Role of starvation in detachment of Pseudomonas aeruginosa biofilms
by Baochuan Huang

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Chemical Engineering
Montana State University

Abstract:
Biofilms of Pseudomonas aeruginosa grown in continuous flow reactors spontaneously detached after the flow was stopped and the biofilm stood in a static aqueous environment for three days. The mean areal viable cell density was 9.2 log cfu cm^-2 before stopping flow and 7.9 log cfu cm^-2 after the static period. Similarly, a 1.2 log reduction in areal total cell density was measured between the same time points. The biofilm matrix appeared to progressively dissolve during the static period, as judged visually. Treatment of the biofilm with 5% formaldehdye immediately prior to stopping flow prevented detachment. Treatment with 200 mg/L chloramphenicol, a protein inhibitor, did not prevent detachment. When, instead of stopping medium flow, the flow was switched to a medium lacking carbon, the same extent of detachment still occurred. In static experiments in which concentrated nutrients were periodically amended to the reactor, detachment was largely inhibited. These results point to a role for carbon starvation in triggering the detachment process.

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APPROVAL

of a thesis submitted by

Baochuan Huang

This thesis has been read by each member of the committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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ABSTRACT

Biofilms of *Pseudomonas aeruginosa* grown in continuous flow reactors spontaneously detached after the flow was stopped and the biofilm stood in a static aqueous environment for three days. The mean areal viable cell density was 9.2 log cfu cm^{-2} before stopping flow and 7.9 log cfu cm^{-2} after the static period. Similarly, a 1.2 log reduction in areal total cell density was measured between the same time points. The biofilm matrix appeared to progressively dissolve during the static period, as judged visually. Treatment of the biofilm with 5% formaldehdye immediately prior to stopping flow prevented detachment. Treatment with 200 mg/L chloramphenicol, a protein inhibitor, did not prevent detachment. When, instead of stopping medium flow, the flow was switched to a medium lacking carbon, the same extent of detachment still occurred. In static experiments in which concentrated nutrients were periodically amended to the reactor, detachment was largely inhibited. These results point to a role for carbon starvation in triggering the detachment process.

INTRODUCTION

Pseudomonas aeruginosa biofilm

Biofilms are microbial aggregates that develop and persist at interfaces in both natural and engineered aquatic environments. Biofilms are composed of microorganisms embedded in the extracellular polymers (polysaccharides, glycoproteins, and proteins) they produce (Christensen, 1990; Costerton 1995). Biofilm bacteria have been shown to predominate in numbers and in metabolic activity in natural (Geesey et al., 1977), industrial, and medical (Khoury et al., 1992) ecosystems.

Pseudomonas aeruginosa is one important organism that can develop biofilms in natural settings, industrial systems, and even in the human body. Some strains of *P. aeruginosa* can produce large quantities of alginate and therefore have distinctive mucoid colony morphology. These mucoid strains are most commonly isolated from the respiratory tract infections that accompany the genetic disease, cystic fibrosis (CF) (Dogget, 1977). Cystic fibrosis is the most prevalent lethal genetic disease among people of European descent. The strain used for this research work, PAO1, was a non-mucoid strain.

It has been well established that biofilms are far more resistant to biocides and antibiotics than their freely suspended counterparts (Costerton et al., 1987; LeChevallier et al., 1984; Nickel et al., 1985). The physical and biological mechanisms that render biofilm microorganisms less susceptible have yet to be established. One hypothesis is that the bacteria and their exopolysaccharide products significantly reduce the penetration

of antimicrobial agents (Nichols et al., 1989). This transfer barrier is reinforced by the reactions and adsorption that occur between constituents of the biofilm and antimicrobial agents. Models of antimicrobial agent penetration into biofilm have been established (Stewart 1994; Stewart et al., 1995) and are supported by experimental data (Chen et al., 1993; De Beer et al., 1994; Xu et al.,1995). Another hypothesis relates biofilm susceptibility to the specific growth rate and phase in the division cycle of biofilm cells (Evans et al., 1991; Evan et al., 1990; Brown et al.,1990; Duguid et al., 1992).

Biocides and antibiotics are the principle weapons to control biofilms (Srinivasan et al., 1995). However the dead cells of biofilm may still attach to the surface after treatment. If a clean surface is needed rather than an inactive but possibly still intact biofilm, biofilm detachment becomes a crucial process. Furthermore, after biofilm cells detach from a surface and become planktonic cells, they are easier to kill by antimicrobial agents. Therefore biofilm detachment and biofilm resistance are closely related.

Biofilm detachment

Biofilm detachment refers to the interphase transport of biomass particles from an attached microbial film to the fluid compartment bathing the film (Stewart, 1993). Although detachment has not been investigated extensively, literature data from strongly different systems prove that it is the primary process that balances microbial growth and, thereby, determines the extent of biofilm accumulation. During the development of

Pseudomonas aeruginosa biofilm in a rotating drum reactor, 85%-95% percent newly formed biomass of biofilm was released into surrounding medium (Tijhuis et al., 1995).

Bryers has distinguished five categories of detachment processes: erosion, sloughing, human intervention, predator grazing, and abrasion (Bryers,1988). Erosion and sloughing are more important in research. Erosion refers to the continuous removal of individual cells or small groups of cells from the surface of the biofilm. Sloughing, in contrast, is the detachment of relatively large particles of biomass, whose size is comparable to or greater than biofilm thickness. Sloughing is a discrete and random process. Abrasion is caused by collision of solid particles with the biofilm.

Biofilm detachment was initially believed to result from a combination of internal biofilm processes and shear and normal forces exerted by moving fluid on the biofilm (Characklis, W. G. 1981). Several models have been forwarded using empirical mathematical expressions to describe detachment rate (Stewart, 1993; Chang et al., 1988; Rittman, 1982). In different biofilm systems the dominant mechanism of detachment may be different. For biofilms in fluidized reactors, the turbulence and attrition of bed fluidization appear to be dominant mechanisms (Chang et al., 1991; Nicolella et al., 1997). Research on an annular biofilm reactor indicated that detachment rate was directly related to biofilm growth rate and the factors that limit growth rate would also limit detachment rate (Peyton, B. M. et al., 1993). No significant influence of shear stress on detachment rate was observed.

Nutrient concentration has a great influence on biofilm detachment. Some researchers found that cells detached when nutrient was lacking (Marshall, 1988;

Delaquis et al., 1989). Anther study showed that specific detachment rate increased when nutrient was depleted (Sawyer, L. K., 1998). In contrast, James et al. (1995) reported cells detached under high nutrient conditions, although different carbon sources were used for high and low nutrient conditions. Peyton et al. (1993) included nutrient factors as growth rate inhibitor in their detachment model and this growth rate-dependent detachment model fitted data better than others in their experimental system.

Enzymes that may cause biofilm to detach

Extracellular polymers (polysaccharides, glycoproteins, and proteins) anchor cells to a surface. Therefore if these polymers are broken down by enzymes, the biofilm will detach (Aldridge, I. Y. et al, 1994; Brisou, J. F.; Sutherland, I. W., 1995; Wiatr, C. L. 1990). Combining biofilm-degrading enzymes with cell-killing enzymes can remove and deactivate biofilms (Johansen, C., et al., 1997). Studies also show that the detachment of *Streptococcus mutans* biofilm is mediated by an endogeneous surface protein releasing enzyme (SPRE) activity (Lee, S. F. et al., 1996). SPRE can cause monolayer biofilm cells to detach under minimal shear stress.

A key component of the extracellular polysaccharides of *Pseudomonas aeruginosa* biofilm is alginate. Alginate is a linear random polymer of β-1-4-linked D-mannuronic acid and L-guluronic acid (Figure 1). The mannuronate residues are modified to various degrees by *O*-acetyl groups. Alginate has been shown to play an

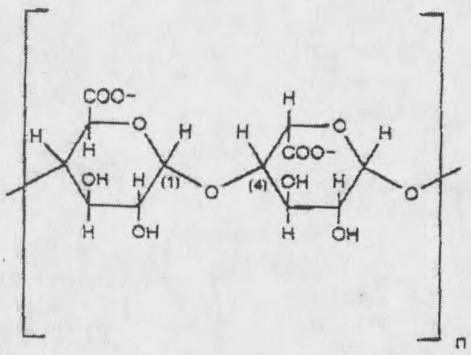

Figure 1. Molecular structure of alginate.

important role in biofilm formation by increasing the adherence of bacteria to the substratum (Ramphal et al., 1985; Mai et al., 1993). It is also involved in the chronic bronchopulmonary infections in cystic fibrosis patients by protecting against antibiotic-meditated and polymorphonuclear leukoycyte - directed phagocytosis and killing (Bayer et al., 1992).

Pseudomonas aeruginosa itself synthesizes an enzyme that can break down alginate molecules - alginate lyase. The gene coding this enzyme is *algL*. This enzyme has been isolated and characterized (Eftekhar et al., 1994; Linker et al., 1984). It has been found that alginate lyase modifies alginate molecules in alginate synthesis. Although some bacteria are capable of digesting alginate and using it as carbon source, it is still unknown if *Pseudomonas aeruginosa* can do so.

The role of *P. aeruginosa* alginate lyase in cell sloughing from agar colonies was investigated (Boyd, A., et al., 1994). Results showed that increased expression of the alginate lyase in mucoid strain 8830 led to alginate degradation and increased cell sloughing. On the contrary, Davies (1996) found that *P. aeruginosa* biofilm did not

detach even when the alginate lyase activity was elevated unless the permeabilizing agents 0.3mM NaCl or SDS (sodium dodecyl sulfate) were present. Adding purified alginate lyase alone to a cell cluster can not cause it to dissolve (Davies, 1996).

Allison et al. (1998) found that *Pseudomonas fluorescens* B52 biofilm lost exopolymers and biomass when subject to starvation. An exopolysaccharide lyase activity was detected in the media taken from dense biofilm cultures. Another researcher reported that *Pseudomonas aeruginosa* biofilm cultured in a flow cell reactor detached after the medium supply was stopped and the biofilm stayed in the static environment for 73 hours (Davies, 1996). It is reasonable to assume that a starvation environment prevailed in this system.

Cell-cell communication signals, such as homoserine lactones, have been found to play an important role in biofilm formation (Davies, 1998). Some research seemed to indicate involvement of signaling in biofilm detachment (Allison, 1998) as well. The addition of *N*-acyl-hexanoyl homoserine lactone to the medium appeared to expedite detachment. One hypothesis is that the signaling molecule may regulate the activity of some degrading enzymes, which cause biofilm to detach.

Thesis goal

The goal of this thesis was to investigate the effect of starvation on biofilm detachment in a static aquatic environment.

MATERIALS AND METHODS

Microorganism and media

The bacterium used in this study was *Pseudomonas aeruginosa* strain PAO1. It is a nonmucoid strain that was isolated from a burned wound.

Glucose minimal medium was used in biofilm growth in two concentrations: 1g/L Glucose minimal medium and 0.1g/L glucose minimal medium (Wentland, 1995). Glucose in the medium must be added separately through a 0.22 μm filter to avoid carbonization during autoclaving.

Table 1. Composition of glucose minimal medium

Chemicals	Strong (g/L)	Standard (g/L)
Glucose	1	0.1
NH_4Cl	0.36	0.036
Na_2HPO_4	13.632	1.3632
KH_2PO_4	6.56	0.656
$MgSO_4 \cdot 7H_2O$	0.056	0.011
Trace	1ml	0.1ml

Table 2: Composition of trace element stock solution

Chemicals	Concentration (mg/L)
$(NH_4)_6Mo_7O_{24}$	8.96
$ZnSO_4 \cdot 7H_2O$	908.8
$MnSO_4 \cdot 7H_2O$	72.96
$CuSO_4 \cdot 5H_2O$	17.92
$Na_2B_4O_7 \cdot 10H_2O$	8.96
$FeSO_4 \cdot 7H_2O$	1017.6
$(HOCOCH_2)_3N$	1280
$Co(NO_3)_2 \cdot 6H_2O$	21.32

Reactor system and operation

Drip flow reactors were used to cultivate biofilms (Fig. 3). Biofilm grows on the inclined surface of inoculated stainless steel slides that are bathed in a dropwise flow of medium. The reactor has four chambers. At the bottom of each chamber resides one stainless steel slide. Sterile medium is pumped continuously (50 mL/hr) onto the elevated end of the slide. Since the reactor is positioned on a slope (10°), the medium flows down the slide surface and then out of the reactor through a drain port and into a waste vessel. The vents on top of the reactor maintain an aerobic environment in the reactor. It usually took four days to develop a mature biofilm on the slide using 0.1g/L glucose minimal medium. Fig. 2 shows the operation of the reactor schematically.

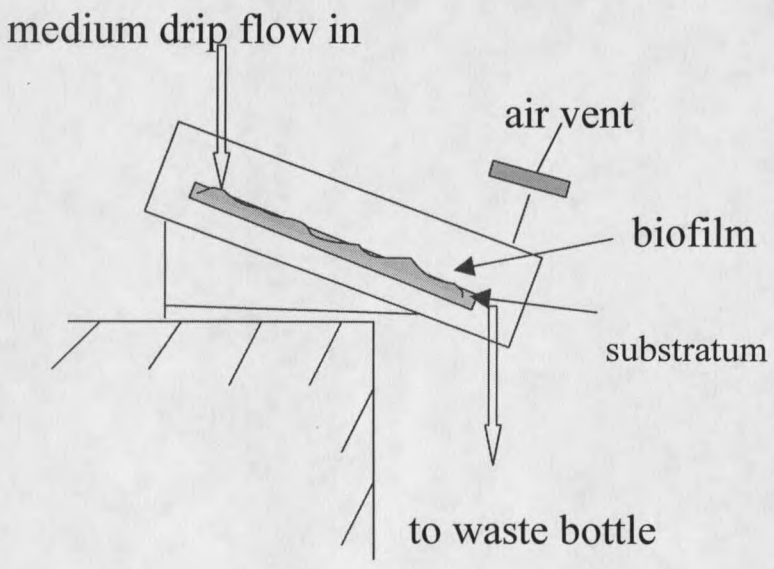

Figure 2. Drip flow reactor

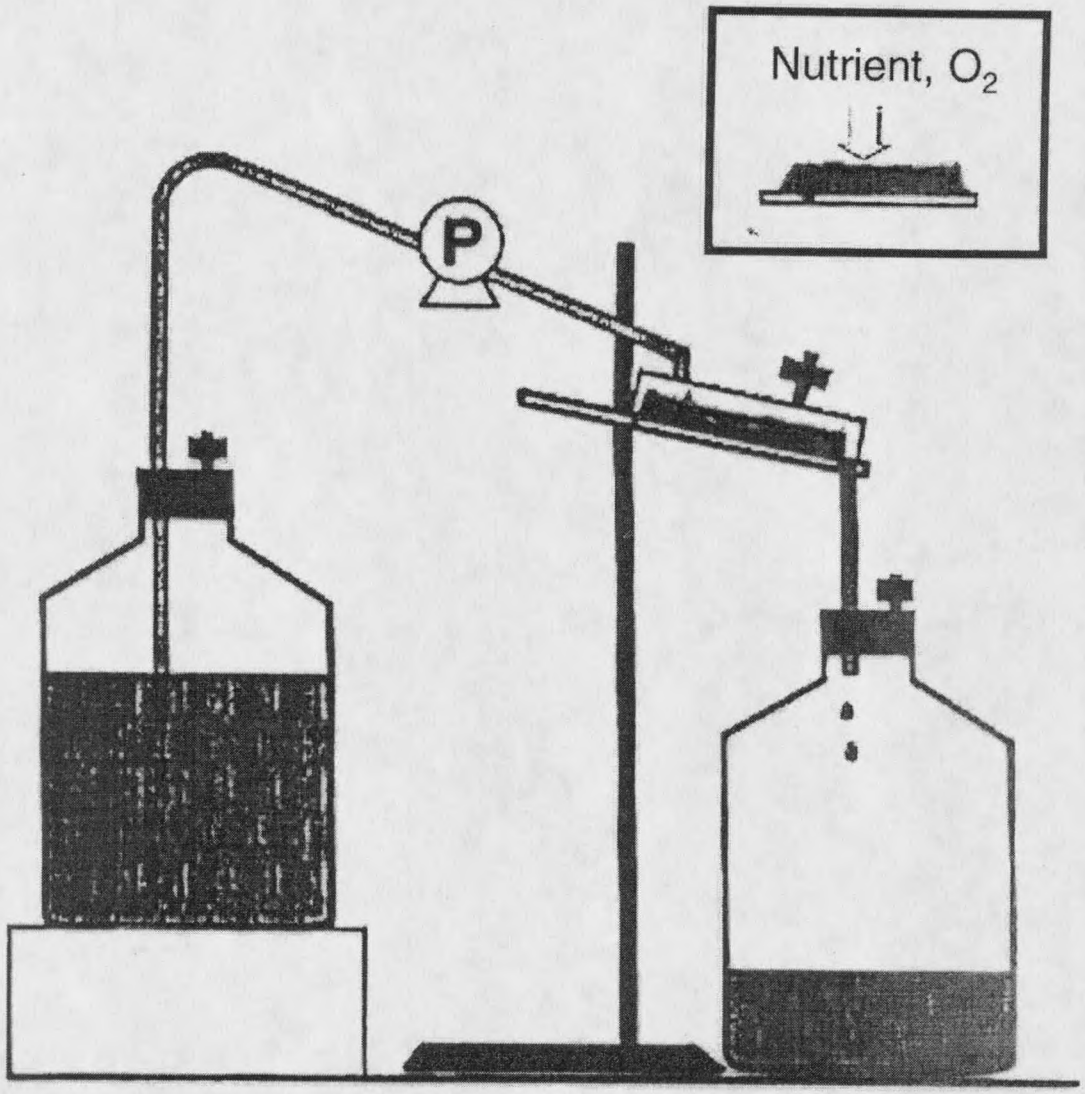

Figure 3. Drip flow reactor biofilm-culturing system

Reactor sterilization

Reactors were sterilized by autoclaving: Treated slides (see below) were fixed to the bottom of the reactor chambers with a small piece of autoclave tape. The reactor lids were laid on top, but left unscrewed. The reactor was wrapped with aluminum foil and autoclaved for 30 minutes. After cooling, all connections were checked to prevent leaks.

Stainless steel (316L) slides pre-treatment

In order to produce steel slides with reproducible surface characteristics, the following treatments were necessary. Slides were first dipped in acetone to remove grease and allowed to air dry, then transferred to fresh PBS 35 (a surface active agent for cleaning and radioactive decontamination of lab glassware and instruments) working solution (1mL PBS in 50mL H_2O) and heated to 50°C for 5 minutes. After being sonicated for 5 minutes, the slides were rinsed with nanopure water and sonicated again for 5 minutes. They were rinsed three more times and allowed to air dry. The next step was to soak the slides in 2.0M HCl solution for two hours, followed by thorough nanopure water rinse and air drying. The slides were then ready for use in the reactor.

Biofilm culture procedure

A planktonic culture was inoculated with PAO1 from an agar plate to a 250mL flask holding 30 mL of 1g/L glucose minimal medium. This culture was incubated at 35°C for 18 hours with shaking. In a biological hood the effluent tubing of the reactor

was clamped off. Fifteen mL of 0.1g/L glucose minimal medium was added to each chamber of the reactor. One mL of planktonic culture was inoculated to each chamber. The reactor then stood for 24 hours at room temperature in a horizontal position with no flow.

After the reactor was connected to a 20 L waste reservoir, the inoculation medium was drained by unclamping the effluent tubing. The influent tubing was connected to a 20 L carboy containing 0.1g/L glucose minimal medium. This medium had been autoclaved for at least 4 hours. The reactor was inclined on a 10° slope. Sterile needles were attached to the end of pump tubing and pierced into the rubber caps on the reactor. A pump (Cole-Parmer Co. Model: 7553-80) fed the medium at a constant rate of 50 mL/hr to each chamber. Biofilm grew for four days at room temperature, which was 22°C.

Detaching process and sampling

After a biofilm developed the following procedure was implemented to bring about detachment. The effluent tube was shut off with a clamp and the reactor was laid flat. Fifteen mL of medium was filled into each chamber of the reactor. Care was taken to prevent hydraulic shock to the biofilm, which might cause biofilm detachment. This addition was therefore allowed to flow slowly along the chamber wall. The biofilm was allowed to detach statically in this aqueous environment for three days (Figure 4).

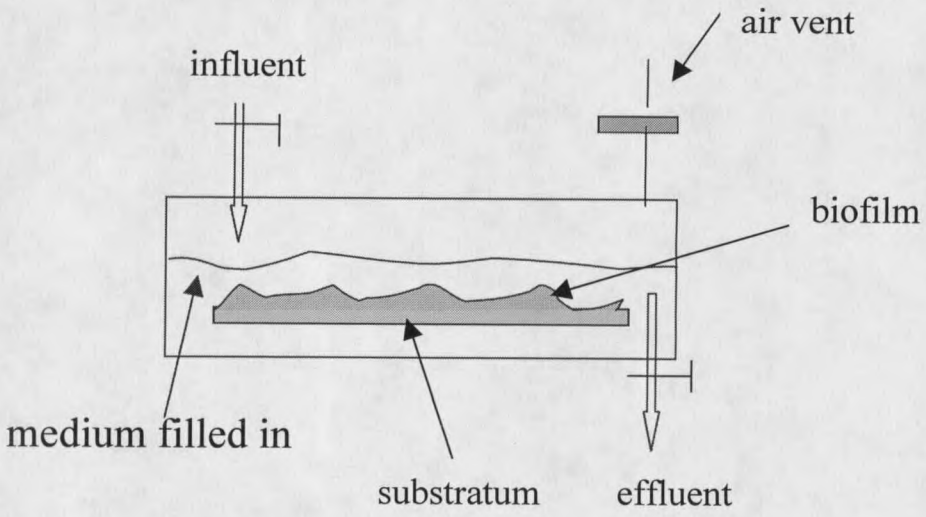

Figure 4. Static biofilm detachment in drip flow reactor

A second kind of biofilm detachment experiment was performed under continuous shear stress. After four days growth the influent tube was switched from medium to sterile nanopure water. The flow speed remained unchanged. The biofilm was allowed to detach for three days.

After detachment the medium in the chamber was drained. The reactor was inclined again and drip-flow continued for 10 minutes at 100 mL/hr to eliminate the cells floating over the biofilm surface. Each slide was transferred with forceps to a 100 mL beaker holding 20 mL PBS. A small plastic scraper (rubber policeman) was used to scrape all the biofilm down into the beaker. The sample was homogenized for 30 seconds using a Ultra-Turrax T25 homegenizer (Janke & Kunkel Co., Staufe i. Br.) and was then ready for assay of total and living cell counts.

Total cell count

A filtration apparatus with chimneys was used to deposit cells on a filter membrane. The stage of the filtration apparatus was first flushed with filter sterilized water. A polycarbonate membrane filter was spread on the stage, shiny side facing up. A chimney was clamped on the stage. The biofilm sample was diluted to the appropriate order of magnitude cell concentration and 1mL of that dilution was dropped slowly and evenly on top of the membrane. Vacuum was applied to the filter for 1 minute. The suction on the filtration apparatus was released. Ten μg/mL DAPI was dropped slowly and evenly on top of the membrane until it was fully covered (about 0.25 mL/filter). After staining for15-20 minutes, the DAPI solution was filtered through. A small drop of immersion oil was placed on a labeled glass microscope slide. The filter membrane was put on top of the immersion oil (shiny side up). Another drop of immersion oil was placed on top of the membrane. A glass cover slip was then placed over the membrane.

Total cell counts were made with epifluorescent microscopy (Olympus BH2-RFCA) using a 100X objective and a 10X ocular. Twenty random fields were chosen to count the cells. The total cells on the membrane were calculated based on the average number of cells in the twenty fields, the area of one field, and the total area of the membrane. In order to make 20 fields representative, they should be selected in a predetermined way (Figure 5).

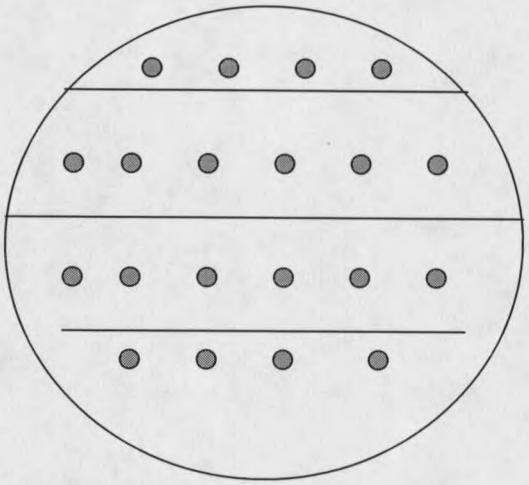

Figure 5. Locations of twenty microscopic fields on the membrane for total cell counts

Living cell count

One mL of the homogenized biofilm sample was utilized to make serial 10 fold dilutions. The dilution tubes of appropriate magnitude were used to do the living cell count. A 100 µl sample of the dilution tube was dispensed in 10µl drops onto R_2A agar plates using a dispenser. After overnight 35°C incubation colonies on the agar were counted. Each colony was taken to represent one colony forming unit (cfu).

Total organic carbon assay

A Dohrmann carbon analyzer (DC-80) was used to measure organic carbon content of a biofilm sample. The basic reaction involves the oxidation of organic carbon by KS_2O_4 to produce CO_2, which was quantified by the detector.

$$OC + O_2 + KS_2O_4 \xrightarrow{uv} CO_2 + H_2O + RX + RS$$

The carbon analyzer was first calibrated with a 10 ppm sucrose standard. Since there were cells and many kinds of long-chain molecules in the biofilm, some tailing occurred due to insufficient oxidization. This tailing can be minimized by limiting the TOC concentration in the sample to a low level.

Cryosectioning and staining of biofilm

Biofilm samples were cryoembedded with Tissue-Tek OCT compound (Miles Inc., Elkhart, IN) as described by Yu et al. (1994). Embedded samples were sectioned using a Leica CM 1800 cryostat (Leica Inc., Deerfield, IL). The 5μm slices were put on Superfrost plus microscopic slides (Fisher Scientific Inc., Pittsburgh, PA).

DAPI is a water soluble fluorescent stain that binds to DNA. 200 μl of 10μg/mL DAPI was dropped on the surface of biofilm section. Staining was performed over a period of 30 minutes and more DAPI was added as needed to replenish that lost by evaporation during this time. The remaining DAPI was drained. The slide was allowed to air dry. Epifluorescent microscopic pictures were taken with a CCD camera program that was connected to a microscope (Nikon Eclipse E800) using a 20X objective and a 10X ocular.

RESULTS

This section reports the results of measurements of the influence of environmental factors on detachment of *Pseudomonas aeruginosa* biofilm. These measurements include areal total cell numbers and areal living cell numbers of the biofilm before and after the detachment as well as biofilm thickness and total organic carbon.

Static biofilm detachment

After three days of static detachment in 0.1 g/L glucose minimal medium, biofilm detachment was reflected in a decrease in the number of cells on the substratum. Table 3 indicates there was a little more than one log reduction (LR) in biofilm living cells, which means more than 90% percent of the cells moved into the planktonic phase after the detachment. The result obtained from living cell counts (LR=1.11) is consistent with that from total cell counts (LR=1.17). It was observed visually that the substratum became visible and the biofilm polymers became increasingly translucent as the static detachment progressed. In the course of performing total cell counts under microscope, it was also observed that the proportion of smaller cells increased after detachment.

Table 3: Areal cell numbers of biofilm before and after detachment in a static aqueous environment for three days.

	Before Detachment	After Detachment	Log Reduction
Total cells ($\log(\#/cm^2)$)	9.56 ± 0.13	8.39 ± 0.17	1.17
Living cells ($\log(cfu/cm^2)$)	9.15 ± 0.20	7.87 ± 0.17	1.28

To further confirm this biomass transfer from the biofilm to the planktonic phase, changes in total organic carbon (TOC) and cell numbers in the two phases were investigated. TOC comes mainly from the cells and extracellular polymeric substances (EPS). Results are shown in Table 4 and Table 5, which indicate that the majority of the biomass (total cell, living cell, TOC) that disappeared from the biofilm phase after detachment transfered to the planktonic phase. The sum of living cells in the two phases did not change significantly. The sum of total cells increased by 119%. TOC increased by 37%.

Table 4: TOC and cell numbers in biofilm and planktonic phases before biofilm detachment.

	Before Detachment		Totals
Phase	Biofilm	Planktonic	
Log_{10} (living cell)	10.44±0.22	9.08±0.11	10.46
Log_{10} (total cell)	10.64±0.12	9.38±0.02	10.66
TOC (mg)	2.25 ±0.41	0.08 ±0.01	2.33

Table 5: TOC and cell numbers in biofilm and planktonic phase after biofilm detachment.

	After Detachment		Totals
Phase	Biofilm	Planktonic	
Log_{10} (living cell)	9.56±0.23	10.46±0.21	10.51
Log_{10} (total cell)	9.65±0.17	10.95±0.39	10.97
TOC (mg)	0.55 ± 0.17	2.65 ±0.79	3.20

Time scale of biofilm static detachment

The extent of detachment in this static situation with time is shown in Figure 6. It is clear that most of the detachment happened within one day, after which there had been a log reduction of 0.99 in living cell density. Log reductions in areal living cell numbers ranged from 1.0 to 1.6 between 1 and 5 days of detachment time. In order to repeat experiments, a three-day detach time was chosen for further work.

Average detachment rate coefficients were calculated using the following equation:

$$\ln(\frac{X}{X_0}) = -k_d * t$$

X (cfu/cm^2): cell density after detachment

X_0 (cfu/cm^2): cell density before detachment

k_d (day^{-1}): detachment rate coefficient

t (day): detaching time

For the first 0.5 day period of time, the average detachment rate coefficient was 3.22 day^{-1}. Over the first 3 days, the detachment rate coefficient value was 0.98 day^{-1}. The detachment rate was much higher in the first 12 hrs than it was over the three day period.

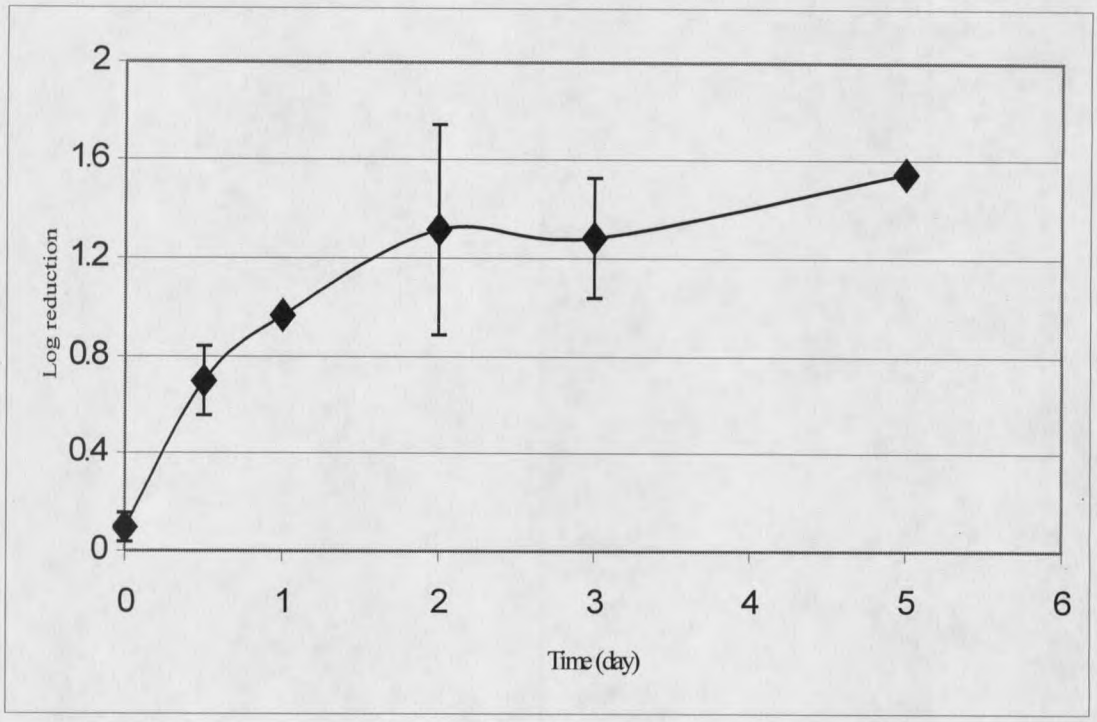

Figure 6. Biofilm detachment as a function of time. Detachment is evaluated as log reduction in areal living cells.

Biofilm detachment under both continuous shear and starvation

Four day old mature biofilms were subjected to glucose starvation by switching the influent medium to the same medium without glucose. Total starvation was implemented in other experiments by switching the influent medium to nanopure sterile water.

Results shown in Table 6 indicate a 1.31 log reduction in total cells after glucose starvation for 3 days and a 1.37 log reduction after total starvation for three days. Statistical analysis of these two groups of data revealed no significant difference (P= 0.49). The results from living cell counts were consistent: a 1.02 log reduction for glucose starvation and a 1.36 log reduction for total starvation (P =0.067).

Table 6: Areal cells remaining in the biofilm after detachment in a flowing environment under starvation conditions.

	No starvation	Glucose starvation	Total starvation
Total cells ($\log(\#/cm^2)$)	9.67 ± 0.21	8.36 ± 0.10	8.30 ± 0.14
Living cells ($\log(cfu/cm^2)$)	9.36 ± 0.12	8.34 ± 0.15	8.00 ± 0.09

Effect of some antimicrobial agents on biofilm detachment

To investigate the relationship between the physiological state of the cells and the detachment process, certain antimicrobial agents were applied in this system. These antimicrobial agents inhibit bacterial growth by different mechanisms. Formaldehyde can kill the cells and deactivate both the intercellular and cytoplasmic enzymes. Sodium azide inhibits respiratory activity of the cells. Chloramphenicol binds ribosomes and stops new protein synthesis. The concentration of each chemical utilized was determined by its effect on the growth curve of PAO1 when added at the early log phase. While 5% formaldehyde and 200 mg/L chloramphenicol totally stopped cell growth, sodium azide of 200 mg/L concentration only retarded the growth (Figure 7). Increasing the concentration of sodium azide to 800 mg/L still did not stop growth completely.

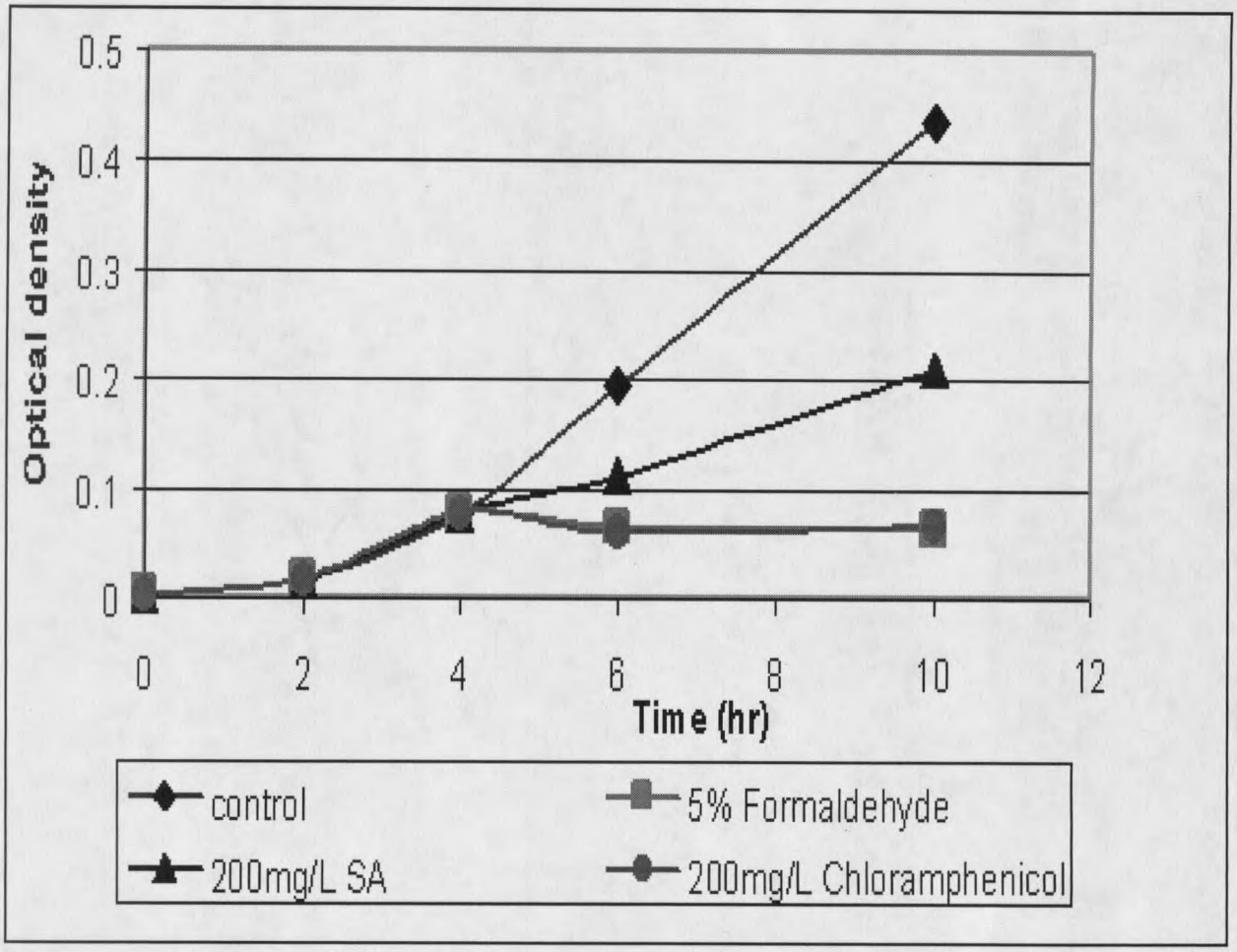

Figure 7: The influence of some chemicals on the growth of PAO1.
Antimicrobial agents were added at time equals 4 hours.

The effects of adding these chemicals before static detachment (with the medium) are shown in Figure 8 and Table 7. While 5% formaldehyde totally stopped subsequent detachment, addition of 200 mg/L sodium azide had no effect on this process. Application of 200 mg/L chloramphenicol seemed to reduce biofilm detachment slightly. These effects were tested for statistical significance (t-test).

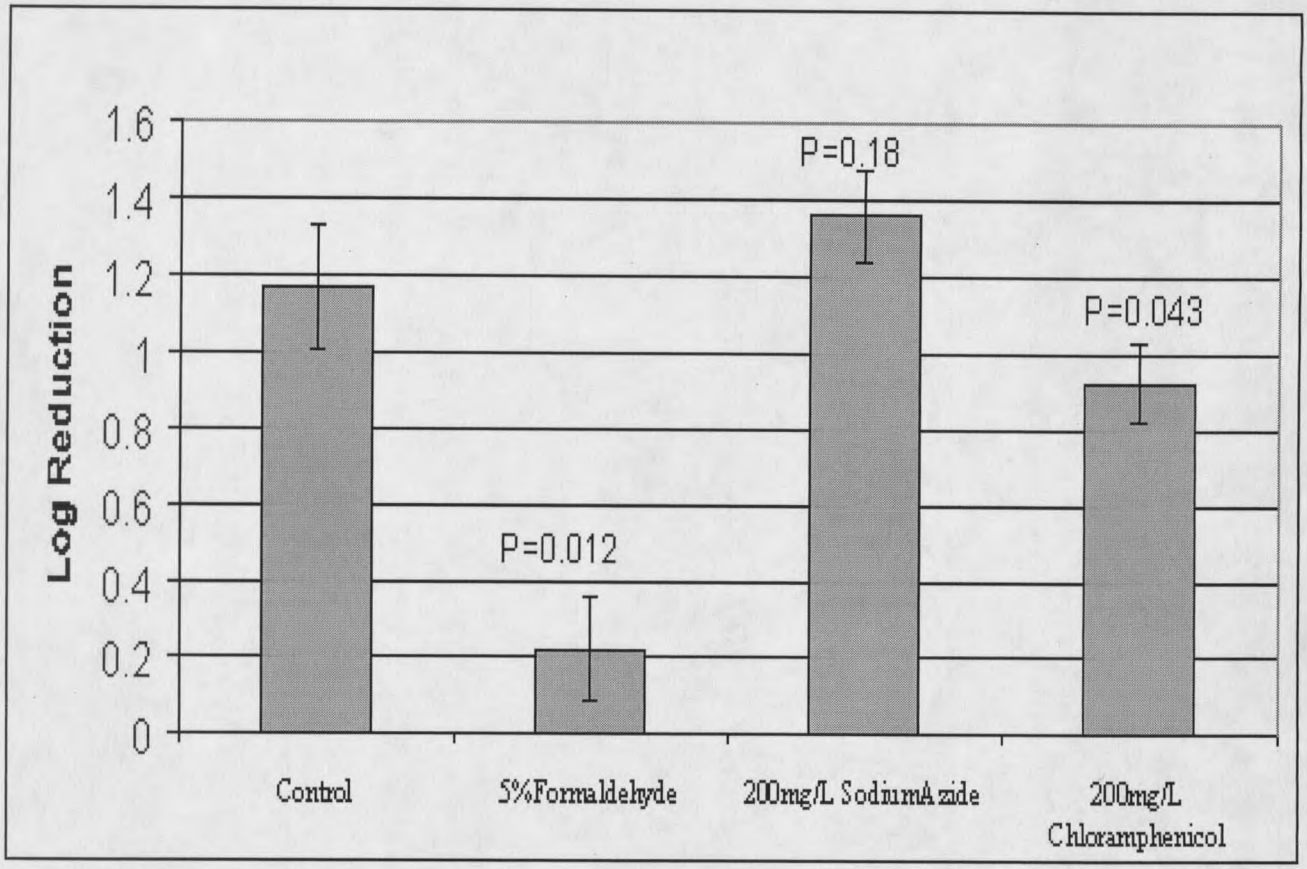

Figure 8. Effect of antimicrobial chemicals on biofilm detachment . The Y axis is the log reduction in areal total cells when certain antimicrobial chemicals were added before static detachment. Control means no chemical was added.

Table 7: Areal total cells and living cells remaining at the substratum after static detachment in the presence of antimicrobial chemicals. Control means no chemical was added.

Chemicals	Total cells (log (#/cm^2))	Living cells (log (cfu/cm^2))
Control (no chemicals)	8.39 ± 0.16	8.32 ± 0.06
5% Formaldehyde	9.34 ± 0.13	4.78 ± 0.19
200 mg/L Sodium azide	8.20 ± 0.12	8.04 ± 0.02
200 mg/L Chloramphenicol	8.64 ± 0.11	8.46 ± 0.18

The changes in areal living cells were also measured, with results shown in Table 7. Five percent formaldehyde killed more than 99.99% cells in the biofilm. Neither 200 mg/L sodium azide nor 200 mg/L chloramphenicol (one dose) killed biofilm cells significantly.

Effect of nutrient amendment on biofilm detachment

To further investigate the role of nutrient starvation in the detachment process, static detachment experiments were performed in which the nutrients were periodically replenished. This was done by adding a small volume of concentrated medium every 12 hours.

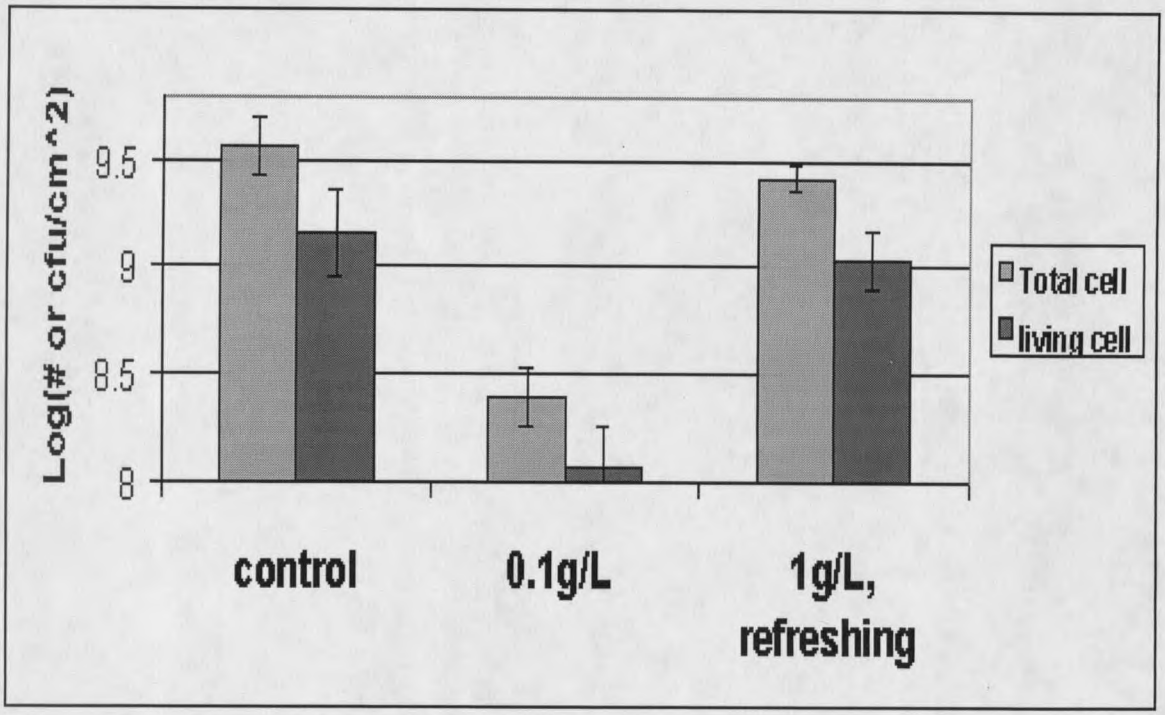

Figure 9. Effect of nutrient amendment on static biofilm detachment. The Y axis represents areal cell numbers remaining at the substratum after static detachment. The bars at right show data from experiments in 1g/L glucose minimal medium with periodic medium amendment. The middle bars show detachment in 0.1g/L glucose minimal medium without medium amendment.

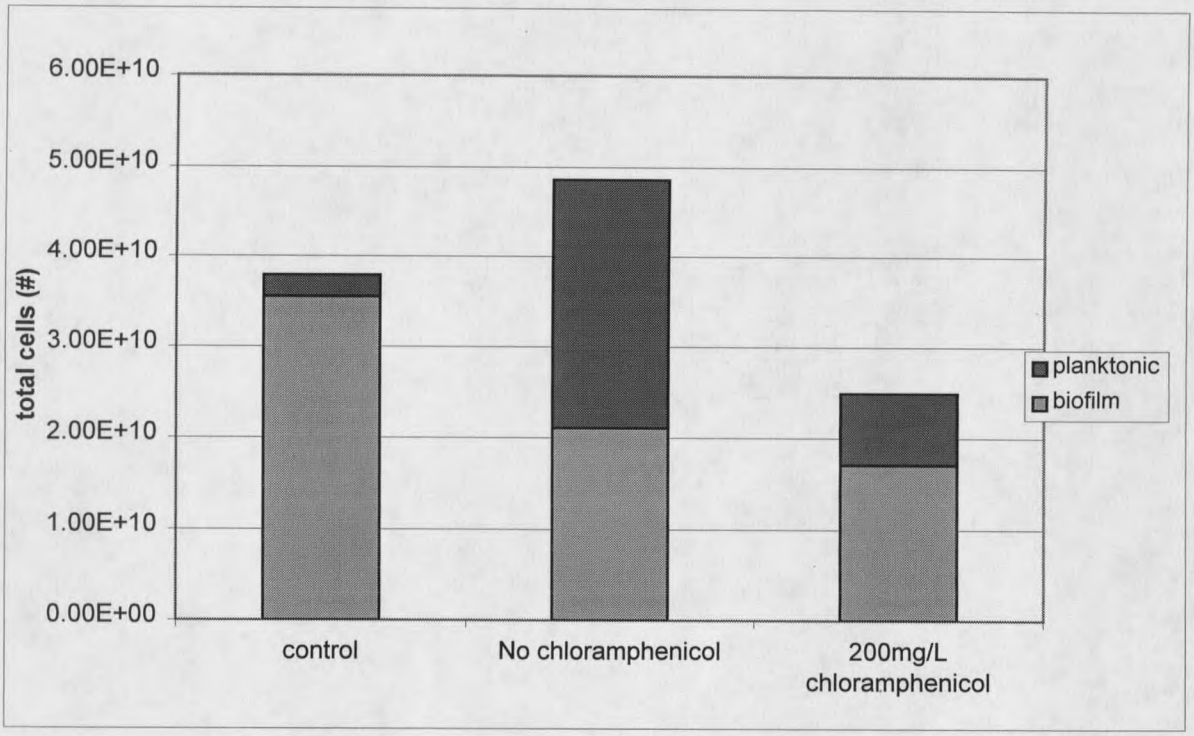

Figure 10. Combined effect of nutrient amendment and chloramphenicol on biofilm detachment. Control represents total cell number before detachment. The middle column is the total cell number after detachment in amended medium without chloramphenicol. The right column is the total cell number after detachment in amended medium with 200 mg/L chloramphenicol. Every 12 hours, 1.5 mL medium was taken out, 0.5 mL 10 g/L glucose minimal medium along with 1.0 ml of 3 g/L chloramphenicol were amended.

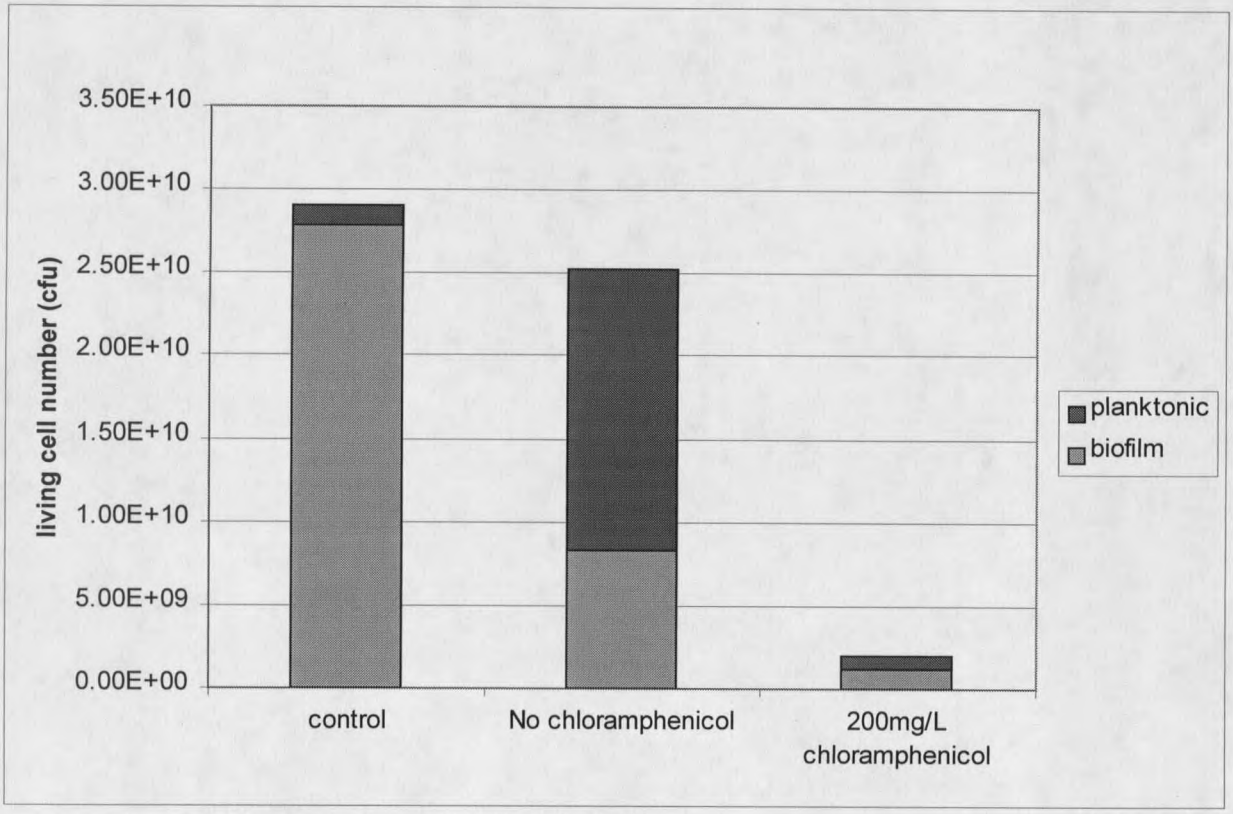

Figure 11. Combined effect of nutrient amendment and chloramphenicol on living cells in planktonic phase and biofilm phase. Control represents living cell number before detachment. The middle column is the living cell number after detachment in amended medium without chloramphenicol. The right column is the living cell number after detachment in amended medium with 200 mg/L chloramphenicol. Every 12 hours, 1.5 mL medium was taken out, 0.5 mL 10 g/L glucose minimal medium along with 1.0 ml of 3 g/L chloramphenicol were amended.

The data in Figure 9 indicates that nutrient addition stopped detachment from occurring. A t-test showed that the total cell numbers in the control and nutrient amended experiment were statistically significantly different ($p < 0.001$). No statistical difference was obtained between the number of areal biofilm cells after detachment in replenished 1g/L glucose minimal medium and the number of cells before detachment ($p = 0.11$).

Figure 10 shows that biofilm detachment was mostly stopped when both nutrient addition and 200 mg/L chloramphenicol were applied in the system. There was no cell growth in both phases, as indicated by Figure 11. About 90% of the living cells were killed.

Biofilm detachment was visualized by microscopic examination of frozen cross sections (Figure 12, 13). The change in biofilm thickness after three days of static detachment was determined by image analysis. Before detachment the average thickness of the biofilm was 334 μm; after three days of static detachment the average thickness had decreased to 27 μm. This is consistent with cell count data showing that over 90% of the cells detached from the surface.

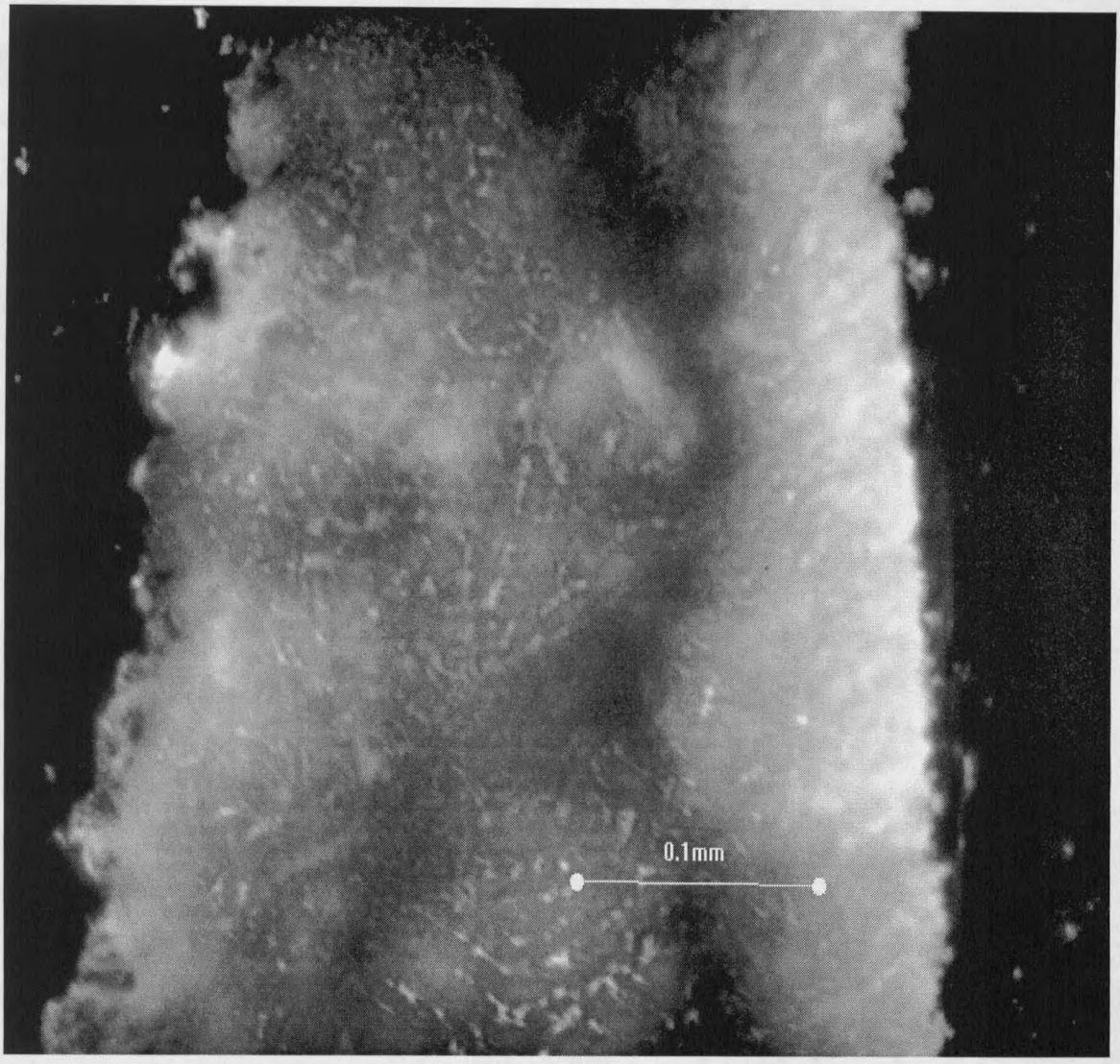

Figure 12. Frozen cross section of *Pseudomonas aeruginosa* biofilm picture before detachment

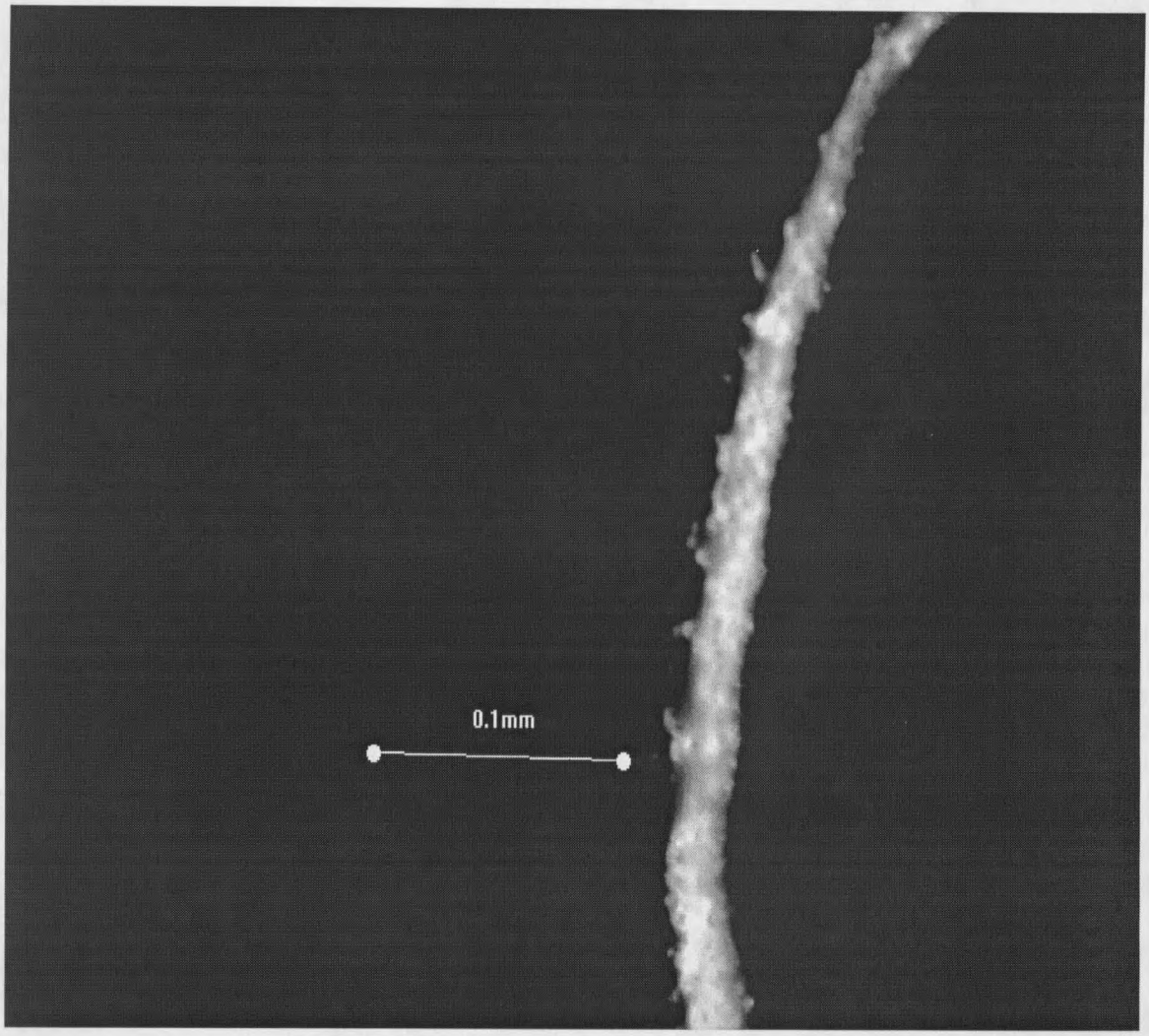

Figure 13. Frozen cross section of *Pseudomonas aeruginosa* biofilm after three days of static detachment

Photo of cells shed from biofilm

To investigate whether the cells detached from biofilm individually or in clusters, microscopic images of the cells that detached from biofilm were taken. Since the cell density was too high, serial dilution of the suspension (to 10^3) was made before it was filtered onto a membrane and stained with DAPI. Figure 14 shows that most of the detached cells were individual cells. However, a few small agglomerates were directly observed in the medium after detachment. Due to dilution they did not appear on the membrane.

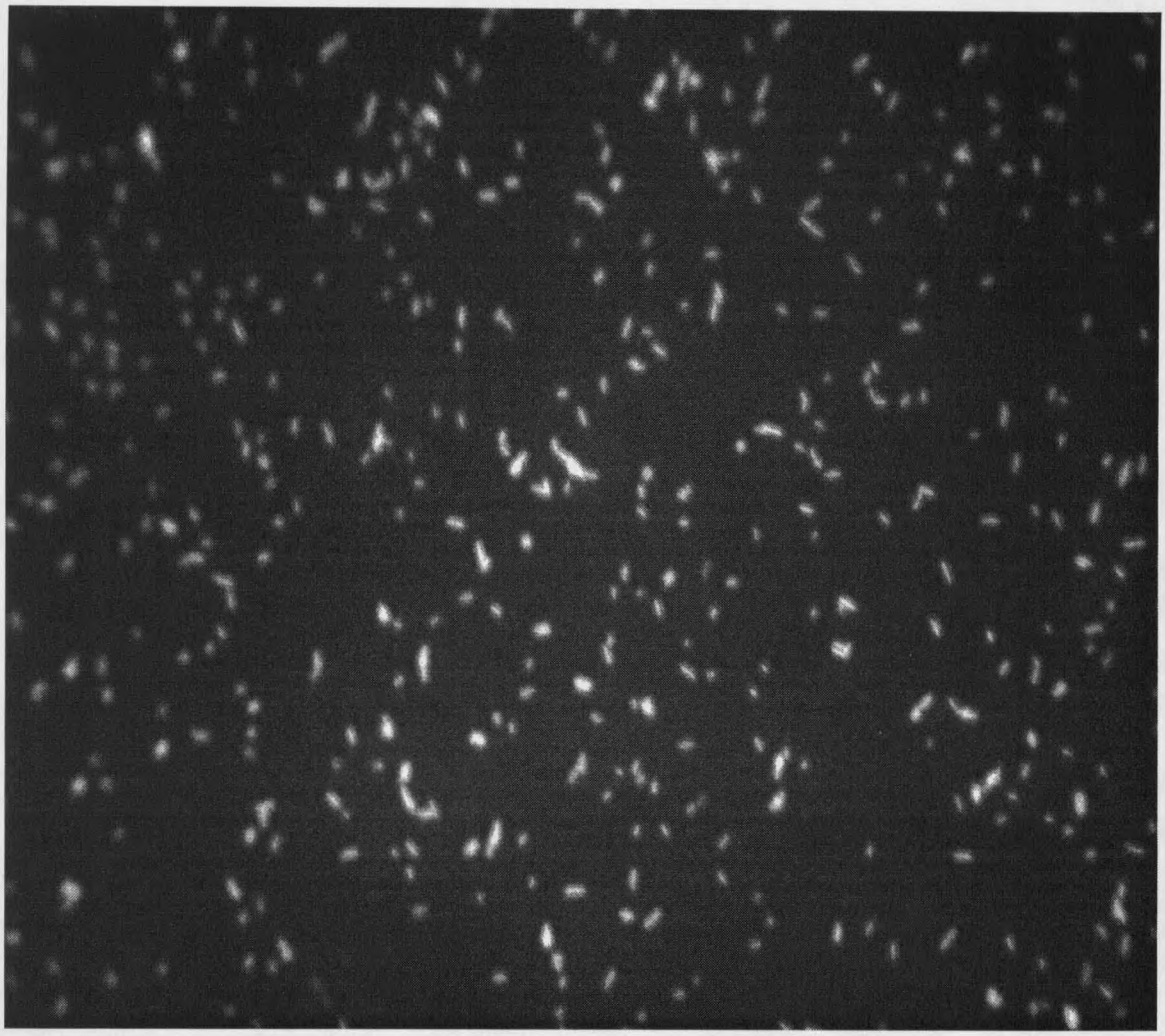

Figure 14. Image of cells detached from biofilm.

DISCUSSION

This chapter summarizes the evidences of static biofilm detachment, discusses the influences of antimicrobial agents on biofilm detachment, analyzes the role of starvation in biofilm detachment. Two models of static biofilm detachment are then put forward and discussed based on the data in this research.

Evidence of static biofilm detachment

The biofilm detachment phenomenon in this research was visible to the naked eye. Before detachment the substratum was covered with chunks of biomass and loosely connected polymer. During detachment, the polymers became progressively more translucent. After detachment the substratum became visible and the polymer disappeared. This observation naturally led to the hypothesis that the polymer was degraded and the biofilm cells were released to the surrounding medium and turned into planktonic cells.

To further confirm and quantify this detachment, several experiments were performed. Ninety percent of the cells in the biofilm were released to the surrounding planktonic phase after three days static incubation. The sum of the number of living cells in two phases remained unchanged in these experiments. However, the sum of the number of total cells in the system increased by 119%. This increase could have been

caused by the growth of planktonic cells, and possibly biofilm cells, due to the availability of dissolved carbon source at the beginning of the experiments. If the number of cells that die is of same order of magnitude as the number of new cells produced by growth, the number of living cells in the system will not change very much. To determine how many new cells can grow out of 0.1g/L glucose minimal medium, a control experiment was performed, in which the reactor holding 15 mL 0.1g/L glucose minimal medium was inoculated with a small amount of bacteria. There was no biofilm in the reactor. After three days incubation at 18°C, the average cell density reached 1.83E+08 cfu/mL. The calculated yield coefficient, $Y_{X/S}$ (number of cells/g glucose), was 1.83E+12. According to this the maximum planktonic growth in the reactor is 2.75E+9 cfu after three days, which is only 10% of the total planktonic cells (2.88E+10 cfu) after detachment. This calculation reveals that most of the planktonic cells in the reactor after three days come from biofilm detachment. The actual planktonic growth in the reactor should be much less than 2.75E+9 cfu because the majority of the carbon source will be used by the high-density biofilm cells. The increase in TOC (by 37%) could be attributed to systematic experimental error. Large molecules such as alginate can not be oxidized completely, so the TOC value measured could be lower than the true value. If large molecules are hydrolyzed to smaller ones after detachment, the apparent total TOC would be expected to increase after detachment, just as was observed.

Frozen cross section pictures of the biofilm directly reveal a ninety percent decrease in biofilm thickness after detachment. This is consistent with results of total and living cell counts.

In summary, a *P. aeruginosa* biofilm incubated under static conditions for three days experienced significant biofilm detachment. This phenomenon was demonstrated by consistent measurements of total cell numbers, viable cell numbers, total organic carbon, biofilm thickness, along with visual observations.

Universality of biofilm static detachment

Although several researchers have reported static biofilm detachment in different experimental systems, it should be pointed out that it is not a universal phenomenon. In this research when 1/50 LB broth medium is used instead of 0.1g/L glucose minimal medium to culture biofilm, biofilm did not detach under same conditions for as long as seven days. Biofilm cultured with 1/50 LB broth appeared to be more compact than biofilm cultured with 0.1g/L glucose minimal medium. Also, when another *Pseudomonas aeruginosa* strain- ERC was used instead of PAO1, the biofilm did not detach. It seems that the structure of biofilm and the components of biofilm polymers are also very important factors.

Influence of antimicrobial agents

Five percent formaldehyde (final concentration) completely inhibited the detachment. The mechanism by which formaldehyde affects biological molecules is quite complex. It kills cells, denatures enzymes, and even creates bonds among cells and polymers. Formaldehyde treatment appears to kill 90% of the bacteria (Table 7), thereby blocking induction of an active detachment process. Formaldehyde might simply have

denatured the already present matrix-degrading enzymes, preventing their slow but continued action on the biofilm. Alternatively, formaldehyde could have crosslinked the matrix components making them resistant to subsequent degradation. Therefore it is hard to reach a specific conclusion out of this. But the formaldehyde result does suggest that static biofilm detachment is not simply a pure physical process. It is a biological process that can be inhibited.

The appliance of 200 mg/L sodium azide did not affect the detachment. Since 200 mg/L sodium azide only moderately inhibited planktonic growth of *Pseudomonas aeruginosa* cells, we hesitate to conclude that respiratory activity of the cells is not needed for detachment.

A parallel experiment with 200 mg/L chloramphenicol led to statistically different detachment ($p = 0.043$) as compared with control. Chloramphenicol is an inhibitor of protein synthesis. This may suggest that stopping new enzyme synthesis reduced detachment to a small extent (20% or so). But this also shows that majority of the detachment was accomplished by enzymes already available at the beginning of detachment. The effect of 200 mg/L chloramphenicol on planktonic *Pseudomonas aeruginosa* is significant. It completely stopped the growth of planktonic cells.

The concentrations of antimicrobial agents were determined by planktonic experiments. Their effects on biofilm cells may be lessened due to the well-known increased resistance of biofilm. This should not be ignored in considering the results. If the physiological state of biofilm cells is less influenced by the antimicrobial agents as compared with planktonic cells, the effect of these antimicrobial agents on detachment

could be underestimated. While 5% percent formaldehyde killed more than 99.99% of biofilm cells, neither 200 mg/L sodium azide nor 200 mg/L chloramphenicol (one dose) killed biofilm cells significantly. We may need further evidence that they do have physiological influence on biofilm cells.

Role of starvation

The static detachment happened in 0.1g/L glucose minimal medium. Since the areal cell density of the biofilm was greater than 10^9 cells/cm^2, it is reasonable to assume that the nutrients were depleted within hours. Therefore this detachment happened in a starvation environment. Did starvation trigger detachment? To answer this question we designed the following experiment. 1 g/L glucose minimal medium was used for detachment with periodic replenishment to prevent starvation. With all other conditions remaining the same, the usual detachment did not occur. This is strong evidence that starvation plays an important role in biofilm detachment. However, there is another question behind this experiment. Since the concentrated medium was amended periodically, how can we know if the new cell growth in biofilm is playing an important role here? Chloramphenicol was then used together with amended medium to control cell growth in the system. Experimental results showed clearly that detachment will not happen if the carbon source is available, if cell growth is completely stopped.

This conclusion is supported by a second type of experiment, in which biofilm detached to the same extent when there was a continuous flow of nanopure water for three days instead of static environment.

Passive model

In this subsection, I present and discuss a model of biofilm detachment that does not require new respiration, protein synthesis, or growth of bacteria to cause detachment. This is therefore termed a passive model of detachment.

It is known that *Pseudomonas aeruginosa* produces alginate lyase, an enzyme that plays an important role in alginate synthesis by modifying alginate molecules. This enzyme is surely capable of chopping the intercellular alginate into smaller molecules. Since EPS are highly complex polymers, there should exist other hydrolytic enzymes that can degrade them. If some of these enzymes resides on the cell membrane or is released into surrounding medium, it will continuously hydrolyze EPS in the extracellular space. If a carbon source is available, newly synthesized polymers can replenish the polymer digested and thereby biofilm structure is maintained. However, if the carbon source is shut off, the extant EPS will be continuously digested into smaller molecules by these degradative enzymes. When the molecular length of polymers is not long enough to keep cells together, detachment happens.

The results from this project seem to support this model. In the chloramphenicol experiment, biofilm detachment happened even when protein synthesis was blocked. To rationalize the result of the formaldehyde experiment, in which detachment was prevented, we hypothesize that 5% formaldehyde denatured degradative enzymes. In medium amendment experiments the replenishment of concentrated medium stopped the detachment. The passive model can explain these phenomena well. Whether static detachment happens or not depends on the amount and status (molecular length and

structure) of EPS, which is determined by the amount of newly synthesized EPS. If carbon source is available, biofilm structure is maintained because newly synthesized EPS can replenish the lost polymer. Otherwise static detachment happens.

One piece of evidence from the literature is consistent with this model. Alginate lyase activity was detected at low level in a flow cell biofilm culture during the development of static detachment (Davies, D., 1995).

Cell-cell signaling model

This subsection discusses a model of biofilm detachment that depends on an active biological induction.

The assumption for this model is: There is some signaling molecule in the biofilm that triggers the elevated synthesis of alginate lyase or the release of this enzyme into the medium when its concentration accumulates to certain level. The environmental factors that may elevate the concentration of a signaling molecule include starvation and cell density (quorum sensing).

David Davies (presentation, 1999) discussed this model in detail and suggested that a homoserine lactone acted as the signaling molecule. Although some experiments (Allison, D. G., 1998) indicated a weak relationship between homoserine lactone and biofilm detachment, there is still no evidence that detachment happens once homoserine lactone concentration reaches a certain level.

The experimental results from this project do not support the cell-cell signaling model of biofilm detachment.

First, biofilm detached to the same extent in both static system and flow system when a starvation environment was created. In the flow detaching system the signaling molecule is not likely to accumulate because the flow of nanopure water may continuously convey the signaling molecule out of the reactor. However, no conclusive statement is made here since little is known about the mass transfer of signaling molecule in biofilm.

Another weak point in this comparison should not be ignored either. While there is no shear stress in static detachment, shear does exist for flowing detachment. Shear strain may play a role in detachment.

Following experimental design might solve the second problem. Instead of flowing nanopure water onto the slope of coupons, the reactor could be laid flat. A small ring would be placed over the outlet of the reactor so that the reactor will hold 15 mL medium before overflow takes place. Then nanopure water would be pumped into the reactor. The flow speed is slow (50 mL/hr) so that shear is negligible. The starvation condition is also maintained. The data from this system could give us more convincing evidence.

Second, the results from nutrient-amended experiments shows that detachment did not happen in a static environment if the carbon supply was maintained. The signaling molecule, if there is any, was allowed to accumulate in this system. Since in each medium amendment (every 12 hrs) 5 mL biofilm culture was first taken out then 5 mL concentrated medium (1g/L glucose minimal) was filled in, the signaling molecule would be diluted by 1/3 every 12 hours. This dilution effect would become negligible if

we use 0.5 mL 10 g/L glucose minimal medium for each medium amendment and do not take out the old culture.

Physiological change of bacteria during starvation

When nutrient in the environment is depleted, the physiological states of bacteria cells change dramatically and turn gradually into stationary phase. Most of these changes are genetically regulated. It is still unknown how these genetic regulations are related to biofilm detachment.

An important difference of cells subject to starvation is their smaller size. This trait may be a passive change reflecting the tendency of cells entering stationary phase to complete the last round of cell division, but not increase their mass significantly (Neidhardt, F. C. et al., 1990). This was supported by direct microscopic observation that the average cell size after static starvation seemed to be smaller than that before detachment. This decrease in cell size may influence biofilm structure, which ultimately affects biofilm detachment.

CONCLUSIONS

Based on the experiments conducted with *P. aeruginosa* (PAO1) pure culture biofilm, the following conclusions are drawn.

1. More than 90 percent of biomass detaches from *P. aeruginosa* biofilm during static incubation for 3 days.
2. Starvation is one of the factors affecting biofilm detachment.
3. Biofilm detachment can be blocked by an increased nutrient level.
4. *P. aeruginosa* biofilm detaches under flowing conditions when subjected to starvation.

RECOMMENDATIONS FOR FUTURE WORK

Future research work may be continued in the following possible directions.

Influence of antimicrobial agents

The continued search for an antimicrobial agent that will inhibit biofilm static detachment is a good way to understand the detachment mechanism. Work should continue with new agents. We can then explain this detachment based on the specific mechanism of that antimicrobial agent. One difficulty is that some antimicrobial agents themselves cause a biofilm to detach.

Enzyme activity measurement

If we can measure the degradative enzyme activity, such as alginate lyase, in a biofilm culture, tracking the activity change while detachment develops may give us useful information. If the activity remains stable during detachment, it may serve as further evidence for the passive model of detachment. If an increase of enzyme activity was detected consistently during static detachment, some regulatory mechanism may be involved. PAO1 is a nonmucoid strain of *P. aeruginosa.* The alginate lyase activity is lower than that of the mucoid strain. This may cause difficulty in measuring enzyme activities.

Another alternative way to investigate the role of alginate lyase is to purify this enzyme and find cations that inhibit its activity significantly. Then such a cation can be

added to the detachment system to measure its influence. The potential problem for this experiment is that these same cations may change the biofilm structure.

Signaling molecules

A simple way to investigate the involvement of a reported signaling molecule in static detachment is quite straightforward. This chemical can be added at the start of static detachment to see if it expedites the detachment. Even if it does, evidence should be given that the chemical does not influence biofilm in other ways before any conclusion is made.

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APPENDICES

(Raw Data)

1. Biofilm living cell densities before and after three day detachment

No.	Before ($log(cfu)/cm^2$)	After ($log(cfu)/cm^2$)
1	9.28	7.65
2	9.37	7.86
3	8.95	8.12
4	9.01	7.93
5		7.81

2. Biofilm total cell densities before and after three day detachment

No.	Before ($log\#/cm^2$)	After ($log\#/cm^2$)
1	9.64	8.55
2	9.41	8.39
3	9.64	8.22

3. Number of living cells in biofilm phase and planktonic phase before and after three day detachment

No	Before detachment (log(cfu))		After detachment (log(cfu))	
	PlanktonicPhase	BiofilmPhase	PlanktonicPhase	BiofilmPhase
1	9.15	10.43	10.49	9.58
2	9.00	10.46	10.42	9.54
3			10.03	9.11

4. Number of total cells in biofilm phase and planktonic phase before and after three day detachment

No	Before detachment (log#)		After detachment (log#)	
	PlanktonicPhase	BiofilmPhase	PlanktonicPhase	BiofilmPhase
1	9.37	10.64	10.99	9.65
2	9.39	10.46	10.91	9.33
3			10.28	9.43

5. TOC of planktonic phase and biofilm phase before and after detachment

No	Before detachment (mg)		After detachment (mg)	
	PlanktonicPhase	BiofilmPhase	PlanktonicPhase	BiofilmPhase
1	0.084	1.84	3.26	0.44
2	0.083	2.26	1.75	0.54
3	0.066	2.66	2.93	

6. Raw data of areal living cell density during three days of static detachment

Time (days)	Log Reduction No. 1	2	3
0	0.12	0.03	0.14
0.5	0.76	0.80	0.54
1	0.89	1.04	
2	0.97	1.79	1.19
3	1.22	1.08	1.55
5	1.54		

7. Raw data of optical density measurements of planktonic cells when antimicrobial agents were added at fourth hour.

Time(hrs)	control	5%Formaldehyde	Sodium Azide 200mg/L	Chloramphenicol 200mg/L
	(Optical density)			
0	0.003	0.003	0.003	0.003
2	0.018	0.018	0.018	0.018
4	0.078	0.078	0.078	0.078
6	0.196	0.065	0.113	0.061
10	0.437	0.063	0.21	0.066

8. Total cell density of biofilm before and after static detachment when certain antimicrobial chemicals were added at beginning

No.	before detachment Units	after detachment	Formaldehyde 5%	Sodium Azide 200mg/L	Chloramphenicol 200mg/L
			log((#/cm^2))		
1	9.64	8.55	9.49	8.06	8.74
2	9.41	8.39	9.32	8.29	8.65
3	9.64	8.23	9.14	8.25	8.53

9. Living cell density of biofilm before and after static detachment when certain antimicrobial chemicals were added at beginning

No.	before detachment Units	after detachment	Formaldehyde 5%	Sodium Azide 200mg/L	Chloramphenicol 200mg/L
			log((cfu/cm^2))		
1	9.34	8.26	4.99	8.06	8.57
2	9.55	8.31	4.63	8.03	8.31
3	9.27	8.38	4.71	8.03	8.51

10. Living cell density of biofilm after detachment in different mediums

No	0.1g/L glucose minimal log((cfu/cm^2))	1g/L glucose minimal, replenishing log((cfu/cm^2))
1	7.93	8.95
2	7.86	8.89
3	8.12	9.1
4	8.31	9.18

10. Total cell density of biofilm after detachment in different mediums

No	0.1g/L glucose minimal log((#/cm^2))	1g/L glucose minimal, replenishing log((#/cm^2))
1	8.4	9.4
2	8.55	9.35
3	8.39	9.44
4	8.22	9.49

11. Number of total cells in two phases after detachment in amended medium and 200mg/L chloramphenicol.

	control		No chloramphenicol		200mg/L chloramphenicol	
No.\phases	Biofilm	planktonic	biofilm	planktonic	Biofilm	planktonic
1 (log10(#))	10.64	9.37	10.32	10.43	10.17	9.97
2	10.46	9.39	10.33	10.44	10.27	9.81
3					10.27	
average	10.55	9.38	10.33	10.44	10.23	9.89
total_cells(#)	3.55E+10	2.40E+9	2.11E+10	2.75E+10	1.71E+10	7.82E+9

12. Number of living cells in two phases after detachment in amended medium and 200mg/L chloramphenicol.

	Control		No chloramphenicol		200mg/L chloramphenicol	
No.\phases	Biofilm	planktonic	biofilm	Planktonic	Biofilm	planktonic
1 (log10(cfu))	10.43	9.15	9.90	10.24	8.87	9.02
2	10.46	9.00	9.94	10.22	8.97	8.80
3					9.37	
average	10.45	9.08	9.92	10.23	9.07	8.91
total_cells(cfu)	2.79E+10	1.19E+9	8.33E+9	1.69E+10	1.17E+9	8.13E+8

13. Measures of biofilm thickness before and after detachment

No.	Before detachment (μm)	After detachment (μm)
1	285	25
2	309	44
3	330	28
4	343	29
5	355	22
6	382	13