Segregational Instability of a Recombinant Plasmid pDML6 in *Streptomyces lividans*

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Segregational instability of a recombinant plasmid, pDML6, encoding extracellular β-lactamase in *Streptomyces lividans* PD6 was characterized by growth kinetic analysis. The quantitative determination of the plasmid harbored in the mycelia was evaluated with mycelia fragmented mechanically, and also with colonies regenerated from protoplasts. Conditions for the formation of protoplasts and regeneration of protoplasts were established. The maximal specific growth rates of the host strain and the plasmid-harboring strain in a chemically defined medium without selection pressure were the same. The probability of plasmid loss from the harbouring cells was higher at higher growth rates. Mathematical models for the prediction of cell growth, substrate uptake, and accumulation of the cloned gene product were developed.

The production of a cloned gene product (CGP) in a recombinant microorganism is dependent on the stability and expression of the cloned gene in the host strain. High plasmid copy number can increase the gene dosage, which gives rise to an increase in the production of CGP. However, the stability of the recombinant plasmid in the host strain is a very critical factor in maintaining productivity (2). Plasmid stability is determined by the structural (25) and segregational characters of the plasmid. When the recombinant strain does not have *par* genes for the active partitioning of the plasmid in cell division (16), or the recombinant strain grows without pressure for selection of the cloned gene, the appearance of plasmid-free cells in prolonged cultivation is inevitable. Furthermore, if the specific growth rate of plasmid-free cells is higher than that of plasmid-harboring cells, the plasmid-free cells eventually will dominate the population. This is attributed to a precipitous decline in the productivity of CGP (8). A number of strategies, where culture conditions are crucial to the optimal production of CGP, for improving plasmid stability or providing advantages for the plasmid-harboring cells have been proposed (11).

*Streptomyces* is an important group of microorganisms producing valuable metabolites. They are also considered as alternative bacterial hosts for the production of autologous and heterologous proteins (4, 5, 10, 12, 14, 18, 22). However, genetic instability in the *Streptomyces* is evident even in chromosomal DNA (1, 3, 19, 24). Quantitative analysis of the segregational instability of a plasmid in *Streptomyces* spp. has been attempted (20). The mycelia of *Streptomyces* spp. are so filamentous and highly branched that a lump of mycelia can develop a colony. It is also not well understood whether or not recombinant plasmids are evenly distributed over the mycelia. We tried to determine the instability of a recombinant plasmid, pDML6, encoding β-lactamase in *Streptomyces lividans* PD6.

**MATERIALS AND METHODS**

**Microorganisms and Culture Conditions**

*Streptomyces lividans* TK24 was used as a plasmid free strain (7) and *Streptomyces lividans* PD6 was used as a strain harboring a recombinant plasmid, pDML6, which encoded extracellular β-lactamase (4). The strains were maintained on a rich agar medium (TSB) consisting of 0.25% glucose, 1.7% tryptone, 0.3

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Key words: *Streptomyces lividans*, β-lactamase, segregational plasmid stability, kinetics, modelling
% soytime, 0.5% NaCl, 0.25% K$_3$HPO$_4$, and 1.5% agar (pH 7.0). Fifty µg/ml of thiostrepton was added to the TSB agar medium in order to give a selective advantage to the PD6 strain. Spores formed on the TSB agar medium were harvested and suspended in TSB containing glycerol (20%), then kept in a deep freezer (−70°C) until use. Seed and main cultures were carried out with a chemically defined medium (CDM) consisted of 0.2% glucose, 0.2% NH$_4$Cl, 0.5% NaCl, 0.01% K$_2$HPO$_4$, 0.03% MgSO$_4$·7H$_2$O, 0.001% FeSO$_4$·7H$_2$O, 0.0002% MnCl$_2$·4H$_2$O, 0.001% CaCl$_2$·2H$_2$O, 0.0004% CuSO$_4$·5H$_2$O, 0.0004% CoCl$_2$·6H$_2$O, and 0.002% ZnSO$_4$·7H$_2$O (pH 7.4). Frozen spore suspensions were thawed in a water bath (30°C) then inoculated into seed culture media containing 10 µg/ml of thiostrepton. The seed culture was carried out using baffled flasks and a rotary shaking incubator for 2 days at 30°C. The seed culture was inoculated into the main culture medium to give a 5% (v/v) inoculum size. The main cultures were performed in 2-liter culture vessels (Korea Fermentor Co.) where agitation and aeration rate were set at 350 rpm and 0.5 vvm, respectively. Culture temperature was maintained at 30°C and pH at 7.2 by automatic addition of 1 N HCl or 1 N NaOH. The dilution rate was controlled by continuous feeding of fresh medium using a peristaltic pump.

**Analytical Methods and Detection of Plasmids in Cells**

Cell mass was analyzed by cell weight dried at 80°C for 12 hours and expressed as dried cell weight (DCW). Glucose concentration in the culture broth was analyzed by the dinitrosalicylic acid method (13). The activity of β-lactamase was analyzed by the iodometric method where one unit of β-lactamase was defined as the amount of enzyme required to hydrolyze 1 µmol of penicillin G in 1 minute at 30°C (23). Harboring of recombinant plasmids in mycelia was analyzed using the following procedure: The culture broth with mycelia was gently homogenized and the homogenized fragments of mycelia were diluted and plated on TSB agar medium. The colonies developed on the plates were transferred, by the replica technique, to plates of the TSB agar medium where 50 µg/ml of thiostrepton was added. In parallel, protoplasts were prepared with a lysozyme treatment, which was modified from the method of Hopwood et al. (6) Mycelia obtained from the main culture were washed twice with a 0.3 M sucrose solution. The washed mycelia were resuspended in the P buffer solution containing 2 mg/ml of lysozyme, and then incubated for 1 hour at 30°C. The protoplasts were regenerated on R2YE regeneration agar medium and regenerated colonies were transferred by the replica technique as described above. The stability of a recombinant plasmid was determined as the fraction of plasmid harboring cell population to the total cell population in the cultures.

**General Structure of Models**

Mathematical models for CGP production kinetics using recombinant *S. lividans* were based on the assumption that the predominating factor influencing the growth kinetics of *S. lividans* PD6 is substrate limitation where substrate inhibition and product inhibition for cell growth were not taken into account. It was assumed that plasmid harboring cells lost plasmids and turned into plasmid free cells with a probability (k) of plasmid loss. A diagrammatic flow chart of plasmid harboring cells and plasmid free cells is shown in Fig. 1.

![Diagrammatic flow chart of changes in plasmid harboring cells and plasmid free cells in recombinant *S. lividans*](image)

The general equations for growth rates of plasmid-harboring cells and plasmid-free cells are given as Eqn. (1) and (2). The rate of substrate uptake and the rate of product formation are given as Eqn. (3) and (4).

\[
\frac{dX^+}{dt} = \mu_{m^+} \cdot \frac{S}{K_S + S} X^+ - k \cdot \mu_{m^+} \cdot \frac{S}{K_S + S} X^+ \tag{1}
\]

\[
\frac{dX^-}{dt} = \mu_{m^-} \cdot \frac{S}{K_S + S} X^- - k \cdot \mu_{m^-} \cdot \frac{S}{K_S + S} X^- \tag{2}
\]

\[
\frac{dS}{dt} = - \frac{1}{Y_{X/S}} \left( \frac{dX^+}{dt} + \frac{dX^-}{dt} \right) \tag{3}
\]

\[
\frac{dP}{dt} = Y_{P/X^+} \cdot \frac{dX^+}{dt} \tag{4}
\]
The maximum specific growth rates of plasmid-harbouring cells (PD6) and plasmid-free cells (TK24) can be estimated from a washout experiment (17). If the segregational instability during a short period of culture time is negligible and D>D, in the chemostat culture, the culture biomass decrease according to the Eqn. (5). The slope of the logarithmic plot is (μn-D), which gives the value of μn. Changes in the concentrations of plasmid-harbouring cells, plasmid-free cells, and total cells (X*) in chemostats can be expressed as Eqn. (6), (7), and (8), respectively. When plasmid-harbouring cells and plasmid-free cells were grown with different growth rates steady-state conditions were not obtained. But, when μ+ is equal to μ- and the total cell mass (X+ + X- = X*) is constant throughout the culture, μ+ = μ- = D, and Eqs. (9) and (10) are obtained. The probability of plasmid loss (k value) can be obtained from integration of Eqn. (9) or (10). The fraction of the plasmid-harbouring cell population to the total cell population is represented in Eqn. (11) and the changes in the fractions versus culture time are given in Eqn. (12). The k value can be calculated by Eqn. (13).

\[
\frac{dX}{dt} = (\mu_n - D)X \\
\frac{dX^+}{dt} = \mu^+ \cdot X^+ - k \cdot \mu^- \cdot X^- - D \cdot X^+ \\
\frac{dX^-}{dt} = \mu^- \cdot X^- + k \cdot \mu^+ \cdot X^+ - D \cdot X^- \\
\frac{d(X^*)}{dt} = \mu^+ X^+ + \mu^- X^- - D(X^*) \\
\frac{dX^+}{dt} = -k \cdot D \cdot X^+ \\
\frac{dX^-}{dt} = k \cdot D \cdot X^- \\
F = \frac{X^+}{X^*} \\
\frac{dF}{dt} = \frac{1}{X^*} \cdot \frac{dX^+}{dt} = -k \cdot D \cdot F \\
\ln(F_2) - \ln(F_1) = -k \cdot D (t_2 - t_1) = -ln2 \cdot k \cdot Gn
\]

RESULTS AND DISCUSSION

Evaluation of Kinetic Parameters

Data for the changes of glucose, mycelia, and β-lactamase in batch cultures of the plasmid-free strain (TK24) and plasmid-harbouring strain (PD6) are shown in Fig. 2. The production of extracellular β-lactamase, the cloned gene product, in the culture of strain PD6 followed the growth-associated type although the enzyme was not produced in the culture of the plasmid-free strain (TK24). The specific growth rates of both strains in the exponential growth phase were calculated to be 0.133 h⁻¹. The cell mass yield to the glucose consumption (Y_W) and product yield to the cell mass (Y_p/k) in the batch cultures were estimated to be 0.45 g·g⁻¹ and 7.3 unit·g⁻¹, respectively. The μ_n values for the plasmid-harbouring strain and plasmid-free strain were estimated to be 0.139 h⁻¹ by the washout method (Fig. 3). Segregational plasmid instability is thought to be determined by two factors: one is the probability of generation of

![Fig. 2. Batch culture kinetics of Streptomyces lividans strain TK24 (plasmid-free strain) and PD6 (recombinant plasmid-harbouring strain) on a chemically defined minimal medium with 1% glucose.](image)

○: glucose, ●: D.C.W., △: β-lactamase activity(BLA)
plasmid-free cells from plasmid-harboring cells, and the other is different growth rates of the two cell types (9). We found that the segregational instability of the plasmid pDML6 in *S. lividans* was mainly determined by the probability of plasmid loss (k).

**Differential Counting of Plasmid-Harboring Cells and Determination of the Probability of Plasmid Loss**

Differentiation of plasmid harboring mycelia from plasmid free mycelia was attempted with mycelia growing at the dilution rate of 0.106 h⁻¹ in a continuous culture. Intact mycelia, protoplasts, and regenerated cells were compared microscopically (Fig. 4(A)-4(C)). Branching mycelia were changed to protoplasts by lysozyme treatment and protoplasts were regenerated, developing red (a) and yellow (b) colonies. However, the two different colony types showed similar characteristics in the plasmid-harboring test. The fraction of plasmid-harboring cells to the total population was determined by fragmented mycelia and by regenerated cells. Results are compared in Fig. 5. The fraction of plasmid harboring cells to the total population obtained from the protoplast method declined the culture time elapsed. However, the fraction obtained from fragmented mycelia was slightly higher than that obtained from the protoplast method. These results suggest that mycelia were not sufficiently fragmented and not as small as the protoplasts. We concluded that the protoplast method was a more reliable method for the quantitative determination of plasmid-harboring cells.

The change in the fraction of plasmid-harboring cells with generation (Gn) was calculated from glucose limited chemostat where dilution rates were maintained at 0.062, 0.106 and 0.128 h⁻¹ respectively. As shown in Fig. 6, the probability of plasmid loss (k) was calculated to be 0.011, 0.013, and 0.034 for dilution rates of 0.062, 0.106 and 0.128 h⁻¹ respectively. This indicates that the probability of recombinant plasmid pDML6 loss in
S. lividans is higher at higher dilution rates. It has been previously reported that stability of high copy number plasmids was improved with increasing dilution rate (21). However, the probability of plasmid pDML6 loss in S. lividans was found to be high compared with Escherichia coli (15).

Model Prediction and Comparisons with Batch Culture Experimental Data

The kinetic parameters used in model simulations are shown in Table 1. The probability of plasmid loss (k value) used in the simulation was 0.034 because the specific growth rate during the exponential growth phase of the batch culture was about 0.133 h⁻¹. Comparisons between model predictions and experimental data for biomass production, glucose utilization, and β-lactamase production in a batch culture are shown in Fig. 7. Agreement between the experimental data and model predic-

![Graph showing kinetics of instability of a recombinant plasmid pDML6 in Streptomyces lividans PD6 grown in continuous cultures at different dilution rates.](image)

**Fig. 6.** Kinetics of the instability of a recombinant plasmid pDML6 in Streptomyces lividans PD6 grown in continuous cultures at different dilution rates. • D=0.062 h⁻¹, ▲ D=0.106 h⁻¹, ■ D=0.128 h⁻¹

**Table 1. Growth parameters of Streptomyces lividans TK24 and its transformant PD6.**

<table>
<thead>
<tr>
<th>Constant</th>
<th>Description</th>
<th>Value</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>µ_m</td>
<td>Maximum specific growth rate</td>
<td>0.139 h⁻¹</td>
<td>*Experiment</td>
</tr>
<tr>
<td>µ_n</td>
<td>Maximum specific growth rate</td>
<td>0.139 h⁻¹</td>
<td>*Experiment</td>
</tr>
<tr>
<td>K_s</td>
<td>Substrate limitation constant for growth</td>
<td>0.025 g L⁻¹</td>
<td>Assumed</td>
</tr>
<tr>
<td>Y_v_b</td>
<td>Cell mass yield</td>
<td>0.45 g g⁻¹</td>
<td>*Experiment</td>
</tr>
<tr>
<td>Y_p_b</td>
<td>Product yield</td>
<td>7.3 Unit g⁻¹</td>
<td>*Experiment</td>
</tr>
<tr>
<td>k</td>
<td>Probability of plasmid loss</td>
<td>0.034</td>
<td>*Experiment</td>
</tr>
<tr>
<td>F_0</td>
<td>Initial plasmid-harboring fraction</td>
<td>1.0</td>
<td>Assumed</td>
</tr>
</tbody>
</table>

* Determined by the wash-out method in a continuous culture
b Determined by a batch culture
c Determined by a continuous culture

The marker +, −, and * indicate plasmid-harboring cells, plasmid-free cells, and the sum of them, respectively.

Acknowledgement

This study was supported by the Korea Science and Engineering Foundation, for which authors are very grateful.

We would like to express our appreciation to Professor Jin Ho Seo and Professor Sunghoon Park for helpful suggestions and discussions.
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