Plasmid Retention and Gene Expression in Suspended and Biofilm Cultures of Recombinant *Escherichia coli* DH5α(pMJR1750)

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Differences in plasmid retention and expression are studied in both suspended and biofilm cultures of *Escherichia coli* DH5α(pMJR1750). An alternative mathematical model is proposed which allows the determination of plasmid loss probability in both suspended batch and continuously fed biofilm cultures. In our experiments, the average probability of plasmid loss of *E. coli* DH5α(pMJR1750) is 0.0022 in batch culture in the absence of antibiotic selection pressure and inducer. Under the induction of 0.17 m*M* IPTG, the maximum growth rate of plasmid-bearing cells in suspended batch culture dropped from 0.45 h^{-1} to 0.35 h^{-1}, and the β-galactosidase concentration reached an experimental maximum of 0.32 pg/cell 4 hours after the initiation of induction. At both 0.34 and 0.51 m*M* IPTG, growth rates in batch cultures decreased to 0.16 h^{-1}, about 36% of that without IPTG, and the β-galactosidase concentration reached an experimental maximum of 0.47 pg/cell 3 hours after induction.

In biofilm cultures, both plasmid-bearing and plasmid-free cells increase with time reaching a plateau after 96 hours in the absence of both the inducer and any antibiotic selection pressure. Average probability of plasmid loss for biofilm-bound *E. coli* DH5α(pMJR1750) population was 0.017 without antibiotic selection. Once the inducer IPTG was added, the concentration of plasmid-bearing cells in biofilm dropped dramatically while plasmid-free cell numbers maintained unaffected. The β-galactosidase concentration reached a maximum in all biofilm experiments 24 hours after induction; they were 0.08, 0.1, and 0.12 pg/cell under 0.17, 0.34, and 0.51 m*M* IPTG, respectively.

Key words: plasmid retention • gene expression • biofilm • β-galactosidase • segregational instability • *Escherichia coli*

INTRODUCTION

The applications of recombinant DNA techniques have created a new era in biotechnological research and development. Many successful examples are reported on the use of recombinant DNA cultures in agriculture, in health care, and in the commercial production of pharmaceuticals and special chemicals. Plasmids containing genes of interest can be expressed in different desired procaryotic and eucaryotic organisms.[15,23] However, there are two impediments to widespread commercial utilization of genetically engineered expression systems: (1) the unstable characteristics of plasmids under different culture conditions; and (2) the restrictive regulations which constrain the use of recombinant strains within natural environments. This latter restriction is mainly due to a lack of knowledge regarding the fate of recombinant DNA released in a natural ecosystem.

Two mechanisms will lead to the loss of the original plasmid DNA and subsequent cloned gene expression: structural and segregational instability. *Structural* instability arises from physical changes in the plasmid DNA molecule which usually involves a deletion and/or insertion of a segment of plasmid DNA, or the rearrangement of DNA sequences within the plasmid. *Segregational* instability is the result of random, unequal partitioning of plasmid DNA molecules between daughter cells upon division, potentially leading to the generation of a cell containing no plasmids. Typically, plasmid-free cells exhibit higher turnover rates than their plasmid-bearing counterparts, thus plasmid-free cells will dominate plasmid-bearing cells after several generations.[10,16,19,25,40]

Significant research has focused on those factors that control plasmid segregational and structural stabilities in close systems, (e.g., well-controlled suspended pure culture bioreactors).[26,36,39,45,46] The mathematical model of plasmid loss in batch culture was first proposed by Imanaka and Aiba in 1981.[23] They derived an expression to describe plasmid loss in batch cultures based upon the following assumptions: (1) host cells grow exponentially; (2) those cells that lose all plasmids cannot regain plasmids (i.e., conjugation and transformation are ignored); (3) the probability of losing the plasmid per cell division is constant; and (4) the initial plasmid-free cell concentration is zero. The relation between the plasmid-bearing cell fraction, generation number and probability of plasmid loss is:

$$F(n) = \frac{1 - \alpha - p}{1 - \alpha - p \cdot 2^{n(\alpha+p-1)}} \qquad (1)$$

However, in open environmental systems, a majority of the microbial activity occurs at an interface, within thin

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biological layers known as biofilms.[5,7] Release of plasmid-recombinant microorganisms, either inadvertently or intentionally, to an open ecosystem mandates research into those fundamental organism/plasmid processes that influence plasmid retention, stability, and transfer.

Immobilization and Plasmid Stability

That immobilization might stabilize a plasmid-bearing population in suspension can be shown mathematically.[2] Plasmid persistence in suspended cultures has been observed in cases where the plasmid-bearing cell was at a growth rate disadvantage[1,24]; this observed persistence has been directly attributed to biofilm formation and cell sloughing from the film.[14] Inloes et al.[24] reported the maintenance of a plasmid-containing strain of *E. coli* in the absence of antibiotic selection pressure when immobilized in a hollow fiber membrane.

The effect of immobilization on plasmid stability has been investigated by de Taxis du Poët et al.[11] Plasmid-bearing *E. coli* were immobilized in κ-carrageenan beads that were subsequently fluidized in a chemostat operated at a volumetric residence time of 15 minutes. Cells extracted from the beads and resuspended were shown to have the same plasmid-loss frequency as suspension-cultured cells. However, it was reported that gel immobilization enhanced the stability of the plasmid-bearing population.

Nasri et al.[32] extended this analysis to three genetically differrent *E. coli* hosts (HB101, W3101, and B) using the same plasmid (pTG201), and again reported that, without antibiotics, the fraction of cells in the beads carrying the plasmid was greater than one would find in suspension cultures. In free suspension, all strains studied exhibited varying degrees of plasmid instability; when immobilized, all three strains exhibited stable plasmid maintenance for the duration of the culture. When plasmid-bearing and -free cells were co-immobilized, plasmid-free cells did not overrun the culture. This last result suggests that increased plasmid stability may not be due to plasmid transfer between cells.

Sayadi et al.[38] repeated Nasri's work with just *E. coli* B (pTG201) but under different growth nutrient limitations. This third study found a decreasing specific growth rate increased plasmid copy number and cloned gene activity but decreased stability. They also found that immobilization, in the absence of antibiotic selection, increased stability of the plasmid under glucose, nitrogen, or phosphate limitations, but not for magnesium limited growth. Unfortunately, all the above reports are difficult to interpret. The gel-immobilized cultures were grown in a chemostat operated at a volumetric residence time that was much higher than possible for the suspension cultures used in their comparison. None of the three studies determined the intrinsic growth rates of the cells inside the gel beads. Thus, it is difficult to ascertain whether the apparent increase in stability is due to decreased growth rate in the bead resulting from nutrient mass transport limitations, plasmid transmission between cells, or other mechanisms.

Many studies of plasmid transfer rates for suspended cells indicates that transfer can occur at significant rates under a variety of conditions and organisms,[28,29,39,41,42] including *Pseudomonas* grown in the presence of competing organisms from a natural ecosystem.[39] Studies of plasmid transfer in aquatic systems show that transduction,[29] transformation,[41,42] and triparental mating[20] are all possible means of plasmid movement in these systems. Studies of *Streptococcus faecalis*[8,9] further indicated that conjugative plasmids that transfer at a relatively low frequency in suspension (10^{-6} per donor), when immobilized to a surface, exhibit significantly higher transfer frequencies ($>10^{-4}$ per donor).

There are a number of strategies to improve plasmid stability in recombinant bacterial systems. It comes as no surprise that immobilization might be responsible for alterations in cellular behavior. Many different immobilized cell systems have exhibited altered metabolism. Studies done with immobilized *Saccharomyces* have indicated alterations in productivity,[44] macromolecular composition,[12] and regulation of glycolytic oscillations.[13] *E. coli* has been found to exhibit changes in optimal growth conditions and product yields,[21] while *Bacillus* has exhibited an altered cell morphology upon immobilization.[33] In studies of plasmid transfer in sterile soil, Graham and Istock[17,18] found that transformation occurred in *B. subtilis* in the absence of plasmids or transducing bacteriophage. Similarly, transduction[34,49] and conjugation[34,45] have been observed for soil microorganisms in natural biofilms. Kumar and Schügerl[27] provide an excellent review of the observed increases in plasmid retention, cloned gene expression, and system protein productivity seen in a variety of immobilized cell (bacterial, yeast, and animal cell) systems. Unfortunately, no concise explanation exists for these observed improvements in plasmid stability.

This brief section indicates the ubiquitous occurrence of plasmid movement when considering the fate of genetically engineered DNA in the natural environment. These transfer processes occur in both gram-positive and -negative organisms, in suspended and biofilm-bound communities. Quantification of the risks involved in the release of plasmid-bearing cells to the environment, be it inadvertent or intentional, will therefore require quantitative, mechanistic information regarding both the survival and mobility of the original host/plasmid system in these environments (suspended and immobilized) and the frequency of plasmid transfer from the original host to indigenous microorganisms.

Factors Affecting Plasmid Retention and Expression in Immobilized Cultures

Experimental observations of the effects of immobilization, within artificially formed gel bead carriers, on

recombinant plasmid stability can be summarized as follows:

1. plasmid vectors exhibit higher stability in a reactor system, even in the absence of antibiotic selection;
2. reduction or elimination of plasmid structural instabilities[38]; and
3. plasmid copy number maintained or increased.[32]

Several hypotheses have been suggested to explain the effects of immobilization on plasmid stability, including:

1. structure of the immobilizing gel separates the two populations, negating competition;
2. the close proximity of immobilized cells plus less freedom of motion could promote transfer of plasmid DNA between populations by either conjugation (if a mobilizing factor is present) or transformation; and/or
3. mass transfer limitations on nutrients may create lower growth rates in the interior of a gel, thus promoting increased plasmid stability in those regions.

Similar mechanisms may serve to affect plasmid retention in biofilms naturally formed by attaching bacteria. Blenkinsopp and Costerton[4] report that biofilms, formed under natural conditions, create highly heterogeneous microstructures. Use of confocal laser-scanning microscopy,[31] coupled with fluorescent probe molecules (for Eh, pH, cell viability, cell species number concentration, matrix permeability), clearly indicate that adjacent microcolonies within biofilms may experience radically different environments. Localized concentrations of calcium may promote plasmid DNA incorporation by transformation. Dense glycocalyx structures surrounding established colonies may exhibit different mass transfer properties than adjacent sections of biofilm, segregating bacteria into discrete microcolonies much the same way as in gel beads. One distinction between biofilm-bound cells versus artificially immobilized recombinants, is that naturally attaching bacteria must metabolically produce the encapsulating extracellular polysaccharide glycocalyx; an added burden that artificially immobilized cells do not bear. Metabolic effects of cell replication and extracellular polysaccharide production may affect plasmid segregation differently than in either planktonic or gel-immobilized populations. The effects of polysaccharide synthesis on membrane integrity could also influence both the conjugation and transformation of plasmid DNA.

Another factor influencing plasmid retention and transfer in a biofilm is the continual intraphase transport of cells at the biofilm–fluid interface. Biofilms can be reinoculated with specific community members which is not likely in gel bead systems. Rochell et al.[36] studied plasmid transfer from two donor *Pseudomonas* spp. cultivated wtih two recipient *Pseudomonas* spp. within biofilms in a laboratory reactor system. Results indicated recipient strains survived within the biofilm but not in free suspension. Transconjugants were detected in all cases, despite the fact that donor and recipient cells were inoculated at opposing ends of the substratum used. The authors attributed this observation to cell movement from the biofilm to the liquid phase and redeposition.

Clearly, altered plasmid retention in immobilized or biofilm populations of recombinant cells cannot be explained by any single mechanism. Research has yet to focus on developing methods and systems to assess those environmental and biological factors governing plasmid segregational stability in biofilm populations. Therefore, it is necessary to establish both a mathematical model and experimental protocol with which to describe and quantify the rates of plasmid loss in both suspended and biofilm cultures.

In this study, we propose an alternative approach to calculate plasmid loss probability, in both suspended batch and continuously-fed biofilm cultures, which will be compared to the results calculated by Eq. (1). The effects of culture growth mode (suspended versus biofilm) on plasmid retention and gene expression are investigated here as a function of the level of cloned gene induction.

MATHEMATICAL CONSIDERATIONS

Batch Suspension Culture

The net accumulation of freely suspended plasmid-bearing and plasmid-free cells in a batch culture can be described by two processes: cell growth and growth-related plasmid loss,

$$\frac{dX^+}{dt} = \mu^+X^+ - p\mu^+X^+ \tag{2}$$

$$\frac{dX^-}{dt} = \mu^-X^- + p\mu^+X^+ \tag{3}$$

Let $\chi = X^-/X^+$, and apply the product rule of differentiation, yielding,

$$\frac{dX^-}{dt} = X^+\frac{d\chi}{dt} + \chi\frac{dX^+}{dt} \tag{4}$$

Substituting Eqs. (2) and (3) into (4) and rearranging yields,

$$\frac{d\chi}{dt} = (\mu^- - \mu^+ + p\mu^+)\chi + p\mu^+ \tag{5}$$

During the exponential growth phase, $\mu^- = \mu_m^-$ and $\mu^+ = \mu_m^+$. Therefore, Eqs. (2) and (5) can be rewritten as:

$$\frac{dX^+}{dt} = (\mu_m^+ - p\mu_m^+)X^+ \tag{6}$$

$$\frac{d\chi}{dt} = (\mu_m^- - \mu_m^+ + p\mu_m^+)\chi + p\mu_m^+ \tag{7}$$

Plotting dX^+/dt versus X^+, the slope is:

$$m^+ = \mu_{m^-}^+ - p\mu_m^+ \tag{8}$$

Plotting $d\chi/dt$ versus χ, the slope and intercept are, respectively,

$$m = \mu_m^- - \mu_m^+ + p\mu_m^+ \quad (9)$$

$$b = p\mu_m^+ \quad (10)$$

Solving Eqs. (8), (9), and (10), $\mu_m^+ = m^+ + b$, $\mu_m^- = m^+ + m$, and $p = b/(m^+ + b)$.

Biofilm Culture

Accumulation of cells within a biofilm culture is the net result of several processes, including: the deposition of suspended cells from the liquid phase, cell replication and extracellular polymer production, and biofilm detachment due to shear stress.[6,7] Deposition of suspended cells can be ignored, because cells were not supplied to the reactor after inoculation and the residence time was maintained much less than the generation time of the culture. Assume the existence of plasmid will not affect the adhesion or detachment of the host cells, and the detachment rate constant is the same under same hydrodynamics condition. Therefore, the net accumulation rates of plasmid-bearing and plasmid-free cells in the biofilm can be expressed as the combination of cell growth, plasmid loss, and biofilm detachment:

$$\frac{dB^+}{dt} = \mu^+B^+ - p\mu^+B^+ - k_{det}B_d^+ \quad (11)$$

$$\frac{dB^-}{dt} = \mu^-B^- + p\mu^+B^+ - k_{det}B_d^- \quad (12)$$

Assuming the distributions of plasmid-bearing and plasmid-free cells within the biofilm are uniform with depth, then one can assume that cells are detached in the same ratio as exists in the biofilm. Defining $\beta = B^-/B^+ = B_d^-/B_d^+$, and substituting into Eq. (12),

$$\frac{dB^-}{dt} = B^+\frac{d\beta}{dt} + \beta\frac{dB^+}{dt} \quad (13)$$

Substituting Eqs. (11) and (12) into (13) and rearranging,

$$\frac{d\beta}{dt} = (\mu^- - \mu^+ + p\mu^+)\beta + p\mu^+ \quad (14)$$

The specific growth rates for the two populations can be expressed as:

$$\mu^+ = \frac{\mu_m^+ S}{K_S + S} \qquad \mu^- = \frac{\mu_m^- S}{K_S + S}$$

Thus, Eq. (14) can be rewritten as:

$$\frac{d\beta}{dt} = \left(\frac{\mu_m^- S}{K_S + S} - \frac{\mu_m^+ S}{K_S + S} + p\frac{\mu_m^+ S}{K_S + S}\right)\beta + p\frac{\mu_m^+ S}{K_S + S} \quad (15)$$

Plotting $d\beta/dt$ versus β, the slope and intercept will be:

$$m' = (\mu_m^- - \mu_m^+ + p\mu_m^+)\frac{S}{K_S + S} \quad (16)$$

$$b' = p\frac{\mu_m^+ S}{K_S + S} \quad (17)$$

Solving Eqs. (16) and (17) to get

$$p = \frac{b'}{m' - b'}\frac{\mu_m^- - \mu_m^+}{\mu_m^+}. \quad (18)$$

MATERIALS AND METHODS

Bacterial Strain and Plasmid

E. coli DH5α (kindly donated by Dr. Vickers Burdett, Department of Microbiology and Immunology, Duke University) was selected for this study, because it can form biofilm efficiently under low substrate concentrations and does not naturally produce β-galactosidase. Its genotype is *Ø80dlacZΔM15*, *Δ(lacZYA-argF)*, *U169*, *deoR*, *recA1*, *endA1*, *hsdR17*, *supE44*, *thi-1*, *gyrA96* (nalidixic acidresistant), *relA1*. Plasmid pMJR1750 is a 7.5-kb plasmid consisting of an ampicillin-resistant marker, a strong promoter, *tac*, a repressor gene, $lacI^Q$, and the *lacZ* gene which encodes β-galactosidase. The β-galactosidase promoter can be induced by various inducers, including isopropyl β-D-thiogalactoside (IPTG). Hence, cells harboring the plasmid which express β-galactosidase form blue colonies on plate medium containing 5-bromo-4-chloro-3-indol-β-D-galato-pyranoside (X-gal), whereas plasmid-free cells form white colonies.

Batch Culture

The fermentation medium used was M9 minimal medium containing 0.2% glucose, 0.4% casamino acid, and 0.01% thiamine. The batch culture was performed in the absence of ampicillin in a Biostat M fermentor (B. Braun, Allentown, PA) of 1-L working volume at pH 7.0 and 37°C. Dissolved oxygen was maintained above 50% of saturation. One percent (v/v) of exponentially growing cells resuspended in identical medium was used to inoculate the fermentation vessel to minimize the lag phase. Ten milliliters of cell suspension was sampled every hour for analysis. For induction experiments, IPTG was added when the absorbance at 600 nm reached about 0.8.

Biofilm Formation System

Biofilms of *E. coli* DH5α(pMJR1750) were cultivated in a parallel-plate flow cell which is constructed of optically clear polymethylmethacrylate (PMMA). The detailed reactor design is illustrated in Figure 1 and its pretreatment protocol can be found elsewhere.[22] The inoculum was centrifuged from an overnight suspension culture which was selectively cultivated under 100 μg/mL ampicillin, then resuspended into about 10^8 cells/mL with sterile M9 minimal medium. The flow cell reactor was inoculated by recirculating the inoculum through the cell for 2 hours at a flow rate of 45 mL/min. After in-

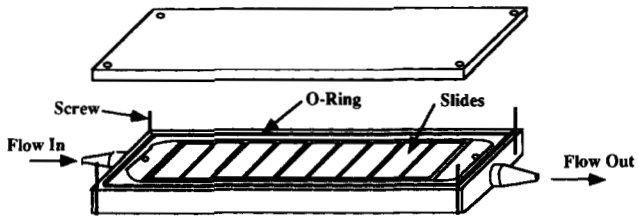

Figure 1. Schematic drawing of the parallel-plate flow cell.

oculation, the inoculum container was removed from the recycle loop and replaced with a small mixing vessel (100 mL) and the loop rinsed with sterile M9 minimal medium supplemented with 50 mg/L glucose, 100 mg/L casamino acid, and 25 mg/L thiamine, and recirculation flow resumed. Fresh nutrient solution was delivered to the mixing vessel to affect an overall system dilution rate of 4 h^{-1}.

The system was oxygenated with pure oxygen to prevent oxygen limitation. System dilution rate was maintained well in excess of the growth rate of *E. coli* DH5α, which served to minimize cell growth in the fluid phase. The mixing vessel and connecting tubing were replaced with sterile versions every 12 hours to minimize the biofilm growth outside the flow cell. A micro flow-thru dissolved oxygen probe (Lazar Research Lab., Inc., Los Angeles, CA) was placed in the recirculation loop to monitor dissolved oxygen of medium leaving the flow cell.

A slide with accumulated biofilm was removed from the flow cell reactor every 12 hours and replaced with a clean slide. Biofilm on the removed slide was scraped completely into 50 mL autoclaved M9 minimal medium and vortexed at maximum for 5 minutes to completely disrupt all bacterial aggregates. The resultant suspension of biofilm material was used in the analyses detailed below.

Measurement of Plasmid Stability

Segregational instability was determined by cell growth on LB-agar plates that contained 40 μg/mL X-gal, 40 μg/mL IPTG, and 50 μg/mL nalidixic acid. Biomass samples from batch and biofilm cultures were suitably diluted with sterile M9 minimal medium and spread on the plates to form between 30 and 300 colonies per plate. The number of viable plasmid-bearing and plasmid-free bacteria were determined by averaging the blue and white colony-forming units (CFU), respectively, on three plates. The probability of plasmid loss per cell division was calculated with Eq. (1) and the alternative approach described in this study. Eq. (1), nonlinear in p, was solved by a ZREAL subroutine from the IMSL mathematical library. Time derivatives of the ratio of plasmid-free to plasmid-bearing cells for both suspension batch and biofilm cultures ($d\chi/dt$ and $d\beta/dt$, respectively) in Eqs. (7) and (15) are calculated from a least-squares, second-order polynomial fit. Structural stability of the plasmid was checked periodically throughout an experiment by horizontal electrophoresis. No plasmid structural modification was found in any experiments.

β-Galactosidase Assay

Samples obtained from batch and biofilm cultures were centrifuged at 1000g for 15 minutes at 5°C. Cell pellets were resuspended in 1 mL TEP buffer (10 mM Tris; 1 mM EDTA, pH 8.0; 1 mM PMSF, phenylmethylsulfonyl fluoride) then disrupted using two 30-second pulses by a Kontes Micro-Ultrasonic Cell Disrupter (Vineland, NJ) set at 30% output. The sonicated cellular homogenate was transferred to microcentrifuge tubes and chilled on ice for 10 minutes then centrifuged at 5000g for 10 minutes to pellet cell debris. The β-galactosidase activity was determined by the rate of hydrolysis of the colorless compound, o-nitrophenyl-β-D-galactoside (ONPG), to the yellow chromophore, o-nitrophenol (ONP).[30] Cell extract, 200 μL, was mixed with 2.5 mL reagent A (0.1M Na_2HPO_4, adjusted to pH 7.3 with 0.1M NaH_2PO_4), 100 μL reagent B (3.6M β-mercaptoethanol), 100 μL reagent C (30 mM $MgCl_2$), and 200 μL reagent D (33.2 mM ONPG in reagent A) in a disposable cuvette.

After vortex mixing, the cuvette was placed in a Milton Roy Spectronic 1201 Spectrophotometer (Rochester, NY), and the change in the absorbance at 410 nm monitored over a 2-minute interval. The activity was calculated using Beer's law where 1 unit of β-galactosidase activity was defined as the amount of enzyme that can hydrolyze 1 μmol ONPG in 1 minute at pH 7.3 and 25°C. The amount of β-galactosidase was determined by calibrating activities of aliquots of standard β-galactosidase solutions (5 Prime → 3 Prime, Inc., Boulder, CO).

RESULTS

Segregational Instability of Plasmids

Batch Culture

Three individual experiments were performed to study of the probability of plasmid pMJR1750 loss; typical experimental results are presented in Figure 2. Apparent in Figure 2 is that the plasmid-free cells grow faster than plasmid-bearing cells in suspension without any antibiotic selection. The percentage of plasmid-free cells is initially less than 0.02% of the culture, but increases to 1.3% by the end of experiment. Table I summarizes the parameters and probabilities of plasmid loss calculated by the classic model and the alternative approach proposed here. The average probability of plasmid loss calculated using Eq. (1) is 0.0009 and that by the alternative approach is 0.0022.

Biofilm Culture

Figure 3 depicts the net accumulation of the plasmid-bearing and plasmid-free *E. coli* DH5α(pMJR1750)

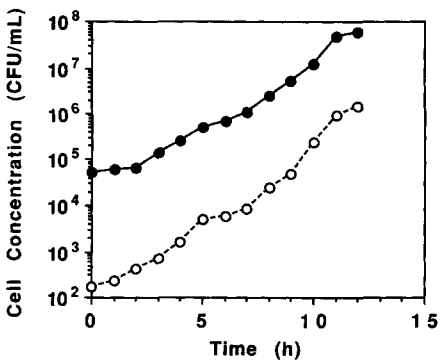

Figure 2. Population dynamics of plasmid-bearing (●) and plasmid-free (○) planktonic cells of *E. coli* DH5α(pMJR1750) in a batch culture at 37°C, pH 7.0.

cells in biofilms cultivated at a carbon-to-nitrogen ratio of 0.07. Both plasmid-bearing and plasmid-free cells increase slowly during the first 36 hours, then enter an apparent maximum accumulation phase, and finally reach a plateau after 96 hours. Without antibiotic selection, the plasmid-free cell percentage increases from 1% to 27%. The probability of plasmid loss in biofilm was calculated by Eq. (18) using the average μ_m^+ and μ_m^- determined from batch cultures. Table II lists the parameters and probabilities of plasmid loss for three independent biofilm experiments. The plasmid loss probabilities range from 0.013 to 0.021, with an average of 0.017 ±0.004.

β-Galactosidase Expression

Batch Culture

Three different IPTG concentrations were added to suspended batch cultures. Figure 4 shows the population dynamics of plasmid-bearing cells after induction. The growth rate of plasmid-bearing cells is 0.45 ±0.06 h^{-1} with no IPTG present. Once IPTG was added, the growth rate decreased. Under 0.17 m*M* IPTG, the growth rate drops to 0.35 ±0.05 h^{-1}, about 77% of the uninduced growth rate. At both 0.34 and 0.51 m*M* IPTG, the growth rates decreased to 0.16 ±0.03 h^{-1}, about 36% of that without IPTG. The β-galactosidase production in batch cultures of *E. coli* DH5α(pMJR1750) is shown in Figure 5, expressed on amount of a single plasmid-bearing cell. Under 0.17 m*M* IPTG, the β-galactosidase concentration reaches its maximum of 0.32 pg/cell in the experiment 4 hours after induction. At 0.34 and 0.51 m*M* IPTG, the β-galactosidase concentration reaches a peak specific production of 0.47 pg/cell, 3 hours after induction.

Biofilm Culture

Figure 6a and b shows the biofilm net accumulation of plasmid-bearing and plasmid-free cells in response to induction. Plasmid-bearing cells can reach a plateau if no IPTG is added. But, once IPTG was added, the plasmid-bearing biofilm cell density dropped dramatically, because cell growth rates decrease due to induction. Conversely, plasmid-free biofilm cells continued to increase and were not affected by IPTG level. Figure 7 is the β-galactosidase production in biofilm cultures of *E. coli* DH5α(pMJR1750). The β-galactosidase concentration can reach its maximum in each experiment about 24 hours after induction. The β-galactosidase concentrations are 0.08, 0.1, and 0.12 pg/cell at 0.17, 0.34, and 0.51 m*M* IPTG, respectively.

DISCUSSION

Comparison of Probability Calculation in Batch Cultures

Three possible reasons exist to explain the differences in probabilities of plasmid loss calculated by these two methods. First, Imanaka and Aiba assumed the initial plasmid-free cell concentrations were zero. However, it is difficult to cultivate an inoculum of 100% plasmid-bearing cells even under a high selection pressure. The traditional way to attain 100% plasmid-bearing culture is to start with the single plasmid-bearing cell, but this leads to a long lag phase in the culture. Second, calculations of p are sensitive to the amount of data collected; the smaller the generation number, the larger the resultant value of p. Third, although exponentially growing cells from identical medium are used as inoculum in all experiments, some initial lag phase is still inevitable.

Feasibility of Probability Calculation in Biofilm Cultures

The approach derived for biofilm cultures assumes the cell concentration in the fluid phase is negligible, and

Table I. Parameters and probabilities for suspended batch culture of *E. coli* DH5α(pMJR1750).

	Imanaka and Aiba[23]				This study			
Experiment	n[a]	$F(n)$[a]	α	p[b]	m^+ (h^{-1})	m (h^{-1})	b (h^{-1})	p
A	8	0.9793	1.17	0.0023	0.41	0.07	0.0004	0.0010
B	9	0.9747	1.25	0.0017	0.52	0.13	0.0003	0.0006
C	8	0.9774	1.15	0.0027	0.41	0.06	0.0005	0.0012
Average				0.0022 ±0.0005				0.0009 ±0.0003

[a] Determined by the final data point of experiment.
[b] Solved by the ZREAL subroutine from the IMSL mathematical library.

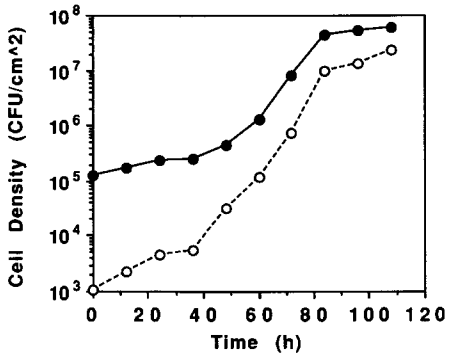

Figure 3. Net accumulation of plasmid-bearing (●) and plasmid-free (○) cells of *E. coli* DH5α(pMJR1750) in a biofilm culture grown continuously at 37°C, pH 7.0, Re = 20, and residence time in system = 0.00625 h.

hence, redeposition of detached cells can be ignored. To validate this assumption, samples from the fluid phase were plate-counted. Figure 8 shows the plasmid-bearing and plasmid-free cell concentration in the fluid phase downstream of the flow cell increases in accordance with biofilm accumulation, but the cell concentration never exceeded 10^5 cells/mL. Because the dilution rate for the biofilm system is 4 h^{-1}, the only source of cells in the fluid phase is by way of the detachment from the biofilm. The redeposition of these detached cells can be examined by the sticking efficiency,[7] which is defined as the ratio of the deposition rate to the flux of cells to the substratum. Average sticking efficiencies for *E. coli* DH5α(pMJR1750) to glass surface under 45 mL/min is 2.5×10^{-7}. This means that less than 3 cells were attached irreversibly for every 10^7 cells transported to the glass surface. Therefore, the amount of detached cells that could be redeposited onto the biofilm surface can be neglected as compared with the biofilm density.

The second assumption regards the even distribution of plasmid-bearing and plasmid-free cells within biofilm. The validity of this assumption was examined by comparing the ratios of plasmid-free to plasmid-bearing cells in both the biofilm and in cells detached from the film and re-entrained into the liquid phase (Fig. 9). The ratios are approximately the same for the first 48 hours, but after that, as the biofilm accumulation approaches its maximum, the amount of plasmid-free cells detached into the liquid phase relative to plasmid-bearing cells increases dramatically—more so than in the overall biofilm. Early in the biofilm accumulation, the plasmid-bearing cells initially dominate the surface. However, the plasmid-free cells grow at a faster rate and, during the course of the experiment, dominate the upper layers of the biofilm, which is reflected in the higher numbers of plasmid-free cells re-entrained into the liquid phase. This prospect will negate the validity of certain assumptions made in the mode derived here. Further study using BIOSIM,[35] an interactive program for the simulation of dynamics of mixed culture biofilm systems, will be modified to allow the prediction of spatial distributions of plasmid-bearing and plasmid-free strains in biofilms, which will be discussed in a subsequent article.

Probabilities of Plasmid Loss Between Batch and Biofilm Cultures

In the experiments above, the probability of plasmid loss in a biofilm culture was greater than that observed in a suspended batch culture. The probability of plasmid loss during cell division has been reported to be affected by many factors including copy number, medium composition, and growth rates.[42] Some models suggest the probability of plasmid loss is a function of copy number which may vary during cell division; the higher the copy number, the smaller the probability. Copy number variance between batch and biofilm cultures may be a key factor affecting our estimated loss probabilities. Further determination of plasmid copy numbers in batch and biofilm cultures is required in order to explain the differences in plasmid loss between batch and biofilm cultures. The media used for batch and biofilm cultures are the same, except for the glucose concentrations, which can lead to different growth rates. In addition, the production of extracellular polymer in biofilm cells may compete with plasmid maintenance/replication for metabolic intermediates and energy sources—a competition suspended cells may not experience.

Plasmid Retention in Response to Induction

Many researches have reported that induction will reduce specific growth rates of plasmid-bearing cells dramatically.[3,45] In our experiments, the growth rates of *E. coli* DH5α(pMJR1750) decreases 36% to 77% depending upon the level of induction. In batch cultures, an increase in the relative amount of plasmid-free cells resulted from the reduced growth rate of plasmid-bearing cells. Growth rates of plasmid-free cells are unaffected by induction. In biofilm cultures, upon induction, the percentage of plasmid-free cells increases due to the reduced growth rates of plasmid-bearing cells. While the

Table II. Parameters and probabilities for biofilm culture of *E. coli* DH5α(pMJR1750).

Experiment	Sticking efficiency	m' (h^{-1})	b' (h^{-1})	p
A	2.07×10^{-7}	0.0187	0.0015	0.013
B	1.92×10^{-7}	0.0058	0.0007	0.021
C	3.50×10^{-7}	0.0115	0.0012	0.018
Average	$(2.50 \pm 0.87) \times 10^{-7}$			0.017 ±0.004

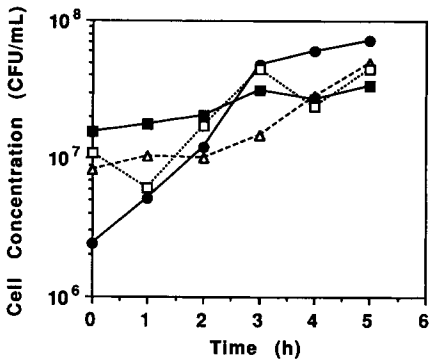

Figure 4. Plasmid-bearing cell concentration profile of *E. coli* DH5α(pMJR1750) in response to various levels of induction by IPTG: (●) 0 m*M*; (△) 0.17 m*M*; (■) 0.34 m*M*; (□) 0.51 m*M*.

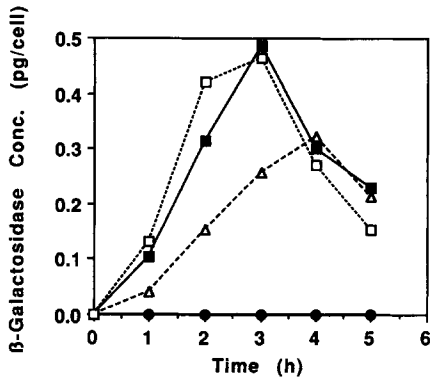

Figure 5. β-Galactosidase production in batch cultures of *E. coli* DH5α(pMJR1750) under different induction levels: (●) 0 m*M*; (△) 0.17 m*M*; (■) 0.34 m*M*; (□) 0.51 m*M* IPTG.

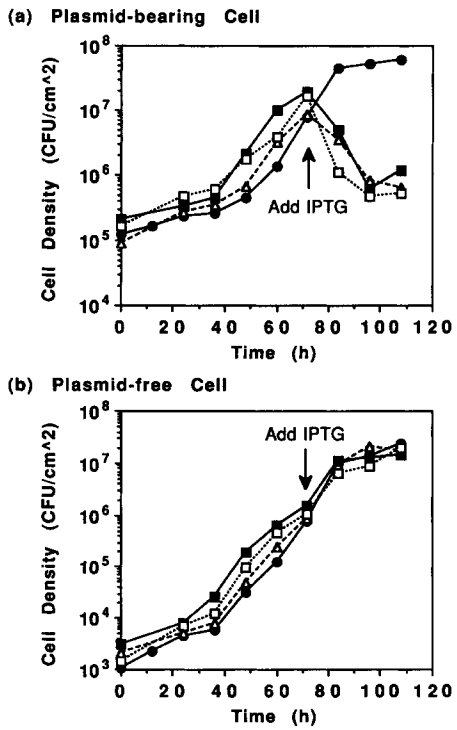

Figure 6. Biofilm net accumulation of *E. coli* DH5α(pMJR1750) in response to the induction by IPTG: (●) 0 m*M*; (△) 0.17 m*M*; (■) 0.34 m*M*; (□) 0.51 m*M*.

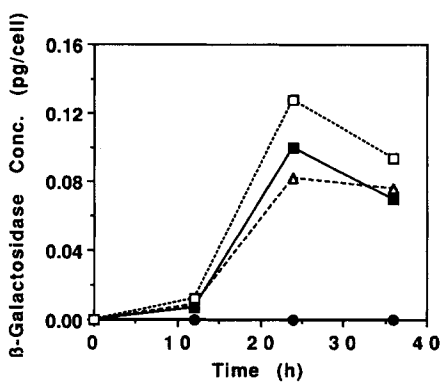

Figure 7. β-Galactosidase production in biofilm cultures of *E. coli* DH5α(pMJR1750) under different induction levels: (●) 0 m*M*; (△) 0.17 m*M*; (■) 0.34 m*M*; (□) 0.51 m*M* IPTG.

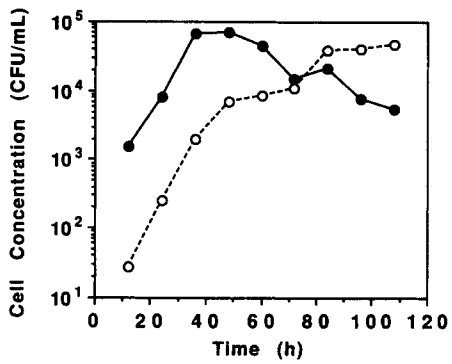

Figure 8. Concentration profile of plasmid-bearing (●) and plasmid-free (○) cells of *E. coli* DH5α(pMJR1750) in the liquid phase of biofilm reactor.

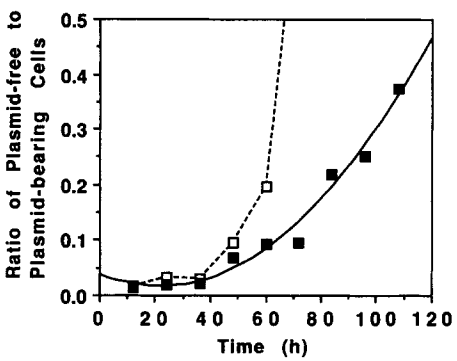

Figure 9. Comparison of ratios of plasmid-free to plasmid-bearing cells between biofilm and detached cell in liquid phase: (■) biofilm; (□) detached cells.

biofilm community loses both plasmid-bearing cells and plasmid-free cells due to detachment during early phases of development, the plasmid-bearing cells soon become relegated to the depths of the biofilm due to the faster growing plasmid-free cells. Consequently, shear removal begins to affect plasmid-bearing cells less than plasmid-free cells. Unlike biofilm cultures, batch cultures cannot operate for a long time due to the depletion of substrate and, in continuous cultures, segregation will eliminate all plasmid-bearing cells eventually.

β-Galactosidase Expression Between Batch and Biofilm Cultures

The β-galactosidase concentrations in suspended cells increase with IPTG concentration and time but drop at the end of each experiment which is consistent with other reports.[3,46] Maximum β-galactosidase concentrations increase from 0.32 to 0.47 pg/cell when IPTG concentration increases from 0.17 to 0.34 m*M*, but higher IPTG concentrations (0.51 m*M*) produce no further increase in protein production. This suggests the repressor, *lacI*^{Q}, has been saturated by IPTG. Optimal inducer concentration is dependent on the host, repressor, promoter, and cloned-gene protein. In biofilm cultures, the β-galactosidase concentrations are very low after the first 12 hours, because plasmid-bearing cells may take a longer time to adjust their metabolic pathway and to induce enzyme production. The different IPTG concentrations did not affect the maximum β-galactosidase concentration. This is because the IPTG was fed continuously and the repressor was assumed saturated 24 hours after induction. The maximum β-galactosidase concentrations in suspended cells are obviously higher than those in biofilm cells. Cell volume of suspended cells were also larger. However, this does not necessarily mean the ratio of clone-gene protein to total protein, a parameter normally used to quantify gene expression, in suspended cells is higher than in biofilm cells. Unfortunately, in our experiments, we cannot differentiate the total protein from plasmid-bearing or plasmid-free cells. Further study using plasmid pTKW106,[46] which contains a *par*B locus, will eliminate the evolution of plasmid-free cells, allowing us to estimate β-galactosidase fractions based on total protein.

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NOMENCLATURE

B^+, B^-	cell density of plasmid-bearing and plasmid-free biofilm (cell L^{-2})
B_d^+, B_d^-	cell density of detached plasmid-bearing and plasmid-free biofilm (cell L^{-2})
b, b'	intercepts from Eqs. (10) and (15) (t^{-1})
$F(n)$	plasmid-bearing cell fraction at nth generation
K_S	saturation constant ($M\ L^{-3}$)
k_{det}	detachment rate constant (t^{-1})
m^+, m, m'	slopes from Eqs. (6), (7), and (15) (t^{-1})
n	generation number
p	probability of plasmid loss per cell per cell division
S	limiting substrate concentration ($M\ L^{-3}$)
X^+, X^-	suspended plasmid-bearing and plasmid-free cell concentration (cell L^{-3})
α	ratio of specific growth rates of plasmid-free to plasmid-bearing cells
β	ratio of biofilm cell density of plasmid-free to plasmid-bearing cells
μ^+, μ^-	specific growth rate of plasmid-bearing and plasmid-free cells (t^{-1})
μ_m^+, μ_m^-	maximum specific growth rate of plasmid-bearing and plasmid-free cells (t^{-1})
χ	ratio of suspended cell concentration of plasmid-free to plasmid-bearing cells

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