Study of *Ralstonia pickettii* Biofilm Structure Formed in
A Flow Cell

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by
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Goals

The goals of this study is to understand the process of the biofilm establishment, to observe how cells are accumulated, and the structural difference from time to time by using a phase contract microscope and staining techniques.
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I. Introduction

A biofilm is a naturally occurring aggregation of microorganisms attaching to a surface in the aqueous environment. It is known for its slimy and high persistence. As it was first described by Van Leeuwenhoek as irreversibly associated with a surface, biofilm is a matrix of primarily polysaccharide material enclosed with bacteria that cannot be simply removed by gentle rinsing. As a population of microorganisms, biofilm is a role model for studies of biofilm surface architecture, genetic expression, microbial-microbial interaction, cell-cell communication, as well as toxicity resistance.
1.1 Significance of Biofilm

Environmental

As a mechanism for microorganisms to survive in the environment, biofilm can be established both inter- and intra species. Once it is exposed to a surface with sufficient moisture and nutrient, biofilm will be universally formed. The immobilization of the cells creates a polysaccharide layer coated protection from the harmful environment as well as providing the optimal growth condition for survival of the microorganism. This specific ability has been applied to in wastewater treatment plants and in situ bioremediation on toxic pollutants. The highly packed microorganisms trap or utilize the nutrients, chemicals, even heavy metal compounds in the liquid passing by. Compare to the traditional pump and treat process, the tremendous benefits come with the relative low operation costs, high efficiency, as well as less chances for secondary contamination. A recent approach of utilizing biofilm is to use it as a biobarrier on land to protect soil or groundwater from hazardous contaminants.

Human Health

The high resistance and survival potential of microorganisms within a biofilm has also cost many serious human health problems. From simple gunk on human tooth to serious Cystic Fibrosis respiratory illness, biofilm plays the primary role, which contributes to the difficulties of removal and curing. The finding of biofilm formation in water purification system and other medical devices such as urinary catheters, hemodialysis equipment has raised the great concern of human health issue. According to the Center for Disease Control and Prevention, an estimation of two-third of the physician encountered bacterial infections are in relation to biofilm.

Economical

Besides the concerns of health have been raised, biofilm has been problematic from the standing point of economics as well. The formation of biofilms makes sterilization process harder in food industry. Microorganisms within biofilms are more persistent to disinfectants, cleaning detergents, etc. The corrosion caused by attachment of the
biofilms proposes another big issue in industry and economics. Billions of dollars has been spent on removal of the biofilm on equipment surface. Even in fuel utilizing field, biofilm can be problematic too. For example, the hulls of ships are prone to form biofilms. The biofilm layer on the surface of hulls will increase the friction between the ship and water. Therefore to run a ship as fast as before biofilm was formed on the hulls, more fuels have to be used to supply the energy.

Biofilm can be both beneficial and harmful to human society. Detailed studies have to be done to take the advantages of biofilm as well as minimization of its damages.
1.2 How is biofilm formed?

Unlike the planktonic cells we have always observed, biofilm is a collection of microbe populations firmly packed together. Depends on the surface materials for attachment, the complexity of a biofilm can vary. Although the architecture of a biofilm can be very different from one organism or another and from one environment to another, the process and necessary components are the same. See Fig. 1.

In order to form biofilm, planktonic cells have to be immobilized first. In order for the single-celled organism to exhibit the mode of attached state, a quorum sensing mechanism has to be activated within the cell. With the switching of the mode, the cell undergoes a phenotypic shift where genes will be up- and down- regulated to suite the biofilm formation condition. These initial adherences is held by a weak and reversible force, commonly known as van der Waals forces. To further stabilize the adhesion between the cells and surface, other elements such as pili, extracellular polysaccharide, etc. Once the first colonization has established, more cells will anchor themselves to the adhesion matrix. Biofilm will then be formed.

1.3 Single Organism Biofilm Formation in A Flow Cell: Theory

A constant flow is supplied in a flow cell. Innoculants will grow within this space when the nutrients, surface, and flow rate are allowed. In order to accumulate cells, flow rate has to be smaller than growth rate.
II. Experimental Setup

Fig. 2

The flow cell is placed under a phase contrast light microscope. Nutrients will be pumped through by a peristaltic pump. It is designed at a simple flow without recycling.
2.1 Materials

2.1.1 Flow cell

The FC 71 flow cell was purchased from Biosurface Technology Corporation (BST). It is constructed as a small chamber with 2 coupons for biofilm attachment.

Fig. 3

“The FC 71 Flow Cell is a flat plate flow cell designed to accommodate coupons of various materials to study biofilm formation and response to these materials.”

The surface-adherence property gives biofilms the ability to grow on almost every surface when growth condition is thriving, including medical devices and water purification system, etc. Therefore, the surface area plays an important role in bacterial attachment, which results in biofilm formation. The flow cell came with polycarbonate coupons, which supply a good surface area for biofilm formation. Coupons made by other materials are also available at BST. It must be considered that the processes and structures of the biofilm will be different respect to different materials.

2.1.2 Inoculants

*Ralstonia pickettii*, Gram negative bacterium obtained from ROME lab (Dr. Terry Marsh) was used to inoculate the flow cell.
In this study, single microorganism *Ralstonia pickettii* was grown in the flow cell with 1/3 TSB (Tryptic Soy Broth) then spike with cupric sulfate in a final concentration of 200 μg/mL. *Ralstonia pickettii* is a Gram negative rod shaped bacterium. It was also known as *Pseudomonas (Burkholderia) pickettii*, can be isolated from a wide range of environment, from human upper respiratory system to Super-fund site. It is a known for its capability to form biofilm in highly purified water system and copper accumulation (*Adley et. al.*). To study the biofilm structural shifts before and after copper compound was added, a flow cell system was located under phase contract microscope for a ten day’s observation (*Konstantinidis et. al.*).

### 2.1.3 Pump and inoculating tools

To manager the flow rate, Watson-Marlow’s 101U peristaltic pump was applied directly with Master Flow tubing. It has to be calibrated before using.

Sterile syringes and needles: B.D. syringes: two 60ml and one 5ml.

B.D. needles: 22G1/2 preferred.

### 2.1.4 Microscopes

Olympus Phase-contract BH2-RFCA microscope. The microscope was connected to a television set and a VHS recorder. The establishing of *Ralstonia pickettii* biofilm within the flow cell was recorded.

And Nikon Eclipse E600 assembled with Cannon DS6031 digital camera for higher resolution picture taken.
Both camera operates similarly. The only difference is the camera and video adaptors. Both of them have mercury lights for fluorescence purpose.

2.1.5 Medium

Tryptic Soy Broth (TSB). The formula of mixing is listed on the tag (30g in 1 L of water). Only 1/3 of the concentration is needed. The bacterium strain is environmental, the high concentration of TSB would inhibit the growth of environmental microorganisms. Thus 1/3 TSB was preferred to full strength of TSB.

1000mL Sterile Water For Injection made by Abbott Laboratory was used as medium reservoir. The water bag was connected to the flow cell by a sterile Microdrip Infusion Set, made by Ventrex Laboratories Inc. Detail see Fig. 5.

Fig. 5* The two parts are connected by poking the Microdrip into the bottom of the water bag.
2.1.6 Stains

Many types of stains can be used in order to distinguish cells from background. However, cells within the flow cell have to be kept alive. Thus, any chemicals that can kill the cells cannot be applied, including methylene blue in simple stain, crystal violet in Gram stain.

A good stain for cell extracellular capsules is Indian ink, which gives a darker background to contrast with the brighter capsules. Another one is BacLight Live/Dead stain, which tests the cell viability.
2.2 Assembling

2.2.1 Cleansing

1. Put all coupons in a small beaker. Immerse all in de-ionized water. Pour lab soup in to obtain a final concentration of approximately 1:10. Sonic for 10 minutes.

2. Repeat the same process for all other parts, including screws and tubing. Due to the size of the parts, microwave for 20 minutes is recommended instead of sonic.

3. Acid bath all non-metal parts except tubing for at least 3 hours in 1% HCl solution.

4. Pump 1% HCl solution through tubing several times then pump clean de-ionized water through for rinsing.

5. Reassemble the flow cell then autoclave for 20 minutes (see assembling part). Wrap all open ends with aluminum foil. Also cover the flow cell with a piece of aluminum foil. Put everything in an aluminum foil tray for autoclaving. The autoclave in the 3357 Engineering Building was used. For operation details please contact Joseph Nguyen. After autoclaving, the tray was quickly removed and put into oven to bake at 50 degree Celsius over night for ridding all moisture.

2.2.2 Flow cell

Stable flow cell on the bottom plate by screws. Locate the two coupons in the coupon holding spots. A thin layer of leakage preventing grease was applied surrounding the edge of the flow cell. Cover slip was then carefully positioned right on top of the coupons. Attach the gasket and push lightly on the edge where flow cell and gasket meets as well as the edge where cover slip and gasket meets. Do not break the cover slip.
Last put the top plate on top of the gasket. Screw on the complete the flow cell
assembling. To prevent leakage and efficiently tighten the screws, all screws were firstly
screwed on lightly. Then tightened as pairs located opposite to each other.

In order to connect the Master Flow tubing to the flow cell, one 1/5 inch Tygon
tubing was used as connector at the inflow end. The outflow end was attached with
another 1/5 inch Tygon tubing, which was directed to the waste tank.

Fig. 4

2.2.3 Media Preparation

A 100mL 3X TSB was prepared in a 150mL serum bottle (washed and acid bathed).
The bottle was sealed with a black stopper and aluminum crimp top. It was then put in a
tray with bottle covered with water to latent the temperature changes during autoclaving.
The media stock was then autoclaved as liquid for 15-20 minutes (Sterilization time, not
including heating and cooling time. The complete autoclaving process takes
approximately 1 hour.) This nutrient stock will be added into the water bag after
everything is completely assembled. Detail see procedure.
2.2.4 Final Assembling

Gloves were worn to prevent contamination. The system was constructed with a commercially sold sterile water bag, a sterile tubing line can be found in any nursing catalog. A peristaltic pump was added to pump the media through at a velocity of 5ml/3hr. Using a 50mL syringe inject the 100mL 3X TSB into the sterile water bag after letting out approximately 100mL of sterile water to obtain a final 1/3 TSB concentration. Ethanol sterilization at the rubber insertion points were also used to prevent unwanted contamination. The flow cell was then stabilized on the stage of the microscope.

Fig. 5
III. EXPERIMENTAL PROCEDURES

3.1 Procedures

1. Grow a fresh 5mL *Ralstonia pickettii* culture in 1/3 TSB overnight (12-16 hours) at 30 degree Celsius shaker.

2. Assemble all parts together as described in the final assembling.

3. Use a graduate cylinder collect at the waste end approximately 100 mL sterile water flowed out of the water bag. (Note: everything so far are still sterile. Be careful do not touch any open ends.) This process can be achieved by using the pump or gravity flow.

4. Stop the pump or clamp the tubing when 100 mL of water is collected

5. Ethanol washing the nutrient stock (3X TSB) stopper. Then flame it to further sterilize it and keep air around hot to prevent contamination. Put together a 60mL syringe and 22G1 ½ needle and stab into the nutrient stock. Withdraw 60mL. Meanwhile, ethanol sterilize (no flaming) the injection rubber dot on the water bag. Inject the 60mL of 3X TSB into the water bag. Ethanol sterilize the rubber. Repeat with another sterile syringe and needle until 100mL of 3X TSB is injected into the water bag. (Note: because the serum bottle is sealed, do push the same amount of air in first before withdrawing. The small gage of the needle will help keep the rubber injection end sealing.) The final concentration of the medium is 1/3X TSB.
6. Adjust the flow rate by changing the numbers on the pump. (5mL/3 hr-5mL/hr)

7. Allow the nutrient to fill the flow cell chamber. Then stop the pump and clamp the microdrip.

8. Use a 5mL syringe with 22G1½ needle to withdraw all *R. pickettii* culture. Inject all from the injection site into the chamber. Ethanol sterilize the injection site before and after.

9. Look under the microscope and make sure cells are observed. Run the pump to adjust the amount of cells in the tubing and the chamber. Try to maximize the amount of cells in the chamber while minimizing the amount of cells in the tubing (between the injection end and the flow cell).

10. Stop the pump and clamp the tubing. Incubate the inoculated chamber at room temperature for 12-18 hours.

11. After a 12-18 hour incubation, start the pump and unclamp the tubing at a constant flow rate. Observe the flow cell under the microscope daily.

12. Take pictures with/without stains.
3.2 Picture Acquisition

The details on picture taking are presented below.

* The pictures were taken by using ZoomBrowser EX, a picture editing software for Cannon digital camera.

1. Double click ZoomBrowser EX to open the file (Fig. 7).

Fig. 7
2. On the left side of the panel, there is a choice of Camera & Memory Card (Fig. 8).
3. By clicking on Camera & Memory Card, the panel shows the option below the choice, including Remote Shooting. This is located as the last choice on this panel (Fig. 9).

Fig. 9
4. Click the Remote Shooting, 2 smaller windows will pop out (Fig. 100). Turn on the camera, then click “Connect” on the small window says “Connect to Camera”?

Fig. 10
5. After the camera is connected, a small window labeled as Shooting-RemoteCapture will appear. The Release button is used to take pictures (Fig. 11).

Fig. 11
6. When the object on the microscope is in focus, blacken out the eye piece lenses. Click Release to take the picture (Fig. 12).

Fig. 12
7. The taken picture will appear on the other small window labeled “Save-RemoteCapture”. Pictures can then be viewed or deleted (Fig. 13).

Fig. 13
IV APPENDIXES

4.1 Experimental results

A volume of 1ml approximately $10^{10}$ *Ralstonia pickettii* culture was inoculated at the inoculation site on the tubing. Cells were then flushed into the chamber and incubated at room temperature for 26 hours before the flow started. Cell accumulations were expected to be observed in 48 hours. However, few rod shaped materials were seen under the 100X magnification lens. Indian ink was applied to stain the bacteria capsule for clearer resolution. The rough background image gave a much stronger signal, which made it even harder to distinguish cells from the coupon surface. After another 36 hours running, increasing amount of the cell debris was observed. I have also observed an interesting clump of object. It was in yellowish orange color formed by rod shaped materials, which was very likely to be cells. This could also be the indication of biofilm formation. However, to ensure the cell growth, another 5mL of *Ralstonia pickettii* overnight culture was injected by using a Harvard Syringe Pump 11 at a rate of 0.5mL/hr. Twelve hours later, there were observably more flowing cells in the chamber. Also, cell clustering was also seen on the surface of the coupon where there were “valleys”. The effluent cell density was determined as 2.64E10 CFU/mL.

All of the images were captured on the VHS tape. However, the resolution appeared to be low, which cannot be re-exported onto a computer for further process.

The structural formation was only seen on the coupon that was close to the influent end. On the surface of the coupon which was next to the outflow end less attached cells were observed.
Therefore, the set up was moved on to Nikon Eclipse E600 phase contract microscope assembled with Cannon DS6031 digital camera (Fig. 5) 3 days after the 2\textsuperscript{nd} inoculation.

Several set of pictures of the flow cell coupon surface were taken with common light, however the turn outs were not convincing (Fig. 14).

Fig. 14

10X magnification. Coupon surface. 3 days after 2\textsuperscript{nd} inoculation.

100X magnification. Coupon surface. 3 days after 2\textsuperscript{nd} inoculation.

Cell debris and cells
To obtain a more definite result, I applied BacLight Live/Dead stain. It is a fluorescing stain tells whether the cells are viable. The stain contains two different fluorescing stains, one is SYTO 9 green-fluorescent nucleic acid, the other one is red-fluorescent nucleic acid, propidium iodine. The SYTO 9 green-fluorescent nucleic acid stains viable and non-viable cells by penetrating both intact and damaged cell membrane. However, propidium iodine stains cells with damaged cell membrane only, which causes a reduction of green fluorescing in the nonviable cells. Thus, if a cell appears to be red, it is dead. If a cell appears to be green, it indicates the cell is still alive. Before stain was added, it was observed the coupon itself did not fluorescing. Please see protocol for more detail.

Fig. 15 10X magnification. Edge of the coupon after Live/Dead Stain.
Fig. 16 Top: 10X magnification coupon surface after applying live/dead stain. Bottom: 100X mag. Coupon surface after applying live/dead stain. Both were taken 4 days after second inoculation.

* All green rods are cells in the lower picture.
Fig. 17 Top: 100X magnification coupon surface after applying live/dead stain. Bottom: 100X mag. Coupon surface after applying live/dead stain. Both were taken 4 days after second inoculation.

* All pictures at 100X magnification have a blurriness.
Fig. 17 Top: 100X magnification coupon surface after applying live/dead stain. Bottom: 100X mag. Coupon surface after applying live/dead stain. Both were taken 5 days after second inoculation.

* More dead cells appeared. The blurriness increased and so did the fluorescence.
Fig. 18 Top: 10X the coupon surface on the influent site.
Bottom: 10X the coupon surface on the effluent site.
Both were taken 5 days after 2nd inoculation.

* The coupon surface on the effluent site appeared to be much more regular than the the one that was on the influent site. Also less cells were detected on the effluent site coupon surface.
4.2 Experimental conclusions

According to the observation results, it can be concluded that the cell attachment was particularly important. It was very possible during the process of flushing the cells into the chamber also flushed many cells out of the chamber. The results have seen after the second inoculation may prove the possibility. The increasing amount of cell debris was an indication of increasing cell activity. Also, the clustering pattern of the cells have shown that the attachment of the cells starts at relatively flat area. The surrounding rising areas were necessary to slow down the local flow rate for cells to settle.

There are many possibilities can be concluded from the results. As it was mentioned in the results that there was an increasing of blurriness as time proceeded. This may be explained by the biofilm bulking with the increasing amount of the extracellular polysaccharides being produced, which caused a focus difference on the phase contract microscope.

Another explanation is that the Live/Dead stain reacted with poly-carbonate coupon, causing the background fluorescing and interfere with my final reading.
From the results, I am able to conclude that cell attachment and accumulation was occurring and the structure did change from time to time. This is a good indication of biofilm formation.

### 4.3 Future work

- Addition of negative control for comparison to limit the background noise and determine whether Live/Dead stain is a good choice.
- Addition of cupric sulfate to determine whether there is a structural shift
  - Comparison between 2 flow cells: set up two exactly same flow cells at the same time with exactly same conditions except one is fed with 1/3 TSB and the other one is fed with 1/3 TSB + cupric sulfate. A different rate of biofilm formation is expected due to the toxicity of cupric sulfate. As a result, a different architecture of the biofilms shall be observed as well.
  - And establish biofilm first then adding cupric sulfate: when a biofilm is established, the structural shift would happen due to the flow but not significant enough. However, if cupric sulfate is added. It is significant to see whether the structural shift is great or not. If there is a significant shift of the biofilm architecture, it means the toxic impact of cupric sulfate is great. If the structure does not change much or changed then the biofilm is restored, it means by forming biofilm itself is an advantage for cells to survive in a toxic environment.
CMEIAS modification: Center for Microbial Ecology Image Analysis System.

Invented by Dr. Frank Dazzo at Michigan State University. It is a great tool for image editing.

4.4 Live/Dead Baclight Bacterial Viability Kits

See attached pages 1-8.
V. REFERENCES


Adley C.C. and Saieb FM. **Biofilm formation in high purity water: Ralstonia pickettii a Special Case for Analysis.** 2005 *Ultrapure Water Journal* Jan/Feb 14-17.


Anneberg/CPB  
http://www.learner.org/channel/courses/biology/index.html

Center for Biofilm Engineering, Montana State University  
http://centerforgenomicsciences.org/research/images/bio_01_large.jpg  
http://www.erc.montana.edu/CBEssentials-SW/bf-basics-99/bbasics-02.htm

MicroMem Analytical  
http://www.micromemanalytical.com/bacAA/bactAA.htm

Wikipedia  
http://en.wikipedia.org/wiki/Biofilm

Hermann Eberl, Laurent Demaret, Antonjia Duvnjak, Messoud Efendiev. **Biofilm Modeling.**  
http://ibb.gsf.de/homepage/laurent.demaret/biofilm_poster.pdf

J. B. Xavier, A. M. Reis, A. Schnell, S. Wuertz, E. S. Gilbert, S. E. Cowan, J. D. Keasling, D. C. White, J. S. Almeida. **Quantification of Biofilm Morphology.**  