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## Plasmid Stability in PHA-Producing Recombinant *Escherichia coli* Strains

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**Abstract:** Polyhydroxyalcanoates (PHAs) are biodegradable polyesters synthesized by numerous prokaryotic organisms from renewable carbon sources. In this study, the stability of three plasmids (pBHR68, pBHR71 and pBHR77), containing the PHA biosynthesis genes from *Ralstonia eutropha* and *Pseudomonas aeruginosa*, was investigated in recombinant *E. coli* JM101 and DH10B cultures performed at 37°C for 96 h. After 72 h of cultivation, cell growth was resumed due to ampicillin depletion, which allowed the recovery of plasmid-free cells. All plasmids showed almost 100% stability for the first 24 h and up to 30-35 generations. The most stable culture, *E. coli* strain JM101 harboring the plasmid pBHR68, achieved 50% after 110 generations of growth, while, a similar reduction was observed in the other cultures within 60-80 generations. The best performance of the JM101 strain cultures concerning plasmid stability can be attributed to its best adaptability to the environmental conditions and stability of plasmid-host system.

**Key words:** Plasmid stability, biopolymer, PHA, polyhydroxyalcanoate, recombinant *Escherichia coli*

### INTRODUCTION

Genetic engineering techniques have provided the biotechnology industry with an increasing number of modified microorganisms capable of producing a wide range of valuable substances, which are synthesized by genes cloned into expression vectors (Zhang *et al.*, 1996). These expression vectors (or plasmids) are semi-autonomous genetic elements that replicate independently of the host cell chromosome (Bingle and Thomas, 2001).

One of the most important factors that determine the ability of a recombinant cell to express its genes is the capacity of retaining plasmids, which is called plasmid stability. It can be affected by diverse factors such as the difference in specific growth rates of plasmid-bearing and plasmid-free cells (Nancib and Bordrant, 1992; McDermott *et al.*, 1993); genetic characteristics of strains (Spalding and Tuite, 1989); structural characteristics of plasmids (Fiedler and Skerra, 2001); nutritional stress (Matsui *et al.*, 1990; Gupta *et al.*, 1995). Thus, a certain probability of vector loss per generation (cell duplication) must be taken into consideration.

Segregational instability results from plasmid loss at cellular division due to mother cell failure to distribute replicated plasmids between daughter cells. Among the techniques applied to prevent such instability is the use of partitioning (Meacock and Cohen, 1980; Niki and

Hiraga, 1997), high copy number plasmids (Ryan and Parulekar, 1991; Summers, 1998) and post-segregational killing of plasmid-free cells (Gerdes *et al.*, 1986, 1997).

Paradoxically, high copy number plasmids might bring instability due to gene over expression. As a consequence, diverse methods for culture stabilization have been considered. In order to control plasmid-free cell population, environmental selective pressure (use of selective media) has been widely employed, even though it has not been always effective (Zhang *et al.*, 1996). Likewise, many mathematical models that govern the process of plasmid loss have been developed (Shoham and Demain, 1991; van der Sand *et al.*, 1995; Gupta *et al.*, 2001) and have helped to optimize cell growth and gene expression in recombinant strain fermentations.

In order to succeed, models have to consider not only the difference between specific growth rates of plasmid-bearing and plasmid-free cells but also the segregation of plasmids at cell division. These parameters were first estimated by Moser (1958) and are often taken into account, even though refinements are continuously made.

Polyhydroxyalcanoates (PHAs) are biodegradable, thermoplastic polymers synthesized by numerous prokaryotic organisms and stored in cytoplasmic inclusion bodies as a carbon/energy reserve material. For their physicochemical properties, some of them have been considered promising substitutes for petrochemical-based

polymers. For successful implementation of PHA production processes, optimization of fermentation conditions is required (Lee, 1996; Lee and Choi, 1999).

Thus, the stability of recombinant strains (*E. coli* JM101 and DH10B), carrying plasmids involved in the synthesis of polyhydroxyalkanoates (PHAs), was investigated. Besides, plasmid stability in a complex medium, which had never been studied, was also evaluated.

## MATERIALS AND METHODS

This study was conducted between 2003 and 2004 at Federal University of Santa Catarina, Brazil.

**Strains and plasmids:** The plasmids and strains employed are described in Table 1, as well as their relevant genotypic characteristics.

**Competent cell preparation and *E. coli* transformation:** The competent cells were prepared using a method based on membrane permeabilization by a calcium chloride solution (Hanahan, 1983) and transformed by plasmid insertion, according to a classic methodology (Sambrook and Russell, 2001).

**Culture media and conditions:** Inocula were prepared by transferring a loopful of cells from a freshly grown agar plate culture to 125 mL Erlenmeyer flasks containing 25 mL of Luria-Bertani (LB) medium (Sambrook and Russell, 2001) and ampicillin (50 mg L<sup>-1</sup>) on a rotary shaker at 37°C and 150 rpm until optical density (OD<sub>600</sub>) 1.2. A 2% (v v<sup>-1</sup>) inoculum, grown as described above, was transferred into flasks in order to perform the cultures properly said, which, in turn, were carried out in 500 mL baffled Erlenmeyer flasks containing 100 mL of LB medium and ampicillin (50 mg L<sup>-1</sup>) for 96 h under the same conditions. Ampicillin was previously sterilized by filtration.

Table 1: Relevant genotypic characteristics of the plasmids and *E. coli* strains employed

Strain or plasmid	Relevant genotypes	References
<b>Plasmids</b>		
pBHR68	<i>amp<sup>r</sup></i> , PHA operon from <i>R. eutropha</i>	(Spiekermann <i>et al.</i> , 1999)
pBHR71	<i>amp<sup>r</sup></i> , <i>phaC1</i> from <i>P. aeruginosa</i>	(Langenbach <i>et al.</i> , 1997)
pBHR77	<i>amp<sup>r</sup></i> , <i>phaC1</i> gene from <i>P. aeruginosa</i> and PHA operon from <i>R. eutropha</i>	(Antonio <i>et al.</i> , 2000)
<b><i>E. coli</i> strains</b>		
DH10B	-	(Stratagene, La Jolla, USA)
JM101	<i>thi-1</i>	(Stratagene, La Jolla, USA)
<i>R. eutropha</i> operon- <i>phaA</i> , <i>phaB</i> e <i>phaC</i> : genes coding for acetoacetyl-CoA reductase, β-cetotiolase and PHA synthase from <i>R. eutropha</i> , respectively; <i>phaC1</i> : gene coding for PHA <sub>MCL</sub> synthase from <i>P. aeruginosa</i> ; <i>amp<sup>r</sup></i> : gene coding for ampicillin resistance; <i>thi-1</i> : gene coding for thiamine		

**Analytical procedures:** Cell growth was estimated by measuring the optical density of culture samples at 600 nm. Maximum specific growth rates of plasmid-bearing cells (μ<sup>+</sup>) and plasmid-free cells (μ<sup>-</sup>) were calculated from the slope obtained by plotting biomass on a logarithmic scale and time on an arithmetic scale. The parameter α (segregation coefficient) represents the ratio of μ<sup>-</sup> to μ<sup>+</sup>. The probability of plasmid loss per generation (p) was calculated from the slope obtained in a semi-log plot of plasmid stability vs. time. Plasmid-free (X<sup>-</sup>) and plasmid-harboring (X<sup>+</sup>) cell concentrations were obtained by multiplying cell concentrations (X) by the corresponding fraction.

**Plasmid stability:** In order to evaluate segregational plasmid stability (triplicate analyses), samples were taken from liquid cultures in 6 h intervals up to 36 and 24 h intervals from 48 to 96 h. After appropriate dilutions with sterilized saline solution, aliquots were plated on LB/agar medium with and without ampicillin (50 mg L<sup>-1</sup>). Bacterial colonies were counted after 24 h of incubation at 37°C. Plasmid stability (% plasmid-bearing cells) was defined as the ratio of the number of colonies grown on a medium with ampicillin to the colonies grown on a medium without the antibiotic.

To estimate the number of generations, cells were serially sub-cultured in test tubes containing 10 mL of LB medium. Cells were grown (until reaching) to an OD<sub>600</sub> of approximately 1 and appropriate volumes were transferred to a fresh medium so that the initial OD<sub>600</sub> could be restored after 10 generations.

## RESULTS

Figure 1 supplies the biomass concentration, measured by the absorbance, while Fig. 2 and 3 show the variation of the cells cultures with and without plasmid, vs. time, respectively. Figure 4 indicates the counting forming units of colonies vs. time. Figure 5 show the percentages of the number of cells harboring plasmids vs. time, while Fig. 6 indicates the relation among the fraction of cells harboring plasmid (F) vs. number of generations (n). Overall, low cell growth was observed in all experiments (Fig. 1 and Table 2). For Plasmid-bearing cells

Table 2: Maximum specific growth rates (μ<sub>max</sub>), the probability (p) of plasmid loss per generation and the coefficient of segregation (α)

Strain and plasmid	μ <sub>max</sub> (h <sup>-1</sup> )	(p) generation <sup>-1</sup>	α
JM101 (pBHR68)	0.167	0.0010	1.64
JM101 (pBHR71)	0.250	0.0013	1.86
JM101 (pBHR77)	0.288	0.0015	1.95
DH10B (pBHR71)	0.192	0.0017	1.99
DH10B (pBHR77)	0.315	0.0019	2.10

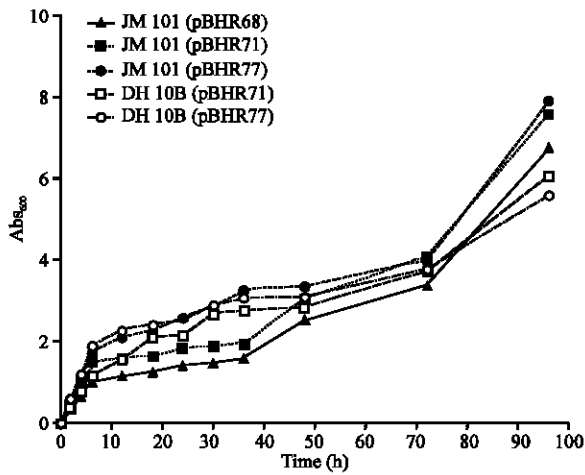


Fig. 1: Cell growth vs. time in cultures of *Escherichia coli* DH10B and JM101, harboring the plasmids pBHR68, pBHR71 and pBHR77

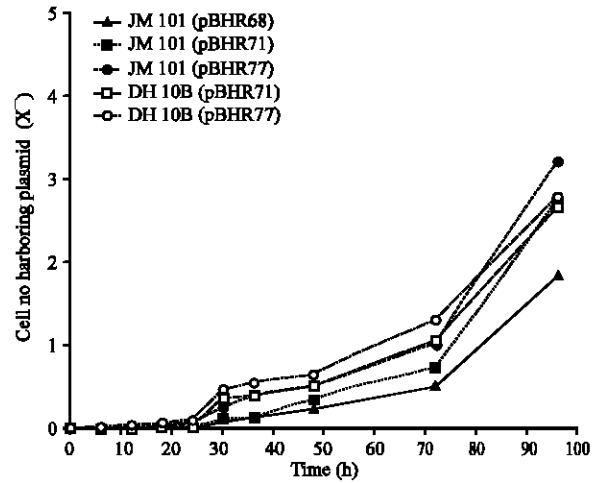


Fig. 3: Dynamics of plasmid-free cells in cultures of *Escherichia coli* DH10B and JM101, no harboring the plasmids pBHR68, pBHR71 and pBHR77

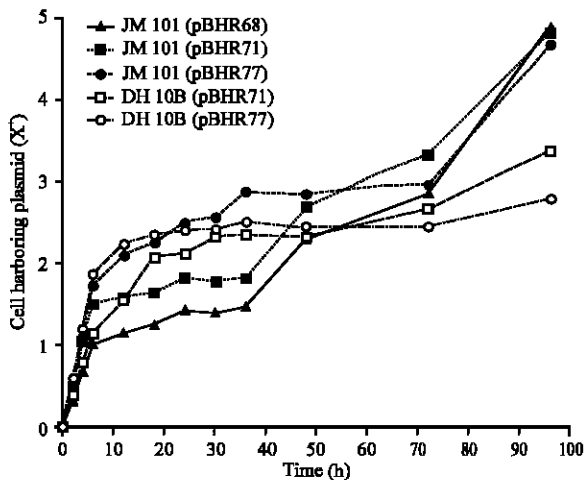


Fig. 2: Dynamics of plasmid-bearing cells in cultures of *Escherichia coli* DH10B and JM101, harboring the plasmids pBHR68, pBHR71 and pBHR77

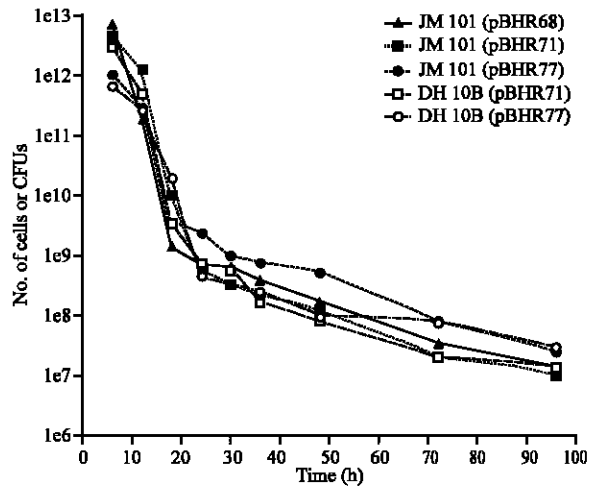


Fig. 4: Number of Colony Forming Units (CFU) of *Escherichia coli* DH10B and JM101, harboring the plasmids pBHR68, pBHR71 and pBHR77 vs. time

(Fig. 2), a 6 h exponential growth phase took place followed by a stationary phase that continued until 48 h, and a new increase of absorbance after 72 h. The concentration of cells without plasmids is stagnated at zero until 24 h. After this, lose of plasmids is more evident with an abrupt increase after 72 h of culture (Fig. 3). The Colony Forming Units (CFU) harboring plasmid decrease with time (Fig. 4).

The practically 100% stability until 24 h (Fig. 5) represents 30-35 generations (Fig. 6), according with biomass growth measured. In 48 h, time normally adopted by the authors for the accomplishment of batch cultures,

the presented stability still was sufficiently raised, having been, in average, of 89% for *E. coli* JM101 and 80% for *E. coli* DH10B. In 96 h, the minimum stability detected was 50% in *E. coli* DH10B (pBHR77). The studied strains and plasmids reach a stability of 50% between 60-80 generations, except for *E. coli* JM101, harboring the plasmid pBHR68, which reaches this value after 110 generations. The minimum of 10% of stability is reached between 75-100 generations, except for *E. coli* JM101 (pBHR68) that reaches this value around 140 generations after the start of the culture (Fig. 6).

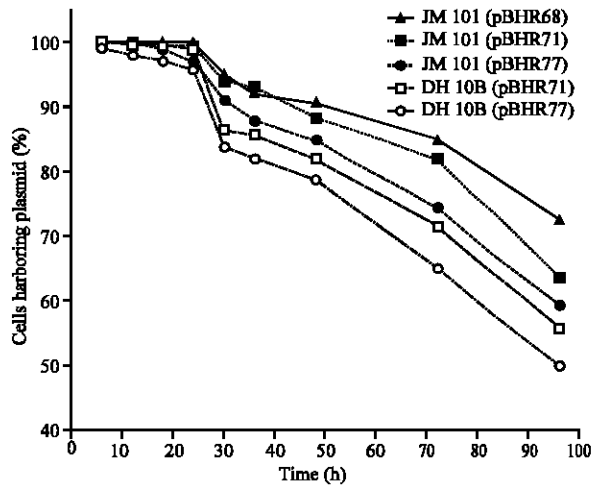


Fig. 5: Plasmid stability (%) vs. time in cultures of *Escherichia coli* DH10B and JM101, harboring the plasmids pBHR68, pBHR71 and pBHR77

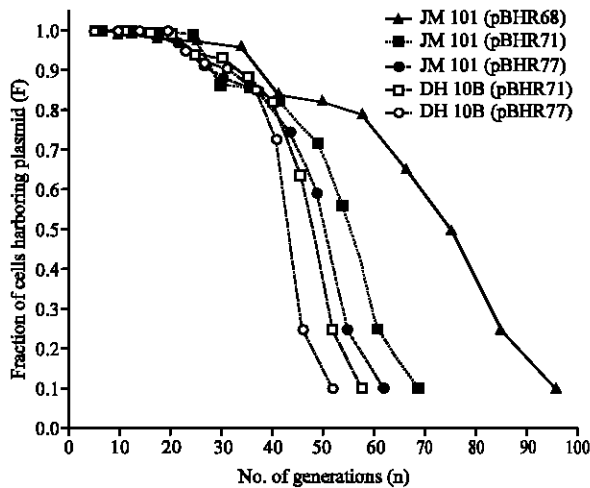


Fig. 6: Fraction of plasmid-bearing cells (F) vs. number of generations (n)

Table 2 shows the maximum specific growth rate ( $\mu_{max}$ ), the probability (p) of the cell lose the plasmid during the cellular division, and the coefficient of segregation ( $\alpha$ ). According to the  $\alpha$  values obtained, the relation between the specific rates of cells without plasmid with that ones that contains them tends to those that does not harbor plasmids, due the presented values have been higher that 1. Almost all the obtained values meet inside of the typical interval of variation, among 1 and 2.

**DISCUSSION**

The growth curve of *E. coli* strains DH10B and JM101, harboring the plasmids pBHR68, pBHR71 and

pBHR77 had a well-defined exponential phase of 6 h, a long stationary phase (almost 40 h) and rise of absorbance after 72 h. This rise suggests a reduction in the concentration of ampicillin in the medium, probably due to its degradation by penicillinase, allowing the recovery of the plasmid-free bacteria. It should be mentioned that the plasmids carry the genes that code for penicillinase. Moreover, cells containing plasmids grew slower during the growth phase in comparison with plasmid-free cells (Fig. 2 and 3), which might be explained by the difference of the specific growth rates of the subpopulations (Table 2). According to Lee *et al.* (1994) such an increase in plasmid-bearing cell concentrations can be attributed to accumulation of reserve materials (PHAs), which changes cellular morphology, affecting absorbance measurements.

As cells grew older, they probably started accumulating PHAs and the number of Colony Forming Units (CFU) of cells harboring plasmids decreased (Fig. 4). This reduction underlines the hypothesis that cells start to accumulate polymer instead of increase the number of colonies. Lee *et al.* (1994) describes the behaviour of recombinant *E. coli* cells grown in 96 h cultures aimed at producing PHAs as follows: up to 48 h, younger cells replicate in a medium rich in nutrients and accumulate low amounts of PHAs. As cells grow older and the culture medium becomes poor in nutrients, PHA cell content increases towards its maximum.

In this study we did not measured the accumulated PHA, however the plasmid pBHR68 is able to accumulate much higher amounts of PHAs (Langenbach *et al.*, 1997; Antonio *et al.*, 2000). Our results present this plasmid disparate as the most stable among the studied ones (Fig. 5 and 6). This form, we can associate the PHA production with the plasmid stability. The segregational plasmid stability studies were conducted longer (96 h) in comparison with previous studies (24 and 48 h), allowing very meaningful profiles. Recombinant *E. coli* strains JM101 and DH10B containing plasmids pBHR68, pBHR71 and pBHR77 proved to be more stable (100% stability within 24 h from inoculation) than the recombinant *E. coli* strains grown in LB medium at 37°C previously studied by Gupta *et al.* (1995), in which stability had achieved 80% at 24 h. However, recombinant cells employed by these authors had grown faster, corroborating the fact that the probability of plasmid loss is directly proportional to specific cell growth rates.

In light of industrial applications, the results obtained seem to be very interesting. In the studied recombinant strain cultures, plasmid stability remained at 100% up to 25 and 40 generations of growth (excluding pre culture stage). These are promising results taking into account that Imanaka and Aiba (1981) reported that at least 25

generations of growth (including pre culture stage) are required to keep plasmid stability at high levels. Moreover, the recombinant *E. coli* JM101 cultures showed 50% stability after 60-80 and 110 generations for the plasmids pBHR71 and pBHR68, respectively, while Lee *et al.* (1994) had obtained values between 5-50% after 110 generations of growth in recombinant cell cultures in LB media with and without glucose. The segregation coefficients ( $\alpha$ ) obtained, in the range from 1 to 2, are in agreement with literature values (Imanaka and Aiba, 1981; Shoham and Demain, 1991). The best performances of JM101 cultures concerning plasmid stability might be mainly attributed to its best adaptability to the environmental conditions. It has been reported that strain adaptability is influenced either positively or negatively by the chemical composition of the culture medium employed.

Regarding the plasmid-host systems, Emerick *et al.* (1984) observed that temperature affects significantly the biosynthetic pathways and regulatory systems in recombinant cell fermentations. Even though the optimal growth temperature for *E. coli* wild strains is 37°C, optimal recombinant protein production is achieved at 30°C. Likewise, the optimal growth temperature for *R. eutropha* (Lütke-Eversloh and Steinbüchel, 1999; Wang and Yu, 2000) and *P. aeruginosa* (Timm and Steinbüchel, 1990; Ballistreri *et al.*, 2001), PHA synthase gene donors, is 30°C. However this effect is contradictory. While Gupta *et al.* (1995) and Oscan *et al.* (1992) observed that high temperatures elevated stability, Aiba and Koizumi (1984) and Son *et al.* (1987) obtained less stable cultures at high temperatures. Thus, these results indicate that not only temperature but also plasmid-host systems are expected to govern the process of plasmid loss.

The insertion of large foreign pieces of genetic material can reduce bacterial plasmid stability (Warnes and Stephenson, 1986). However the plasmid pBHR68 (8.20 Kb), bigger than the plasmid pBHR71 (4.66 Kb), presented the best performance concerning stability (72% after 96 h). We believe that the presence of the entire PHA synthesis operon (*phaC*, *phaA* and *phaB* from *R. eutropha*) on the plasmid, may have brought more stability to the system. Antonio *et al.* (2000) showed that plasmid pBHR70, containing only the *phaC* from *R. eutropha*, produces insignificant amounts of PHAs which probably is due an instable plasmid-host systems.

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