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RECOMBINANT PLASMID RETENTION AND EXPRESSION IN BACTERIAL BIOFILM CULTURES

James D. Bryers and Ching-Tsan Huang

The Center for Biofilm Engineering, A National Science Foundation, Engineering Research Center, Montana State University, Bozeman, MT 59717, USA

ABSTRACT

Any exposure of plasmid recombinant microorganisms to an open system environment, either inadvertently or intentionally, mandates research into those fundamental organism:plasmid processes that influence plasmid retention, transfer and expression. In open environmental systems a majority of the microbial activity occurs associated with an interface, within thin biological layers consisting of the cells and their insoluble extracellular polymer, layers known as *biofilms*. Thus any study regarding the fate of recombinant DNA sequences in an open system must consider processes that affect plasmid retention and expression in a biofilm culture.

Biofilm cultures were cultivated in a parallel-plate flow cell reactor using *E. coli* DH5α which contained a recombinant plasmid with a plasmid stability factor, *parB*, (pTKW106) or without (pMJR1750). Using ß-galactosidase as inducible reporter protein, plasmid retention and gene expression of pMJR1750 and pTKW106, in suspended versus biofilm cultures, were studied under different carbon to nitrogen ratios and plasmid induction levels. Recombinant biofilm formation under these environmental conditions was also investigated. Biofilm net accumulation rate of *E. coli* DH5α (pTKW106) decreases with increasing induction levels. The ß-galactosidase production and ratios of ß-galactosidase to total protein increase with increasing induction levels. Synthesis rates of total RNA, ß-galactosidase mRNA and rRNA in biofilm cultures of *E. coli* DH5α (pTKW106) increase after induction by IPTG.

KEYWORDS

Bacterial biofilms, plasmid loss, mathematical model, plasmid expression.

INTRODUCTION

Applications of recombinant DNA techniques have created a new era in biotechnological research and development. Numerous examples are reported on the successful use of recombinant DNA cultures in agriculture, in health care, and in the commercial production of pharmaceuticals and special chemicals. Plasmids are extrachromosomal DNAs which possess their own origins of replication (replicons) and are capable of self-duplication. They usually encode no essential functions and can be transferred between cells. Recombinant plasmids containing heterologous genes of interest can be expressed in different desired procaryotic and eucaryotic organisms (Georgiou, 1988; Lauffenburger, 1987). 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)* restrictive governmental regulations which constrain the use of recombinant strains within natural environments. 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 (Ensley, 1986). Restrictions on the release of recombinant plasmid DNA are due mainly to a lack of knowledge regarding the fate of recombinant DNA in a natural ecosystem. Antibiotic selection is employed currently to reduce the number of or eliminate any plasmid-free cells that may arise in the course of commercial cultivation, but this is extremely expensive and not always 100% effective. Besides, overuse of antibiotics can lead to new resistant strains.

Significant research has focused on those factors that control plasmid segregational and structural stabilities in close systems, *i.e.*, in well-controlled suspended pure culture bioreactors (Rochelle *et al.*, 1989; Wood *et al.*, 1990; Shoham and Demain, 1991; Birnbaum and Bailey, 1991; Stephens *et al.*, 1992). However, in an open environmental system, the majority of microbial activity is associated with an interface, within thin biological layers known as biofilms. Exposure of plasmid recombinant microorganisms to an open system, either inadvertently or intentionally, mandates research into those fundamental organism/plasmid processes that influence plasmid retention, stability and transfer in biofilm systems.

Biofilm Formation

When solid surfaces are submerged in an aquatic environment, suspended microbial cells attach to the surface, the immobilized cells grow, replicate, and secrete extracellular polymers that surround the cells within a gelatinous matrix; the collective surface community referred to as a biofilm (Characklis and Marshall, 1990). Although the term is usually applied to bacterial cells and their extracellular polymers, any biologically active layer of cells (microbial, plant or mammalian) can be considered a biofilm (Bryers, 1987). Biofilms are three dimensional gelatinuous structures consisting bacterial cells entrapped within a matrix of insoluble extracellular polysaccharides secreted by the bacteria themselves. Biofilm formation is the net result of several processes occurring simultaneously, including: suspended cell deposition, attached cell metabolism, and biofilm removal processes (Bryers, 1987). Deposition of cells onto a substratum includes several individual processes: the macromolecular organic preconditioning of the target surface; cellular transport from the bulk phase to the solid substratum; and reversible and irreversible cell adhesion to the surface. Cellular growth, substrate conversion, endogenous decay, and extracellular polymer (EP) production collectively comprise biofilm metabolic processes. Biofilms can be removed from the surface through chemical or physical challenges, abrasion, shear-related detachment, and sloughing.

Plasmid DNA Basics

A plasmid is an extrachromosomal, circular, and double stranded DNA molecule which is found in both procaryotic and eucaryotic organisms. It can be used as a cloning vehicle for the isolation of specific DNA sequences. Plasmid DNAs usually encode no essential functions, and bacteria lacking them usually multiply normally. There are two essential features of plasmids: replication and mobilization. Replication of plasmid DNA is carried out by subsets of enzymes used to duplicate the chromosome. Different plasmids can duplicate themselves to a different extent in different host cells. Copy number, the number of plasmid molecules per host-cell chromosome (which is controlled by a region of plasmid DNA including the origin of DNA replication), can vary from one to 700 per cell (Sambrook *et al.*, 1989). Plasmids can be transmitted to new hosts through conjugation, transformation and transduction. Under natural conditions, many plasmids are transmitted to new hosts by conjugation, a procedure by which donor cells can transfer genes to recipient cells (Hayes, 1952). However, plasmid vectors in common use are incapable of directing their own conjugation because of the lack of the *tra* gene. They can move from one cell to another in the form of pure DNA. A bacterium absorbs pure DNA from its surroundings and functionally integrates the exogenous DNA into its own genetic material in a process called transformation. Bacterial genes can also be passively carried from one bacterium to another by phage particles, a process called transduction (Watson, *et al.*, 1987). Desirable characteristics for useful vectors include: *(1)* high copy number, *(2)* possession of several unique restriction endonuclease cleavage sites, *(3)* small size, *(4)* genetic stability, *(5)* a screening marker, and a simple procedure to transfer into the host.

Mechanisms for Plasmid Instability. Two mechanisms will lead to the loss of the original plasmid DNA and subsequent cloned gene expression: *(1)* segregational instability and *(2)* structural instability. *Structural* instability arises from a physical change of the plasmid DNA molecule which usually involves the deletion

and/or insertion of a segment or segments of plasmid DNA, or the rearrangement of DNA sequences within the plasmid. *Segregational* instability is the result of random, uneven partitioning of plasmid DNA molecules between daughter cells upon division, potentially leading to the generation of a cell containing no plasmids. Structural instability is more insidious because it can cause the loss of the desired gene or the expression of the cloned-gene protein while the other plasmid DNA, including the selection markers, are retained and expressed. Significant research has found, both experimentally and theoretically, that plasmid maintenance and cloned gene expression reduce the overall growth rate of the plasmid-bearing cell relative to plasmid-free cell (Uhlin, 1978; Peretti and Bailey, 1987).

Immobilization and Plasmid Stability. That immobilization might stabilize a plasmid-bearing population in suspension can be shown mathematically (Ollis, 1982). Plasmid persistence in suspended cultures has been observed in cases where the plasmid-bearing cell was at a growth rate disadvantage (Adams *et al.*, 1979) and has been directly attributed to biofilm formation and cell detachment from the biofilm (Dykhuizen and Hartl, 1983). Inloes *et al.* (1983) reported the maintenance of a plasmid-containing strain of *E. coli* in the absence of selection pressure when immobilized in a hollow fiber membrane.

The effect of cell immobilization in gel beads on plasmid stability has been investigated by de Taxis du Poët *et al.* (1986). 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, yet it was reported that immobilization enhanced the stability of the plasmid-bearing population. Nasri *et al.* (1987) extended this analysis to three genetically-different *E. coli* hosts (HB101,W3101, and B) using the same plasmid (pTG201), and again reported that, without antibiotics, the fraction of cells carrying plasmid in the beads was greater than one would find in suspension cultures. In free suspension, all planktonic cells exhibit 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 coimmobilized, plasmid-free cells did not overrun the culture. Their results suggest that increased plasmid stability was not due to either plasmid transfer between cells or to increases in copy number. Sayadi *et al.* (1989) 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.

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 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 to indigenous microorganisms from the original host.

MATHEMATICAL CONSIDERATIONS

A simple binary strain population model has been derived (Huang *et al.*, 1993) that predicts the time-dependent concentrations of plasmid-bearing and plasmid-free cells during biofilm formation. The model accounts for the growth rate of both the bearing and free strains, the detachment rate from the biofilm of both populations, plus segregational loss from the plasmid-bearing population resulting in an increase in plasmid-free cells. Deposition of suspended cells is ignored in our model because once surface inoculation was complete, suspended bacterial cells were not supplied to the reactor. In addition, during biofilm formation, the residence time in the reactor was maintained much lower than the generation time of the culture thus essentially eliminating suspended cell replication in the fluid phase. Thus, any suspended cells leaving the reactor effluent must originate only from the biofilm due to the detachment process. We also assume that the existence of the plasmid does not affect the rate of adhesion or detachment of the host cells and that the detachment rate was the same for both plasmid-bearing and plasmid-free cells under same hydrodynamic conditions. Details as to the derivation of the model describing plasmid loss in a biofilm population due to segregation can be found in Huang *et al.* (1993). Results of that model indicate that the probability of plasmid loss can be calculated from:

$$p = \left[\frac{b'}{m'-b'}\right] \frac{(\mu_m^- - \mu_m^+)}{\mu_m^+} \qquad (1)$$

where $$m' = (\mu_m^- - \mu_m^+ + \mathbf{p}\, \mu_m^+) \frac{S}{K_S+S} \quad (2)$$

$$b' = \mathbf{p}\, \frac{\mu_m^+ S}{K_S+S} \quad (3)$$

where μ^+ and μ^- are the specific growth rate ($time^{-1}$) for plasmid-bearing and plasmid-free cells; Ks = the half saturation coefficient ($mass\text{-}length^{-3}$); and $\mathbf{p}$ is the probability of plasmid loss.

MATERIALS AND METHODS

Bacterial Strain and Plasmids

E. coli DH5α (kindly donated by Dr. Vickers Burdett, Department of Microbiology, Duke University) was selected for this study since this strain could form biofilms efficiently under low substrate concentrations and since it did not naturally produce the reporter protein, ß-galactosidase. Its genotype was *ø80dlacZΔM15, Δ (lacZYA-argF), U169, deoR ,recA1, endA1, hsdR17, supE44, thi-1, gyrA96, relA1.* Plasmid pMJR1750 is a 7.5-kb plasmid consisting of an ampicillin resistance marker, a strong promoter (*tac*), a repressor gene, ($lacI^Q$), and the *lacZ* gene which encodes for ß-galactosidase. The ß-galactosidase promoter can be induced by various chemical inducers, including isopropyl ß-D-thiogalactoside (IPTG). Recombinant cells that express ß-galactosidase form blue colonies on a plate medium containing 5-bromo-4-chloro-3-indol-ß-D-galato-pyranoside (X-gal) and IPTG whereas plasmid-free cells form white colonies. Plasmid pTKW106 (Wood *et al.*, 1990) is a 11.9-kb plasmid consisting of a kanamycin resistance marker, a strong promoter (*tac*), a repressor gene ($lacI^Q$), a *lacZ* gene which encodes for ß-galactosidase, and the *parB* locus.

Biofilm Formation System

Biofilms of *E. coli* DH5α were cultivated in a parallel-plate flow cell which was modified from our previous design to allow for more biofilm sampling area (Huang *et al.*, 1993). The schematic experimental set-up for inoculation and biofilm formation and the operating conditions are presented elsewhere in greater detail (Huang *et al.*, 1993). Inoculum was centrifuged from an overnight suspension culture which was grown in M9 minimal medium supplemented with 2 g/L glucose, 100 mg/L thiamine and trace elements ($FeCl_3 \cdot 6H_2O$, 27 mg/L; $MnCl_2 \cdot 4H_2O$, 10 mg/L; $ZnCl_2$, 2 mg/L; $CuCl_2 \cdot 2H_2O$, 0.85 mg/L; $CoCl_2 \cdot 6H_2O$, 2.5 mg/L) and was selected under 100 µg/mL ampicillin. Cell pellets were resuspended to about 10^8 cells/mL with sterile M9 minimal medium. The flow cell reactor was inoculated by recirculating inoculum through the reactor cell for 2 hours at a flow rate of 45 mL/min. Once the inoculation period was completed, the inoculum container was removed from the recycle loop and replaced with a small mixing vessel (*ca.*100 mL), the loop rinsed with sterile medium and recirculation flow resumed. After inoculation, no suspended cells were introduced to the system; any suspended cells leaving in the reactor effluent must originate from the biofilm due to detachment process. In separate experiments, medium at different C/N ratios was delivered to the mixing vessel at a volumetric feed rate to affect an overall system dilution rate of 4 h^{-1}; a dilution rate which was much greater than the maximum growth rate of *E. coli* DH5α. Medium used for biofilm growth was similar to that for seed culture except that 50 mg/L glucose and 1 mg/L thiamine were used in the absence of ampicillin selection. C/N ratios of 0.07, 1, 5, and 10 were affected by appropriately varying the NH_4Cl concentrations. The system was oxygenated with pure oxygen to prevent oxygen limitation. The mixing vessel and connecting tubing were replaced with sterile versions every 12 hours to minimize the biofilm growth outside the flow cell.

The flow cell contained a total of 17 slides resulting in 17 biofilm samples taken in the following progression: two slides each for times 0, 24, 36, 48, 60, 72, and 84 ; and one slide at times 96, 108 and 120 hours. Slides removed were replaced with clean slides but replacement slides were never used for biofilm samples. Biofilm on the removed slides was scraped completely into 50 mL autoclaved identical medium and vortexed at maximum for 5 min to completely disrupt all bacterial aggregates. 2 mL of resultant suspension of biofilm material was used directly for viable cell count. The remaining 48 mL biofilm suspension was divided into two parts for the analyses of total polysaccharide and protein.

In separate experiments to quantify the effects of heterologous gene expression in biofilm bound cells, different levels of the inducer, IPTG, were applied for time periods up to 72 hours, to biofilms cultivated in the absence of inducer. *E. coli* DH5α with *parB* stabilized PTKW106 was used in these experiments to eliminate the development of a plasmid-minus population due to segregational loss; thus estimates of 'per cellular contents' of any parameter would truly relate to only plasmid-bearing cells. In these induction experiments, a majority of glass slides were retained for sampling during the induction. Two glass slides with accumulated biofilm were removed from the bioreactor every 12 hours and replaced with clean slides but were never used for further samples. Biofilm from one of the slides was scraped into 50 mL of autoclaved medium and vortexed for 5 min. 1 mL of the resultant biomass suspension was used for viable cell counts. The remaining 49 mL were used to determine total protein content and ß-galactosidase activity. The second slide sampled at each time point, was used for RNA pulse-labeling to determine the synthesis rates of total and messenger RNA.

Analytical Methods

Viable cell count. Suitably dilute biofilm sample suspensions were plated on LB agar plates containing 40 µg/mL IPTG and 40 µg/mL X-gal. 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.

Total biofilm polysaccharide. The total polysaccharide concentration per surface area was determined by phenol-sulfuric acid method (Dubois *et al.*, 1956) with sodium alginate as standard. Biofilm samples were centrifuged then resuspended in 1 mL 0.05% NaCl solution. After adding 0.05 mL 80% phenol, 5 mL concentrated sulfuric acid was added instantaneously. Samples were allowed to stand at room temperature for 10 min, then shaken in a water bath at 25 to 30 °C for 15 min. The resultant yellow-orange color, measured at 490 nm, was directly proportional to polysaccharide concentration.

Total biofilm protein. Biofilm samples were collected by centrifuging and resuspended in 1 mL TEP solution (10 mM Tris; 1 mM EDTA, pH 8.0; 1 mM PMSF, phenylmethylsulfonyl fluoride), then cells were disrupted using two 30 sec pulses by a Konets Micro-Ultrasonic Cell Disrupter (Vineland, New Jersey) set at 30% of maximum output. After microfuging, 200 µL crude cell extract was used for total protein assay (Sigma Kit No. 690).

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 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 Eqn. (1). Structural stability of the plasmid was checked periodically throughout an experiment by horizontal electrophoresis. No plasmid structural modification was found in any experiments.

Total protein and ß-Galactosidase assay. Samples obtained from batch and biofilm cultures were centrifuged at 1,000 g for 15 min 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 sec pulses by a Kontes Micro-Ultrasonic Cell Disrupter (Vineland, New Jersey) set at 30% output. After microfuging for 5 min, 200 µL crude cell extract were used for the total protein assay using a Sigma Diagnostic Kit 690. Another 200 µL crude cell extract was used for the determination of ß-galactosidase activity as per the method of Miller (1972). The amount of ß-galactosidase was determined by calibrating activities of aliquots of standard ß-galactosidase solutions (5 Prime → 3 Prime, Inc., Boulder, Colorado).

Pulse-labelling of RNA. To ensure the penetration of a radioactive precursor of RNA into the biofilm cells, a slide with accumulated biofilm was placed in a petri dish and submerged in 5 mL medium containing 20 µCi mL^{-1} of 5,6- ^{3}H uracil for 15 min. After labeling, 0.25 mL stop solution (200 mM Tris-Cl, pH = 8.0, 20 mM EDTA, 20 mM sodium azide, 20 mM aurintricarboxylic acid) was added to terminate RNA synthesis. The labeled biofilm was scraped into 50 mL sterile M9 minimal medium and placed on ice until RNA isolation.

Total RNA isolation. Rapid isolation of total RNA from *E. coli* was as per Ausubel (1991). Reagents used for RNA isolation were made RNase-free using the method of Berger and Kimmel (1987). Lysosyme was used to strip off cell walls and the resultant protoplasts were lysed with detergent. Diethylpyrocarbonate (DEPC), a potent inactivator of ribonuclease, was added to the lysate. Salt was then added to co-precipitate

the predominant component of the supernatant and was recovered by ethanol precipitation. RNA concentration was determined spectrophometrically at 260 nm where 1 unit of absorbance equated to 40 μg mL^{-1} RNA.

Determination of total and ß-galactosidase messenger RNA synthesis rates. Total RNA synthesis rates were determined by adding 20 μL pulse-labeled RNA directly to 5 mL liquid scintillation cocktail. The synthesis rate of messenger RNA was found by hybridizing *lacZ* which was immobilized on nitrocellulose membranes with radioactive RNA. *lacZ* was isolated from pMC1871 (Casadaban *et al.*, 1983) using the method of Maniatis (Sambrook *et al.*, 1989). The isolated DNA fragment (*lacZ*) was bound to 0.45 μm nitrocellulose filter paper (S&S BA85) using a Scleicher and Schuell Minifold II Slot-Blot system. To ensure a linear relationship between input cellular RNA and the radioactivity of DNA-RNA hybrids, the ratio of cellular RNA to DNA locus was kept less than 6 for *lacZ* (Wood and Peretti, 1990). After hybridization, the nitrocellulose membrane was dissolved with 1 mL ethylene glycol monoethylether (Sigma Cellusolve™) for 15 min in a disposable liquid scintillation vial and then 5 mL cocktail was added. Radioactivity was measured by a Packard 1900CA Tri-Carb liquid scintillation analyzer. Since ß-galactosidase-mRNA is rapidly degraded after synthesis and its average cellular half-life is about 1.5 min, only the radioactivity from mRNA synthesized within one half-time period could be measured. Results are reported by averaging three separate assays.

RESULTS

Effects of C/N on Biofilm Formation

Three individual sets of experiments were performed and the average biofilm net accumulation of plasmid-bearing under different carbon to nitrogen ratios is shown in Figure 1. Plasmid-free cell concentrations were not published for lack of space but are available upon request. At C/N ratios of 0.07 and 1, biofilm cell densities of both plasmid-bearing and plasmid-free populations exponentially increased throughout the experiment with no apparent steady-state. Plasmid-bearing cells reached ~ $5x10^7$ cells/cm^2 at the termination of both experiments. At a C/N ratio of 5, however, the rate of biofilm accumulation of plasmid-bearing cells decreased at 84 hours and reached a steady-state about 10^7 cells/cm^2. At a C/N ratio of 10, the biofilm accumulation of plasmid-bearing cells did not continue to increase after 72 hours, and stayed at $4x10^6$ cells/cm^2 for the remaining period of experiment.

The C/N ratios of the growth medium affected not only the accumulation rate of plasmid-bearing and plasmid-free cells, but also extracellular polysaccharide (EP) production. Figure 2 illustrates the total polysaccharide production per unit area in *E. coli* DH5α (pMJR1750) biofilm under different C/N ratios. In biofilm cultures, we observed the level of EP per area increased with increasing C/N ratios and increased significantly at latter stages of those experiments at high C/N ratios. These results are consistent with several reports which used C/N ratio to enhance biofilm cell EP production (Mian et al., 1978; Tam and Finn, 1977; Williams and Wimpenny, 1978).

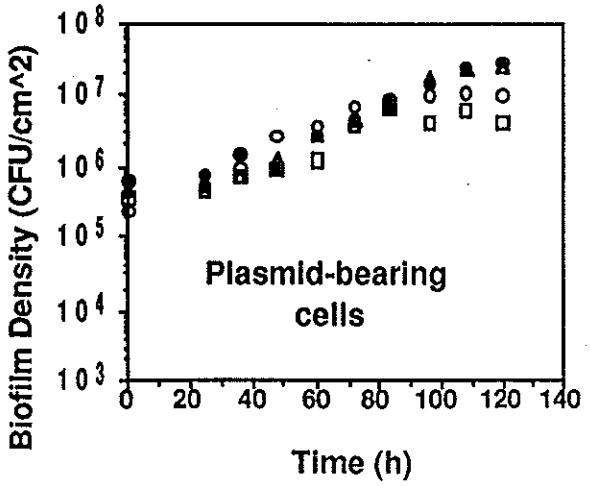

Fig. 1. Net biofilm accumulation of *E. coli* DH5α (pMJR1750) under different carbon:nitrogen ratios: (●) C:N = 0.07; (Δ) C:N = 1.0; (○) C:N = 5; (□) C:N = 10.

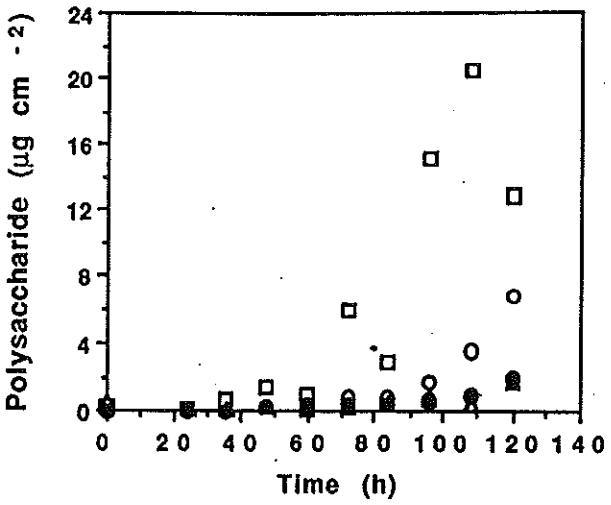

Fig. 2. Biofilm total polysaccharide at different carbon to nitrogen ratios: (●) C:N = 0.07; (Δ) C:N = 1.0; (O) C:N = 5; (□) C:N = 10.

At C/N ratios of 0.07 and 1, the ratios between EP and protein never exceeded 2.5 µg alginate equivalent/µg protein, while biofilm EP to protein ratios at C/N ratios of 5 and 10 reached maximum levels of 7 and 12 µg alginate equivalent/µg protein, respectively. Similar values of 4 to 6 µg carbohydrate/µg protein for biofilm bound cells at C/N ratio of 5.5 were recently reported by Vandevivere and Kirchman (1993). Obviously, medium C/N ratios had a direct impact on the cellular and extracellular composition of a biofilm.

Effects of C/N on Plasmid Stability

Table 1 lists the parameters required for the calculation of the plasmid loss probability (**p**) and the average values of **p** in biofilm cultures of *E. coli* DH5α (pMJR1750), under different medium C/N ratio. The less stable plasmid characteristics at higher C/N ratios might result from the nutrient competition between plasmid-bearing and plasmid-free cell growth. At a C/N ratio of 0.07, the maximum growth rate ratio of plasmid-free to plasmid-bearing cells (μ^-/μ^+) is 1.16. Under C/N at 10, the μ^-/μ^+ is 1.37. This result is consistent with the report from Sayadi and co-workers (1989). They found the stability of pTG201 in *E. coli* B was strongly affected by the nutrient depletion. The μ^-/μ^+ of *E. coli* biofilm cells was 1.08 when glucose limited while the ratio was 1.16 under ammonium limited. Mason and Bailey (1989) used *in vitro* enzyme activity assays to examine the influence of plasmid presence on the glucose metabolism in recombinant *E. coli* DH5α. They reported the activity of fructose 1,6 diphosphate was lower in plasmid-bearing cells than in the plasmid-free host. Fructose 1,6-diphosphatase catalyzed the dephosphorylation of fructose 1,6-diphosphate to fructose 6-phosphate and was an irreversible reaction involved in the glyconeogenic pathway. It was reasonable to speculate that decreased enzyme activities imply decreased reaction rates. In other words, precursors from TCA cycle for amino acid biosyntheses were less in plasmid-bearing cells than those in plasmid-free cells. EP production might be the second reason leading to the higher probability of plasmid loss at higher C/N ratios. At higher C/N ratios, a higher percentage of glucose metabolism was used in the EP production and made less glucose available for cell growth and plasmid expression. Hence, the probabilities of plasmid loss at high C/N ratios are higher than those at low C/N ratios.

ß-Galactosidase Expression

Batch suspended culture. Three different IPTG concentrations were added to suspended batch cultures. 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 mM 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 mM IPTG, the growth rates decreased to 0.16±0.03 h^{-1}, about 36% of that without IPTG. The ß-galactosidase production in batch cultures of *E. coli* DH5α (pMJR1750) is shown in Figure 3, expressed on the amount of a single plasmid-bearing cell. Under 0.17 mM IPTG, the ß-galactosidase concentration reaches its maximum of 0.32 pg per cell in the experiment 4 hours after induction. At 0.34 and 0.51 mM IPTG, the ß-galactosidase concentration reaches a peak specific production of 0.47 pg per cell, 3 hours after induction.

TABLE 1. PARAMETERS REQUIRED FOR THE CALCULATION OF PROBABILITY OF PLASMID LOSS AND ITS AVERAGE VALUES UNDER DIFFERENT CARBON TO NITROGEN RATIOS

C/N mass ratio	m' (h^{-1})	b' (h^{-1})	μ^+_m[a] (h^{-1})	μ^-_m[a] (h^{-1})	μ^-_m/μ^+_m	Probability of plasmid loss p (average±standard error)
0.07[b]	0.0314	0.0004	0.45	0.52	1.16	0.013±0.011
	0.0091	0.0014				
	0.020	0.0009				
1[c]	0.0086	0.0014	0.45	0.52	1.16	0.020±0.006
	0.0214	0.0018				
	0.0398	0.0040				
5[d]	0.0140	0.0037	0.32	0.41	1.28	0.122±0.021
	0.0118	0.0033				
	0.0184	0.0101				
10[d]	0.0289	0.0120	0.27	0.37	1.37	0.388±0.125
	0.0174	0.0082				
	0.0155	0.0094				

a: growth rate calculation can be found in Huang *et al.*, (1993).
b: slope and intercept were determined by the first 60 hour data
c: slope and intercept were determined by the first 72 hour data
d: slope and intercept were determined by 120 hour data

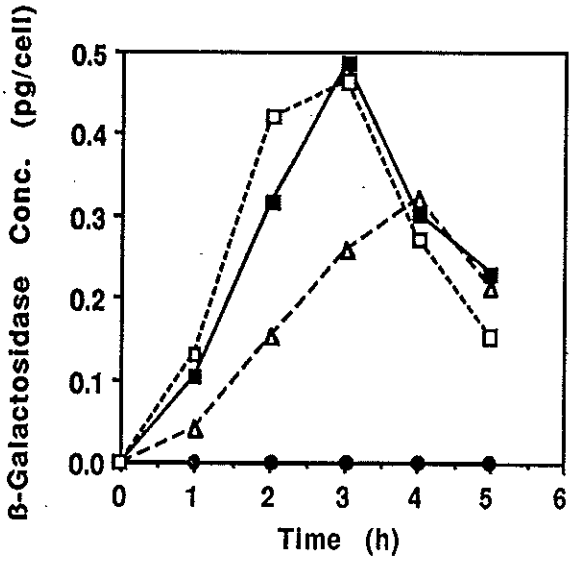

Fig. 3. ß-Galactosidase production in batch suspended cell cultures of *E. coli* DH5α (pMJR1750) under different induction levels: (●) 0 mM; (Δ) 0.17 mM; (■) 0.34 mM; and (□) 0.51 mM IPTG.

Biofilm Culture. Figure 4 is the ß-galactosidase production in biofilm cultures of *E. coli* DH5α (pMJR1750). The ß-galactosidase concentration can reach its maximum in each experiment about 24 hours after induction. The ß-galactosidase concentrations are 0.08, 0.1, and 0.12 pg per cell at 0.17, 0.34 and 0.51 mM IPTG, respectively.

Total RNA and Total RNA Synthesis Rates

Figure 5 shows the total RNA content for *E. coli* DH5α (pTKW106) in response to induction by IPTG at different levels. Total RNA levels remained esstentially constant in the control and only showed a slight increase at IPTG levels of 0.17 mM. At 0.34 mM IPTG, total RNA increased 3-fold over the first 12 hours to a level that remained steady for the remainder of the experiment. Higher levels of IPTG caused a five-fold increase in cellular RNA followed by a decline to a level 1.6 times higher than the original.

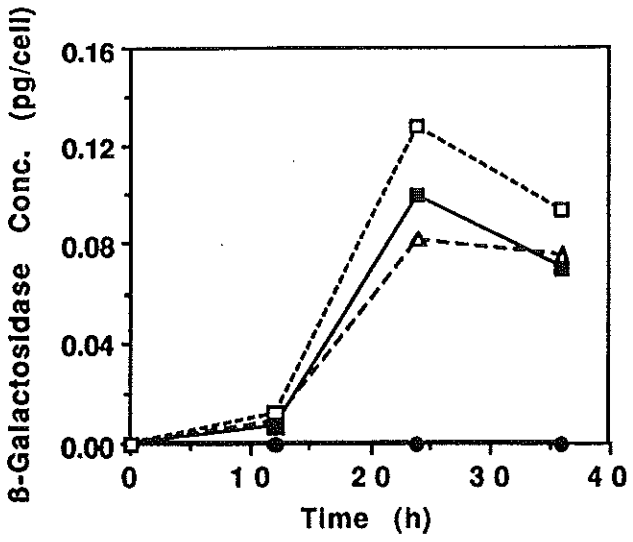

Fig. 4. ß-Galactosidase production in biofilm cultures of *E. coli* DH5α (pMJR 1750) under different induction levels: (●) 0 mM; (△) 0.17 M; (■) 0.34 mM; and (❑) mM IPTG.

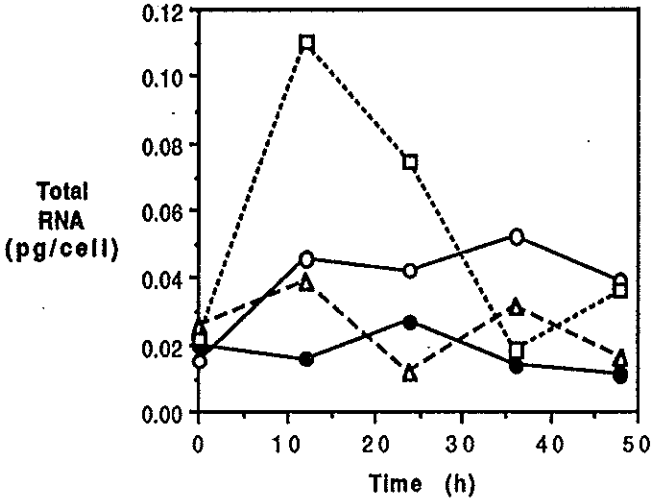

Fig. 5. Total RNA content of biofilm cells of *E. coli* DH5α (pTKW106) in response to different levels of induction: (●) 0 mM; (○) 0.17 M; (■) 0.34 mM; and (❑) 0.50 mM IPTG.

Total RNA synthesis rates responded more dramatically to IPTG exposure than did total RNA levels (Figure 6). The control case indicated a constant RNA synthesis rate of $8.2 \pm 1.1 \times 10$ dpm min-1 cell-1. Addition of IPTG accelerated rates of RNA synthesis in all experiments and, irrespective of the IPTG levels, after 48 hours of exposure to IPTG, all experiments illustrated the same RNA synthesis rate.

ß-Galactosidase mRNA Synthesis Rates

ß-galactosidase mRNA synthesis rates in biofilm *E. coli* DH5α (pTKW106), in reponse to different induction levels are shown in Figure 7. At 0.17 mM IPTG, no obvious increase occurred during the first 24 hours after induction; then ß-galactosidase mRNA rates increased 6-fold after 36 hours of induction. At 0.34 mM of IPTG, the ß-galactosidase mRNA synthesis rate increased 5-fold after 12 hours and at 0.51 mM IPTG, the ß-galactosidase mRNA synthesis rates increased continuously after induction to an 18-fold increase, 36 hours after induction.

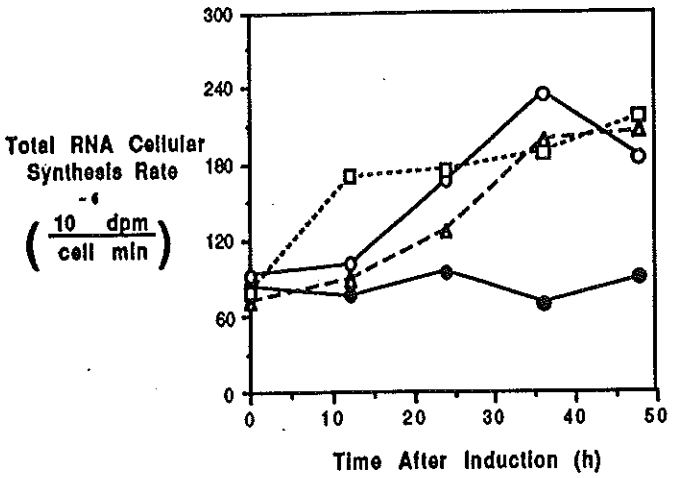

Fig. 6. Total RNA synthesis rate biofilm cells of E. coli DH5α (pTKW106) in response to different levels of induction: (●) 0 mM; (○) 0.17 M; (■) 0.34 mM; and (□) 0.50 mM IPTG.

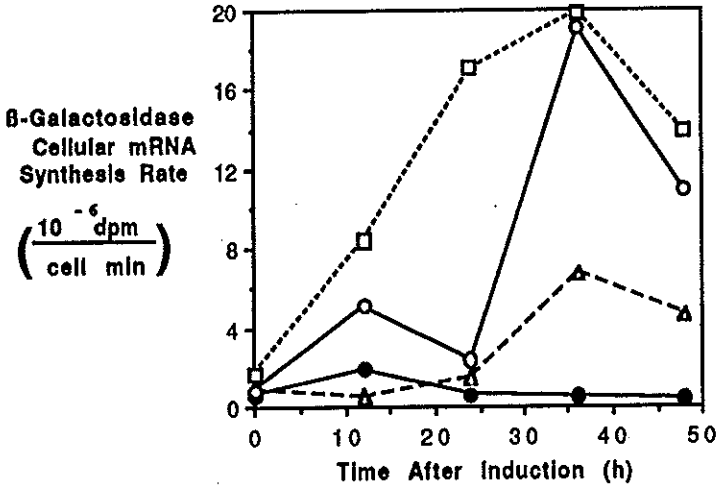

Fig. 7. Rates of ß-galactosidase mRNA synthesis on a per cell basis for biofilm cells of *E. coli* DH5α (pTKW106) in response to different levels of induction: (●) 0 mM; (○) 0.17M; (■) 0.34 mM; and (□) 0.50 mM IPTG.

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