Effects of Medium Carbon-to-Nitrogen Ratio on Biofilm Formation and Plasmid Stability

Ching-Tsan Huang,[1] Steven W. Peretti,[2] and James D. Bryers[1]*
[1]The Center for Biochemical Engineering, Duke University, Durham, North Carolina 27708-0277, and [2]Department of Chemical Engineering, North Carolina State University, Raleigh, North Carolina 27695-7905

Received June 22, 1993/Accepted February 17, 1994

Biofilm formation and plasmid segregational instability in biofilm cultures of *Escherichia coli* DH5α (pMJR1750) were investigated under different medium carbon-to-nitrogen (C/N) ratios. At C/N ratios of 0.07 and 1, net accumulation of both biofilm plasmid-bearing and plasmid-free cells continued throughout the entire experiment without attaining any apparent steady state. At C/N ratios of 5 and 10, net biofilm cell accumulation for the two populations reached apparent steady states after 84 and 72 h, respectively. At C/N ratios of 0.07 and 1, polysaccharide production increased slowly and reached about 2 μg alginate equivalent/cm^2 by the end of both experiments. At a C/N ratio of 5, polysaccharide increased significantly after 84 h, reaching about 7 μg alginate equivalent/cm^2 prior to termination. At a C/N ratio of 10, polysaccharide increased significantly after 72 h and reached 21 μg alginate equivalent/cm^2 at 108 h. At C/N ratios of 0.07 and 1, protein production reached 6.5 and 4 μg/cm^2, respectively. At C/N ratios of 5 and 10, protein production increased slightly for the first 84 h and reached a maximum at 108 h, at 3 and 2 μg/cm^2, respectively, then decreased over the last 12 h of the experiment. Ratios of polysaccharide to protein increased with increasing C/N ratios. At C/N ratios of 0.07 and 1, the ratios between extracellular polysaccharide (EP) and protein were no more than 2.5 μg polysaccharide/μg protein, whereas those at C/N ratios of 5 and 10 increased to about 7 and 12 μg polysaccharide/μg protein, respectively.

Probabilities of plasmid loss in the biofilm cultures increased with increasing C/N ratios. At C/N ratios of 0.07, 1, and 5, the probabilities of plasmid loss were 0.013 ± 0.011, 0.020 ± 0.006 and 0.122 ± 0.021, respectively. At a C/N ratio of 10, the probability of plasmid loss was significantly higher, reaching 0.388 ± 0.125. The increase of probability of plasmid loss at higher C/N ratios results from competition between cell replication and extracellular polysaccharide production.

Key words: biofilm formation • *Escherichia coli* • C/N ratio • plasmid retention • extracellular polysaccharide

INTRODUCTION

Genetically engineered expression systems have received extensive attention due to their tremendous application potential in such areas as agriculture, health care, pharmaceutical, and specialty chemicals. There are two impediments to the widespread commercial utilization of recombinant plasmid DNA: (1) instability of the plasmid under different culture conditions and (2) restrictive governmental regulation constraining the use of recombinant strains within open environments. The first limitation results from either structural or segregational instability of the plasmid which led to the loss of target gene expression. The second limitation is due mainly to a lack of knowledge regarding the fate of recombinant plasmid DNA released to a natural ecosystem. Due to the obvious biotechnological incentives, significant research has focused on those factors that control plasmid segregational and structural stability in closed systems. Environmentally well-controlled reactor systems used to study plasmid loss are almost exclusively directed toward freely suspended cultures.[8,11,13,16,18]

However, in open environmental systems, most, if not all, microbial activity is associated with an interface within thin biological layers known as biofilms. Biofilms consist of microbial cells and their secreted insoluble extracellular polymers. The rate and extent of the processes occurring within biofilms are frequently controlled by the micro- and macroscopic properties associated with the composition of biofilms. The microbial extracellular polymer matrix plays an important role in the interaction between a bacterial cell, its nearest neighbor, and the substratum.

A study of plasmid stability in biofilm cultures would provide information necessary to understand the fate of a recombinant strain released to an open environment. This information is crucial for governmental agencies to judiciously regulate the use of recombinant DNA within natural ecosystems. Recently, Goodman and co-workers[4] reported conjugative plasmid transfer between *Vibrio* S14 strains and *Escherichia coli* using a broad-host-range plasmid RP1. However, the fate of plasmid DNA in natural environments is still not well quantified. Research published earlier[6] combined with that reported here comprises a first step in the study of plasmid fate within biofilm communities. In our previous study,[6] we proposed a mathematical model to predict the probability of plasmid loss in biofilm cultures. That work suggested that a commercially unstable plasmid was more unstable in biofilm cultures than in suspended cultures. Results in that work suggested that in the biofilm

* To whom all correspondence should be addressed at current address: The Center for Biofilm Engineering, Montana State University, Bozeman, MT 59717.

CCC 0006-3592/94/030329-08

mode of growth, cells preferentially channel energy to synthesize and secrete extracellular polysaccharide (EP) rather than to express a heterologous plasmid-encoded protein. To verify this hypothesis, a series of biofilm cultures were grown at different C/N ratios, a parameter known to affect the relative amounts of cells and extracellular polymer in a biofilm. Here we report the effects of the nutrient medium carbon-to-nitrogen (C/N) ratios on plasmid stability and biofilm formation in a pure culture.

MATHEMATICAL MODEL

Accumulation of biofilm cells is the net result of several processes, including the deposition of suspended cells from the liquid phase, attached cell growth and replication, extracellular polymer production, and biofilm detachment due to growth-related forces and to fluid shear stress.[1,2] 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 the same hydrodynamic conditions.

Therefore, the net accumulation rates of plasmid-bearing and plasmid-free cells in the biofilm can be expressed as the combination of attached cell growth, plasmid loss, and biofilm cell detachment:

$$\frac{dB^+}{dt} = \mu^+ B^+ - p\mu^+ B^+ - k_{det} B_d^+ \tag{1}$$

$$\frac{dB^-}{dt} = \mu^- B^- + p\mu^+ B^+ - k_{det} B_d^- \tag{2}$$

where B^+ and B^- are the overall biofilm cell density (cells/length^2) with and without plasmid, respectively; μ^+ and μ^- are the specific growth rate (time^{-1}) for plasmid-bearing and plasmid-free cells; p is the probability of plasmid loss; k_{det} is the detachment rate constant (time^{-1}); and B_d^+ and B_d^- are plasmid-bearing and plasmid-free biofilm cell density (cells/length^2) detached from biofilm surface. Assuming the distributions of plasmid-bearing (B^+) and plasmid-free (B^-) cells within the biofilm are uniform spatially with depth, then one can assume that cells of each population detach in the same ratio as that which exists throughout the biofilm, i.e., there is no spatial stratification of strains with depth. Thus we can define $\beta = B^-/B^+ = B_d^-/B_d^+$ and differentiate β with time to get

$$\frac{dB^-}{dt} = B^+ \frac{d\beta}{dt} + \beta \frac{dB^+}{dt} \tag{3}$$

Substituting Eqs. (1) and (2) into (3) and rearranging yield

$$\frac{d\beta}{dt} = (\mu^- - \mu^+ + p\mu^+)\beta + p\mu^+ \tag{4}$$

Using Monod-like rate dependencies on limiting substrate, 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. (4) can be rewritten as

$$\frac{d\beta}{dt} = \left(\frac{\mu_m^- S}{K_S + S} - \frac{\mu_m^+ S}{K_S + S} + p\frac{\mu_m^+ S}{K_S}\right)\beta + p\frac{\mu_m^+ S}{K_S + S} \tag{5}$$

Plotting $d\beta/dt$ versus β, the slope and intercept can be determined as

$$m' = (\mu_m^- - \mu_m^+ + p\mu_m^+)\frac{S}{K_S + S} \tag{6}$$

$$b' = p\left(\frac{\mu_m^+ S}{K_S + S}\right) \tag{7}$$

Solving Eqs. (6) and (7) for the plasmid loss probability p yields

$$p = \frac{b'}{m' - b'}\frac{\mu_m^- - \mu_m^+}{\mu_m^+} \tag{8}$$

MATERIALS AND METHODS

Bacterial Strain and Plasmid

Escherichia 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, β-galactosidase. Its genotype was *φ80dlacZΔM15*, *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-resistant marker, a strong promoter (*tac*), a repressor gene, (*lacI*^{Q}), and the *lacZ* gene which encodes for β-galactosidase. The β-galactosidase promoter can be induced by various chemical inducers, including isopropyl β-D-thiogalactoside (IPTG). Recombinant cells that express β-galactosidase form blue colonies on plate medium containing 5-bromo-4-chloro-3-indol-β-D-galactopyranoside (X-gal) and IPTG whereas plasmid-free cells form white colonies.

Biofilm Formation System

Biofilms of *E. coli* DH5α (pMJR1750) were cultivated in a parallel-plate flow cell (Fig. 1) which was modified from

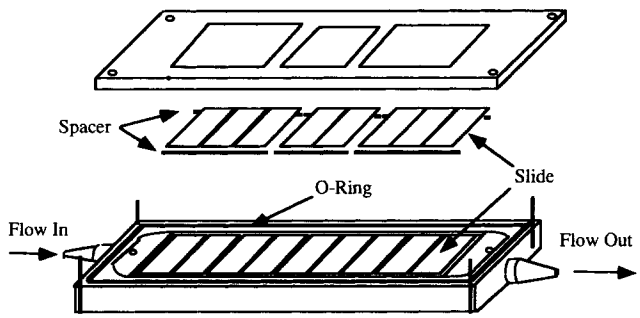

Figure 1. Schematic drawing of a parallel-plate flow cell biofilm study reactor.

our previous design to allow for more biofilm sampling area.[5] The schematic experimental setup for inoculation and biofilm formation is illustrated in Figure 2, and the operating conditions are listed in Table I. 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 h at a

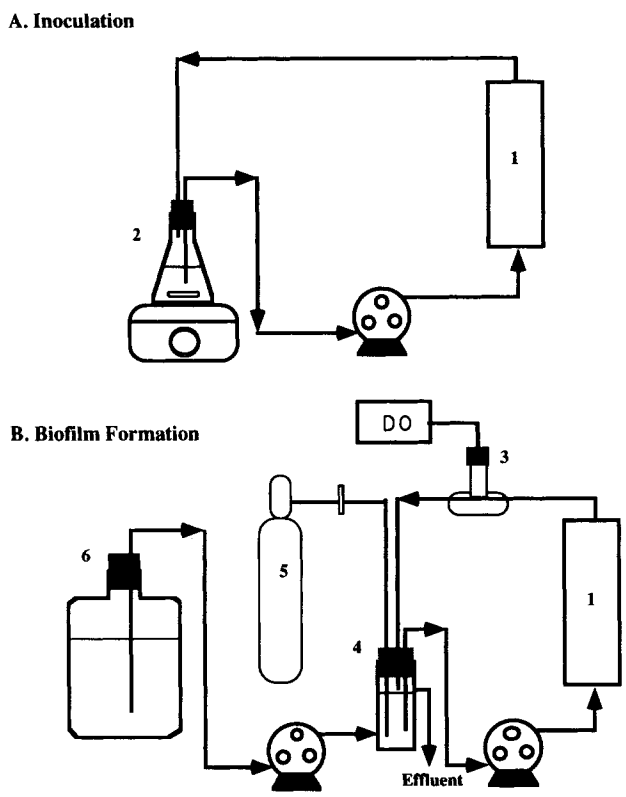

Figure 2. Schematic experimental setup for biofilm formation system: (1) flow cell reactor; (2) seed culture flask; (3) oxygen probe; (4) mixing vessel; (5) oxygen cylinder; (6) nutrient reservoir.

Table I. Operating conditions for biofilm formation experiments.

Flow recirculation rate	45 mL/min
Fluid velocity	1 cm/s
Reynolds number	20
Shear stress	2.7 N/m^2
Medium feeding rate	6.7 mL/min
System residence time	15 min
Inlet glucose concentration	50 mg/L

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 the 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 the 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. The 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 h 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: 2 slides each for times 0, 24, 36, 48, 60, 72, and 84, slide at times 96, 108, and 120 h. 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. Two milliliters of resultant suspension of biofilm material was used directly for viable cell counts. The remaining 48 mL biofilm suspension was divided into two parts for the analyses of total polysaccharide and protein.

Analytical Methods

Viable cell count: Suitably dilute biofilm sample suspensions were plated on Luria–Bertani (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 polysaccharide was determined by the phenol-sulfuric acid method[3] with sodium alginate as standard. Biofilm samples were centrifuged and then resuspended in 1 mL 0.05% NaCl solution. After adding 0.05 mL of 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 protein: Biofilm samples were collected by centrifuging and resuspending in 1 mL TEP solution [10 m*M* Tris; 1 m*M* ethylenediaminetetraacetic acid (EDTA), pH 8.0; 1 m*M* phenylmethylsulfonyl fluoride (PMSF)]; then cells were disrupted using two 30-s pulses by a Konets Micro-Ultrasonic cell disrupter (Vineland, NJ) set at 30% of maximum output. After microfuging, 200 μL crude cell extract was used for total protein assay (Sigma Kit No. 690).

RESULTS AND DISCUSSION

Effects of C/N Ratio on Biofilm Formation

Three individual sets of experiments were performed, and the average biofilm net accumulation of plasmid-bearing and plasmid-free cells under different C/N ratios are shown in Figures 3A and B. 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 ~5 × 10^7 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 h and reached a steady state at 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 h, and stayed at 4 × 10^6 cells/cm^2 for the remaining period of the experiment.

Similar results were also found in the biofilm accumulation of plasmid-free cells. At C/N ratios of 0.07 and 1, a steady state was not observed since these experiments were terminated prematurely. However, at C/N ratios of 5 and 10, an apparent plateau was observed which could be brought about by the shear stress exerted by the flowing fluid and the depletion of the growth-limiting substrate for both plasmid-bearing and plasmid-free cells.

The C/N ratios of the growth medium affected not only the accumulation rate of plasmid-bearing and plasmid-free cells but also EP production. Figure 4 illustrates the total polysaccharide production per unit area in *E. coli* DH5α (pMJR1750) biofilm under different C/N ratios. At C/N ratios of 0.07 and 1, the EP production increased slowly and reached 2 μg alginate equivalent/cm^2 by the end of an experiment. At a C/N ratio of 5, EP increased obviously after 84 h and reached 7 μg alginate equivalent/cm^2 prior to termination. At a C/N ratio of 10, EP increased significantly after 72 h and reached about 21 μg alginate equivalent/cm^2 at 108 h, then dropped back to about 13 μg alginate equivalent/cm^2 due to biofilm sloughing at the end of the experiment.

The effect of medium C/N ratios on total cellular protein production is shown in Figure 5. There were no obvious increases of protein production at the first 84 h for C/N ratios of 0.07 and 1. Cell protein levels increased significantly after 84 h and reached 6.5 and 4 μg/cm^2 for C/N ratios of 0.07 and 1, respectively, by the end of the experiments. At a C/N ratio of 5, there was a slight increase for

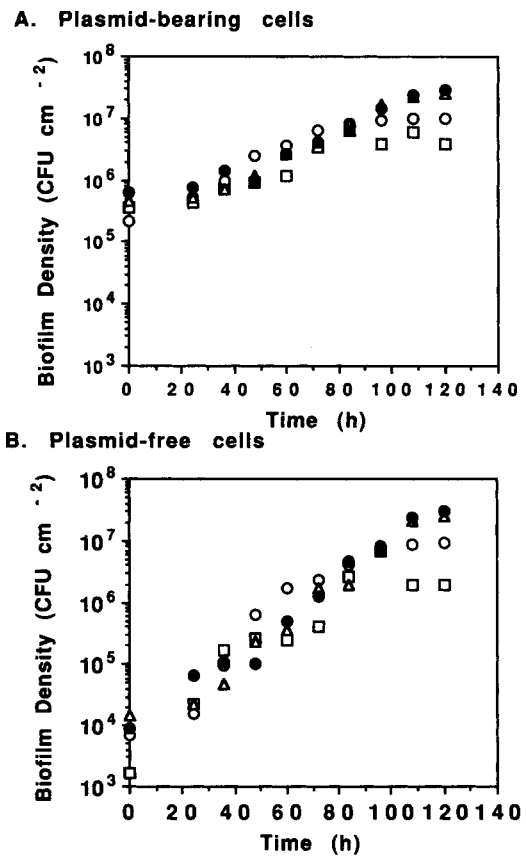

Figure 3. Net biofilm accumulation of (A) *E. coli* DH5α (pMJR1750) and (B) *E. coli* DH5α (no plasmid) under different carbon-to-nitrogen ratios: (●) C/N = 0.07; (△) C/N = 1; (○) C/N = 5; (□) C/N = 10. All data points represent the average of three replicate experiments.

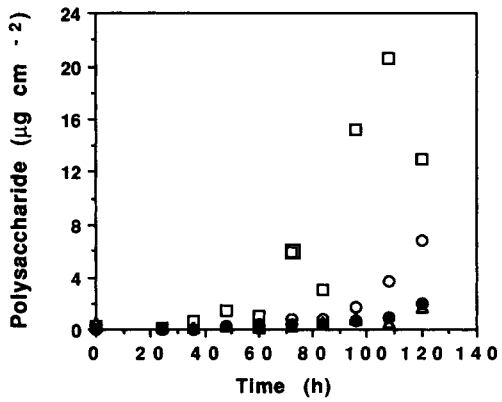

Figure 4. Biofilm total polysaccharide at different carbon-to-nitrogen ratios: (● C/N = 0.07; (△) C/N = 1; (○) C/N = 5; (□) C/N = 10. All data points represent the average of three replicate experiments.

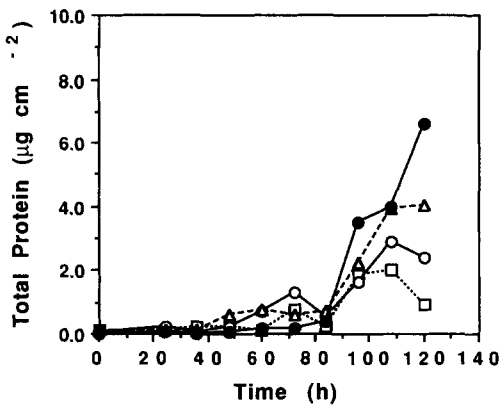

Figure 5. Biofilm total protein production in *E. coli* DH5α (pMJR1750) at different carbon-to-nitrogen ratios: (●) C/N = 0.07; (△) C/N = 1; (○) C/N = 5; (□) C/N = 10. All data points represent the average of three replicate experiments. Lines are provided to illustrate trends.

the first 84 h and an obvious increase thereafter. The maximum protein level, 3 $\mu g/cm^2$, was reached at 108 h, then dropped a little at the end of the experiments. A similar result to the experiment at a C/N ratio of 5 was found for a C/N ratio of 10. The cellular protein level reached its maximum of 2 $\mu g/cm^2$ at 108 h and decreased to about 1 $\mu g/cm^2$ at the end.

In biofilm cultures, we observed the level of EP per area increased with increasing C/N ratios and increased significantly at later 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.[10,14,17] Figure 6 presents the ratio of biofilm EP to biofilm cell protein in response to different C/N ratios. At C/N ratios of 0.07 and 1, the ratios between EP and protein never exceeded 2.5 μg alginate equivalent/μg protein, whereas 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 a C/N ratio of 5.5 were recently reported by Vandevivere and Kirchman.[15] These suggested medium C/N ratios had a direct impact on the biofilm composition.

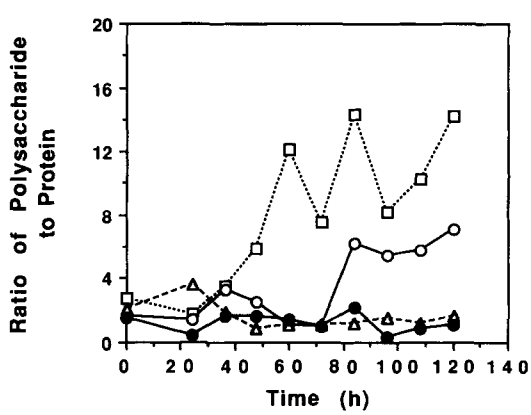

Figure 6. Ratio of total polysaccharide to total protein in *E. coli* DH5α (pMJR1750) at different carbon-to-nitrogen ratios: (●) C/N = 0.07; (△) C/N = 1; (○) C/N = 5; (□) C/N = 10. Lines are provided to illustrate trends.

Effects of C/N Ratio on Plasmid Stability

Probability of Plasmid Loss

Table II lists the parameters required for the calculation of the plasmid loss probability (p) and the average values of p in biofilm cultures under different medium C/N ratios. The probability of plasmid loss was 0.013 ± 0.011 at a C/N ratio of 0.07 and increased with increasing C/N ratios. Probabilities of plasmid loss were estimated at 0.020 ± 0.006, 0.122 ± 0.021, and 0.388 ± 0.125 at C/N ratios of 1, 5, and 10, respectively. The less stable plasmid characteristics at higher C/N ratios might result from the nutrient competition between plasmid-bearing and plasmid-free cell growth. Table II shows that the maximum growth rates of plasmid-bearing and plasmid-free cells decreased and the difference between these two populations enlarged with C/N increasing. 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 the C/N ratio of 10, μ^-/μ^+ is 1.37. This result is consistent with the report from Sayadi and co-workers.[12] They found the stability of pTG201 in *E. coli* B was strongly affected by nutrient depletion. The μ^-/μ^+ of *E. coli* B cells was 1.08 under glucose-limited conditions while the ratio was 1.16 if ammonium limited. Mason and Bailey[9] 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 the tricarboxylic acid (TCA) cycle for amino acid biosyntheses were less in plasmid-bearing cells than those in plasmid-free cells. The 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 EP production which made less glucose available for cell growth and plasmid expression. Hence, the probabilities of plasmid loss at high C/N ratios were higher than those at low C/N ratios.

Applicability of Plasmid Loss Model

In our previous work,[6] we mentioned that possible stratification between the ratios of plasmid-free to plasmid-bearing cells in the biofilm and detached biofilm could reduce the validity of assuming uniform spatial distribution of plasmid-bearing and plasmid-free cells with biofilm depth. The va-

Table II. 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 ref. 8 and results are based upon the data from this study.
[b]Slope and intercept were determined by the first 60 h of data.
[c]Slope and intercept were determined by the first 72 h of data.
[d]Slope and intercept were determined by 120 h of data.

lidity 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. Figure 7 illustrates the comparison of these ratios at different C/N ratios. Note that biofilm concentrations of plasmid-free (B^-) and plasmid-bearing (B^+) cells were values determined over the entire biofilm sample (i.e., values averaged over the depth of the biofilm)

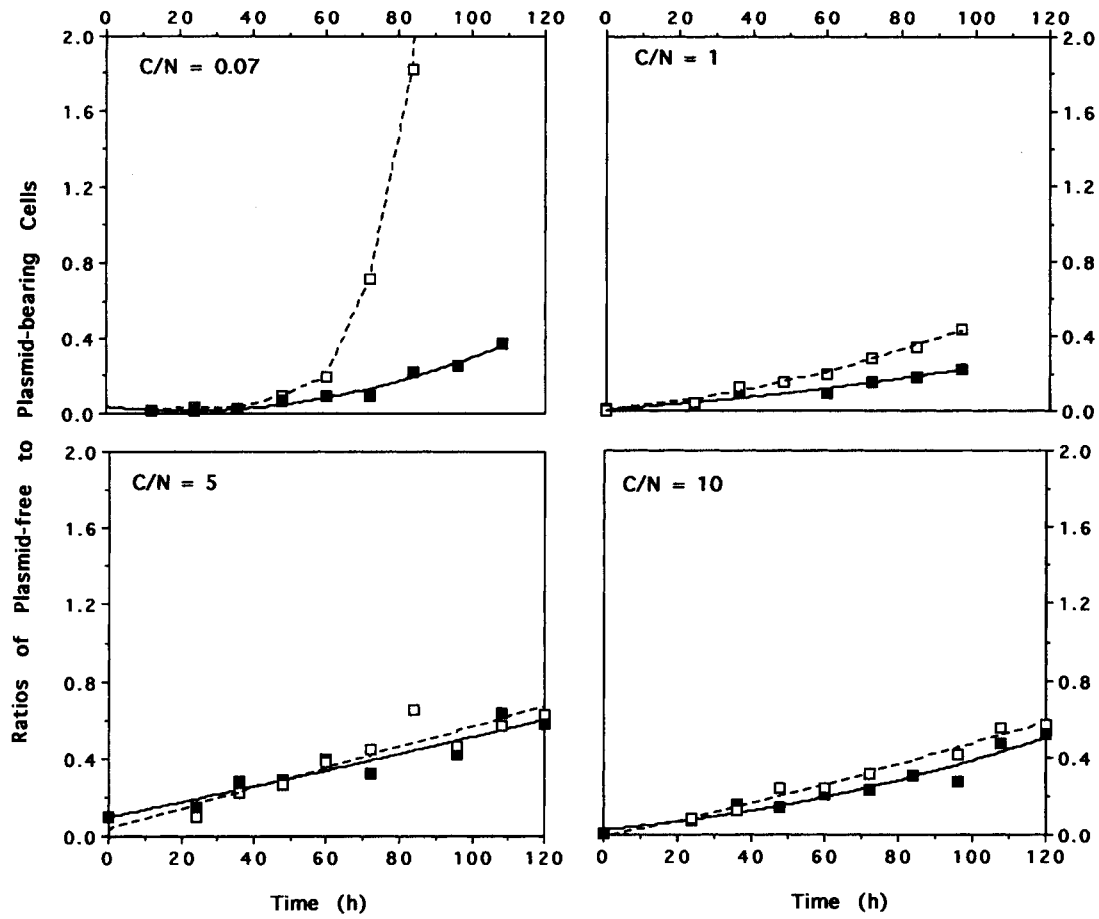

Figure 7. Comparison of ratios of plasmid-free to plasmid-bearing cells between biofilm and detached cells in liquid phase at different C/N ratios: (■) biofilm; (□) detached cells. Lines are provided to illustrate trends.

whereas the concentration of plasmid-free (X^-) and plasmid-bearing (X^+) cells in the liquid leaving the reactor reflect the distribution of cells that detached from the upper layers of the biofilm. For C/N ratios of 0.07 and 1, the ratios were approximately the same for the first 60 and 72 h, respectively, but after that, as the biofilm layer approached its maximum thickness, the amount of plasmid-free cells detached into the liquid phase relative to plasmid-bearing cells increased significantly, more so than in the overall biofilm. Early in the biofilm accumulation, the plasmid-bearing cells initially dominated the surface community, and there was no spatial distribution. However, as the biofilm developed, the plasmid-free cells grew at a faster rate and, over the course of the experiment, began to dominate the upper layers of the biofilm, which was reflected in the higher numbers of plasmid-free cells reentrained into the liquid phase. Based on these findings, the assumption that $B^-/B^+ = B_d^-/B_d^+$ was only valid prior to an elapsed time of 60 h in experiments of C/N = 0.07 and 72 h for C/N = 1. Consequently, p was calculated from B^- and B^+ data for these time ranges for these two experiments.

This discrepancy between B^-/B^+ and B_d^-/B_d^+ was not so pronounced as the C/N ratio increased. The differences in the ratio of plasmid-free to plasmid-bearing cells (suspended vs. biofilm) among C/N ratios of 5 and 10 were much less than that seen at the C/N ratio of 0.07. These results combined with Figure 4 suggest that at increasing C/N ratios, a greater level of polysaccharide production is induced instead of cell replication. If stratification of biofilm cell populations is predicated on a growth rate difference, then as polysaccharide is promoted, cell turnover rates of B^- and B^+ should decrease. A more complex model that accounts for spatial distribution should be developed in the future.

CONCLUSIONS

Under the same glucose feed concentration (carbon source), the experimental results regarding the biofilm cultures of recombinant *E. coli* DH5α (pMJR1750) led to the following conclusions:

1. The proposed mathematical model can predict the plasmid loss probability in developing biofilm cultures within certain restrictions. A uniform spatial distribution of plasmid-bearing and plasmid-free cells in the biofilm was valid at high C/N ratios but was only valid through portions of experiments carried out at low C/N ratios.
2. The increase of plasmid loss probability in biofilm cultures with increasing C/N ratio is due to competition between cell replication and EP production with increased EP production promoted by increased C/N ratios.
3. The ratio of total polysaccharide to total protein increased with increasing C/N ratios.

Financial support from the National Science Foundation (Grant No. BCS-9020502) is greatly appreciated.

NOMENCLATURE

B^+, B^-	cell density of overall plasmid-bearing and plasmid-free biofilm (cells/L^2)
B_d^+, B_d^-	cell density of detached plasmid-bearing and plasmid-free biofilm (cells/L^2)
b'	intercept from Eq. (5) (time^{-1})
K_S	saturation constant (M/L^3)
k_{det}	detached rate constant (time^{-1})
m'	slopes from Eq. (5) (time^{-1})
p	probability of plasmid loss per cell per cell division
S	limiting substrate concentration (M/L^3)
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 (time^{-1})
μ_m^+, μ_m^-	maximum specific growth rate of plasmid-bearing and plasmid-free cells (time^{-1})

References

1. Bryers, J. D. 1984. Biofilm formation and chemostat dynamics: Pure and mixed culture considerations. Biotechnol. Bioeng. **26**: 948–958.
2. Characklis, W. G., Turakhia, M. H., Zelver, N. 1990. Pp. 195–265 In: W. G. Characklis and K. C. Marshall (eds.), Biofilms. Wiley, New York.
3. Dubois M, Gilles K. A., Hamilton, J. K., Rebers, P. A., Smith, F. 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. **28**: 350–356.
4. Goodman, A. E., Hild, E., Marshall, K. C., Hermansson, M. 1993. Conjugative plasmid transfer between bacteria under simulated marine oligotrophic conditions. Appl. Environ. Microbiol. **59**: 1035–1040.
5. Huang, C.-T., Peretti, S. W., Bryers, J. D. 1992. Use of flow cell reactors to quantify biofilm formation kinetics. Biotechnol./Techn. **6**: 193–198.
6. Huang, C.-T., Peretti, S. W., Bryers, J. D. 1993. Plasmid retention and gene expression in suspended and biofilm cultures of recombinant *Escherichia coli* DH5α (pMJR1750). Biotechnol. Bioeng. **41**: 211–220.
7. Huang, C.-T. 1993. Plasmid retention and gene expression in bacterial biofilm cultures. Ph.D. Dissertation, Duke University, Durham, NC.
8. Kumar, P. K., Maschke, H-E., Friechs, K., Schügerl, K. 1991. Strategies for improving plasmid stability in genetically modified bacteria in bioreactors. Trends Biotechnol. **9**: 279–284.
9. Mason, C. A., Bailey, J. E. 1989. Effects of plasmid presence on growth and enzyme activity of *Escherichia coli* DH5α. Appl. Microbiol. Biotechnol. **32**: 54–60.
10. Mian, F., Jarman, T. R., Righelato, R. C. 1978. Biosynthesis of exopolysaccharide by *Pseudomonas aeruginosa*. J. Bacteriol. **134**: 418–422.
11. Rochelle, P. A., Fry, J. C., Day, M. J. 1989. Plasmid transfer between *Pseudomonas* spp. within epilithic films in a rotating disc microcosm. FEMS Microbiol. Ecol. **62**: 127–136.
12. Sayadi, S., Nasri, M., Barbotin, J. N., Thomas, D. 1989. Effect of environmental growth conditions on plasmid stability, plasmid copy number and catechol 2,3-dioxygenase activity in free and immobilized *Escherichia coli* cells. Biotechnol. Bioeng. **33**: 801–808.
13. Saye, D. J., Ogunseiten, O., Sayler, G. S., Miller, R. V. 1987. Potential for transduction of plasmids in a natural freshwater environment: Effect of plasmid donor concentration and a natural microbial

community on transduction in *Pseudomonas aeruginosa*. Appl. Environ. Microbiol. **53**: 987–995.
14. Tam, K. T., Finn, R. K. 1977. Polysaccharide formation by a *Methylomonas*. pp. 58–80 In: P. A. Sandford and A. Laskin. (eds.), Extracellular microbial polysaccharides. Symposium Series 45, American Chemistry Society, Washington, DC.
15. Vandevivere, P., Kirchman, D. L. 1993. Attachment stimulates exopolysaccharide synthesis by a bacterium. Appl. Environ. Microbiol. **59**: 3280–3286.
16. Weinberg, S. R., Stosky, G. 1972. Conjugation and genetic recombination of *Escherichia coli* in soil. Soil Biol. Biochem. **4**: 171–180.
17. Williams, A. G., Wimpenny, J. W. T. 1978. Exopolysaccharides production by *Pseudomonas* NCIB1264 grown in continuous culture. J. Gen. Microbiol. **104**: 47–57.
18. Wood, T. K., Kuhn, R. H., Peretti, S. W. 1990. Enhanced plasmid stability through post segregational killing plasmid-free cells. Biotechnol. Tech. **4**: 39–44.