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ENHANCED PLASMID STABILITY THROUGH POST-SEGREGATIONAL KILLING OF PLASMID-FREE CELLS

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SUMMARY

In order to develop an extremely stable, inducible host/vector system, the following genetic manipulations were made: a *recA* mutation was introduced into the chromosome of the host strain, the plasmid selectable marker was changed from ampicillin to kanamycin, and the *parB* stability locus of plasmid R1 was added to the plasmid. The stability of the new vector, pTKW106, was increased such that the fraction of plasmid-bearing cells present during chemostat fermentations under selective pressure increased from 1.75% to 100% when plasmid protein production was fully induced. At this level of induction, β-galactosidase represents 10% of the total cell protein. In addition, the frequency of generation of plasmid-free cells was shown to decrease from 1.0 per generation to less than 10^{-11} with full promoter induction under non-selective conditions.

INTRODUCTION

Plasmid instability is a primary impediment to industrial utilization of recombinant microorganisms. As the vector becomes more effective in directing protein production, it becomes an increasing drain on cellular metabolism. Betenbaugh et al. (1989) have shown that the growth rate of the plasmid-bearing cell is reduced relative to the plasmid-free cell as cloned-gene expression is increased through either plasmid amplification or promoter induction. Hence, faster-growing, plasmid-free segregants can rapidly outnumber the plasmid-bearing population and greatly reduce the yield of recombinant protein from the culture. In addition, structural instability through homologous recombination events can lead to plasmid derivatives that no longer produce the desired protein.

As a result of the segregational and structural instability, the plasmid initially chosen for our studies, pMJR1750 (Stark, 1987), was extremely unstable in the host strain AMA1004 (Casadaban et al., 1983), even in the presence of antibiotics. This plasmid was chosen to help discern the metabolic impact of recombinant protein expression because it allows transcription of β-galactosidase to be tightly regulated by the addition of IPTG to the fermentation medium. In order to increase the stability of this vector, three changes were made to the host/plasmid system: the *recA* gene was deleted from the host, the antibiotic selection marker was changed from ampicillin (Ap) to kanamycin (Kan), and the *parB* stability locus of plasmid R1 (Gerdes, 1988) was added to the plasmid.

Structural instability should be reduced considerably by the *recA* deletion. This deletion should severely limit homologous recombination between the plasmid and chromosome since Csonka and Clark (1979) have shown the Δ(*srl-recA*)306 mutation used in this work decreases the rate of recombination of the host during conjugation by a factor of 36,000. Additionally, Laban and Cohen (1981) have shown that a *recA* point mutation lowers the frequency of recombination events within a plasmid by 100-fold.

To provide more effective selection pressure, the selectable marker was changed from β-lactamase

(Ap^r) to aminoglycoside 3'-phosphotransferase II (APH, Kan^r). Unlike APH, β-lactamase is transported to the periplasmic space and leaks in sufficient quantity from plasmid-bearing cells to degrade rapidly the ampicillin in the medium (Kemp and Britz, 1987; Pierce and Gutteridge, 1985). The resultant removal of selection pressure allows the plasmid-free cells to dominate the culture (Nishimura et al., 1987). The use of APH should limit the extracellular clearance of antibiotic from the medium.

The main genetic tool used to boost stability of plasmid pMJR1750 is the *parB* locus isolated from the multiple resistance factor R1 by K. Gerdes (1988). This locus stabilizes plasmids in a population by encoding a cell-killing gene (*hok*) whose mRNA is activated only when the cell loses the plasmid. Upon loss of the plasmid, the 52 amino acid Hok protein is expressed, and the cell is rapidly killed due to a collapse of the transmembrane potential and cessation of respiration. Therefore, although this locus does not change the rate of appearance of plasmid-free segregants, it kills them as they are generated. The *parB* locus is the only system known which utilizes this post-segregational killing mechanism.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

The *E. coli* strain AMA1004 (Casadaban et al., 1983) was chosen as a suitable host. Its genotype is: *Δ(lacIPOZ)C29 lacY⁺ hsdR galU galK strA^r leuB6 trpC9830* with the result that AMA1004 cannot produce β-galactosidase due to a stable deletion, but it can produce β-galactoside permease. Hence, this strain can be used conveniently with MacConkey agar plates to indicate cells harboring plasmids expressing *lacZ*. Plasmid-bearing cells form red colonies whereas plasmid-free cells form white colonies.

The tightly-regulated expression vector pMJR1750 (Figure 1) capable of producing large quantities of β-galactosidase was obtained from Stark (1987). It includes the strong *tac* promoter upstream of the β-galactosidase gene as well as the $lacI^Q$ gene for complete repression. Multiple copies of the *tac* promoter arising from multiple copies of the plasmid can titrate the repressor if $lacI^Q$ is not placed on the expression vector (Stark, 1987). It follows that by adding the non-cleavable lactose analog IPTG to the fermentation medium, transcription of *lacZ* can be induced over a wide range. This vector has the additional advantage that its DNA sequence has been completely determined.

Deletion of *recA*

In order to limit homologous recombination, P1 *kc* generalized transduction was used to introduce a *recA* deletion into the host strain AMA1004. The donor strain for the transduction was constructed by Ihara et al. (1985) and consists of the host JC10289 with the genotype Δ(*srl-recA*)306::Tn*10*, and the helper plasmid pKY102 which is $recA^+$ and ampicillin-resistant. The method of Silhavy et al. (1984) was used to perform the transduction of AMA1004 and consists of forming a P1 lysate from the donor strain and transfering this lysate to the recipient strain.

Transductants were selected by tetracycline resistance (15 μg/mL) on LB plates and scored for ultraviolet light sensitivity. The UV sensitivity test involved shining UV light at 260 nm (UVP Inc. Model R-526) from a distance of 3.1 cm from colonies on LB plates for periods of 5, 10, or 15 seconds. The transductant colonies were checked for growth and compared to $recA^-$ and $recA^+$ controls that were also irradiated. The resulting *recA* deletion strain derived from AMA1004 was named BK6.

Insertion of *parB* and Kan^R

In order to obtain the *parB* locus, pKG1022 (Gerdes, 1988) was isolated from CSH50 using chloramphenicol amplification and CsCl centrifugation as described previously (Wood and Peretti, 1989). The recipient vector pMJR1750 was isolated from AMA1004 following transformation with DNA supplied by Stark. The *parB* locus was excised from pKG1022 along with *aphA* (Kan^r) using *Hind*II (Boeh. Mann.), creating a DNA fragment of 1630 bp. The recipient plasmid pMJR1750 (7550 bp) was digested with *Sca*I (Boeh. Mann.) at a unique site, abolishing ampicillin resistance. The two restricted plasmids (2.5 μg each) were dialyzed and blunt-end ligated (Rodriguez and Tait, 1983) using T4 DNA ligase (BRL). BK6 was transformed with the ligated DNA following the method of Maniatis et al. (1982).

Transformants were selected as red colonies on MacConkey-Kan (50 μg/mL) plates and scored for ampicillin sensitivity on MacConkey-Ap (50 μg/mL) plates. The resulting plasmid, pTKW106, is shown in Figure 1. The plasmid size and the orientation of the *parB* locus were checked by restriction mapping with *EcoRI*.

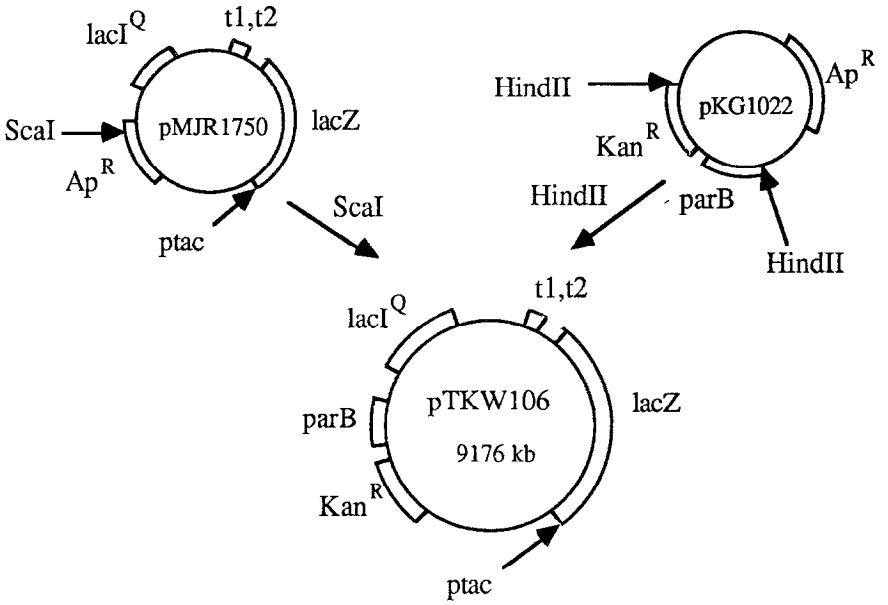

Figure 1. Construction of pTKW106

Fermentations

The stabilities of AMA1004/pMJR1750 and BK6/pTKW106 were evaluated by chemostat fermentations using selection pressure. The media used was M9 minimal medium (Rodriguez and Tait, 1983) containing 0.2 wt% glucose, 0.4 wt% casamino acids and 0.0018 wt% tryptophan (Trp). Kanamycin (sulfate salt, 100 μg/mL) or ampicillin (sodium salt, 400 μg/mL) was used to maintain selection pressure for the appropriate plasmid. IPTG (U.S. Biochemicals) was added to the medium from 0.0 to 7.5 mM, and silicon antifoam was present at 50 μg/mL to minimize foaming.

The strains were grown at a dilution rate of 0.6 hr^{-1} in a 3.0 L Applikon Fermentor with 1L culture volume (Wood and Peretti, 1989). The pH was controlled at 7.0 ± 0.05, and the temperature was maintained at 37.0 ± 0.1°C. The dissolved oxygen levels were monitored and maintained above 60% of saturation. Agitation was constant at 1000 rpm. The culture was assumed to have achieved steady-state after continuous feed of five culture volumes. On-line monitoring of the reactor effluent with a Bausch and Lomb Spec 21 UV spectrophotometer indicated that the culture absorbance had stabilized by that time. The number of viable cells at steady-state was determined by diluting the reactor samples and plating on 10 MacConkey agar plates. Plasmid segregational instability, if present, was indicated by the appearance of white colonies.

SDS-PAGE (Miller et al., 1986) was used to discern the percentage of total protein that was plasmid-mediated for chemostat cultures of BK6/pTKW106 with 1.0 mM IPTG induction. A 12 wt% acrylamide gel was used, and the individual bands were quantitated with a scanning densitometer (Hoeffer).

Specific growth rates were determined for all strains at 37°C using M9C-Trp media with 0.4 wt% glucose. For the plasmid-bearing strains, growth rates were determined in the presence of 0.0 and 0.5 mM IPTG in batch cultures after inoculation with a single, plasmid-bearing colony from MacConkey agar plates. To determine the frequency of appearance of plasmid-free segregants, single colonies of AMA1004/pMJR1750, BK6/pMJR1750, and BK6/pTKW106 were used to inoculate M9C-Trp medium with 0.4 wt% glucose in the absence of selection pressure. During exponential growth, samples were diluted and spread on MacConkey agar plates to determine the fraction of plasmid-bearing cells as a function of time.

RESULTS

Batch Stability

The specific growth rates of the plasmid-bearing strains were lower than those of the plasmid-free hosts (~ 0.9 hr^{-1}), and steadily decreased as production of β-galactosidase was induced with IPTG (Table I). This decrease reflects the metabolic drain of plasmid maintenace and cloned-gene expression within these hosts. These growth rates were determined in order to quantify the probability that plasmid-free segregants would arise. In an exponentially-growing batch population, the frequency of plasmid-free cells arising per cell division, p, can be determined for a population starting with 100% plasmid-bearing cells using the following equation (Imanaka and Aiba, 1981):

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

where F is the fraction of the total population carrying the plasmid, n is the number of cell generations at which F is measured, and α is the ratio of the specific growth rate of the plasmid-free cell divided by the specific growth rate of the plasmid-bearing cell.

The frequency of appearance of plasmid-free segregants (p) was determined to be 0.02 per generation for AMA1004/pMJR1750 in minimal medium without induction (Table 1). The *recA* mutation within BK6 reduced this to 6 x 10^{-4}. Addition of the *parB* locus reduced p to less than 10^{-16} for BK6/pTKW106 since no plasmid instability was detected after 75 generations (Padukone, 1989).

The same behavior was noted for growth in the presence of IPTG. When induced with 0.5 mM IPTG, the frequency of plasmid-free segregation was reduced from approximately 1.0 (theoretical maximum) to 0.22 upon addition of the *recA* mutation. The presence of the *parB* locus further reduced this frequency to less than 10^{-11} (no plasmid instability was detected for over 20 generations).

Strain	IPTG, mM	Specific Growth Rate, 1/hr	loss/generation, p
AMA1004	0.0	0.94	na
BK6	0.0	0.89	na
AMA1004/pMJR1750	0.0	0.88	2 x 10^{-2}
BK6/pMJR1750	0.0	0.80	6 x 10^{-4}
BK6/pTKW106	0.0	0.68	<1 x 10^{-16}
AMA1004/pMJR1750	0.5	-	1.00 (est.)
BK6/pMJR1750	0.5	-	0.22
BK6/pTKW106	0.5	0.61	<1 x 10^{-11}

Table I. Growth Rates and Plasmid Loss Rates in M9C-Trp Medium

Chemostat Stability

To compare the stability of the new expression system, BK6/pTKW106, with the original, AMA1004/pMJR1750, both were cultured individually with selection pressure in a chemostat using

M9C medium at a dilution rate of 0.6 hr^{-1}. The stabilities of these strains at different levels of induction are presented in Table II as the percent of the population bearing plasmids at steady-state. The parent system was very unstable even in the absence of IPTG. No instability was noted for the *parB* system even as the expression of β-galactosidase was increased 460-fold. The SDS-PAGE results indicate that for the chemostat culture of BK6/pTKW106 induced with 1.0 mM IPTG (full induction), β-galactosidase was 10% of the total protein. Furthermore, APH and the *lac* repressor protein were each estimated to be present at 2-5% of the total protein, so the plasmid directed the synthesis of 15-20% of the total cellular protein.

IPTG, mM	% Plasmid-Bearing Cells AMA1004/pMJR1750	% Plasmid-Bearing Cells BK6/pTKW106
0.00	12.72	100
0.01	5.18	100
0.05	0.89	100
0.10	0.05	100
0.50	1.75	100
1.00	-	100
7.50	-	100

Table II. Chemostat Stability for AMA1004/pMJR1750 and BK6/pTKW106

DISCUSSION

The genetic manipulations of the host/plasmid system proved extremely effective in enhancing plasmid stability. The impact of introducing the *recA* mutation is illustrated by the reduction in the frequency at which plasmid-free segregants arise (p) for the two hosts bearing pMJR1750. Under non-inducing conditions, a 33-fold decrease in p was observed for BK6 (*recA*$^-$) as the host relative to AMA1004 (*recA*$^+$). The relative decrease in p due to the *recA* deletion was only five-fold upon IPTG induction of *lacZ*. The *recA* mutation may enhance segregational stability significantly by limiting the formation of plasmid multimers, which form readily in *recA*$^+$ hosts (Bedbrook and Ausubel, 1976). Plasmid multimerization has been shown to increase segregational instability by decreasing the number of partitioning molecules (Summers and Sherratt, 1984).

A dramatic increase in plasmid stability occurred upon addition of the *parB* locus to the expression vector. In the absence of antibiotic selection pressure, p decreased by over ten orders of magnitude for both induced and non-induced conditions. Furthermore, chemostat stability increased to 100% at all levels of transcription induction in the presence of kanamycin using BK6/pTKW106. The original expression system, AMA1004/pMJR1750, was unstable in the chemostat even with the continuous addition of ampicillin at 400 μg/L (1.75% plasmid-bearing at 0.5 mM IPTG). This result underscores the inadequacy of the β-lactamase gene as an effective selective marker for unstable plasmids.

True partitioning loci, which aid stabilization of plasmid maintenance by increasing the fidelity of plasmid partitioning, have not proved fully effective in stabilizing high expression vectors (Skogman et al., 1983). In contrast, the *parB* system, which actively enforces plasmid stability through the post-segregational killing of any plasmid-free segregants that arise, effectively stabilized a fully-induced expression vector directing production of 15-20% of the total cell protein. This result is highly significant since it illustrates the potential of *parB* as a stabilizing element in large-scale production processes where antibiotic addition is undesirable due to its expense and the resulting contamination of

the product stream (Ensley, 1985).

The genetic stabilization strategy employed in this work can be applied to any *E. coli* host/vector system. The *recA* mutation can be conveniently introduced into the host using P1 transduction, and Gerdes (1988) has constructed plasmids with *parB* cassettes which allow easy manipulation of the *parB* gene. This allowed us to replace the β-lactamase gene with the genes for kanamycin resistance and *parB* in a single step. Furthermore, Gerdes has found the *parB* locus to be an effective plasmid-stabilizing element in a number of gram-negative strains, increasing the generality of this approach.

In addition, the host/plasmid system developed here (BK6/pTKW106) is a very attractive model system for kinetic studies of the impact of cloned-gene expression on cell metabolism. Gene expression of an easily assayed enzyme, β-galactosidase, can be varied almost 500-fold in a stable chemostat culture by varying IPTG concentrations in the medium. This allows molecular level responses (mRNA synthesis, ribosome population size, macromolecular stability) to be analyzed over a wide range of conditions in a effort to determine the metabolic processes most significantly affected by cloned-gene expression.

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