the number of generations of cell growth. This 90% point was chosen because it was found easily from a plot of % plasmid-containing cells vs. fermentation time. To find the total number of generations, it was estimated that 8 h of exponential growth occurred during each 12-h shake-flask fermentation when the cloned-gene was not induced (based on observation of cellular OD), and it was estimated that 9 h of exponential growth occurred during each 12-h shake-flask fermentation when the cloned-gene was induced with 2 m*M* IPTG (based on observation of cellular OD). Hence, the total number of stable generations for experiments in which the cloned-gene was expressed (2 m*M* IPTG) was calculated as

$$N = \frac{\text{total h of exponential growth}}{\text{doubling time}} = \frac{k(9\text{ h})}{\ln 2/\mu_{max}} \quad (1)$$

where N is the total number of generations up to the time 90% of the population was plasmid-bearing, and k is the

number of 12-h shake-flask fermentations. For experiments in which the cloned-gene was not expressed (0 m*M* IPTG), 8 h of exponential growth/cycle was used to determine the hours of exponential growth.

In addition, by starting with 100% plasmid-bearing cells in the shake-flask experiments, the frequency of plasmid-free cells arising per cell division for the exponentially-growing batch population, p, was determined using the following equation[7]:

$$F = \frac{(1 - \alpha - p)}{1 - \alpha - p \cdot 2^{n(\alpha+p-1)}} \quad (2)$$

where F is the fraction of the total cellular population carrying the plasmid, n is the number of cell generations at which F is measured, and α is the ratio of the maximum specific growth rate of the plasmid-free cell divided by the maximum specific growth rate of the plasmid-bearing cell. By quantifying the plasmid loss frequency, the *hok/sok* locus may be evaluated more analytically.

To calculate the number of stable generations and values of p, for maximum specific growth rates at 37°C for BK6 and BK6/pTKW106 were determined for each medium supplemented with 0, 0.5, and 2.0 m*M* IPTG by following the increase in optical density at low OD_{600} values (OD_{600} less than 0.6) with a Cary 1 UV/Visible spectrophotometer (growth of BK6/pTKW106 at 30°C in M9C Trp was also determined). The maximum specific growth rates of BK6/pMJR1750 in LB and semidefined media were estimated using those of BK6/pTKW106 (because 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 sample was taken at the end of each fermentation and stored at −20°C for later analysis. The amounts of the thawed samples were adjusted to the same OD (0.5 mL at OD = 5.0) for all the fermentations. Plasmid minipreparations were conducted using the protocol of Rodriguez and Tait,[17] with the modification that an internal reference plasmid (pBR322) was used to gauge slight differences in plasmid isolation efficiency. The GM33/pBR322 cells were added (0.3 mL) to each fermentation pDNA sample and mixed thoroughly before the plasmid isolation protocol. Isolated pTKW106 and pBR322 pDNA were digested with *Eco*RI (NE Biolabs) for 1 h at 37°C. The digested samples were analyzed using a 0.6 wt % agarose gel, and horizontal electrophoresis was conducted at 260 V-h.

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 (for 100% plasmid-bearing cells) was gauged by measuring relative plasmid content for a constant amount of cell mass: the pDNA bands of photographs of the horizontal electrophoresis gels (which were used to determine the mechanism of plasmid instability) were scanned with a densitometer (Bio-Rad GS-670 with Molecular Analyst software). The internal pBR322 band served to make sure differences in copy number were not due to differences in plasmid isolation efficiency.

β-Galactosidase Activity and SDS–PAGE

Specific β-galactosidase enzyme activity was measured using D. M. Miller's method[26] for 100% plasmid-bearing cell populations. A 20-mL sample was placed on ice immediately after removing the sample from the shake-flask (to avoid the degradation of the enzyme). Activity was calculated based upon the conversion rate of uncolored *o*-nitrophenyl-β-D-galactopyranoside (ONPG, Sigma) to yellow *o*-nitrophenol.

SDS–PAGE was used to discern the fraction of β-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.[10] From 20-mL samples, the cellular proteins were isolated by forming a cell pellet at 10 krpm, resuspending in 0.3 mL 2X SDS buffer (10% SDS, 125 m*M* Tris pH 6.8, 15% sucrose, 10% β-mercaptoethanol, 1 m*M* EDTA, 0.05% bromophenol blue, 1 m*M* PMSF) and 0.3 mL of TE (10 m*M* Tris-HCl, 1 m*M* EDTA). The samples were then heated at 95°C for 5 min and sonicated for 30 sec at 35% (Fisher Sonic Dismembrator Model 300). The individual bands of β-galactosidase in the continuous runs were quantified with a laser-scanning densitometer (Molecular Dynamics Personal Densitometer).

RESULTS

Plasmid segregational stability of pTKW106 and pMJR1750 in host BK6 was compared in different media with and without induction of the cloned gene (*lacZ*) to evaluate the effectiveness of the *hok/sok* locus.

Specific Growth Rates and Relative Plating Efficiency

The maximum specific growth rates (μ_{max}) of BK6 and BK6/pTKW106 in the four kinds of media with 0, 0.5, and 2 m*M* IPTG were measured and are listed in Table I. As expected, both the addition of pTKW106 to BK6 and expression of β-galactosidase from pTKW106 lead to a significant decrease in μ_{max}. Adding pTKW106 to BK6 decreased μ_{max} by 22% in LB (1.18 → 0.92/h) and by 34% in M9C Trp (1.05 → 0.69/h). These decreases reveal that plasmid replication and expression of plasmid genes other

Table I. Maximum specific growth rates (μ_{max}) of BK6 and BK6/pTKW106 in various media.

Strain	Temp.	Medium	μ_{max}, h^{-1}
BK6	37°C	LB	1.18
BK6/pTKW106 (*hok/sok*$^+$)	37°C	LB	0.92
		LB/0.5 m*M* IPTG	0.79
		LB/2.0 m*M* IPTG	0.75
BK6	37°C	LB/glucose (2 g/L)	1.21
		LB/glucose (60 g/L)	1.03
BK6/pTKW106 (*hok/sok*$^+$)	37°C	LB/glucose (60 g/L)	0.81
		LB/glu (60 g/L)/2.0 m*M* IPTG	0.73
BK6	37°C	Semidefined medium	1.12
BK6/pTKW106 (*hok/sok*$^+$)	37°C	Semidefined medium	0.61
		Semidefined/0.5 m*M* IPTG	0.64
		Semidefined/2.0 m*M* IPTG	0.58
BK6	37°C	M9C Trp	1.05
BK6/pTKW106 (*hok/sok*$^+$)	37°C	M9C Trp	0.69
		M9C Trp/0.5 m*M* IPTG	0.69
		M9C Trp/2.0 m*M* IPTG	0.65
	30°C	M9C Trp	0.33
		M9C Trp/0.5 m*M* IPTG	0.32
		M9C Trp/2.0 m*M* IPTG	0.31

than *lacZ* are a heavy metabolic burden. This burden includes constitutive expression of the plasmid replication machinery, LacI repressor protein, Kan^R protein, and a β-lactamase deletion polypeptide. Additionally, μ_{max} is decreased due to *hok/sok* killing of plasmid-free cells. Upon induction of *lacZ*, the maximum specific growth rate was decreased another 18% (0.92 → 0.75/h) for BK6/pTKW106 in LB medium supplemented with 2 m*M* IPTG and by 6% (0.69 → 0.65) in M9C Trp medium supplemented with 2 m*M* IPTG.

No significance difference in the plating efficiency of the BK6 and BK6/pTKW106 strains on Mac plates was found after growth in LB medium for the three cell densities checked. For example, at OD_{600} = 2.1, 1.42 ± 0.06 × 10^9 BK6 colonies formed per mL compared to 1.41 ± 0.05 × 10^9 BK6/pTKW106 colonies/mL.

Plasmid Stability

As shown in Figure 1, in the absence of cloned-gene expression, the *hok/sok* system maintained pTKW106 (*hok/sok*$^+$) in BK6 for more than 300 h (on average) for all the media tested. However, plasmid pMJR1750 (*hok/sok*$^-$) was only maintained in host BK6 for about 19 h in LB medium. Hence, plasmid-segregational stability in LB was increased by 14-fold (in terms of generations) after the addition of the *hok/sok* locus.

Plasmid stability is more readily compared at different conditions by converting absolute hours of stability to the number of stable generations; these are reported in Table II along with the calculated plasmid loss frequency, *p*, for each fermentation. As listed in Table II, *p* decreased on the order of 10^{30}-fold upon addition of *hok/sok* to the unstable plasmid. This increase in segregational stability did not depend on the medium used, and it reveals dramatically the stabilization that *hok/sok* provides. It should be noted that very small values of *p* (less than 10^{-12}) are variable since they are calculated by solving nonlinear eq. (2) for *p* (e.g., Newton's method); therefore, the value of *p* depends on the tolerance selected (a tolerance of 10^{-7} was used throughout).

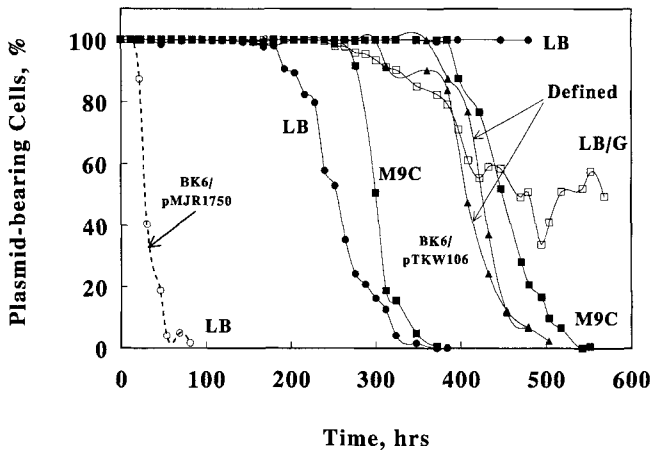

Figure 1. BK6/pTKW106 and BK6/pMJR1750 *hok/sok*- shake-flask stability in different media at 37°C without induction of *lacZ* (0.0 m*M* IPTG) and no antibiotics. Most fermentations performed twice.

Table II. Shake-flask studies of BK6/TKW106 and BK6/pMJR1750 in various media without induction (0.0 m*M* IPTG) at 37°C (no antibiotics).

Strain	Medium	Total time, hr (90% plasmid-bearing)	*N*, Gen. no. (90% plasmid-bearing)	Plasmid-loss mechanism	*p* (plasmid-loss frequency)
BK6/pTKW106 (*hok/sok*$^+$)	LB	192	170	—	10^{-16}
		450	398	Segregational	$<10^{-35}$
	Modified complex medium	324	254	Segregational	$<10^{-36}$
	Semidefined medium	384	224	Structural	$<10^{-36}$
		366	214	Structural	$<10^{-36}$
	M9C Trp	384	255	Segregational	$<10^{-36}$
		281	187	Segregational	$<10^{-30}$
BK6/pMJR1750 (*hok/sok*$^-$)	LB	19	20	Segregational	7×10^{-4}

Gen. no., generation number.
Dual entries indicate that fermentation was performed twice.

To test the *hok/sok* locus under the most stringent conditions possible, β-galactosidase was expressed fully using 2.0 m*M* IPTG.[26] Under these conditions, the plasmid-free host grows 62% faster in LB and M9C Trp than the plasmid-bearing cells (Table I); therefore, plasmid-free cells rapidly take over the fermentation if they are not killed by *hok/sok*. Plasmid stability is shown as a function of time for the shake-flask fermentations in the four media for BK6/pMJR1750 and BK6/pTKW106 in Figure 2, and Table III lists number of stable of generations up to the time at which 10% of the population lost the plasmid. With high β-galactosidase expression, pMJR1750 is extremely unstable and is lost in two generations in both LB and semidefined media. In contrast, the *hok/sok*-stabilized pTKW106 is stable for 35–63 generations in the four media. This result is also reflected in the plasmid loss frequencies: for pMJR1750, *p* was 0.05 in LB compared to 10^{-7} for pTKW106. Therefore, *hok/sok* stabilized the β-galactosidase-expression vector even with high cloned-gene expression.

The type of medium used appears to play a significant role in plasmid stability with high cloned-gene expression (Figure 2 and Table III). Comparing Tables II and III shows

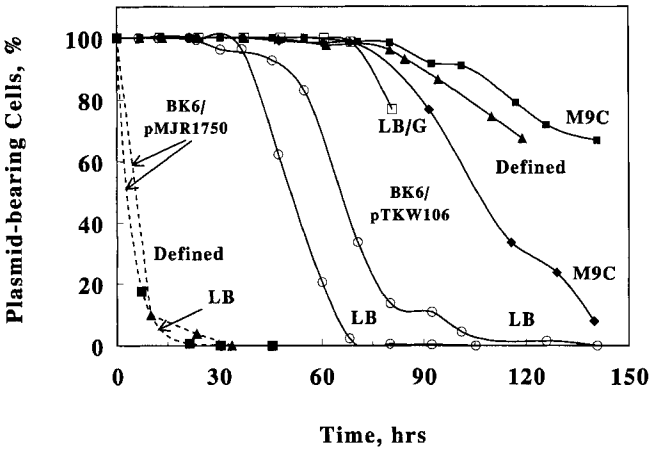

Figure 2. BK6/pTKW106 and BK6/pMJR1750 *hok/sok*-shake-flask stability in different media at 37°C with induction of *lacZ* (2.0 m*M* IPTG) and no antibiotics. Duplicate fermentations shown for some media.

that the number of generations until 90% of the cell population is plasmid-bearing were significantly decreased when 2.0 m*M* IPTG was added to induce β-galactosidase. The average stable generations (gen.) of BK6/pTKW106 were decreased by 88% in LB medium (284 → 35 gen.), 74% for semidefined medium (219 → 57 gen.), and 71% for M9C Trp medium (221 → 63 gen.). Clearly, plasmid segregational stability was superior in M9C Trp (M9C Trp > semidefined medium > LB) for fermentations with constant cloned-gene expression.

The effect of medium on the enhanced stability provided by *hok/sok* is best interpreted in terms of the extent of cloned-gene expression as indicated by specific β-galactosidase activity and fraction of total cell protein that was β-galactosidase. Table IV lists both β-galactosidase-specific enzyme activity and fraction of total cell protein (based on scanning densitometer results of the β-galactosidase band on the SDS–PAGE gel, which is not shown) for the BK6/pTKW106 fermentations in LB, semidefined, and M9C Trp media. Although pTKW106 was least stable in LB 2 m*M* IPTG (35 gen.), β-galactosidase was 15% of total cell protein. In M9C and semidefined media supplemented with 2 m*M* IPTG, pTKW106 was more stable (63 gen. in M9C and 57 gen. in semidefined); however, β-galactosidase was 7% and 6% of total cell protein, respectively. Analogous results were obtained for β-galactosidase-specific activity and the number of generations in which the plasmid was maintained stably: highest β-galactosidase activity and least stability occurred in LB 2 m*M* IPTG medium (Table IV). Good agreement was obtained between the β-galactosidase specific activity and fraction of total cell protein results: the 2.5-fold increase in the β-galactosidase cell-protein fraction in LB IPTG media relative to semidefined IPTG medium is reflected in a 2.2-fold increase in specific activity (Table IV).

Plasmid Structure Examination and Relative Copy Number

To determine the mode of plasmid instability (segregational vs. structural), the plasmid size (structure) was analyzed as

Table III. Shake-flask studies of BK6/pTKW106 and BK6/pMJR1750 in various media with induction (2 m*M* IPTG) at 37°C (no antibiotics).

Strain	Medium/2 m*M* IPTG	Total time, hr (90% plasmid-bearing)	Gen. no. (90% plasmid-bearing)	Plasmid-loss mechanism	*p* (plasmid-loss frequency)
BK6/pTKW106 (*hok/sok*$^+$)	LB	39	33	Segregational	1.4×10^{-7}
		48	36	Segregational	4.2×10^{-8}
	Modified complex medium	77	62	Segregational	2.7×10^{-7}
	Semidefined medium	90	52	Segregational	3.2×10^{-10}
		99	62	Segregational	1.4×10^{-16}
	M9C Trp	77	55	Segregational	3.7×10^{-12}
		101	71	Segregational	3.9×10^{-15}
BK6/pMJR1750 (*hok/sok*$^-$)	LB	2	2	Segregational	5.1×10^{-2}
	Semidefined medium	2	2	Segregational	

Gen. no., generation number.
Dual entries indicate fermentation was performed twice.

a function of time by isolating pDNA from fermentation samples during the course of the experiments and using agarose gel electrophoresis. 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 *Eco*RI, 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 they occurred).

Figure 3 is a representative horizontal electrophoresis gel which shows *segregational* loss of plasmid pTKW106 for fermentations conducted at 37°C in LB and M9C media with 2 m*M* IPTG. As shown, the two pTKW106 bands (from *Eco*RI digestion) decrease gradually over the course of the fermentations (the pBR322 internal standard band migrates with the sixth MW band and is constant); this indicates the plasmid was completely lost during the fermentation and a plasmid-free cell overtook the cell population. These results also corroborate the independent determinations of the fraction of plasmid-bearing cells obtained from red/white colony formation on Mac plates.

Figure 4 is a representative gel that shows that for one of the semidefined fermentations conducted without IPTG at 37°C, β-galactosidase activity decreased as a result of *structural* instability. In this fermentation, a new, 3.8 kb pDNA band (just below the internal standard band) forms and becomes the dominate plasmid as the fermentation continues. This new band is a deletion derivative of pTKW106 and was found for both of the long (average, 219 h), semidefined fermentations that lacked IPTG. After analysis through restriction enzyme digestions (*Bam*HI, *Eco*RI, *Pst*I, *Hin*dIII, *Sma*I, and *Nde*I) and horizontal gel electrophoresis, the deletion derivative was found to contain both the *hok/sok* locus and kanamycin resistance gene, but probably none of *lacZ* gene and only part of the *lacI*^{Q} gene, as shown in Figure 5. Hence, it appears *lacZ* and *lacI*^{Q} are deleted from pTKW106 (based on the disappearance of the *Bam*HI and *Eco*RI sites in this region).

Tables II and III summarize the results of this analysis in terms of the plasmid-loss mechanism for each of the shake-flask fermentations conducted with and without IPTG. For all the batch fermentations except the two conducted in semidefined medium without IPTG, the plasmid loss mechanism was segregational instability (plasmid completely lost without structural modification).

By scanning the pTKW106 band intensities on these gels relative to the constant pBR322 internal standard band, relative copy number was determined for the fermentations. These results are listed in Table IV (and can be roughly estimated from Figs. 3 and 4) for LB, semidefined, and M9C media supplemented with 2 m*M* IPTG. It appears that

Table IV. Fraction of β-galactosidase from SDS–PAGE, specific β-galactosidase activity, and relative copy number of BK6/pTKW106 batch cultures.

Medium (2 m*M* IPTG)	β-gal. (% total cell protein)	β-gal. specific activity, nmol ONPG/(min AU)	Relative Copy no.
LB, 100% plasmid-bearing	14.9	9,076	1.2
Semidefined, 100% plasmid-bearing	6.0	4,118	1.0
M9C Trp, 100% plasmid-bearing	6.9	3,512	1.0

β-gal. β-galactosidase.
Cultures sampled at 24 h in various media supplemented with 2.0 m*M* IPTG at 37°C (no antibiotics).

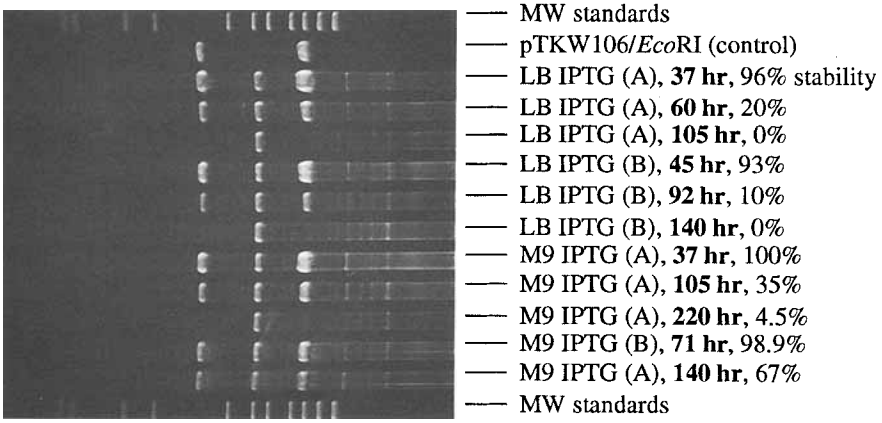

Figure 3. Examination of BK6/pTKW106 shake-flask broth as a function of time to determine the mechanism of plasmid pTKW106 instability in M9C Trp with induction (2.0 m*M* IPTG) and LB with induction (2.0 m*M* IPTG) at 37°C. DNA molecular weight standards from NE Biolabs (lambda *Bst*EII digest, from top right: 8454, 7242, 6369, 5686, 4822, 4324, 3675, 2323, 1929 bp). The pBR322 internal standard (4363 bp) migrates with the sixth MW marker (4324 bp). A and B distinguish duplicate fermentations.

copy number was slightly higher (approximately 20%) for BK6/pTKW106 in LB medium; however, the most significant result is that copy number is relatively constant for these fermentations.

DISCUSSION

This study indicates that the *hok/sok* locus has excellent potential for maintaining plasmids in the absence of antibiotic selection pressure. The insertion of the *hok/sok* locus resulted in more than a 14-fold increase in plasmid segregational stability both for conditions in which β-galactosidase expression was not induced (LB, 284 vs. 20 gen.) and when it was induced fully (LB 2 m*M* IPTG, 35 vs. 2 gen.). Therefore, the *hok/sok* stability locus dramatically increases plasmid segregational stability, even when the cloned gene is constantly expressed at 15% of total cell protein in media that lack antibiotics.

Based on the shake-flask results conducted in the absence of induction of the cloned-gene, there appear to be no media effects on the *hok/sok* system. The *hok/sok* locus maintained the plasmid in the three primary media with almost the same efficiency (LB, 284 gen.; semidefined medium, 219 gen.; M9C Trp, 221 gen.) compared to 20 stable generations for the *hok/sok*$^-$ plasmid. This suggests *hok/sok* can be a reliable and general tool to stabilize plasmids regardless of the medium type used before induction of the cloned gene.

However, media type and extent of cloned-gene expression influenced plasmid stability during growth with constant induction of *lacZ* (2 m*M* IPTG). Cells grown in the minimal medium (M9C Trp) had 1.8-fold greater stability (63 gen.) than cells grown in the rich medium (LB, 35 gen.); however, cells grown in the rich medium expressed 260% more β-galactosidase (based on specific activity). Similar results were found with defined media. Therefore, the effectiveness of the *hok/sok* locus for stabilizing the β-galactosidase-expression vector pMJR1750 is linearly related to the extent of cloned-gene expression. For the three media used, the number of stable generations is inversely proportional to the specific β-galactosidase activity (Table IV, regression coefficient of 0.99). Hence, it appears that the most important parameter for stabilization with *hok/sok* is the extent of cloned-gene expression.

This linear relationship between the extent of cloned-gene expression and *hok/sok*-mediated plasmid maintenance also suggests why the *hok/sok* locus is less effective during high cloned-gene expression. Our hypothesis is that a shortage of ribosomes exists in the plasmid-bearing cell due to high-level expression of the cloned gene (which requires ribosomes); therefore, cells that lose the plasmid (but have been expressing the cloned gene) are unable to synthesize sufficient amounts of the killer protein, with the effect that some plasmid-free daughter cells escape the killing process (*hok/sok* efficiency is decreased) and take over the fermentation. This scenario is likely because efficient translation of the Hok killer protein is completely dependent on translation of the upstream *mok* reading frame that is present on the killer mRNA.[22] It appears that the translation initiation region (TIR) of the *hok* gene may be sequestered in a stable secondary structure of the mRNA which prevents ribosomes from translating the killer message; translation of the upstream *mok* reading frame may open the *hok* TIR so that the killer protein may be translated.[22] Therefore, efficient translation of each Hok polypeptide requires two ribosomes: one translating the Mok polypeptide (and relaxing the mRNA secondary structure) and another translating the Hok killer message.

The idea that there may be a shortage of ribosomes within the recombinant-protein-producing cell has been suggested before.[25,26] For expression of cloned genes, specialized ribosomes have been shown to enhance productivity by supplying an alternative pool of ribosomes specific for the

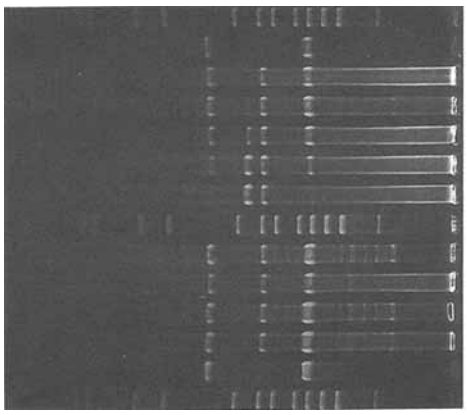

Figure 4. Examination of BK6/pTKW106 shake-flask broth as a function of time to determine the mechanism of plasmid pTKW106 instability in *semidefined medium* with induction (2.0 m*M* IPTG) and without induction (0.0 m*M* IPTG) at 37°C. DNA standards as in Figure 3. B indicates the second of duplicate fermentations.

cloned gene.[11,27] Furthermore, Birnbaum and Bailey have shown that as copy number increases from 0 to 240 (and constitutive expression of β-lactamase on the plasmids increases), the ribosome content, translational proteins, and free 30S and 50S ribosomal subunit pools decrease.[1]

From the current work, the evidence (indirect) that supports inefficient translation (or transcription) of the *hok* mRNA as the cause of eventual segregational instability is based on the change in specific growth rates (μ^+ and μ^-). For BK6/pTKW106 cells that constantly produce β-galactosidase (2 m*M* IPTG), the relative reduction in μ upon adding the plasmid to the host is approximately the same for cells grown in either LB or M9C ($\mu^-/\mu^+ = 1.57$ for LB and 1.62 for M9C). However, there is a substantial difference in the number of stable generations for which the plasmid is maintained in LB vs. M9C (35 gen. for LB vs. 63 gen. for M9C). This indicates that there must be a significant difference in the *rate* the cells lose the plasmid and survive (i.e., the killing efficiency of *hok/sok* as measured here by *p*), since there is very little difference in the ratio μ^-/μ^+ [from eq. (3), the number of generations required for plasmid-free cells to become 10% of the population is a

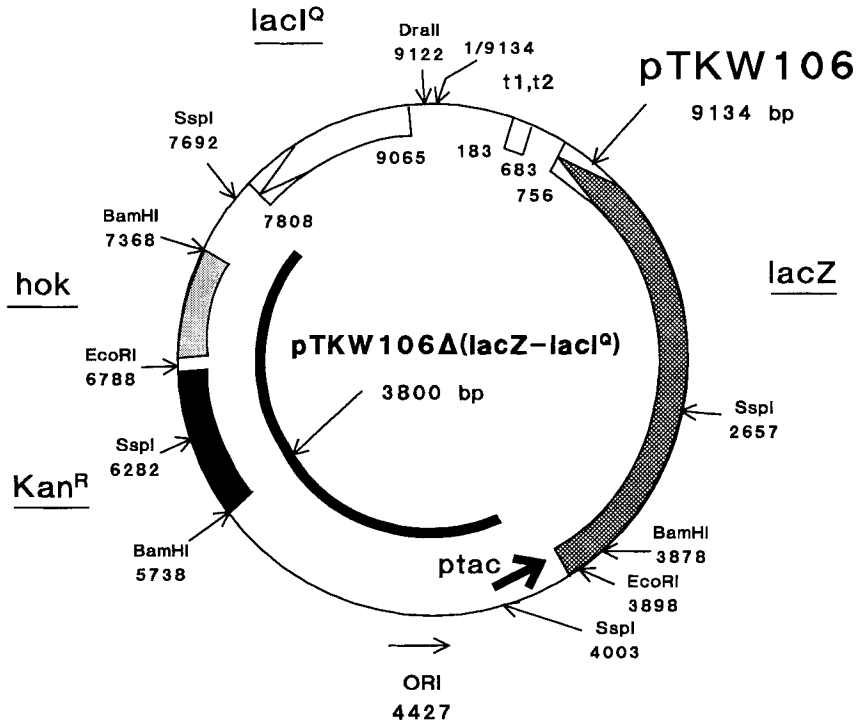

Figure 5. Plasmid maps of the β-galactosidase-expression vector pTKW106 (9134 bp) and the 3800-bp deletion derivative of pTKW106, pTKW106Δ(*lacZ-lacI*^{Q}). The inner black curve indicates the DNA of pTKW106 that is contained in pTKW106Δ(*lacZ-lacI*^{Q}).

function of only μ^-/μ^+ and the plasmid loss frequency, p]. Since gene dosage does not vary very much (copy number is approximately constant for growth in LB and M9C at 37°C), the difference in stability seems to be related to the amount of expression of β-galactosidase (15% total cell protein for LB vs. 7% for M9C).

This conclusion is supported by comparing the stability of BK6/pTKW106 cells in M9C medium. Without IPTG, the relative growth rate for plasmid-free and plasmid-bearing cells is again about 1.5; yet, the cells with *hok/sok* are much more stable than those with *hok/sok* that produce β-galactosidase in M9C Trp (221 vs. 63 gen.). Hence, it appears that β-galactosidase expression may decrease the availability of free ribosomes (since gene dosage is again approximately constant), and this leads to inefficient translation of Hok proteins as a result of strong competition for the finite energy pool and translational machinery of the cell.

The increase in stability due to addition of the *hok/sok* locus suggests that the *hok/sok* locus may be used to prevent plasmid loss in many commercial batch and fed-batch fermentations operated without antibiotic. The *hok/sok* locus in this study ensured at least 200 generations of growth of 100% plasmid-containing cells without induction, so a 100% plasmid-containing seed should be maintained easily for 30 generations of growth for the pre-seed tank and seed tank (as long as a tightly regulated promoter of the cloned gene is used). Since *hok/sok* has been shown to stabilize *fully-induced* cells from 35–63 generations (depending on the medium), the population should be plasmid-bearing throughout the production period. In practice, cells would increase in number slowly during the period of cloned-gene induction in a fed-batch fermentation and not undergo as many generations of exponential growth as the *hok/sok*-containing cells in this study.

CONCLUSIONS

The *hok/sok* locus has great potential for increasing the segregational stability of recombinant plasmids in fed-batch fermentations conducted without antibiotic. We have shown that adding the *hok/sok* locus to the unstable, β-galactosidase-expression vector pMJR1750 dramatically increases plasmid segregational stability both for the case of no induction of the cloned gene *lacZ* (200 vs. 20 gen.) and with constant, full expression of β-galactosidase at 6–15% of total cell protein (33–63 vs. 2 gen.). The number of generations for which the plasmid was stable was found to be linearly dependent on the extent of cloned-gene expression (specific β-galactosidase activity), and the mechanism of plasmid loss for BK6/pTKW106 in these shake-flask experiments was primarily segregational instability.

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