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Plasmid Stability and Cloned-Gene Expression in Continuous Culture of Recombinant *Escherichia Coli* Under Derepressed Condition

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Continuous culture was carried out with a recombinant *Escherichia coli* W3110/pCR185, which encodes *trp*-operon enzymes when the temperature is shifted from 37°C to 42°C. Under derepressed condition of 42°C, plasmlid stability and gene expression were analysed as function of the dilution rate. The stability of plasmid increased with the dilution rate, but maximal levels of gene expression (tryptophan concentration) and plasmid DNA content were obtained at the lowest dilution rate, 0.075 hr^{-1}. The plasmid instability, observed at low dilution rates, could be explained by the unbalanced biosynthetic state of the recombinant cell harboring a high copy number of plasmid.

Recombinant microorganisms become important means for producing valuable biomolecules through the gene dosage effect. Expression of cloned gene, however, is restricted by the exhaustion of cellular resources and plasmid instability which are caused by excessive synthesis of plasmid DNA and plasmid-encoded product. Also cloned-gene expression is known to be dependent strongly on factors such as cultivation conditions (10), bioreactor operation mode (24), and genetic parameters such as promoter strength and plasmid size (2, 4, 18, 25).

In order to apply the recombinant microorganism to practical or industrial use, a kinetic analysis on gene expression and plasmid stability should be done through fermentation experiments under derepressed or induced condition of cloned-gene expression. The use of continuous culture, which provides a controlled and constant environment, has been an important tool in studies on the effect of growth conditions on the cloned-gene expression and plasmid instability. Kinetic studies of recombinant *Escherichia coli* fermentation using chemostat culture have been mainly focused on the p_L and p_R promoters employing temperature-sensitive repressor, *CI857* (15, 23). Little, however, is known about the kinetics of recombinant fermentation employing *trp* promoter- and repressor-related expression system, in which the gene expression is regulated by a *trp* repressor (*trp*R). The *trp* promoter has been known to be a kind of strong promoter used in *E. coli* (17). In our case we had a recombinant *E. coli* strain carrying both *trp* promoter and *trp*-operon gene in ColEl replicon plasmid. This *trp* promoter was regulated by a temperature-sensitive tryptophan repressor, *trp*R^{ts}, the gene of which was located on the host chromosomal DNA.

In this work we investigated the effect of growth rate or dilution rate on plasmid stability and cloned-gene expression during the continuous culture of recombinant *E. coli*. The continuous culture was conducted with various dilution rates under derepressed condition (42 °C) in the absence of selective pressure (antibiotic addition).

MATERIALS AND METHODS

Microorganism and Plasmid

Escherichia coli W3110 (Δ*trp*LD *trp*R^{ts} *tna*$^-$) was used as the host strain throughout this work. This strain has a temperature-sensitive tryptophan repressor which is represented as a *trp*R^+ character at permissive temperatures (below 37°C) and a *trp*R^- character at nonpermissive temperatures (above 40°C). The recombinant plasmid pCRT185, which has a kanamycin-resistant marker and a ColEl replicon, was constructed by subcloning *trp*-operon from pVH5 to pCRT1. Details of host cell and plasmid construction have been previously descri-

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bed (13).

Media and Culture Method

LB (0.2% glucose, 0.5% yeast extract, 1% Bacto-tryptone, 0.5% NaCl) and minimal A media (13) were used for the first and second precultures, respectively. Kanamycin was added as a selective pressure for recombinant cells to the preculture media with a final concentration of 50 μg/ml. The exponentially growing second preculture was inoculated (6%) into the fermentor. The working volume of the fermentor (NBS BioFlo 30, USA) was fixed at 750 ml and the dilution rate was varied from 0.075 to 0.425 hr^{-1}. When the batchwise cultivation reached late log phase, continuous feeding of medium A was started and at the same time (culture time= 0 hr) the culture temperature was shifted from 37°C to 42°C to derepress the *trp*-operon gene on plasmid pCR185.

Analytical Methods

The cell growth was monitored by measuring the optical density at 540 nm (Milton Roy Spectronic 21 spectrophotometer, USA) and converting the measurement to dry cell weight with a calibration curve. The glucose concentration in culture broth was measured with a YSI Glucose Analyzer (Model 23A, USA). The tryptophan concentration produced in the culture medium was determined by the method of Udenfriend and Peterson (27). The culture broth was sampled aseptically, appropriately diluted, and spreaded on the LB plate. After a 24-hr incubation at 30°C, 100~200 colonies were transferred to a minimal agar plate containing kanamycin of 50 μg/ml. The plasmid stability was defined as the ratio of the colony numbers exhibiting tryptophan non-requiring for growth (TRP^+) and kanamycin resistance (Km^R) characters to the total number of colonies replicated. Culture samples with 0.4~0.5 mg dry cell weight were used for the plasmid DNA isolation by the method of Bimboim and Doly (1). After linearizing the plasmid DNA with *Eco*RI treatment, electrophoresis was performed on a 1.0% agarose gel. Ethidium bromide fluorescence of the DNA on gel was photographed with a Polaroid type 665 positive-negative film, and the density of negative film was scanned using a Zeineh Soft Laser Scanning Densitometer. The amount of plasmid DNA was determined by comparing the density of sample DNA with that of λDNA-*Hind*III loaded on the same agarose gel. The plasmid DNA content, C_p (mg-DNA/g-cell), was calculated as follows:

$$C_p = \frac{450}{300} \cdot \frac{W_p}{W_c \cdot p} \cdot 10^{-3}$$

where 450/300 value comes from the plasmid isolation step with 150 μl discarded from the original supernatant volume (450 μl); p is the plasmid stability of sample broth; W_p is the plasmid amount (ng-DNA/300 μl supernatant); W_c is the cell concentration (g/l). The determination of plasmid content was repeated 2~4 times using the same culture sample.

RESULTS AND DISCUSSION

In order to investigate the effect of growth rate on gene expression and plasmid stability of the recombinant *E. coli* W3110/pCRT185, chemostat experiments were carried out at various dilution rates under the derepressed condition of 42°C. Since the maximum specific growth rate (μ_{max}) of the recombinant *E. coli* at 42°C was previously determined as 0.45 hr^{-1} in the batch fermentation with minimal medium (13), continuous culture was carried out at dilution rates below 0.45 hr^{-1}, e.g., 0.075, 0.15, 0.25, 0.35 and 0.425 hr^{-1}. Cultures were performed without a selective pressure (kanamycin addition). Feeding of the fresh medium A was started affter 10 hrs of batchwise cultivation at 37°C and at the same time the culture temperature was shifted to 42°C.

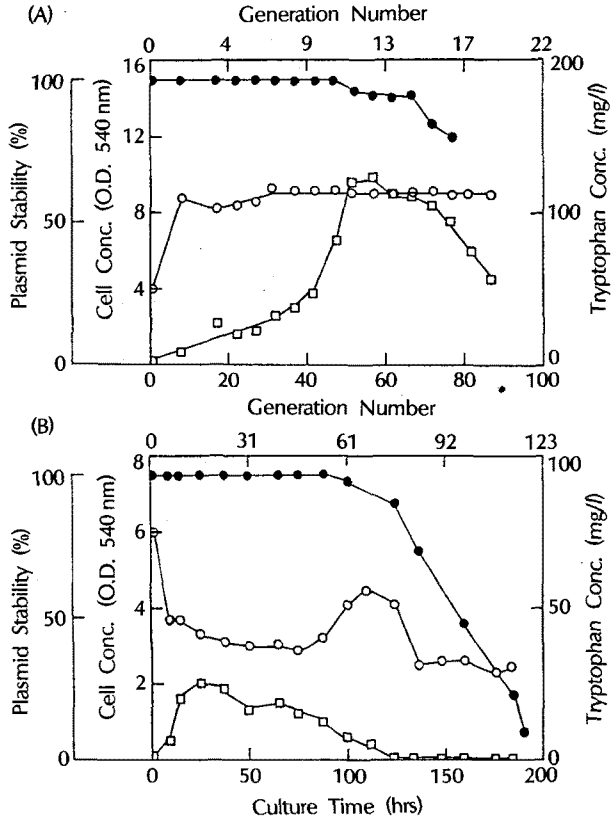

Fig. 1. Profiles of plasmid stability (●), cell concentration (○) and tryptophan concentration (□) in the continuous culture of the recombinant *E. coli* at 42°C.
(A) dilution rate=0.15 hr^{-1}, (B) dilution rate=0.425 hr^{-1}.

Table 1. Cell and tryptophan concentrations during the pseudo-steady state in the continuous culture of the recombinant *E. coli* at the derepressed condition, 42°C

Dilution rate (hr^{-1})	Cell conc. (g/l)	Tryptophan conc. (mg/l)	Period of pseudo-steady state (hrs)
0.075	2.55	350	24
0.15	3.33	110	20
0.25	2.70	40	62
0.35	2.00	38	32
0.425	1.35	22	28

Cell Growth and Trytophan Concentration

Typical results of the continuous cultures under the derepressed condition of 42°C are shown in Fig. 1 (A) and (B) at dilution rates of 0.15 hr^{-1} and 0.425 hr^{-1}, and in Fig. 2 (A) and (B) at dilution rates of 0.35 hr^{-1} and 0.25 hr^{-1}, respectively. During the early period of culture, a transition state for cell growth and tryptophan production was observed. After the transient period, steady states were apparently shown at all dilution rates, and continued about 20 hr at 0.15 hr^{-1} and 62 hr at 0.25 hr^{-1}. During the apparent steady state (pseudo-steady state), the cell concentrations showed 5~10% deviation from the mean value of cell concentration. This pseudo-steady state pattern was observed at other dilution rates, but the duration period of pseudo-steady state varied with the dilution rate (Table 1).

As shown in Fig. 1 and Fig. 2, the tryptophan concentration increased in the early period of culture, and after the pseudo-steady state it gradually decreased. The initial increase of tryptophan production was caused by the temperature shift to 42°C and by the derepression of tryptophan repressor (*trp*R). Since the cellular activity or biosynthetic capability differed with the growth rate, the initial production rate of tryptophan varied with the dilution rate: the higher the dilution rate, the steeper the production curve of tryptophan. The cell concentration increased during the late period of culture at most dilution rates, but the culture time or generation number showing the increase of cell mass varied according to the dilution rate. As illustrated in Fig. 1, when the plasmid stability began to fall (a population of plasmid-free cells began to be detected), the steady state collapsed. Therefore, the reduction in tryptophan concentration during the late period of continuous culture was believed to be mainly due to the consumption of tryptophan by plasmid-free cells, since the host cell (plasmid-free cell) was a tryptophan auxotrophic mutant. The similar unsteady states with respect to the cell growth and product formation in the late chemostat culture were observed at all dilution rates.

It was interesting to note that the profile of cell concentration showed an oscillatory trend even during the period of almost 100% plasmid stability. This oscillation of cell concentration was also reported with other recombinant *E. coli* chemostat culture, and it may have been caused by the population dynamics of plasmid-free and plasmid-carrying cells in the fermentor (6). Even though the plasmid appeared to be 100% stable, a small amount of plasmid-free cells may still have existed in the culture broth and it is very likely that they were not detected in the plate assay employed in this study. Therefore, in order to analyze the plasmid instability accurately, it is desirable to replicate, if possible, more than 200 colonies grown on the LB plate when measuring the plasmid stability with the plate method used in this work.

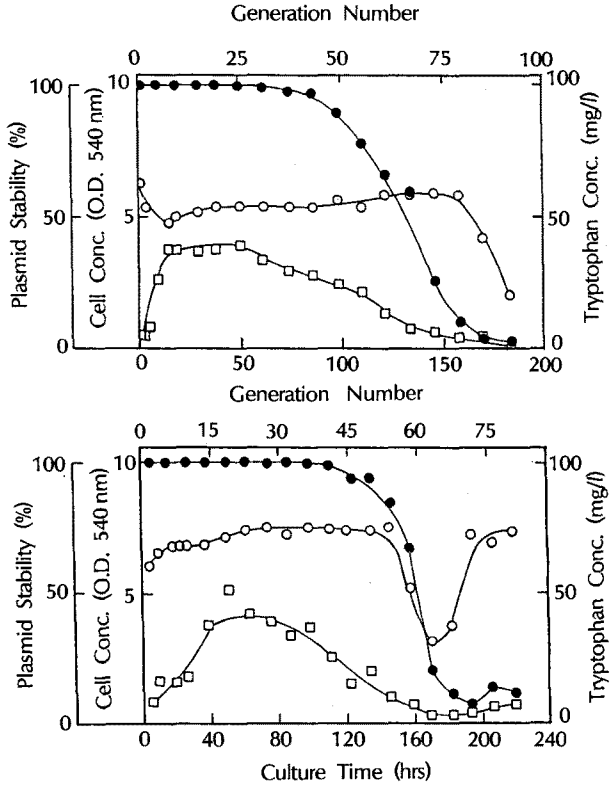

Fig. 2. Profiles of plasmid stability (●), cell concentration (○) and tryptophan concentration (□) in the continuous culture of the recombinant *E. coli* at 42°C.
(A) dilution rate=0.35 hr^{-1}, (B) dilution rate=0.25 hr^{-1}. [data in (B) is taken from ref. 13.]

Gene Expression Rate and Plasmid Content

When the plasmid was maintained 100% stable and the cell concentration showed a constant value, it was assumed that the culture had reached the pseudo-steady state. At the pseudo-steady state, the cell and tryptophan concentrations were averaged at each dilution rate and listed in Table 1, together with the duration

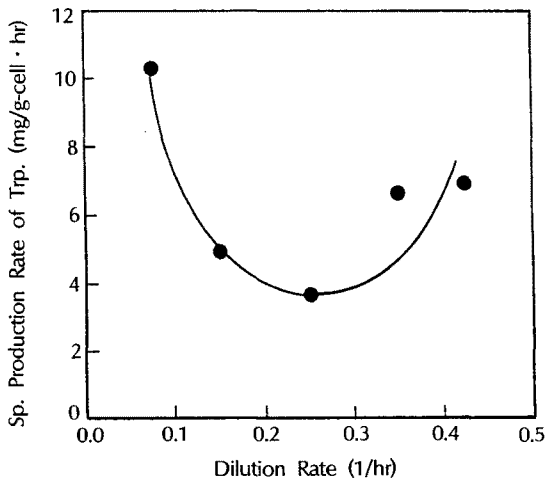

Fig. 3. Effect of dilution rate on specific production rate of tryptophan.

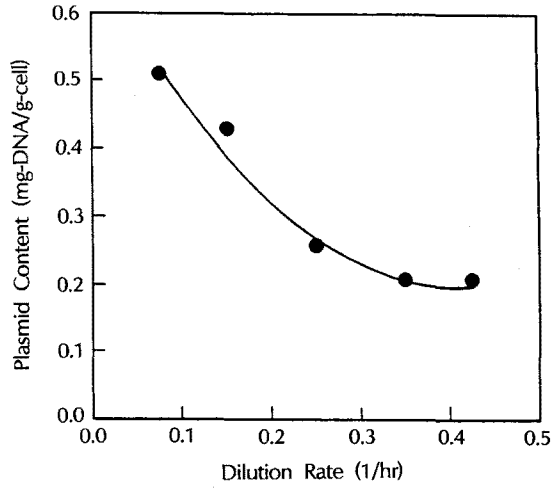

Fig. 4. Effect of dilution rate on plasmid DNA content.

of the pseudo-steady state.

From the average values of cell and tryptophan concentrations, the specific production rates of tryptophan, q_p, were calculated and plotted against various dilution rates in Fig. 3. It is interesting to note that the relationship between q_p and dilution rate is parabolic; at the moderate dilution rate of 0.25 hr^{-1} the lowest q_p value was observed, while at lower and higher dilution rates than 0.25 hr^{-1} as a standpoint, greater q_p values were shown. In the fermentation of conventional non-recombinant microorganisms, amino acids are growth-associated products and the q_p value for amino acids or growth-associated products is known to be directly proportional to the specific growth rate (μ) (16). The discrepancy in the relationship between q_p and μ, especially in the range of low dilution rate is thought as that the copy number of the recombinant plasmid per cell at low growth rates is higher than that at high growth rates. Therefore, the *trp*-operon enzymes encoded by the recombinant plasmid, would be synthesized to a higher concentration at low growth rates, and result in the greater production of tryptophan. To support this quantitative interpretation, the content of plasmid in the cell was measured at various dilution rates.

The culture broth was sampled at least 4 times over the period of pseudo-steady state at different dilution rates and was used to isolate plasmid DNA. At various dilution rates, the plasmid DNA isolated was quantified by comparing its amount with the standard amount of λDNA. The plasmid DNA contents were observed to increase from 0.2 to 0.5 mg plasmid-DNA per g-dry cell weight as the dilution rate decreased (Fig. 4). From the number of plasmid-carrying cells and the molecular weight of pCRT185 plasmid (16.9 kb), the plasmid copy number corresponding to the plasmid content of 0.5 mg at the dilution rate of 0.075 hr^{-1} was evaluated to be about 23 molecules per cell. And the plasmid copy number at the dilution rate of 0.425 hr^{-1} corresponded to ca. 9 molecules per cell. The plasmid copy number of 9~23 copies per cell was well consistent with copy number of ColEI replicon plasmid as 15~20 copies (20). From the result, it seems that the high specific production rate of tryptophan, q_p, at a low dilution rate is due to the enhanced plasmid DNA content or plasmid copy number per cell.

This result agreed with the simulation data (8) and several experimental observations for pBR322 (9) and NR1 (11) plasmids, in which the plasmid copy number at stationary phase (lower growth rate) was several-fold higher than that at exponential growth (higher growth rate). Also Seo and Bailey (21, 22) had found that plasmid content reached its maximum near the lowest dilution rate. Thus, a distinct or optimal growth rate for the maximum plasmid copy number and/or for the maximum gene expression could exist.

Plasmid Stability

From the gel-electrophoretic analysis of the plasmid, in which plasmid DNA was isolated from kanamycin-sensitive cells during the continuous culture, the reduction of plasmid size and alteration of restriction site were not observed (data not shown). Therefore, it could be deduced that the phenotypic instability of the cells, such as the tryptophan requirment for cell growth (TRP^-) and the kanamycin sensitivity (Km^S), was due to the segregation of plasmid pCR185.

To investigate more quantitatively the effect of growth rate on the segregational instability of the plasmid, the fractions of plasmid-harboring cells at various dilution rates were plotted as the function of the generation number in Fig. 5. It was found that the plasmid was considerably more stable at high dilution rates than at

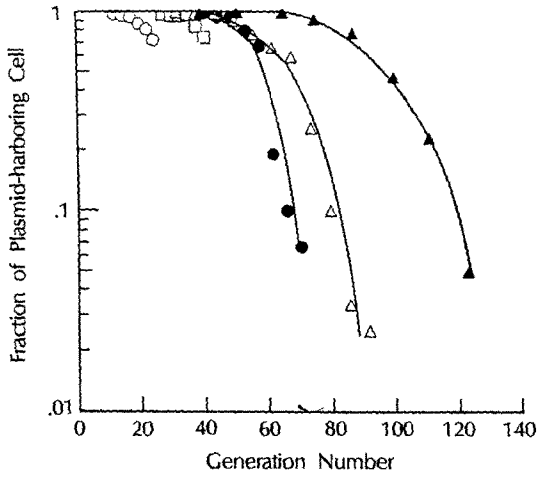

Fig. 5. Plasmid stability as a function of generation number at various dilution rates.
Dilution drate of 0.075 hr^{-1} (○), 0.15 hr^{-1} (□), 0.25 hr^{-1} (●), 0.35 hr^{-1} (△), and 0.425 hr^{-1} (▲).

low dilution rates. The kinetics on plasmid loss and dependency on the dilution rate or growth rate have also been reported by many authors for the low-copy-number and high-copy-number plasmids (3, 5-7, 12, 14, 19, 26).

The fact that the plasmid content increased with the decrease in the growth rate (Fig. 4) could explain the plasmid instability: the appearence of plasmid-free cell was more rapid at the lowest dilution rate where the plasmid DNA content was the greatest. Moreover, the high plasmid content or plasmid copy number may have led the recombinant cells to the unbalanced condition to synthesize excessively the cellular nutrients and cloned-gene product. As a consequence, the plasmid-harboring population was less competitive and was fastly replaced with the plasmid-free cell population as the dilution rate decreased. The unbalanced state or overloaded state of the cell was supported by a two-stage continuous culture experiment, in which the activity of tryptophan synthetase (TSase), one of the *trp*-operon enzymes, per g-cell showed the highest value at the lowest dilution rate (data not shown).

With respect to the experimental results of plasmid instability, we further quantitatively studied in detail the effect of growth rate on the plasmid instability, and the result will be published in the near future. The study was mainly focused on the growth rate difference between the plasmid-free cell and plasmid-containing cell, and the probability of plasmid loss as the key parameters for the plasmid instability.

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