Direct observation of individual cell behavior within a biofilm
by Andrew Ramsey Rice

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

Abstract:
The ability of microorganisms to form biofilms has been well characterized. Bacterial cells can transition from a planktonic state to a sessile state, replicate and subsequently populate a surface. Organisms which are the first to colonize a “clean” surface are referred to as “primary” biofilm cells. The progeny of the first generation of sessile cells are known as “secondary” biofilm cells. This study examined the growth of both primary and secondary biofilm cells.

The organism of interest in this study was Pseudomonas aeruginosa PA01. A parent strain, deficient of the ability to synthesize green flourescent proteins (GFP) and a construct strain which constitutively produced GFP were used. This organism was grown in planktonic and biofilm environments. A parallel plate flow cell reactor was used with a glass substratum for the biofilm experiments. A population of only GFP+ cells was studied for planktonic and primary biofilm experiments, whereas a mix of GFP+ and GFP- cells were used to examine the growth of secondary biofilm cells.

It was found that primary cells apparently undergo a change in phenotype from planktonic to biofilm form and also experience a lag in their growth. This is referred to as a surface associate lag time. Planktonic and secondary biofilm cells both grew at a faster rate than the primary biofilm cells.

The growth of secondary biofilm cells is characterized by replication, emigration, and movement. Each of these behaviors was directly observed for individual cells. A dilute mix of GFP+ cells in a predominantly GFP- biofilm allowed was used. It was discovered that cells which are destined to emigrate are more active in terms of movement than their remaining counterparts. Also, cells emigrate at a median time 1/3 of that of the median time to replication. Finally, at least 1/3 of all cells emigrate which is consistent with the “spreading” mode of biofilm growth of Pseudomonas aeruginosa.

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APPROVAL

of a thesis submitted by

Andrew Ramsey Rice

This thesis has been read by each member of the thesis 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

The ability of microorganisms to form biofilms has been well characterized. Bacterial cells can transition from a planktonic state to a sessile state, replicate and subsequently populate a surface. Organisms which are the first to colonize a "clean" surface are referred to as "primary" biofilm cells. The progeny of the first generation of sessile cells are known as "secondary" biofilm cells. This study examined the growth of both primary and secondary biofilm cells.

The organism of interest in this study was *Pseudomonas aeruginosa* PA01. A parent strain, deficient of the ability to synthesize green flourescent proteins (GFP) and a construct strain which constitutively produced GFP were used. This organism was grown in planktonic and biofilm environments. A parallel plate flow cell reactor was used with a glass substratum for the biofilm experiments. A population of only GFP+ cells was studied for planktonic and primary biofilm experiments, whereas a mix of GFP+ and GFP- cells were used to examine the growth of secondary biofilm cells.

It was found that primary cells apparently undergo a change in phenotype from planktonic to biofilm form and also experience a lag in their growth. This is referred to as a surface associate lag time. Planktonic and secondary biofilm cells both grew at a faster rate than the primary biofilm cells.

The growth of secondary biofilm cells is characterized by replication, emigration, and movement. Each of these behaviors was directly observed for individual cells. A dilute mix of GFP+ cells in a predominantly GFP- biofilm allowed was used. It was discovered that cells which are destined to emigrate are more active in terms of movement than their remaining counterparts. Also, cells emigrate at a median time 1/3 of that of the median time to replication. Finally, at least 1/3 of all cells emigrate which is consistent with the "spreading" mode of biofilm growth of *Pseudomonas aeruginosa.*

CHAPTER 1

INTRODUCTION

It is important to understand how microbial biofilms form because of the complexity, prevalence, and impact of these communities. During the initial events of biofilm formation, the cells which originally colonize a particular surface become the parent organisms of the ensuing biofilm. The behavior exhibited by these cells appears to vary according to species (Lawrence and Caldwell, 1987a). Parent cells can form dense microcolonies and then disperse in a matter of minutes (O'Toole and Kolter, 1998a). Upon replication, daughter cells can pack tightly near the parent cell location, spread out upon the surface, be cast back into the bulk fluid, form long chains of cells, or roll along the substratum (Lawrence and Caldwell, 1987a; Korber *et al.*, 1989; Power and Marshall, 1988; Shapiro and Hsu, 1989; Dalton *et al.*, 1994).

Purpose

The general purpose of this research project was to examine the importance of initial events; or, to determine if the initial colonization pattern of cells had any

bearing upon the biofilm that resulted. This research involved studying the processes which occur after initial events to understand how a biofilm develops from a population of parent cells.

The practice of extrapolating the behavior of individual cells to that of a general population has been suggested by (Gualtieri *et al.*, 1985) in the study of the motion of microorganisms. The consequence of this approach yielded two related areas of results: a quantitative description of individual cell behavior within an emerging biofilm matrix and a more qualitative account of an effect of the phenotypic transition of planktonic cells to sessile cells. When taken together, it is evident that the process of biofilm development is quite dynamic. Cells may have different growth rates depending upon their point of origin. They also are constantly repositioning themselves within the biofilm matrix; some move relatively short distances while others completely relocate over distances of hundreds of microns.

Much of the work responsible for the present scope of knowledge of biofilms was gained through direct observation approaches rather than efforts that were "hypothesis driven" (Costerton, 1999). It has been postulated that further gains in this field will stem from "very basic, extremely cross-disciplinary and sometimes esoteric research" (Palmer and White, 1997). The results of this study follow in this theme and are a product of direct, in situ observation of biofilm development utilizing flow cell and Confocal Scanning Laser Microscopy technology.

Technology

Microscopy and Image Analysis

The microscope is a key instrument for the investigation of bacterial behavior. Various means of employing the microscope in conjunction with the study of sessile organisms have been reviewed (Madigan *et al.*, 1997). Transparent and/or opaque substrata coupled with automatic image analysis is one of the most prevalent combinations currently used in this field of research. The use of various fluorochromes in biofilm research is also becoming a method of choice due to the ability of specific organisms and various physiological parameters to be visualized with differing degrees of sample disturbance (McFeters *et al.*, 1995). This research and others (Kuehn *et al.*, 1999; Davies *et al.*, 1998) are exploiting a new and indispensable technology which utilizes the insertion of genes into target bacteria which code for the production of a green fluorescent protein (GFP). Cells which posses the ability to produce this GFP are in essence, self-labeling. The protein provides a valuable way to observe individual bacteria in a biofilm with minimal to no sample preparation or disturbance.

Computer technology, image analysis techniques and video capture capability have enabled many researchers to conduct experiments that utilize time lapse microscopy to illuminate sequential events in biofilm processes (Mueller, 1996; Power and Marshall, 1988; Shapiro and Hsu, 1989; Lawrence and Caldwell,

1987a; Habash *et al.*, 1997; Dalton *et al.*, 1994). This technique has enabled various modes of "recolonization" to be visualized (Lawrence and Caldwell, 1987a), the discrimination between and quantification of cell growth and attachment in the rate of surface accumulation (Mueller *et al.*, 1992) and the rates of locomotion of bacteria on surfaces (Power and Marshall, 1988).

Confocal scanning laser microscopy (CSLM) has become the premier tool for studying biofilms in three dimensions. The major advantage of this microscope system is the ability to eliminate out of focus haze and produce very thin optical slices. When coupled with automated stages that can step in increments of sub-microns, very accurate three-dimensional images of biofilms can be created (Lawrence *et al.*, 1991). CSLM has been used to determine fractal dimensions of biofilms (Hermanowicz *et al.*, 1996). Three-dimensional imaging of morphological changes in biofilms has been used to illustrate the findings of Dalton *et. al.* (1994). Moller *et. al.* (1996) used fluorescent probes and CSLM to map the location of certain cells within a biofilm matrix. Further applications of CSLM and fluorescent probes have been reviewed by Costerton *et. al.* (1995b).

Parallel Plate Flow Cell

The parallel plate flow cell has also become an important piece of equipment for the study of biofilms. The type that is used most commonly was first described by Sjollema *et. al.* (1989) and modifications of which have been utilized by researchers at the Center for Biofilm Engineering (Camper *et al.*, 1994; Hamilton *et*

al., 1995; Davies *et al.*, 1993; Mueller, 1990; Scheuerman, 1996; Escher, 1986). The primary advantage of such a reactor system is that it allows the researcher to obtain images from biofilms with minimal physical disturbance. Real time studies of biofilm formation can also be carried out in flow cell reactors and time lapse images can be obtained. Many studies which have examined the initial attachment, detachment, and growth of bacterial cells on glass surfaces along with measurements of rates and types of movements have been conducted with a parallel plate flow cell reactor in conjunction with time-lapse microscopy (Korber *et al.*, 1994; Lawrence *et al.*, 1987b; Lawrence and Caldwell, 1987a; Lawrence *et al.*, 1989; Caldwell and Lawrence, 1986; Korber *et al.*, 1989; Lawrence *et al.*, 1991). The research presented in the following chapters was carried out using similar methods.

Organization

The following terms will be used in this report and need to be clarified in advance:

1. Primary biofilm cells: Those cells which are the first to colonize a surface. In essence they are the first generation biofilm cells and have transitioned from a planktonic environment to a sessile state.

2. Secondary biofilm cells: Any generation of cells which arise from primary biofilm cells. These cells do not undergo a change in phenotype for they originate in a sessile state.

3. Emigrate: The action of a cell moving from its previous location. Cells which emigrate do not necessarily detach and partition into the bulk fluid. On the contrary, they usually remain intimately associated with the biofilm, but travel to a new location in what is termed a recolonization event (Lawrence *et al.*, 1987b).

This report is organized in two main sections; each describing a portion of the total research project. Both parts are a contribution to the understanding of biofilm development. Chapter 2 presents the more qualitative exposition of the effects of a change in phenotype upon primary biofilm cells and Chapter 3 is a quantitative description of the behavior of secondary biofilm cells. Two strains of the same organism, *Pseudomonas aeruginosa* PA01, were used in these experiments and are described below in more detail. The experiments that dealt with planktonic and primary biofilm cells used only the GFP+ strain, whereas the secondary biofilm experiments utilized at mix of GFP+ and GFP- strains; with 1% of the cells being GFP+. The content of each of these sections will be condensed and submitted as manuscripts to be published at a later date.

CHAPTER 2

APPARENT SURFACE ASSOCIATED LAG TIME OF PRIMARY BIOFILM CELLS

Background

Biofilms are complex communities of microorganisms pervasive in the natural environment. The current conceptual model of a biofilm portrays an ingeniously complicated multi-species entity where ecological microniches are created and occupied by specific organisms (Costerton *et al.*, 1995b; Costerton, 1995). Bacteria are known to attach to both abiotic materials and other organisms in all manner of aqueous environments. Following attachment of the primary colonizing bacteria, growth of the sessile population and induction of new organisms into the community occurs. The resulting biofilm is an intricate assortment of micro- and macroorganisms (Fletcher, 1996) which can be responsible for many types of human infections, the corrosion of metals, the reduced efficiency of industrial heat dissipation equipment, and the degradation of waste materials (Costerton *et al.*, 1995b).

Because of these pertinent biofilm effects, researchers are interested in knowing how these communities form. The term used to describe the first stages

of biofilm development is "initial events" and has been thoroughly studied but is not well understood. Surface topography can affect the manner in which bacterial cells colonize a substratum (Camper *et al.*, 1994; Scheuerman *et al.*, 1998). Cells which first colonize a particular surface become the parent organisms of the ensuing biofilm. The behavior exhibited by these cells is thought to vary according to species (Lawrence and Caldwell, 1987a). Parent cells can form dense microcolonies and then disperse in a matter of minutes (O'Toole and Kolter, 1998a). Upon replication, daughter cells can pack tightly near the parent cell location, spread out upon the surface, be cast back into the bulk fluid, form long chains of cells, or roll along the substratum (Lawrence and Caldwell, 1987a; Korber *et al.*, 1989; Power and Marshall, 1988; Shapiro and Hsu, 1989; Dalton *et al.*, 1994).

Prior Research

Initial Events Description. One of the most useful results from research regarding initial events in biofilm formation is a conceptual model of biofilm accumulation. The framework for this model was first established by Escher in 1986. When a "clean" substratum first comes in contact with an aqueous environment, a layer of organic molecules is almost instantaneously formed on the surface. This layer is known as a conditioning film and may have a substantial

influence on the subsequent biofilm as many surface bound nutrients can be present in this film (Chamberlain, 1992; Marshall, 1996).

Bacterial cells are then transported from the bulk solution to the substratum. This can also be a rapid process with measurable attachment within as little as 30 seconds (Vanhaecke *et al.*, 1990). Several factors, including flow regime, motility and relative concentration of cells in the bulk fluid influence the rate at which cells are transported to the solid-liquid interface. Once organisms have come into contact with a substratum, there is a two-stage adsorption process: reversible adsorption followed by irreversible adsorption. Reversible adsorption is a weak bonding of the cell to the surface which is facilitated by one or a combination of the following: van der Waals forces, electrostatic and steric interactions, and polymer bridging. The time span of reversible adsorption is on the order of minutes. During this time cells may desorb from the surface and partition back into the bulk solution (Escher and Charaklis, 1990). Desorption of cells may also be a fairly rapid process. In an experiment that measured attachment and detachment during the first 120 minutes of biofilm formation, 60-80% of detaching cells had a surface residence time of less than 10 minutes (Wiencek and Fletcher, 1999).

Irreversible adsorption is a more permanent association of a cell with the substratum and is accomplished by the production of extracellular polymers. Net accumulation of a biofilm involves growth of the population of irreversibly attached cells, and the detachment of surface bound organisms either via erosion, sloughing or abrasion (Charaklis, 1990). Detachment events have usually been

thought of as mechanical failures of the biofilm matrix due to an applied shear force, but recent research has shown that these events may be initiated by enzymatic changes brought about by certain segments of the biofilm population as a response to environmental conditions (Davies *et al.*, 1998)

The processes involved in the initial events of biofilm formation, described above, have been succinctly summarized by (Mueller *et al.*, 1992):

1. Conditioning of the substratum by organic molecules
2. Transport of cells from the bulk water to the solid liquid interface
3. Adsorption of cells on the substratum
4. Transformation of irreversibly adsorbed cells to reversibly adsorbed cells
5. Desorption of reversibly adsorbed cells from the substratum into the bulk water
6. Growth of irreversibly adsorbed cells
7. Erosion of cells from adsorbed colonies into the bulk water.

Attachment. As described previously, the creation of a conditioning layer on a "clean" substratum has been thoroughly investigated and reported (Marshall, 1996). The initial attachment of cells to a substratum is influenced by the nature of the surface to which organisms are adhering. On a topographically homogeneous, stainless steel substratum, *Pseudomonas aeruginosa* displays a completely random spatial pattern of attachment. However, for a slightly rougher substratum, this organism exhibits a degree of aggregation, thereby proving that the topography of the substratum does have an influence on the initial attachment of this organism (Camper *et al.*, 1994).

Further examination of the influence of topography on bacterial adhesion was performed by monitoring the attachment patterns of bacteria to a smooth silicon substratum in a flow cell reactor. The surface was marked by defined grooves creating a well-charactarized structural heterogeneity. Results again showed that substratum topography has a bearing upon the bacterial attachment pattern. Cells were shown to preferentially attach to regions of the surface just downstream of the grooves whereas control experiments run with bacterial sized beads did not show the same pattern (Scheuerman *et al.*, 1998).

Mueller (1996) has reported on a combination of processes in initial events of biofilm formation. Attachment, detachment and growth were observed using time-lapse microscopy and a parallel plate flow cell. Substrata of stainless steel, copper and silicon were exposed to a flowing bulk solution of *Pseudomonas aeruginosa* and nutrient media until a certain surface density was reached to monitor attachment and detachment rates. Subsequently, flow of sterile, cell free, nutrient media was initiated and growth and erosion of the attached cells was monitored. Silicon was the smoothest of the three materials and had the lowest rate of adsorption. Copper was the roughest material and showed the highest rate of irreversible attachment. However, no growth of attached cells on this surface was evident which is a result of the toxic effects of copper. Growth rates on silicon and stainless steel were found to be 0.28 hr^{-1} and 0.33 hr^{-1} respectively. This research showed that the net accumulation of cells on a surface was a result of attachment,

detachment and growth with growth being insignificant in the very early stages of biofilm development (Mueller, 1996)

Direct observation of attachment has been accomplished (Lawrence *et al.*, 1987b). In this report, the characteristic behavior of *Pseudomonas fluorescens* was described. The planktonic cells approach the surface and attach. Rotational behavior was seen for up to half an hour on the surface with rotation gradually slowing and ceasing resulting in a longitudinal irreversible attachment. Rotational behavior was also seen with detaching or emigrating cells prior to moving to a nearby location where the sequence of rotation and attachment was repeated. Following longitudinal attachment, cell division occurred resulting in two daughter cells with a clear space of 0.2 to 0.3 μm. The standard colonization and growth pattern for *P. fluorescens* is adsorption to a surface in single cells, development of 8-16 cell microcolonies, and emigration of the daughter cells to form 8-16 cell microcolonies elsewhere.

Observed differences between planktonic and biofilm cells. There is a wide range of research topics which address various aspects of attachment and subsequent growth of sessile cells. It is known that planktonic organisms are much more susceptible to antimicrobial agents than those in a biofilm (Brown and Gilbert, 1993). Organisms which partition from near starvation conditions in the bulk fluid can flourish on a surface. This behavior is explained by the greater

amount of surface associated nutrients available to sessile cells (Habash *et al.*, 1997; Kjelleberg *et al.*, 1982). These studies illustrate advantages for cells to adhere to surfaces rather than remain in the bulk environment.

One of the first efforts to address differences, or lack thereof, between sessile and planktonic cells was reported by Bakke in 1984. In this work, it was shown that *Pseudomonas aeruginosa* does not behave differently in planktonic cultures than biofilm cultures where diffusional resistance is neglectable. This conclusion allowed the kinetic and stoichiometric parameters for *P. aeruginosa* derived from planktonic cultures to describe steady-state biofilm processes (Bakke *et al.*, 1994). A contradictory finding by (Moller *et al.*, 1995) showed that the relationship between substrate concentration and growth rate for planktonic cells was not observed for sessile cells. A detailed review of surface and planktonic growth has examined several other contradictory findings where growth of a sessile population deviated from that of a planktonic population. The conclusion was that the lack of consistency is due to a variety of experimental conditions and analytical methods. More correlateable research is needed in this area (van Loosdrecht *et al.*, 1990).

Another difference between biofilm and planktonic environments is seen in the composition of a dual species biofilm. Under identical planktonic conditions, *Klebsiella pnuemoniae* has a specific growth rate double that of *Pseudomonas aeruginosa* and therefore controls the population distribution in that environment. However, when the two organisms are present in a biofilm, *K. pnuemoniae* can not

displace *P. aeruginosa* due to the manner in which each behaves in a sessile environment (Stewart *et al.*, 1997).

Phenotypic differences between planktonic and biofilm cells. It is now a widely accepted premise that bacteria undergo phenotypic changes when a transition from a planktonic state to a sessile state occurs. In essence, the switch from a planktonic type organism to a sessile one is mediated by a large number of regulated genes which are either expressed or down regulated depending upon the environment. Adhesion to a surface appears to rapidly initiate the phenotypic expression of a range of genes not used by planktonic organisms (Costerton *et al.*, 1995b; Costerton, 1995; Korber *et al.*, 1995; Costerton and Lappin-Scott, 1995a).

Evidence of this type of behavior was first presented by Dagostino et al. in 1991. Two types of marine bacteria were shown to express unknown genes upon attachment to surfaces via the use of a *lacZ* reporter system. The same organisms did not express these genes while in a planktonic environment (Dagostino *et al.*, 1991). In many cases, genes which are involved in EPS production are up regulated after an attachment event (Fletcher, 1996). Supporting research has shown that new protein synthesis is required for *Pseudomonas fluorescens* to form biofilms on abiotic surfaces. The production of these unknown proteins is part of a speculated common genetic pathway used to form biofilms on a range of abiotic surfaces (O'Toole and Kolter, 1998b).

Specific research in this area has identified a portion of the regulated genes that are being expressed. An example is the *algC* gene which is a key component in the alginate production pathway in *Pseudomonas aeruginosa.* Within the span of minutes after a planktonic cell adheres to a surface, this gene is expressed and eventually results in the abundance of alginate which is a primary constituent of the EPS produced by biofilms of this organism (Davies *et al.*, 1993).

The fact that biofilm cells are markedly different than planktonic cells is not trivial as this may help explain the increased resistance to antibiotics (Costerton *et al.*, 1995b) and other morphological changes (Dalton *et al.*, 1994).

Purpose

To initiate the development of a biofilm, bacterial cells in suspension are transported to a solid/liquid interface and adhere. Once these cells are in a sessile environment, they replicate and cover the surface to form a biofilm. The process of becoming a sessile organism may involve a sweeping number of genetic changes which causes the sessile cell to become phenotypically distinct from its planktonic counterpart (Costerton *et al.*, 1995b). In the experiments described here, *Pseudomonas aeruginosa* PA01 cells underwent this transition and there appeared to be a temporary halt in their growth and division cycle. This lag time may be a result of viable cells directing metabolic energy into other products, such as extracellular materials, rather than into replication (Fletcher, 1996).

The planktonic cells used for the experiments were cultivated in a chemostat reactor and then transferred to a parallel plate flow cell reactor for observation with CSLM. Also, inocula were placed in a batch reactor as a control. Under the conditions of these experiments, the results show that growth of primary biofilm cells is negligible during the first 12 hours following attachment, while no difference in growth is seen in planktonic cells which never became sessile. The growth of secondary biofilm cells was slower than planktonic cells but significantly faster than primary biofilm cells. Nutrient conditions for all three types of cells were held constant.

This finding is a result of direct, in situ observation of biofilm cells in a parallel plate flow cell visualized by confocal laser scanning microscopy. The purpose was to qualitatively compare the growth characteristics of cells in planktonic, primary biofilm and secondary biofilm environments.

Materials and Methods

Overview

The growth of bacteria in sessile and planktonic states was examined in this research project. The original bacterial cells which colonize a clean surface are referred to as "primary" biofilm cells and the subsequent progeny cells are termed "secondary". Both primary and secondary biofilm cells were observed using a parallel plate flow cell reactor and planktonic cells were studied in a standard batch culture. Batch culture experiments were conducted for periods of 6 and 12 hours while flow cell experiments lasted 8.5 and 12 hours.

Organism

The organism used in this study was *Pseudomonas aeruginosa* PA01. *Pseudomonas aeruginosa* is a motile, aerobic, Gram negative rod. This particular strain contained a plasmid that conveyed a resistance to 150 mg/l of carbenicillin and encoded the production of a green fluorescent protein (GFP). The GFP was constitutively expressed, thus enabling each cell to be "self labeling". The excitation wavelength of the GFP was 488-nm and an emission maxima was at 512-nm. This organism was obtained from Dr. Mike Franklin, at Montana State University.

Stock Culture

Stock cultures of the organism were prepared by streaking for isolated colonies on R2A agar (Difco) which contained 150 mg/l of carbenicillin (Sigma). Isolated colonies were then transferred to fresh R2A/carbenicilln agar and incubated until a confluent lawn was present. The culture was harvested, placed in a 2% peptone - 20% glycerol solution and stored in 2.0-ml vials at –70 C. For each experiment, approximately 1.0 ml of frozen stock culture was used as an initial inoculum.

Equipment Preparation

The equipment used in this study included media and waste containers, assorted tubing, a chemostat reactor, a parallel plate flow cell reactor, an inoculation vessel, a bubble trap, and a batch reactor. These components are illustrated in Figures 2.1 through 2.4 and described in following paragraphs.

The nutrient media for each experiment was prepared and sterilized separately from the remainder of the items as explained in the following section. The various vessels and tubing were cleaned by washing with soap and water and sterilized by autoclaving dry for 20 minutes at 121 C and 20 psi. The tubing upstream from each of the reactors was replaced for each experiment.

Media

The same nutrient media was used in the chemostat, flow cell, and batch reactor. The constituents are listed below in Table 2.1:

Table 2.1. Media Composition

Constituent	Amount (grams per liter)
K_2HPO_4	0.70
KH_2HPO_4	0.30
$(NH_4)_2SO_4$	0.10
$MgSO_4 * 7H_2O$	0.01
Glucose	0.40

The potassium salts and ammonium sulfate (Fisher) were added to glass carboys, an appropriate amount of reverse osmosis water was added and the containers were sealed and autoclaved for 15 minutes per liter. The solution was cooled to room temperature before the remaining constituents were added. Proper amounts of glucose (Fisher) and magnesium sulfate (Aldrich) were dissolved in approximately 25 ml of reverse osmosis water and added to solution via syringe and a 0.2-micron syringe filter.

Chemostat Operation

A chemostat was used to grow inocula for both the flow cell and batch culture experiments. The reactor was operated at room temperature with a volume of 500

ml and a 5-hour residence time. The culture in the reactor was well mixed by a magnetic stir bar and plate and aerated by the laboratory pressurized air system through bacterial air vents (Gelmen Sciences). A schematic diagram of the system is shown in Figure 2.1. After sterilizing all reactor components the system was assembled. The chemostat was filled with sterile nutrient media, inoculated with 1.0 ml of frozen stock culture and operated in batch mode for 24 hours. The pump was then turned on to supply media to the reactor and the effluent was pumped to a waste container. The bacteria were grown in a chemostat environment for 48 hours (approximately 10 residence times).

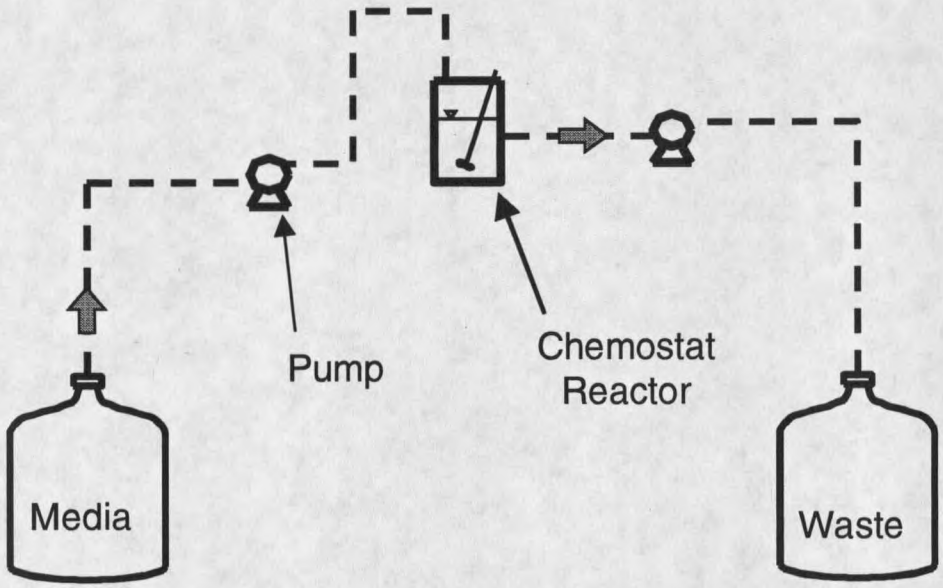

Figure 2.1. Schematic of one chemostat reactor system.

Batch Culture

A batch reactor was utilized with the media described above. 1.0 L of media was sterilized in a 2.0 L Erlenmeyer flask. An inoculum was withdrawn from a 48-hour chemostat culture and placed in the vessel. The batch culture was incubated at room temperature and well mixed using a magnetic stir bar and plate. Population measurements were made at the beginning, middle and end of a 12-hour period.

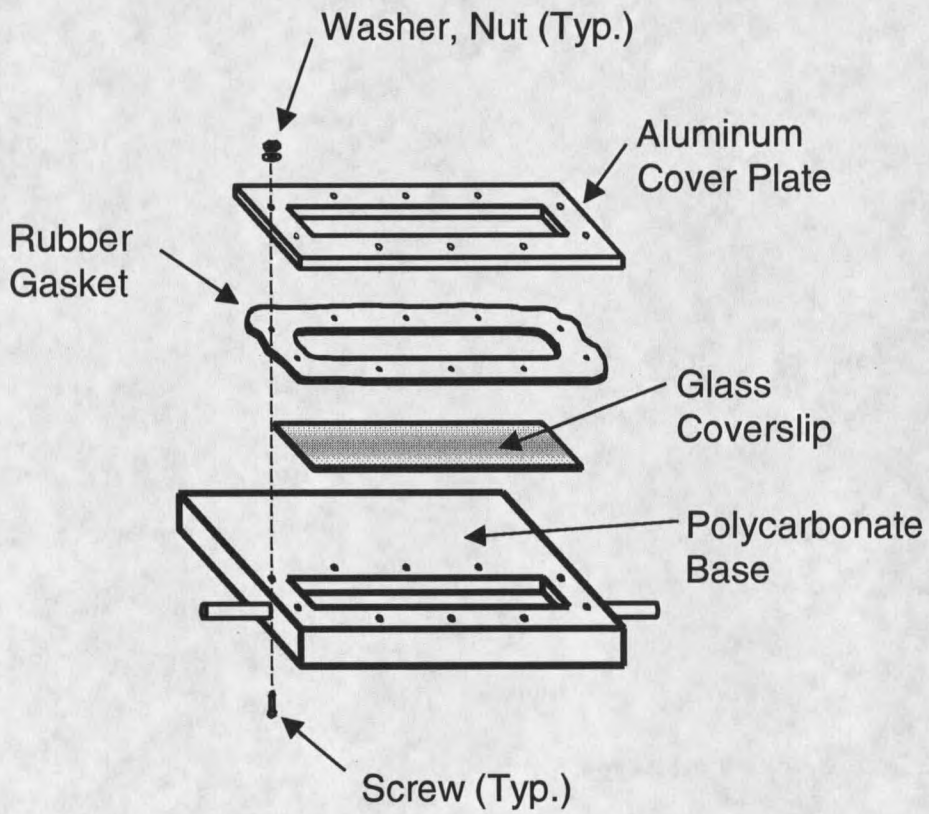

Figure 2.2. Parallel plate flow cell reactor.

Flow Cell Cleaning

Cells growing in an attached or sessile state were observed on the "wet side" of the coverglass in a parallel plate flow cell reactor (Figure 2.2). Cleaning of the flow cell prior to each experiment was carried out as follows:

The polycarbonate base was submersed in 95% ethanol for 30 minutes and then subjected to UV light for 30 minutes. Each experiment utilized a new glass coverslip that was washed twice with antibacterial soap and rinsed twice with 95% ethanol. The coverslip was then autoclaved for 20 minutes and dried at 115 C for 24 hours. Other assorted materials (tubing, coverplate, gasket, screw and nuts) were autoclaved for 20 minutes. After all parts of flow cell were sterilized or cleaned, the flow cell was assembled inside a biological hood.

Flow Cell Operation-Primary Cell Experiments

The entire flow cell reactor system (Figure 2.3) was set up on a laboratory cart to facilitate transportation to and from the microscope lab where the experiments were completed.

Prior to inoculation, the flow cell and all upstream tubing were filled with sterile media to purge air from the system. Approximately 15 ml of inoculum were withdrawn from a 48 hour chemostat culture and placed into the inoculation vessel, upstream of the flow cell. The inoculum was rapidly mixed using a magnetic stir bar and plate. A portion of the stirred inoculum was pumped through the flow cell