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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 recominant 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).

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Key words: *Streptomyces lividans*, β-lactamase, segregational plasmid stability, kinetics, modelling

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 develope 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-lacatmase (4). The strains were maintained on a rich agar medium (TSB) consisting of 0.25% glucose, 1.7% tryptone, 0.3

% soytone, 0.5% NaCl, 0.25% K_2HPO_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℃) until use. Seed and main cultures were carried out with a chemically defined medium (CDM) consisted of 0.2% glucose, 0.2% NH_4Cl, 0.5% NaCl, 0.01% K_2HPO_4, 0.03% $MgSO_4 \cdot 7H_2O$, 0.001% $FeSO_4 \cdot 7H_2O$, 0.0002% $MnCl_2 \cdot 4H_2O$, 0.001% $CaCl_2 \cdot 2H_2O$, 0.0004% $CuSO_4 \cdot 5H_2O$, 0.0004% $CoCl_2 \cdot 6H_2O$, and 0.002% $ZnSO_4 \cdot 7H_2O$ (pH 7.4). Frozen spore suspensions were thawed in a water bath (30℃) 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℃. 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 speed and aeration rate were set at 350 rpm and 0.5 vvm, respectively. Culture temperature was maintained at 30℃ 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℃ 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-lacatmase was analyzed by the iodometric method where one unit of β-lacatmase was defined as the amount of enzyme required to hydrolyze 1 μmol of penicillin G in 1 minute at 30℃ (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℃. 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.

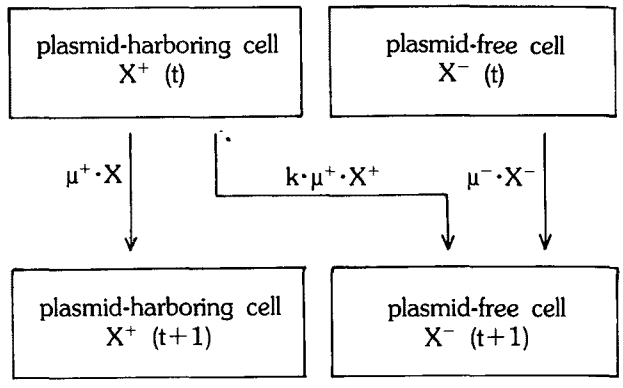

Fig. 1. Diagrammatic flow chart of changes in plasmid harboring cells and plasmid free cells in recombinant *S. lividans*.

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).

$$\frac{dX^+}{dt} = \mu_m{}^+ \cdot \frac{S}{K_S+S} X^+ - k \cdot \mu_m{}^+ \cdot \frac{S}{K_S+S} X^+ \quad (1)$$

$$\frac{dX^-}{dt} = \mu_m{}^- \cdot \frac{S}{K_S+S} X^+ - k \cdot \mu_m{}^- \cdot \frac{S}{K_S+S} X^+ \quad (2)$$

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

$$\frac{dP}{dt} = Y_{p/x^+} \cdot \frac{dX^+}{dt} \quad (4)$$

The maximum specific growth rates of plasmid-harboring 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 $D>D_c$ in the chemostat culture, the culture biomass decrease according to the Eqn. (5). The slope of the logarithmic plot is $(\mu_m - D)$, which gives the value of μ_m. Changes in the concentrations of plasmid-harboring cells, plasmid-free cells, and total cells (X^*) in chemostats can be expressed as Eqn. (6), (7), and (8), respectively. When plasmid-harboring 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, $\mu^+ = \mu^- = D$, and, Eqn. (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-harboring 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).

$$\frac{dX}{dt} = (\mu_m - D)X \quad (5)$$

$$\frac{dX^+}{dt} = \mu^+ \cdot X^+ - k \cdot \mu^+ \cdot X^+ - D \cdot X^+ \quad (6)$$

$$\frac{dX^-}{dt} = \mu^- \cdot X^- + k \cdot \mu^+ \cdot X^+ - D \cdot X^- \quad (7)$$

$$\frac{d(X^*)}{dt} = \mu^+ X^+ + \mu^- X^- - D(X^*) \quad (8)$$

$$\frac{dX^+}{dt} = -k \cdot D \cdot X^+ \quad (9)$$

$$\frac{dX^-}{dt} = k \cdot D \cdot X^+ \quad (10)$$

$$F = \frac{X^+}{X^*} \quad (11)$$

$$\frac{dF}{dt} = \frac{1}{X^*} \cdot \frac{dX^+}{dt} = -k \cdot D \cdot F \quad (12)$$

$$\ln(F_2) - \ln(F_1) = -k \cdot D(t_2 - t_1) = -\ln 2 \cdot k \cdot Gn \quad (13)$$

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-harboring 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 ($Y_{x/s}$) and product yield to the cell mass ($Y_{p/x+}$) in the batch cultures were estimated to be 0.45 $g \cdot g^{-1}$ and 7.3 unit$\cdot g^{-1}$, respectively. The μ_m values for the plasmid-harboring 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

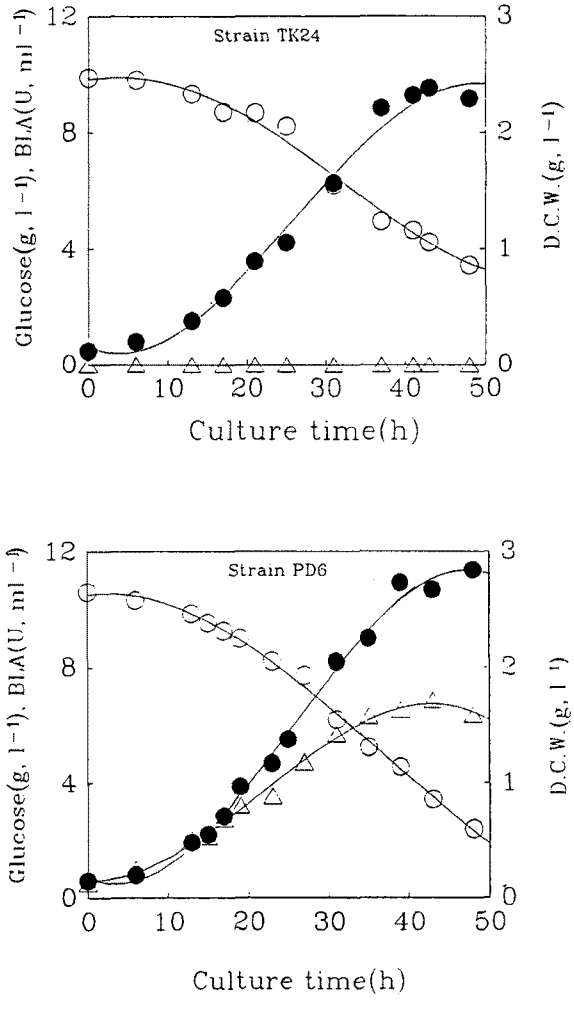

Fig. 2. Batch culture kinetics of *Streptomyces lividans* strain TK24 (plasmid-free strain) and PD6(recombinant plasmid-harboring strain) on a chemically defined minimal medium with 1% glucose.

○; glucose, ●; D.C.W., △; β-lactamase activity(BLA)

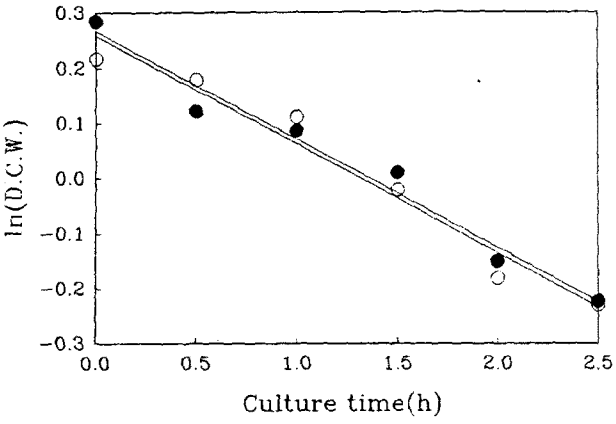

Fig. 3. Washout kinetics of cell mass of *Streptomyces lividans* TK24 and PD6 in continuous cultures where the dilution rate was controlled at 0.334 h^{-1}.
○; *S. lividans* TK24, ●; *S. lividans* PD6

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

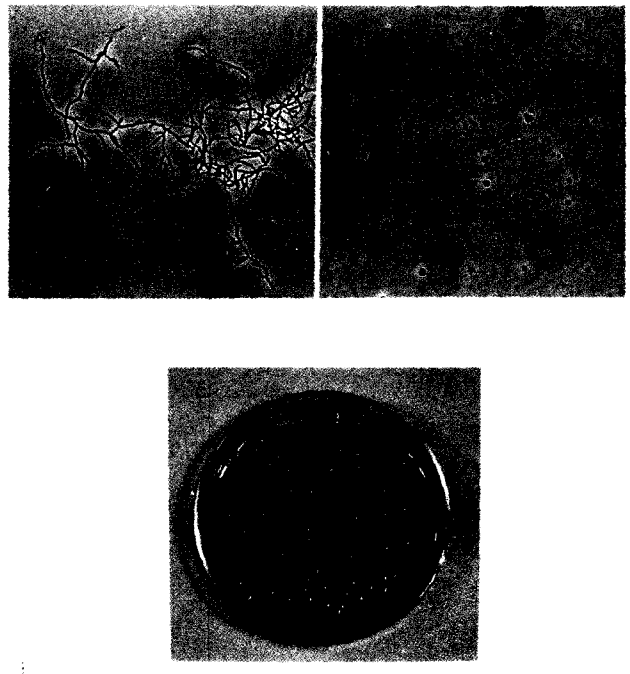

Fig. 4. Photomicrographs of filamentous mycelia(A), protoplast(B), and regenerated colonies on R2YE agar plate (C).

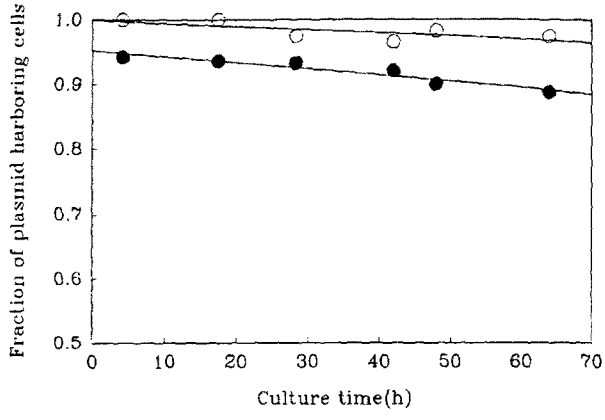

Fig. 5. Comparison of the changes in the fraction of plasmid-harboring cells to total cells of *Streptomyces lividans* PD6 grown in a continuous culture at 0.106 h^{-1}.
The fractions were calculated from mycelial fragments (○) and protoplasts (●).

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 Comparisions 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-lacatmase production in a batch culture are shown in Fig. 7. Agreement between the experimental data and model prediction is good indicating that the parameters used in the modelling were accurate, and the models were properly constructed. The production of CGP β-lactamase is considered to be growth-associated type.

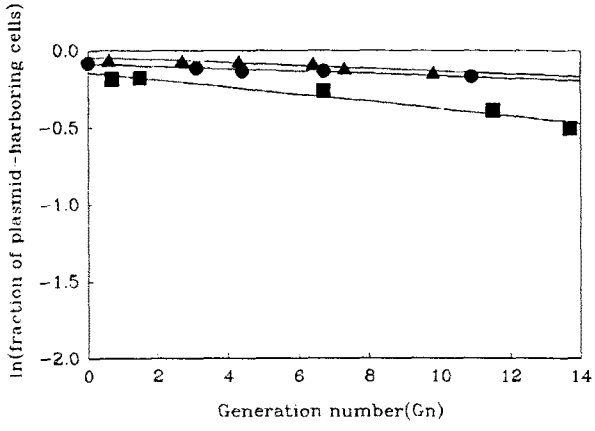

Fig. 6. Kinetics of the instability of a recombinant plasmid pDML6 in *Streptomyces lividans* PD6 grown in continuous cultures at different dilution rates.
●; D=0.062 h^{-1}, ▲; D=0.106 h^{-1}, ■; D=0.128 h^{-1}

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

Constant	Description	Value	Source
μ_m^+	Maximum specific growth rate	0.139 h^{-1}	[a]Experiment
μ_m^-	Maximum specific growth rate	0.139 h^{-1}	[a]Experiment
K_s	Substrate limitation constant for growth	0.025 $g \cdot l^{-1}$	Assumed
$Y_{x/s}$	Cell mass yield	0.45 $g \cdot g^{-1}$	[b]Experiment
$Y_{p/x}^+$	Product yield	7.3 Unit$\cdot g^{-1}$	[b]Experiment
k	Probability of plasmid loss	0.034	[c]Experiment
F_0	Initial plasmid-harboring fraction	1.0	Assumed

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

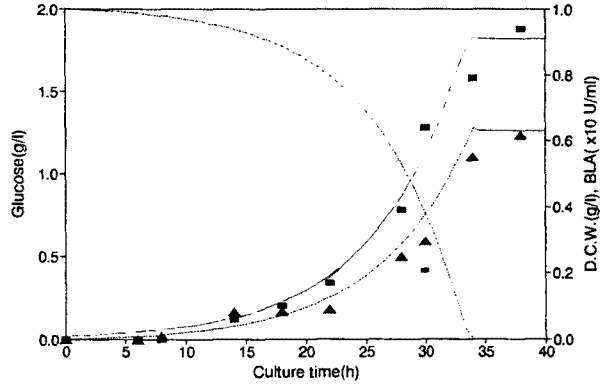

Fig. 7. Comparision of experimental data with mathematical models for biomass, glucose, and β-lactamase production in a batch culture of *Streptomyces lividans* PD6.
□; glucose, ■; D.C.W., ▲; β-lactamase activity

NOMENCLATURE

X : Biomass concentration ($g \cdot l^{-1}$)
X_o : Initial biomass concentration ($g \cdot l^{-1}$)
S : Substrate concentration ($g \cdot l^{-1}$)
S_0 : Initial substrate concentration ($g \cdot l^{-1}$)
μ : Specific growth rate (h^{-1})
μ_{max} : Maxiumum specific growth rate (h^{-1})
t : Culture time (h)
Gn : Generation number
(Gn=0 denotes initiation of continuous operation)
D : Dilution rate (h^{-1})
D_c : Critical dilution rate (h^{-1})
$Y_{x/s}$: Biomass yield ($g \cdot g^{-1}$)
$Y_{p/x}^+$: β-lactamase yield (unit$\cdot g^{-1}$)
K_s : Substrate limitation constant ($g \cdot l^{-1}$)
k : Probability of plasmid loss (dimensionless)
F : Fraction of plasmid-harboring cells (dimensionless)

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

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