

Effect of Temperature on the Stability of Plasmid pTG201 and Productivity of *xylE* Gene Product in Recombinant *Escherichia coli*: Development of a Two-stage Chemostat with Free and Immobilized Cells

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The effect of temperature on the stability of pTG201, a plasmid carrying the *xylE* gene (which encodes catechol 2,3-dioxygenase from *Pseudomonas putida*), and the production of catechol 2,3-dioxygenase in free and immobilized *Escherichia coli* during continuous culture have been studied at various temperatures. Immobilization of cells increased the stability of pTG201 considerably, even under conditions when expression of the *xylE* product was enhanced. Since *xylE* transcription was controlled by the λP_R promoter and *cI857* repressor, increasing derepression temperatures increased catechol 2,3-dioxygenase productivity and decreased pTG201 stability. A two-stage continuous culture system to overcome the impact of the high-level expression of the *xylE* gene on the stability of pTG201 is described. In the first stage, immobilized cells were grown in the repressed state in order to prevent loss of pTG201, whereas in the second stage, cultures were maintained in the derepressed state.

INTRODUCTION

Bacterial plasmids are widely used as cloning vehicles in recombinant DNA research (Hershfield *et al.*, 1974; Tait *et al.*, 1983). However, many studies have shown that newly constructed recombinant plasmids are not maintained stably when recombinant microorganisms are grown in the absence of antibiotic selection (Noack *et al.*, 1981; Skogman *et al.*, 1983). Commercial production of plasmid-coded gene products in continuous culture is possible only if the recombinant plasmid can be stably maintained in cells (Imanaka & Aiba, 1981). A variety of experimental approaches to overcome plasmid instability have been proposed (Uhlin *et al.*, 1979; Zurita *et al.*, 1984; Tucker *et al.*, 1984; De Taxis du Poët *et al.*, 1986). The stability of plasmids in host cells may be affected by several factors, such as physiological consequences of expression of genes on the plasmid. Imanaka *et al.* (1980) and Caulcott *et al.* (1985) have shown that a high level of foreign gene expression may be detrimental to the carriage of plasmids. The use of temperature-sensitive systems offers the possibility of switching on the synthesis of recombinant proteins at will (Caulcott & Rhodes, 1986). Plasmids carrying the powerful λP_R promoter which is controllable by the product of the temperature-sensitive λ repressor gene *cI857* have been developed. This repressor, which blocks transcription of the cloned gene, is denatured at temperatures above 37 °C and causes the overproduction of biomolecules from any gene when regulated by P_R following derepression. Siegel & Ryu (1985) attempted to use a two-stage continuous culture process coupled with a temperature-sensitive expression system to obtain high expression from a cloned gene for prolonged periods despite instability caused by the increased expression. Such a system offers the possibility of minimizing the destabilizing effects of high expression, but the levels of production are not reproducible and cannot be maintained for a prolonged period. One cause is the instability of plasmids in the first stage.

Abbreviation: C23O, catechol 2,3-dioxygenase.

As a model system, we have used the recombinant plasmid pTG201, which is a pBR322 derivative carrying the *Pseudomonas putida xylE* gene, encoding catechol 2,3-dioxygenase, under the transcriptional control of λP_R and *cI857* repressor. In order to achieve high yields of *xylE* gene product together with high stability of pTG201, we have used a two-stage system to separate the phases of growth and product synthesis as suggested by Siegel & Ryu (1985). Cells were cultivated at 31 °C in the first chemostat and were continuously transferred to the second stage where the culture temperature (42 °C) denatured the repressor and caused efficient expression. However, to avoid the instability of pTG201, immobilized recombinant cells were used in the first stage. As immobilization considerably increases the stability of recombinant plasmids even under conditions when expression of cloned gene product is enhanced (Nasri *et al.*, 1987), plasmid-carrying cells could be maintained completely stably during continuous culture for a prolonged period in the first stage.

In this paper, we describe the effects of temperature on pTG201 stability and catechol 2,3-dioxygenase production in both free and immobilized cells during one-stage and two-stage continuous cultures without selection pressure.

METHODS

Bacterial strains and plasmid. The bacterial strains used were *Escherichia coli* W3101 (*recA13 trpE* NaI^r) (Kopecko *et al.*, 1976) and *E. coli* B. Plasmid pTG201 is a pBR322 derivative carrying the *Pseudomonas putida xylE* gene, coding for catechol 2,3-dioxygenase, under transcriptional control of the λP_R promoter and *cI857* repressor. Plasmid pTG201, a generous gift from Transgene SA, France, was constructed by D. F. Gaffney (personal communication).

Media. The medium used throughout the experiments was L broth (LB: 1%, w/v, tryptone, 0.5% yeast extract, 0.5% NaCl). For immobilized cells, LB medium was supplemented with 0.1 M-KCl. Agar (1.5% w/v) was added to LB medium to prepare LB agar plates.

Immobilization procedure. Immobilized growing cells were prepared using the carrageenan method (Takata *et al.*, 1977). A 0.5 ml volume from a preculture was added to 9.5 ml 2.1% (w/v) K-carrageenan E407 (a generous gift from CECA France) and suspended by stirring at 42 °C. This mixture was pumped at a rate of approximately 2 ml min⁻¹ into 40 ml LB medium containing 0.3 M-KCl, to form beads with a diameter of about 3 mm.

Cultivation conditions. Continuous cultures were carried out in 50 ml volumes in 100 ml glass vessels maintained at a fixed temperature, with an aeration rate of 170 ml min⁻¹. For free cultures, experiments were generally started with an overnight batch culture in 20 ml LB medium containing 50 µg ampicillin ml⁻¹. The chemostat was then inoculated with 0.4 ml of this culture. After batch growth for 4–5 h, fresh medium flow was started at the desired dilution rate.

For immobilized cultures, about 10 ml gel beads was transferred to the chemostat, and incubated with 40 ml growth medium supplemented with 0.1 M-KCl. Continuous flow of LB medium was initiated immediately.

For two-stage continuous culture, immobilized recombinant *E. coli* cells were produced in the first stage. Cells released from gel beads were continuously transferred to the second stage. The first chemostat was inoculated as for the single continuous immobilized system. The second stage was initially filled with 50 ml fresh LB medium. The temperatures of the first and the second stage were maintained at 31 °C and 42 °C, respectively.

Plasmid stability test. To determine the proportion of cells in the culture containing the plasmid, samples were withdrawn from the continuous cultures and spread on to LB agar plates. After overnight incubation at 37 °C, the proportion of cells containing pTG201 (*xylE*⁺, *Ap*^r) was calculated after spraying the colonies with an aqueous solution of 0.5 M-catechol. A yellow colour indicates a catechol 2,3-dioxygenase-producing colony (Zukowsky *et al.*, 1983).

Solubilization of gel beads. Gel beads were removed from the fermenter at regular intervals and then washed twice in 0.8% NaCl and dissolved in 1% sodium citrate.

Assay for activity of catechol 2,3-dioxygenase (C230; EC 1.13.11.2). The conversion by C230 of catechol to 2-hydroxymuconic semialdehyde was determined spectrophotometrically as described by Sala-Trepat & Evans (1971).

DNA isolation. Whole-cell lysates were prepared by the method of Eckhardt (1978), as modified by Projan *et al.* (1983).

Gel electrophoresis and determination of plasmid copy number. Electrophoresis of whole cell lysates was done on horizontal 1% (w/v) agarose slab gels. DNA bands were visualized under UV light after staining with ethidium bromide. The DNA content of bands was determined by microdensitometer tracing of photographic negatives.

The number of pTG201 copies per cell was calculated by using the following equation (Projan *et al.*, 1983): $C_p = D_p M_c / D_c M_p$, where C_p indicates plasmid copies per cell, D_p and D_c are the amounts of plasmid and chromosomal DNA in the gel, M_p is the molecular mass of plasmid, and M_c that of the total chromosomal DNA per cell.

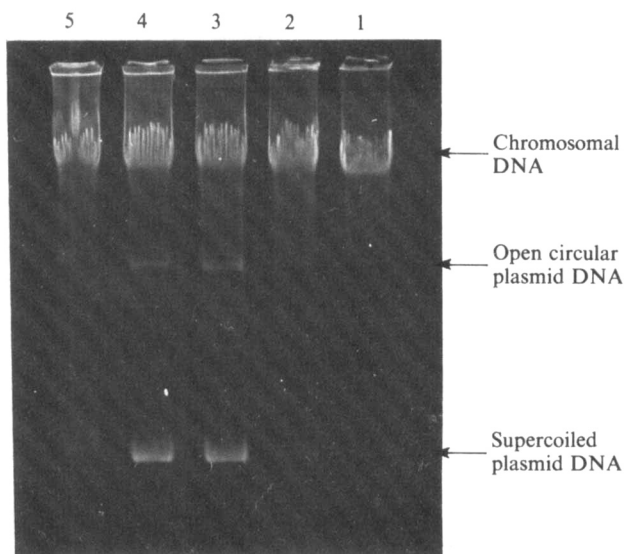


Fig. 1. Plasmid copy number of *E. coli* W3101(pTG201) at various stages of growth: (1) early exponential, (2) mid-exponential, (3) late-exponential, (4) stationary, (5) decline.

RESULTS AND DISCUSSION

The stability of pTG201 and C23O productivity were tested in both *E. coli* B and *E. coli* W3101 cells in continuous cultures. The copy number of pTG201 per cell was 40–50 in *E. coli* B and 70–80 *E. coli* W3101. The copy number of pTG201 in both strains was determined at different stages of bacterial growth; it was maximal during the stationary phase (Fig. 1). Several reports have shown that the plasmid copy number may influence the stability of plasmids (Jones *et al.*, 1980). Therefore, to start from a culture containing the maximum copy number of pTG201, precultures were allowed to grow for at least 12 h.

Effects of temperature on the stability of pTG201 and C23O production in free cells

The dilution rate in these experiments was about 0.9 h^{-1} .

Fig. 2(a) shows that the stability of pTG201 in *E. coli* B was strongly affected by the culture temperature. The initial percentage of cells carrying pTG201 at 31, 37 and 42 °C was 97, 85, and 94%, respectively. At 31 °C, the plasmid was moderately stable: after 83 generations more than 82% of the cells were *xylE*⁺. However, at higher temperatures the plasmid was extremely unstable, since at 37 and 42 °C only 40 and 35% of the cells still expressed C23O activity after about 87 and 67 generations, respectively. The rate of appearance of pTG201-free cells, and the concentrations of viable cells in the effluent at steady-state, are shown in Tables 1 and 2, respectively.

Similar experiments to examine the effect of temperature on the stability of pTG201 were done with *E. coli* W3101. At 31 °C, pTG201 was extremely stable (no pTG201-free cells were found after 280 generations). In contrast, after about 250 generations at 37 and 42 °C, 12 and 48% of cells, respectively, had lost pTG201 (data not shown).

The effect of culture temperature on C23O production by *E. coli* B was also investigated (Fig. 2b). C23O formation does occur at 31 °C, even though the promoter P_R is maintained in a repressed state by the *cI* gene product at this temperature. At higher temperatures, a more than fivefold increase in C23O activity was obtained: the activity increased significantly for the first 25 generations and then decreased (Fig. 2b). This decrease in C23O activity was correlated with the decrease in the proportion of cells containing pTG201.

Two explanations for the loss of pTG201 from *E. coli* B cells may be considered: (1) because of the absence of a partition mechanism in pTG201, which is necessary for stable inheritance of the plasmid in a population of dividing cells (Meacock & Cohen, 1980; Nordström *et al.*, 1980),

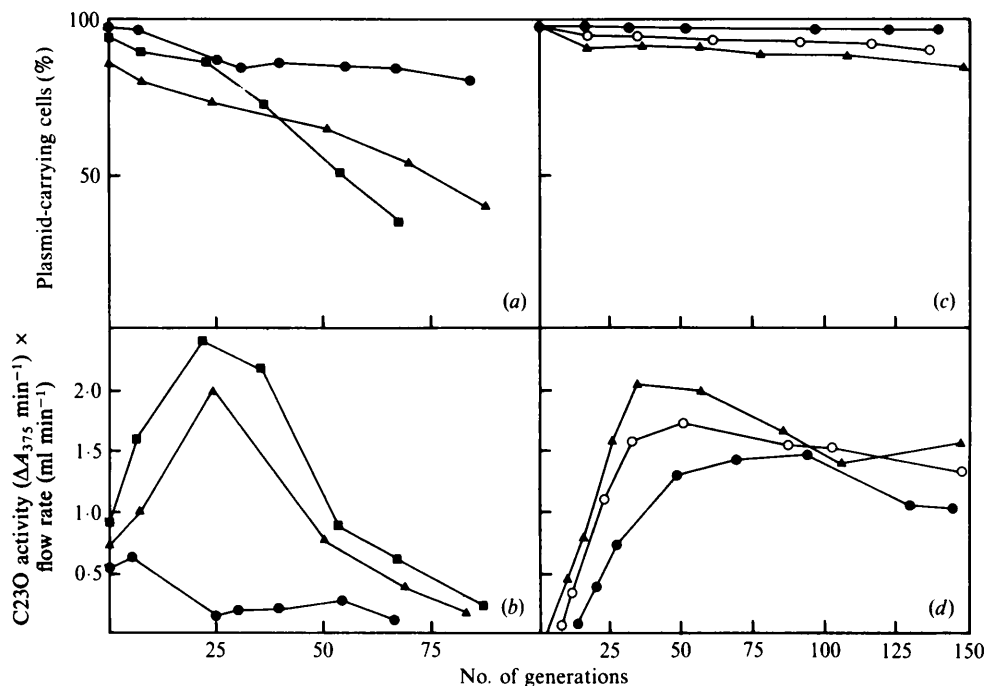


Fig. 2. Effect of temperature on the stability of plasmid pTG201 (a, c) and C23O productivity (b, d) in continuous cultures of free (a, b) and immobilized (c, d) *E. coli* B(pTG201). ●, 31 °C; ○, 34 °C; ▲, 37 °C; ■, 42 °C.

plasmid-free cells may be generated from plasmid-containing cells; (2) differences in growth rate between plasmid-free and plasmid-containing cells. The results in Table 3 indicate that the growth rates of pTG201-containing *E. coli* B are lower than those of pTG201-free cells. Therefore, when pTG201-free cells appear in the culture (due to the spontaneous plasmid loss through segregation during cell division), they will compete with pTG201-containing cells, and after prolonged incubation, overgrow the culture. The differences in growth rate between pTG201-free and pTG201-containing cells increase with increasing temperature (Table 3).

Effects of temperature on pTG201 stability and C23O production in immobilized cells

For this study, continuous fermentations were conducted only at 31, 34 and 37 °C, because the gel beads are dissolved at higher temperatures. The dilution rate of the immobilized cell cultures was 3.2 h⁻¹. Due to the high dilution rate, the cells which are continuously released from the gel beads cannot divide in the chemostat. Consequently, information concerning the evolution of the system as a function of time can be obtained from the released cells.

Fig. 2(c) shows the very high stability of pTG201 in immobilized *E. coli* B cells when compared to that with free cells. At 31 °C the initial percentage of plasmid-bearing cells was 98% and remained nearly the same after about 140 generations. Plasmid stability decreased relatively at higher temperatures, but was still high even at derepression temperatures of 34 and 37 °C (Fig. 2c). Immediately after initiation of a chemostat population and for the first 15 generations, the frequency of plasmid-containing cells decreased and then remained nearly constant. The percentages of plasmid-bearing cells after 140 generations at 34 and 37 °C were 91 and 88%, respectively (initial percentage 98%). This increased pTG201 stability in immobilized cells may have resulted from absence of competition between plasmid-free and plasmid-containing cells within the gel beads and/or to the mechanical properties of the gel bead system, which may only allow a limited number of cell divisions to occur in each clone of cells before the clone escapes from the gel bead. Such escaped populations are washed out by flow before much

Table 1. Rate of loss of pTG201 in *E. coli B* per cell generation

The results are ranges, taken from three independent experiments.

Temp. (°C)	Rate of loss of pTG201 (%)	
	Free cells	Immobilized cells*
31	0.18-0.22	0
34	ND	0.16-0.20
37	0.34-0.57	0.38-0.57
42	1.25-1.35	†

ND, Not determined.

* Rate of loss of pTG201 from cells grown in gel beads for the first 16 generations.

† Gel beads are dissolved at 42 °C.

Table 2. Numbers of viable *E. coli B* cells

Viable cell number of immobilized cells is expressed per ml of gel, and that of free and released cells per ml of liquid medium. The results are means of three independent experiments. The standard errors were within 3% of the mean.

Temp. (°C)	Free cells	Released cells	Immobilized cells
31	1.6×10^9	1.8×10^8	1.5×10^{10}
34	ND	1.8×10^8	1.5×10^{10}
37	3.0×10^9	1.8×10^8	1.4×10^{10}
42	1.9×10^9	†	†

ND, Not determined.

† Gel beads are dissolved at 42 °C.

Table 3. Effect of temperature on growth rate of plasmid-free and plasmid-containing *E. coli B*

The results are means of three independent experiments. The standard errors were within 3% of the mean.

Temp. (°C)	Growth rate (h ⁻¹)		Ratio a/b
	pTG201-free (a)	pTG201-containing (b)	
31	1.37	1.36	1.01
37	2.61	2.45	1.07
42	1.66	1.48	1.12

cell division can occur, ensuring that any cured cells that arise are unable to take over the culture. The lost clones are continuously being replenished by new ones which reside deeper in the gel bead and whose growth was initially retarded by nutrient or oxygen deprivation. These are made accessible at the bead surface due to the sloughing of the outer matrix as it is disrupted by the escaping clones (De Taxis du Poët *et al.*, 1986; Nasri *et al.*, 1987).

The rate of loss of pTG201 from free *E. coli B* cells at 37 °C was similar to that of immobilized cells (Table 1) at the same temperature for the first 20 generations. These results indicate that cells immobilized in gel beads exhibit the same behaviour as free cells during growth in continuous culture. At higher temperatures (37 °C), the cells grew rapidly in the gel beads, while at lower temperatures (31 °C), growth was slower, until a steady state was reached. The maximum number of living cells in gel beads was similar at the three temperatures used (Table 2). The concentration of viable released cells in the effluent at steady-state was also similar at each temperature.

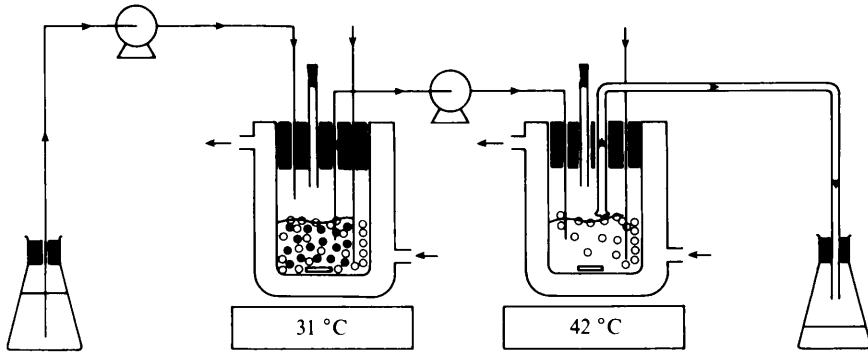


Fig. 3. Schematic representation of the configuration of the two-stage continuous culture.

The production of C23O at different temperatures, as a function of generations of cells released from gel beads, is shown in Fig. 2(d). C23O production increased with increasing temperature, but not as markedly as observed in the free continuous system.

No loss of pTG201 was detected in *E. coli* W3101 after 250 generations, even at 31 and 34 °C. Since pTG201 was more stable in *E. coli* W3101 than in *E. coli* B under both free and immobilized continuous culture conditions, the former strain was used for all subsequent work.

Two-stage continuous cultures

The instability of pTG201 appeared to be associated with expression of the *xyIE* gene. In order to minimize the deleterious effects of high levels of expression of cloned gene product, we separated the phases of growth and expression of *xyIE* gene. In the first stage, recombinant cells were grown in the repressed state, and then continuously transferred to the second stage where derepression was induced (Fig. 3).

The above results, and previous work in our laboratory (De Taxis du Poët *et al.*, 1986; Nasri *et al.*, 1987), show that immobilization of cells offers certain advantages: (a) higher stability of pTG201; (b) higher productivities due to high cell concentrations obtained in the gel bead and the higher productivity of released cells from the gel beads when compared to that obtained with free-cell culture. The increase in the cell concentration through immobilization may lead to an increase in the overall production of cloned gene products. We therefore developed an immobilized system to produce released cells, in the repressed state, in the first stage. After immobilization, the gel beads were transferred to the chemostat and the continuous flow of LB medium was immediately initiated. The dilution rate in both stages was about 3.45 h^{-1} , and the temperatures in the first and second stages were 31 and 42 °C, respectively. Samples were withdrawn from both stages and assayed for pTG201 stability and C23O activity (Fig. 4). After 230 generations, 3% of the cells had lost the plasmid in the derepressed culture, whereas in the repressed culture, no loss of pTG201 was observed after a similar number of generations (Fig. 4a). This difference was due to segregation of plasmid-free cells in the second stage. We found no pTG201-free segregants even after 400 generations in the first stage. Production of C23O of both uninduced and induced cultures increased significantly and then declined during the first 50 generations (Fig. 4b). No change in C23O activity was observed during extended experiments (100–230 generations). The C23O activity of released cells in the second stage was five times higher than that in the first stage (Fig. 4b). Furthermore, derepression by temperature-shift was not accompanied by a decrease in the number of living cells (data not shown).

In conclusion, such a two-stage process coupled with a temperature-sensitive expression system offers the following advantages: (1) high stability of the recombinant plasmid in the first stage, since in contrast to a free system, the immobilized system shows total stability of pTG201

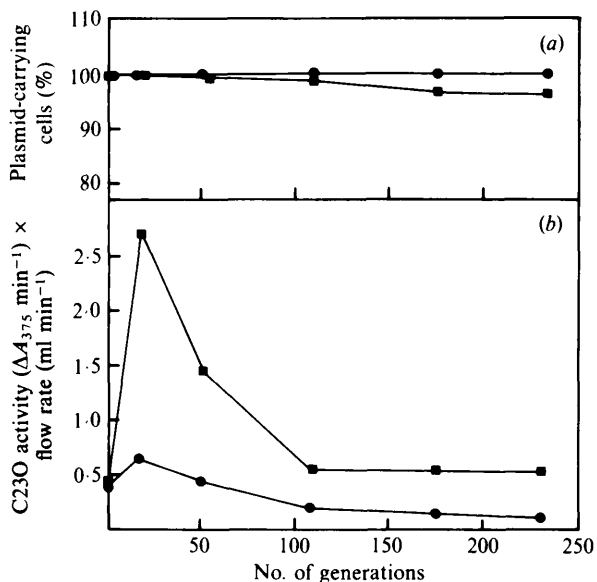


Fig. 4. Stability of pTG201 (a) and C230 productivity (b) in a two-stage continuous culture of *E. coli* W3101(pTG201). ●, First stage; ■, second stage.

in continuous culture, without selective pressure, for an extended number of generations; (2) high cell concentration in the first stage. Previous reports (Shinmyo *et al.*, 1982; Wada *et al.*, 1980; Wang & Wang, 1983) have shown that through immobilization, a significantly higher cell concentration can be obtained when compared to free-cell fermentations. These two factors therefore lead to an increase in the overall productivity of cloned gene products in the second stage.

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