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

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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

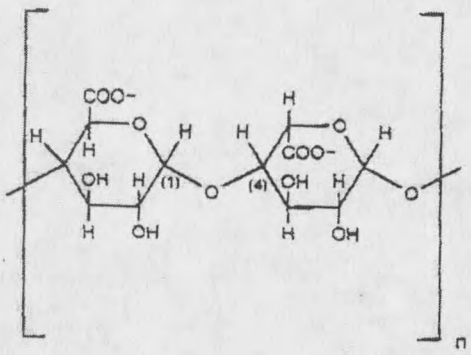

Figure 1. Molecular structure of alginate.

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.

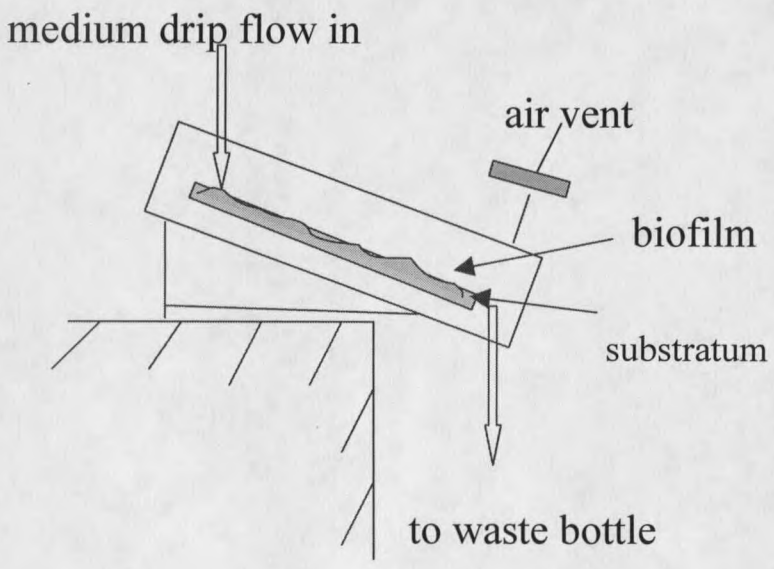

Figure 2. Drip flow reactor

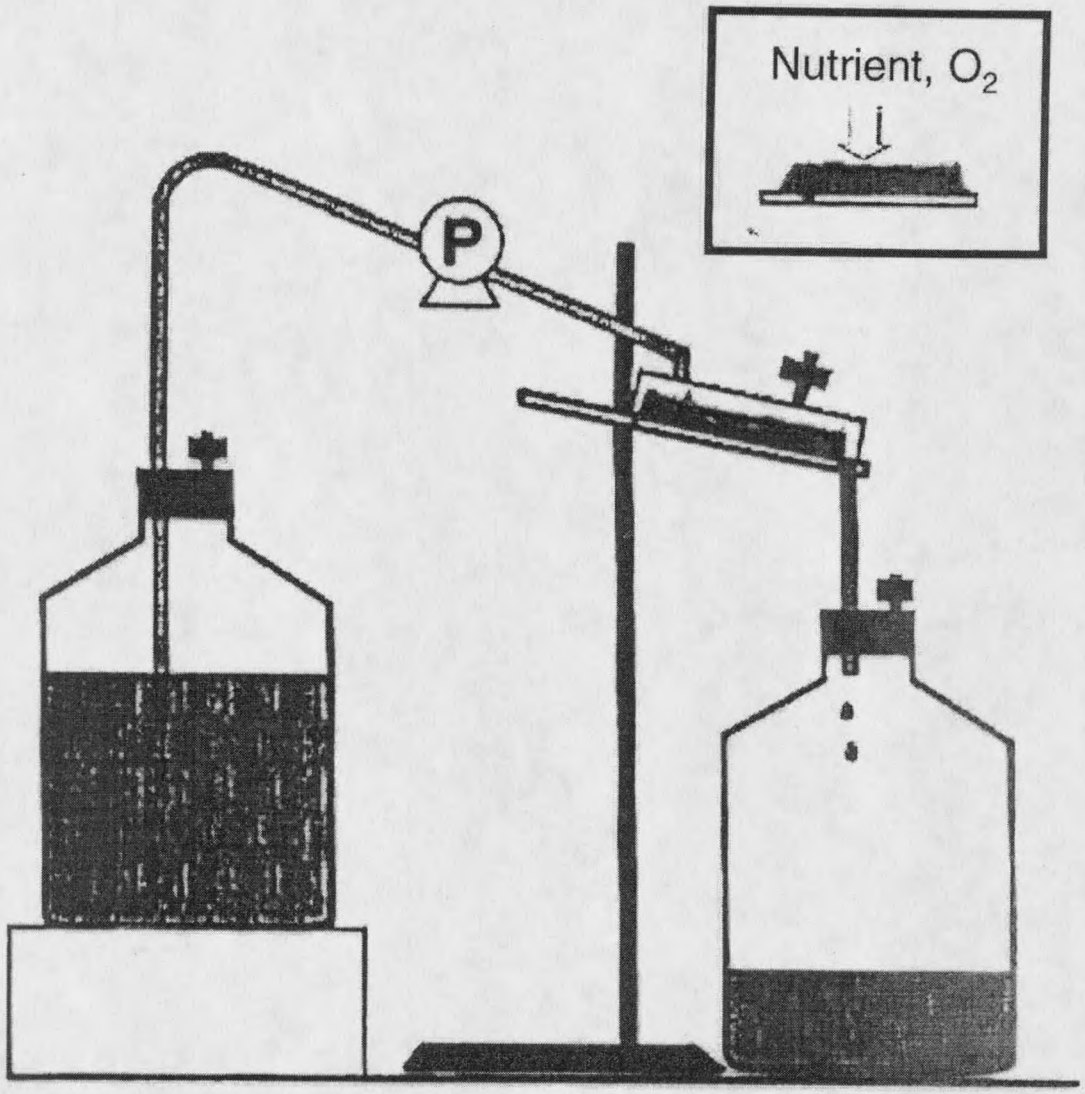

Figure 3. Drip flow reactor biofilm-culturing system

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).