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EFFECTS OF INDUCER LEVELS ON A RECOMBINANT BACTERIAL BIOFILM FORMATION AND GENE EXPRESSION

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SUMMARY

A segregationally stable host-plasmid system, *E. coli* DH5α (pTKW106), was used to study the effect of induction on the accumulation rate of cells and gene expression in biofilm cultures. Isopropyl ß-D-thiogalactoside (IPTG) was used to induce the expression of ß-galactosidase from the plasmid. The biofilm cell net accumulation rates decreased with increasing induction levels. At 0.17 and 0.34 mM of IPTG, the biofilm cell net accumulation rates ranged between 17 and 30% when compared to the uninduced case. At 0.51 mM of IPTG, the biofilm cell density never increased. At 0.17 and 0.34 mM of IPTG, ß-galactosidase contents reached maxima 36 hours after induction with both amounts representing about 7.5% of total protein. At 0.51 mM of IPTG, ß-galactosidase production reached its maximum, about 16% of total protein, 48 hours after induction. The ß-galactosidase mRNA synthesis rates increased with increasing inducer levels. Maximum ß-galactosidase mRNA synthesis rates were reached 36 hours after induction for each IPTG concentration.

INTRODUCTION

Recombinant DNA techniques have been successfully employed in agriculture and in health care, as well as in the commercial production of pharmaceuticals and specialty chemicals. Two impediments to widespread utilization of a recombinant strain are segregational instability of the recombinant plasmid and poor gene expression under different environmental conditions. Numerous studies have reported the many factors affecting gene expression but the majority of studies have been confined to suspended cell cultures (Glick and Whitney, 1987; Bentley *et al.*, 1991; Wood and Peretti, 1991). However, in an open environmental system, the majority of microbial activity is located at interfaces in thin layers known as biofilms. A biofilm is a gelatinous matrix consisting of bacterial cells and their secreted insoluble extracellular polymers. In the past, biofilm research focused on the control of biofilm formation. Recently, the significance of the biofilm mode of bacterial growth in biotechnological applications has received increased attention (Bryers, 1990; Melo *et al.*, 1992). Advantages of using biofilm cultures versus suspended cultures include: ease of biomass-liquid separation, increase of cell concentration, and improvement of over-all system productivity. The combination of

genetic engineering and biofilm cultures can open a new page in biotechnological applications in removing organic or inorganic contaminants from wastewater. These potential applications include *in situ* bioremediation of such hazardous wastes as 4-methyl benzoate and polychlorobenzene (Dwyer and Timmis, 1990; Nußlein *et al.*, 1992).

In a previous paper (Huang *et al.*, 1993), we reported on plasmid retention and gene expression in biofilm cultures of *Escherichia coli* DH5α hosting a segregationally unstable plasmid, pMJR1750. We found plasmid retention in a biofilm culture decreased while gene expression increased with increasing levels of inducer. Unfortunately, this unstable plasmid expression system was not suitable for a quantitative study of gene expression. Parameters related to cloned-gene protein synthesis using the unstable plasmid could not be specified on a per cell basis and thus could not reflect the real influence of inducer levels on gene expression. This earlier study with an unstable plasmid was also biased by the nutrient competition between plasmid-bearing and plasmid-free cells. Here, we report the gene expression of a recombinant bacterial biofilm culture exposed to different inducer levels. A genetically-stable plasmid containing the *parB* locus is used which guaranteed post-segregational killing of plasmid-free cells (Gerdes, 1988). Thus, this system allowed us to study the impact of cloned-gene expression on cell metabolism within biofilm-bound cultures.

EXPERIMENTAL METHODS

Bacterial strain, plasmid and media. *E. coli* DH5α was selected for this study since it could form biofilms efficiently under low substrate concentrations and since it did not naturally produce ß-galactosidase. Plasmid pTKW106 (Wood *et al.*, 1990) is a 11.9-kb plasmid consisting of a kanamycin resistant marker, a strong promoter (*tac*), a repressor gene, (*lacI* ^{Q}), a *lacZ* gene which encoded for ß-galactosidase, and *parB* locus. M9 minimal medium supplemented with 50 mg L^{-1} glucose, 100 mg L^{-1} casamino acid, and 2 mg L^{-1} thiamin was used to culture biofilms for each experiments.

Biofilm Formation System. Biofilms of *E. coli* DH5α (pTKW106) were cultivated in a parallel-plate flow cell (Huang *et al.*, 1993). In each experiment, biofilms were cultivated in the absence of the inducer, isopropyl ß-D-thiogalactoside (IPTG), for up to 72 hours; after that point, medium containing IPTG was used. Biofilm thickness at 72-hour accumalation was about 50 μm and internal diffusion limitation was not significant (Huang, 1993). Dilution rate was held at a value of 4 h^{-1} to minimize suspended cell growth. After 72 hours, two glass slides with accumulated biofilm were removed every 12 hours and scraped into 50 mL M9 medium for analysis.

Viable cell count. Suitably dilute biofilm sample suspensions were plated on LB agar plates containing 40 μg mL^{-1} IPTG and 40 μg mL^{-1} 5-bromo-4-chloro-3-indol-ß-D-galacto-pyranoside (X-Gal). The number of viable plasmid-bearing and plasmid-free bacteria were determined by averaging the blue and white colony forming unit (CFU), respectively, on three plates. No plasmid-free cells were found in all experiments.

Total protein and ß-galactosidase activity. Scraped biofilm samples were collected by centrifuging and then resuspended in 1 mL TEP solution (10 mM Tris-Cl, pH 8.0; 1 mM EDTA, pH 8.0; 1 mM PMSF, phenylmethylsulfonyl fluoride). After disrupting cells with ultrasound, the crude cell extract was used for total protein assay using a Sigma Diagnostic Kit No. 690 and for the ß-galactosidase activity assay as per Miller's method (1972). The amount of ß-galactosidase was determined by calibrating activities of standard ß-galactosidase solutions (5 Prime → 3 Prime, Boulder, Colorado).

Pulse-labeling of RNA. To ensure the penetration of a radioactive precursor of RNA into biofilm cells, a slide with accumulated biofilm was put in a disposable petri dish and submerged in 5 mL medium containing 20 μCi mL^{-1} 5,6-^3H 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.
Determination of ß-galactosidase messenger RNA synthesis rates. The synthesis rate of messenger RNA was measured according to Wood and Peretti's method (1991). Radioactivity was measured by a Packard 1900CA Tri-Carb Liquid Scintillation analyzer. Since the ß-galactosidase mRNA was rapidly degraded after synthesis and the average half-life time was about 1.5 min (Cannistraro and Kennell, 1979), only the radioactivity from mRNA synthesized within a half-life time period could be measured. Results are reported by averaging three separate assays.

RESULTS

Biofilm Cell Accumulation

Net accumulation of cells in the different biofilm experiments for different inducer levels is shown in Figure 1. The uninduced culture accumulated cells at a rate of 4.83±0.65 x 10^6 cell h^{-1}. At 0.17 and 0.34 mM of IPTG, the biofilm cell net accumulation rates ranged between 8.03±0.71 x10^5 and 1.41±0.36 x10^6 cell h^{-1}, respectively; about 17 and 30% of the control case. Cultures exposed to the highest inducer level (0.51 mM) exhibited no net accumulation of cells. Since suspended cells were washed out of the system, reattachment to the surface was not likely. In addition, flow rates were held constant for all the experiments and the rate of detachment should be approximately the same between each experiment. Therefore, differences in accumulation rates should reflect differences in cell growth rates.

ß-Galactosidase Activity

Figure 2 illustrates the results of ß-galactosidase activity in biofilm *E. coli* DH5α (pTKW106) under the different induction levels of IPTG. ß-galactosidase activity was zero without induction. At 0.17 and 0.34 mM of IPTG, the ß-galactosidase activity increased, reaching maxima at 0.027±0.006 and 0.036±0.005 pg $cell^{-1}$, respectively, 36 hours after induction, then levels decreased slightly. At 0.51 mM of IPTG, the active fraction of cellular ß-galactosidase increased with increasing time and reached a maximum of 0.050±0.003 pg $cell^{-1}$ 48 hours after induction. At 0.51 mM IPTG, about 16% of the total protein was ß-galactosidase 48 hours after induction, compared to about 7.5% at 0.17 and 0.34 mM of IPTG after the same period. It should be emphasized that active ß-galactosidase levels were assayed rather than measuring the broad indicator of protein production which would have included the total pool of ß-galactosidase enzyme. This total pool would include both active and inactive (denatured) protein as well as degraded polypeptides derived from ß-galactosidase. Consequently, total ß-galactosidase protein level is underestimated.

ß-Galactosidase mRNA Synthesis Rate

The ß-galactosidase mRNA synthesis rates in biofilm *E. coli* DH5α (pTKW106) in response to different induction levels of IPTG are shown in Figure 3. Only a

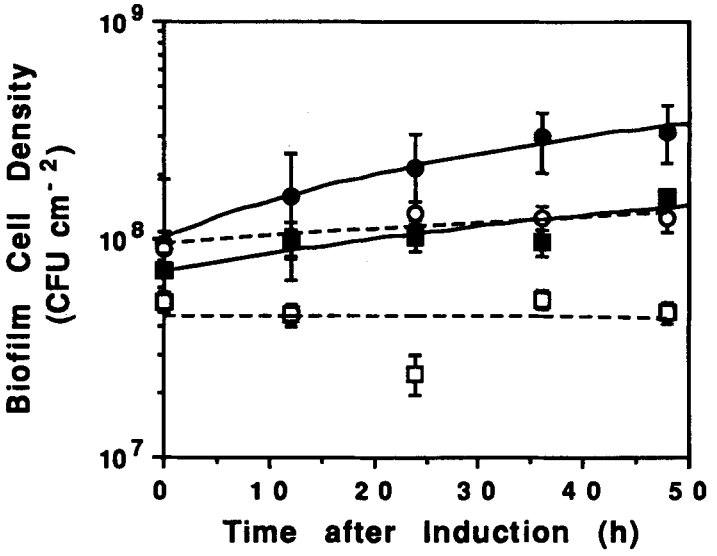

Figure 1 Biofilm net accumulation of *E. coli* DH5α (pTKW106) in response to different levels of IPTG. (●) 0.00 mM; (○) 0.17 mM; (■) 0.34 mM; (□) 0.51 mM.

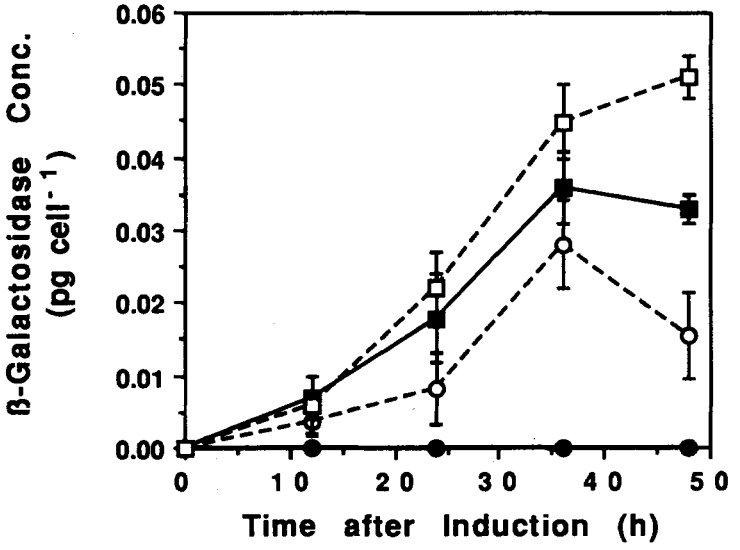

Figure 2 ß-Galactosidase production of *E. coli* DH5α (pTKW106) in response to different levels of IPTG. (●) 0.00 mM; (○) 0.17 mM; (■) 0.34 mM; (□) 0.51 mM.

background radioactivity was detected in the non-induced control experiment. At 0.17 mM of IPTG, no obvious increase in the ß-galactosidase mRNA synthesis rate was observed during the first 24 hours after induction. The ß-galactosidase mRNA synthesis rate increased significantly 36 hours after induction; an approximate 6-fold increase compared to that without induction. At 0.34 mM of IPTG, the ß-galactosidase mRNA synthesis rate increased 5 fold after 12 hours induction and 18 fold 36 hours after induction. At 0.51 mM of IPTG, the ß-galactosidase mRNA synthesis rate increased continuously after induction with an 18-fold increase 36 hours after induction.

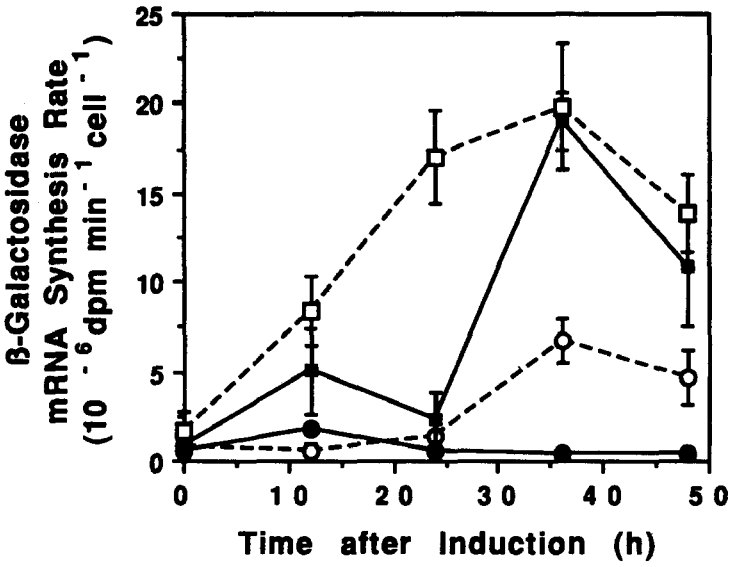

Figure 3 Rates of ß-galactosidase mRNA synthesis for *E. coli* DH5α (pTKW106) in response to different induction levels of IPTG. (●) 0.00 mM; (○) 0.17 mM; (■) 0.34 mM; (□) 0.51 mM. Error bar represents standard error.

DISCUSSION

Effects of Induction Levels on Biofilm Cell Accumulation

Biofilm accumulation rates decreased with increasing inducer levels. With biofilm detachment rates assumed essentially identical in all experiments, these results indicate biofilm cell growth was hindered due to the extra metabolic burden created under induction. For a *E. coli* DH5α (pTKW106) *parB* stabilized expression system, nutrient competition between plasmid-bearing and plasmid-free cells did not exist. However, the metabolic stress which a plasmid imposes under induction is likely to reduce the growth rate of its host. This metabolic stress includes the sequestering cellular resources for the transcription and translation of the cloned gene, and sometimes, the toxicity of cloned gene products. These observations were consistent with the results found in suspension cultures. Wood and co-workers (1990) reported that there was 10% decrease in the specific growth rate of planktonic *E. coli* BK6 (pTKW106) under 0.5 mM IPTG induction. Bentley *et al.* (1991) also found the growth rate of planktonic *E. coli* DH5αF'IQ (pKK262-1) decreased from a value near 0.45 h^{-1} to a value of 0.18 h^{-1} at the induction level of 2.1 mM of IPTG.

Effects of Induction Levels on Inducible Protein Production

Protein synthesis is limited by the amount of its mRNA found in the cell. mRNA synthesis is catalyzed by RNA polymerase which is usually abundant at all growth stages (Gausing, 1980). Therefore, the synthesis rate of mRNA for an inducible protein is mainly determined by the amount of gene available for transcription. In our system, as $lacI^Q$ repressor is bound by the inducer, RNA polymerase would bind the operator and

the *lacZ* gene on pTKW106 is transcribed to synthesize ß-galactosidase mRNA. Any limitation of ß-galactosidase production may be due to the nearly complete titration of $lacI^Q$ repressor. Compared with continuous suspended cultures using the same plasmid, the saturation of $lacI^Q$ did not occur until the inducer level was higher than 1 mM of IPTG (Wood and Peretti, 1991).

The ß-galactosidase mRNA synthesis rate increased 4 fold under 0.17 mM IPTG and increased almost 12 fold under 0.34 and 0.51 mM IPTG, 36 hours after induction. However, the ß-galactosidase production in biofilm cultures did not increase in proportion to the increase of mRNA synthesis rate. These results suggest, that although the synthesis rates of ß-galactosidase mRNA are high at higher expression, the synthesized mRNA is much less stable than those synthesized in low expression.

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