INVESTIGATING THE EFFECT OF SURFACTIN ON MEMBRANE POTENTIAL OF *BACILLUS SUBTILIS* BIOFILM

By

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To my dear parents, my friends and everyone who helped me in my life

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ABC	ATP-binding cassette
APG-4	Asante Potassium Green-4
ATCC	American Type Culture Collection
CF	Cystic fibrosis
$DiSC_3(5)$	3,3’-dipropylthiadicarbocyanine iodide
ECM	Extracellular matrix
EPS	Extracellular polysaccharides
MEA	Microelectrode arrays
MRSA	Methicillin-resistant *Staphylococcus aureus*
ThT	Thioflavin T
WT	Wild type

Abstract of Thesis Presented to the Graduate School
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Major: Biochemistry and Molecular Biology

Forming biofilms is very common for most bacterial cells. A recent study first reported that membrane potential of *Bacillus subtilis* biofilm had oscillation behavior. This study also indicated biofilm formation is related to the membrane potential.

Surfactin is a quorum sensing factor produced by *Bacillus subtilis.* It was reported to be very important in *Bacillus subtilis* biofilm formation and can cause potassium leakage across the lipid bilayer membrane. Surfactin has the potential to link the membrane potential with the biofilm formation.

To further investigate the relationship between the membrane potential and the biofilm formation, surfactin was chosen to test the effect on the membrane potential of *Bacillus subtilis* biofilm.

To detect the change of the membrane potential, we chose to use established voltage-sensitive fluorescent dyes, ThT and $DiSC_3(5)$ to represent the electrical potential of the inner membrane.

In this project, we demonstrated that surfactin could make the membrane potential of *Bacillus subtilis* biofilm more negative by causing the leakage of potassium.

Moreover, the effect of surfactin on the membrane potential was shown to be concentration related. Biofilm formation experiments of WT and the srfAA mutant further indicated that surfactin might have a dual function in *Bacillus subtilis* biofilm formation.

CHAPTER 1
INTRODUCTION

1.1 *Bacillus subtilis* Biofilm

1.1.1 Definition of Biofilm & ECM

A biofilm is a bacterial community that is enclosed by an extracellular matrix and can adhere to surfaces (Costerton et al., 1995). Forming biofilm, and associating to surface and interface is very common for the most bacterial cells in nature. *Bacillus subtilis, Pseudomonas aeruginosa,* and *Staphylococcus aureus* are good examples (Vlamakis et al., 2013; Klausen et al., 2003; Singhal et al., 2011;). Biofilm requires the production of extracellular matrix (ECM) for formation and maintenance of structured multicellular communities (López et al., 2010). The ECM, also called as extracellular polysaccharides(EPS), is produced by the cells, and it shows significant ability in regulating gene expression and coordinating cell behavior in bacterial biofilms (Steinberg et al., 2015).

1.1.2 Functions of Biofilm

By forming biofilms, bacteria have the ability to adhere to almost every surface (López et al., 2010). Moreover, forming biofilms offers other benefits for bacteria. One of the most significant biofilm benefits is that biofilm can provide protection for bacterial cells from the environment. For example, the *P.aeruginosa* biofilm can dilute some antibiotics, and the ECM of *P.aeruginosa* biofilm has the ability to trap antimicrobial agents (Mah et al., 2001; Anderson et al., 2008). Biofilms can also benefit cells by protecting them from short-term severe environments, such as pH shifts, shear forces, and dehydration. Biofilms have even been found on radiation sources (Flemming et al., 1993; Lessel et al. 1975). Besides the protection benefits, biofilms can help bacterial

cells in exchanging nutrients and removing potentially toxic metabolites as well (Davey et al., 2000). Most bacterial cells are likely to form biofilms in natural settings based on these benefits.

1.1.3 Benefits and Problems Associated with Biofilm

Antibiotics have been one of the most important treatments for fighting infectious disease caused by bacterial cells. The bacteria, then, have two ways to deal with antibiotics. First, bacterial cells can contain antibiotic resistant genes and thus have antibiotic resistance. MRSA is a very good example of this situation. MRSA contains multiple antibiotic resistant genes and survives most known antibiotic treatment (Fitzgerald et al., 2001). Second, bacteria can survive from antibiotics by residing within biofilms. Bacteria growing within a biofilm can have up to 1,000-fold resistance to antibiotics compared to the same species grown planktonically (Gilbert et al., 1997). For example, *P.aeruginosa* biofilm has much higher tolerance to antibiotics than planktonic *P.aeruginosa* (Mah et al., 2001). This antibiotic resistance makes it much harder for people to cure diseases associated with biofilms. It is important to note that biofilms can also benefit human beings. *Bacillus subtilis* biofilm is a well-known example. *Bacillus subtilis* biofilm can promote plant growth and protect plants from an extensive collection of pathogens (Arkhipova et al., 2005; Choudhary et al., 2009). *Bacillus subtilis* is widely used as a biofertilizer to promote the growth of crops (Lucy et al., 2004).

1.1.4 Components and Structure of *Bacillus subtilis* Biofilm

Biofilm is composed of water, cells, extracellular matrix, and some DNA and RNA from lysed cells. Water is the main component, and it usually makes up over 90%, or up to 97% (Schmitt et al., 1999; Zhang et al., 1998). As for dry weight, the largest component is the cells. The extracellular matrix composes almost the rest of the biofilm.

In the bacteria biofilm, exopolysaccharides, proteins and nucleic acids are the most extensively studied components of the extracellular matrix (Branda et al., 2005). Exopolysaccharides in biofilm ECM were thought to impact bacterial virulence and promote capsule formation. The exopolysaccharides are made up of glucose, galactose, and N-acetylgalactosamine. In the *Bacillus subtilis* biofilm, the *eps*A-O operon is in charge of producing the exopolysaccharides (Chai et al., 2012). For proteinaceous components, amyloid fibers are mostly found to be the primary member in both Gram-positive and Gram-negative bacteria (Steinberg et al., 2015). In the *Bacillus subtilis* biofilm ECM, TasA amyloid fibers are the best-characterized functional amyloid fibers, and these amyloid fibers are attached to the cell wall (Branda et al., 2006; Chai et al., 2013). TasA amyloid fibers can provide structural integrity and work together with other ECM components to intermediate adhesion between cells (Romero et al., 2010). Another relevant biofilm ECM component is extracellular genomic DNA (eDNA). It has been found in many different bacterial biofilms and is very important for the young biofilm structure. eDNA shows important functions in regulating the cell surface performance, promoting cell adhesion and interaction with other biofilm ECM components (Okshevsky et al., 2015a; Okshevsky et al., 2015b; Das et al., 2013). eDNA, which is not correlated with cell lysis in biofilm, is also produced by *Bacillus subtilis*, although it is only present about 0.1 µg/mL (Lorenz et al., 1991; Zafra et al., 2012).

1.1.5 Metabolic Oscillation within Biofilm

Researchers in 2015 found a very interesting phenomenon called metabolic oscillation during *Bacillus subtilis* biofilm formation within a microfluidic device. The device is continuously flowing with media. The oscillation in biofilm growth happened

spontaneously and had a relatively stable rhythm (Liu et al., 2015). The mechanism behind this behavior of *Bacillus subtilis* biofilm is still unknown. This novel discovery may lead us to understand the intracellular metabolic activity within the biofilms and find a new way to control the development of biofilms.

1.2 Communication within Biofilm

1.2.1 Quorum Sensing System

Bacteria cells are not only living individually, but also in bacterial communities. They need to communicate within the single-species community or even among different species. Bacterial cells have many ways to communicate with each other, and quorum sensing is one of the most important ones. Quorum sensing has been studied less than 40 years. It was first discovered and reported in a luminous marine bacteria *Vibrio fischeri* in 1979 (Nealson et al., 1979). Quorum sensing is the chemical communication that requires small chemical molecules called autoinducers. Autoinducers are produced and released by cells and detected by corresponding proteins in cells. These proteins can respond to autoinducers, and activate or inactivate downstream events (Waters et al., 2005). Quorum sensing system widely exists in both Gram-negative bacteria and Gram-positive bacteria, although the mechanisms may have some differences. Many bacteria have more than one quorum sensing system.

For the quorum sensing system in the Gram-positive bacteria, peptides are chosen as the autoinducers for quorum sensing. The ATP-binding cassette (ABC) transporters in the Gram-positive bacteria secrete these peptides. The two component adaptive response proteins of the Gram-positive bacteria are used to detect these peptides and affect downstream activation or deactivation. A membrane sensor and a cognate response downstream regulator compose the two component adaptive

response proteins. The membrane sensor, usually a kinase, can detect the extracellular concentration of the autoinducers. When the concentration reaches a certain level, the membrane sensor will phosphorylate the cognate response downstream regulator. The regulator can further control the downstream activities (Kleerebezem et al., 1997; Hoch et al., 1995; Lazazzera et al., 1998).

1.2.2 Electrical Signaling in Bacteria

Electrical signaling is very common in nature; the nervous system is the best-known example. The nervous system can transport orders or stimulus fast and precisely via electrical signaling, and this is very important for animals. When it comes to bacteria, however, the electrical signaling has long been neglected compared to chemical signaling. Since electrical signaling in bacteria is not well studied, there is little research on the subject. Researchers have expressed and identified a voltage-gated sodium channel in *Bacillus halodurans* called NaChBac. NaChBac is a transmembrane protein which contains one six-transmembrane segment. NaChBac is activated by voltage, and it will be obstructed by calcium channel blockers (Ren et al., 2001). These characteristics enable it to serve in electrical signaling though further study is required. Still, there are also some examples directly showing the signaling function of the ion channel. In 2015, researchers reported a bacterial potassium channel YugO enables electrical communication in *Bacillus subtilis* biofilm (Prindle et al., 2015). This will be discussed below.

1.2.3 Membrane Potential Oscillation within *Bacillus subtilis* Biofilm

In 2015, a group of researchers reported that when they measured the thioflavin T (ThT) fluorescence quantitatively, they surprisingly found the fluorescence within the *Bacillus subtilis* biofilm showed oscillation behavior. This oscillation happened

spontaneously, and the whole biofilm edge showed oscillation at the same time and rate, as presented in Figure 1-1 (Prindle et al., 2015). They called this behavior a biofilm membrane potential oscillation. To further investigate the membrane potential oscillation, they used extracellular fluorescence dye APG-4 to report the potassium concentration in the media. This revealed potassium release. The potassium release led them to investigate the potassium channel YugO. The YugO is the only experimentally described potassium channel in *Bacillus subtilis.* It contains an intracellular TrkA domain, which is in charge of gating the potassium flux. Moreover, the YugO potassium channel is necessary for *Bacillus subtilis* biofilm formation (Lundberg et al., 2013; Cao et al. 2013). With several tests and comparison with *yugO* deletion mutant and *trkA* deletion mutant, the researchers successfully demonstrated the YugO channel gating could promote electrical communication within the *Bacillus subtilis* biofilm (Prindle et al., 2015).

During *Bacillus subtilis* biofilm formation, the growth rate also showed oscillation. This is called metabolic oscillation. The metabolic oscillation in *Bacillus subtilis* biofilm also happened spontaneously, and the whole biofilm edge showed oscillation at the same time and rate (Liu et al., 2015). This characteristic seems to be very similar to the membrane potential oscillation. Researchers compared the oscillation in membrane potential and growth rate within the *Bacillus subtilis* biofilm and found that they are inversely correlated, as shown in Figure 1-2 (Prindle et al., 2015). Moreover, the YugO channel that can promote electrical communication within the *Bacillus subtilis* biofilm, is also necessary for biofilm formation. This indicates that there might be a close relationship between biofilm formation and membrane potential. There might also be

some other factors besides the YugO potassium channel that can link membrane potential and biofilm formation together.

1.3 Surfactin

Surfactin is a cyclic lipopeptide lactone secreted by *Bacillus subtilis.* It contains two acidic amino acids (glutamate and aspartate) besides five nonpolar residues and one 3-hydroxy fatty acid (Kakinuma et al., 1969). The structure of surfactin is presented in Figure 1-3 (Kraas et al., 2010). Surfactin is produced by nonribosomal peptide synthetases SrfAA, SrfAB, SrfAC and a protein with high homology to external thioesterases of type II called SrfD (Kraas et al., 2010). These enzymes are encoded by corresponding genes that are organized in the srfA operon (Pratap et al. 2013).

To link membrane potential and biofilm formation together, surfactin needs to participate in biofilm formation and have the ability to influence membrane potential.

Surfactin is necessary for the formation of *Bacillus subtilis* biofilm, despite the fact that its function can be partly replaced by molecules produced by other soil bacteria, such as nystatin from *Streptomyces noursei* (López et al., 2009). Surfactin is well known as a quorum sensing factor of *Bacillus subtilis* and plays a critical role in the biofilm formation of *Bacillus subtilis* by activating a protein kinase (López et al., 2010). Researchers found that surfactin can be sensed by KinC, which is a membrane histidine kinase (López et al., 2009). KinC is a member of bacterial sensor histidine kinases family. In prediction, KinC has a PAS-PAC sensor domain and two cross-membrane segments containing seven extracellular residues (Mascher et al., 2006; Taylor et al. 1999). KinC can sense surfactin and phosphorylate Spo0A. Spo0A plays a dual signaling role, and the phosphorylation of Spo0A is critical for the biofilm formation. When the Spo0A~P level is low, phosphorylated Spo0A will activate the *eps*A-O operon

and *tapA-sipW-tasA* operon. The activation of *eps*A-O operon can promote exopolysaccharides production, and the activation of *tapA-sipW-tasA* operon can stimulate TasA amyloid fiber production. When the Spo0A~P level is high, phosphorylated Spo0A will inactivate these operons, stop ECM production and trigger sporulation (Aguilar et al., 2010; Rubinstein et al., 2012; McLoon et al., 2011). When ECM reach a certain level, cells can grow fast in the ECM environment, and the bacterial biofilm can develop by repeating this cycle. In this way, surfactin can promote biofilm formation.

Surfactin also has potential to influence membrane potential. The membrane potential is affected by the stable potassium flux rate through the cell membrane. If a factor has the ability to change the potassium flux rate through the cell membrane, it will probably influence membrane potential, like the YugO potassium channel. Surfactin can cause potassium leakage across the lipid bilayer membrane, which will increase potassium flux through the cell membrane and lead to low intracellular potassium concentration (Sheppard et al., 1991), as shown in Figure 1-4. This provides the possibility for surfactin to influence the membrane potential within the *Bacillus subtilis* biofilm. Moreover, the kinase KinC can also respond to the lowered intracellular potassium concentration (McLoon et al., 2011). Taking all these results together, surfactin, intracellular potassium concentration, and KinC seem to have a functional linkage among them.

The oscillation in fluorescence and growth rate within the *Bacillus subtilis* biofilm both happened spontaneously and have a relatively stable rhythm (Liu et al., 2011; Prindle et al., 2011). Thus, a strong mechanism to synchronize them is required. A

quorum sensing system seems to have good potential. The system mainly uses the extracellular concentration of autoinducer to regulate the transcription of target genes. The extracellular concentration of autoinducer can be considered uniform in a relatively long distance, so this might be the key reason to explain how *Bacillus subtilis* is able to communicate over such long distance. Moreover, as an autoinducer in a quorum sensing system, surfactin can not only turn on or off the target gene, but also control the transcription level of it. Meanwhile, this quorum sensing system is thought to have a negative feedback loop like most quorum systems, although it is not well understood in surfactin quorum sensing system. A negative feedback loop can control the production of surfactin dynamically. When extracellular surfactin concentration is high, the loop should have a strong effect and inhibit surfactin production. When extracellular surfactin concentration is low, the loop should have a weak effect, and the surfactin production will increase. If surfactin could increase the membrane potential of *Bacillus subtilis* biofilm, the membrane potential would correlate with the extracellular surfactin concentration. These characteristics can provide strong support to the oscillation behavior.

Surfactin seems to be a very promising candidate that can help us to decipher the correlation between membrane potential and the biofilm formation.

1.4 Motivation of This Project

1.4.1 Goal of This Project

Investigating the relationship between membrane potential and biofilm formation offers us a new way to study biofilm formation.

Surfactin can cause potassium leakage across the lipid bilayer membrane, and play a critical role in the *Bacillus subtilis* biofilm formation. Thus surfactin has good

potential to influence membrane potential and link membrane potential and biofilm formation together, but it has not yet been directly reported to be able to influence membrane potential.

The goal of this project is to study the effect of surfactin on membrane potential.

1.4.2 Device and Reporter

To investigate the effect of surfactin on the membrane potential of *Bacillus subtilis* biofilm, the change of membrane potential needs to be measured in appropriate devices. The microfluidic devices were designed and made by our lab (Son et al., 2014). Commercial microfluidic slides (ibidi Inc.) were also used. To report the change of membrane potential, membrane potential was recorded time by time. Two established voltage-sensitive fluorescent dyes, thioflavin T (ThT) and 3,3'-dipropylthiadicarbocyanine iodide ($DiSC_3(5)$) were used to detect membrane potential (Kralj et al., 2011; Strahl et al., 2010). The dyes are positively charged and can enter cell membrane. The inner membrane of bacteria has negative electrical potential (Hosoi et al., 1980) and thus will retain the dyes. When the membrane has more negative electrical potential, it will contain a higher concentration of these fluorescent dyes, and so have brighter fluorescence.

1.4.3 KCl Shock to Verify Dyes Can Report Membrane Potential

Although ThT and $DiSC_3(5)$ were used to detect membrane potential in previous studies, we conducted a KCl shock experiment to verify that these two dyes can reliably report the membrane potential within the *Bacillus subtilis* biofilm. The idea of this experiment is to use high concentration of potassium (300mM) to interrupt the normal potassium flux, thereby lowering the membrane potential. Then we can see whether the dye reports this change.

1.4.4 Use Surfactin to See Its Influence on The Membrane Potential

Extra surfactin was added, and we studied the effect on membrane potential and the change of membrane potential oscillation. Meanwhile, APG-4 was used to measure the extracellular potassium concentration to verify that surfactin can cause potassium leakage. The srfAA gene deletion mutant was tested to see if biofilm formation and membrane potential could be controlled by supplying it with surfactin.

1.5 Significance of Studying Biofilm

Studying biofilm formation is very important. On the one hand, there are still many unknown mechanisms and characteristics about biofilms, so studying biofilm can help us to understand more about biofilm itself. On the other hand, studying biofilm can also benefit humanity. If we understand how cells form biofilms and find a way to stop the formation of pathogenic biofilms, we will largely improve the treatment of many diseases associated with biofilms such as cystic fibrosis (CF) lung disease caused by *P. aeruginosa* (Collins 1992). Moreover, we could also help developing biofilms that can benefit humanity. For instance, *Bacillus subtilis* biofilm can promote plant growth and protect plants from an extensive collection of pathogens. If we can control *Bacillus subtilis* biofilm formation and maintain it in the best concentration range, agriculture will be benefited.

For this project, we studied *Bacillus subtilis* biofilm. *Bacillus subtilis* is the best-studied Gram-positive bacteria. It is observed in many different environments and has long been used as a model Gram-positive bacterium (Earl et al., 2008; Kunst et al., 1997).

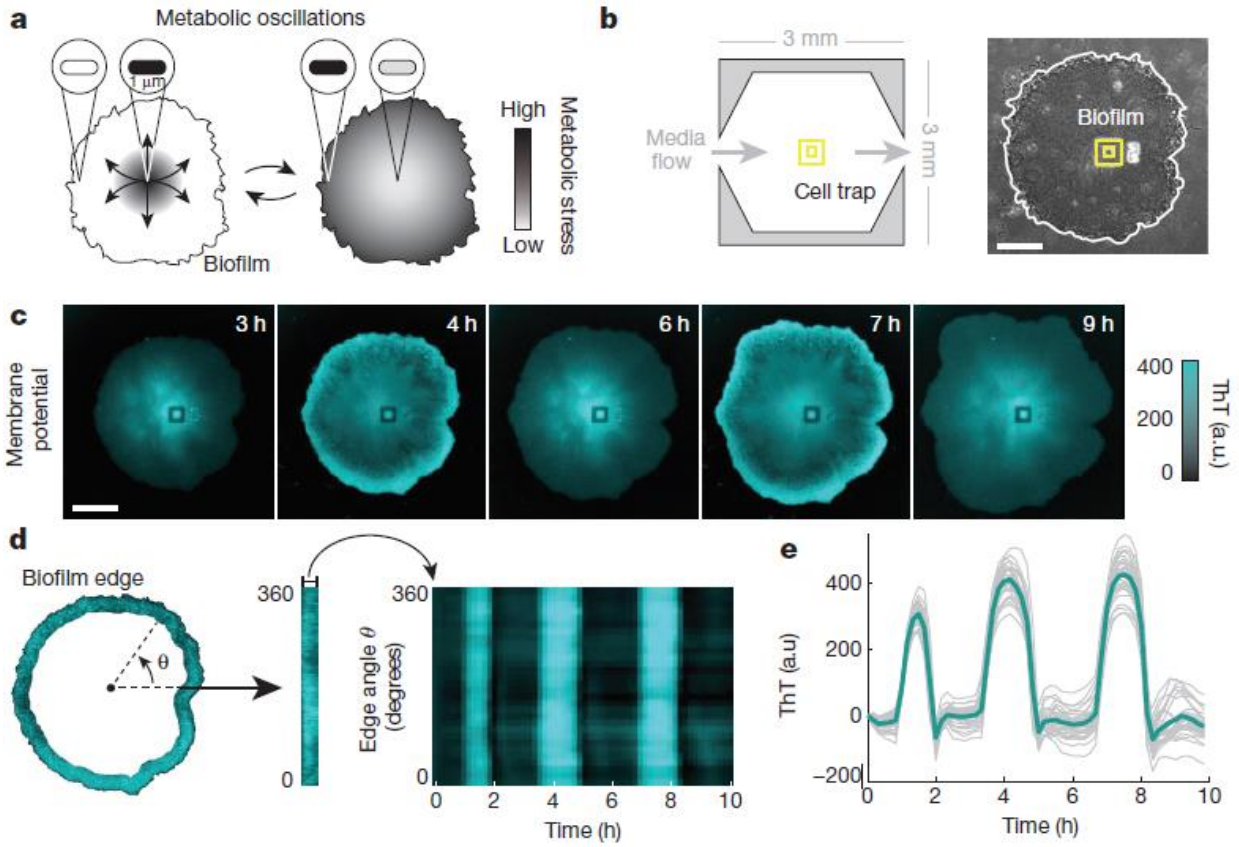


Figure 1-1. Membrane Oscillation within *Bacillus subtilis* biofilm. a) Metabolic oscillation within the *Bacillus subtilis* biofilm, b) Left is the diagram of the microfluidic device. Right is the phase contrast image of a biofilm. Scale bar is 100 µm. c) Membrane potential oscillation, within the *Bacillus subtilis* biofilm, reported by ThT. Scale bar is 150 µm. d) Left is the edge region of the biofilm in c. Right is the plot of fluorescence within edge region over time. e) Time traces plot of the data of the heat map shown in d. (Reproduced with permission from Reference [Prindle et al., 2015]; License Number: 4213850812388)

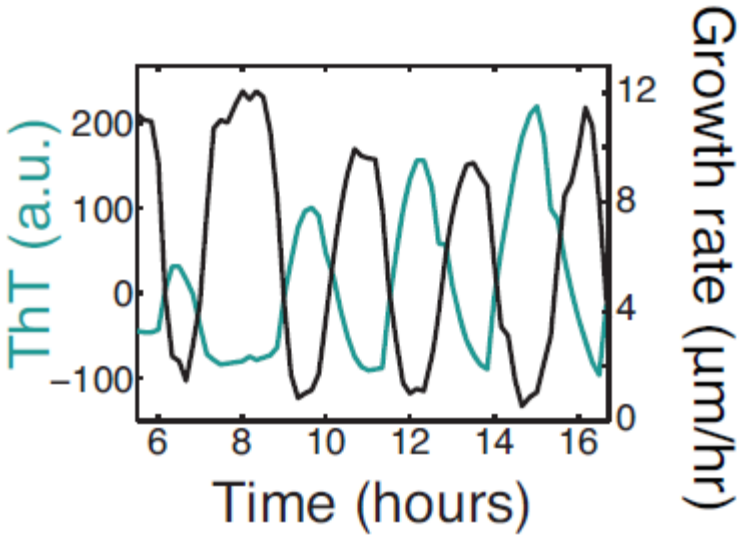


Figure 1-2. Oscillations in ThT and growth rate. The blue line is the time trace of membrane potential within the biofilm. The membrane potential is represented by the fluorescence of ThT. The black line is the time trace of the growth rate of the biofilm. (Reproduced with permission from Reference [Prindle et al., 2015]; License Number: 4213850812388)

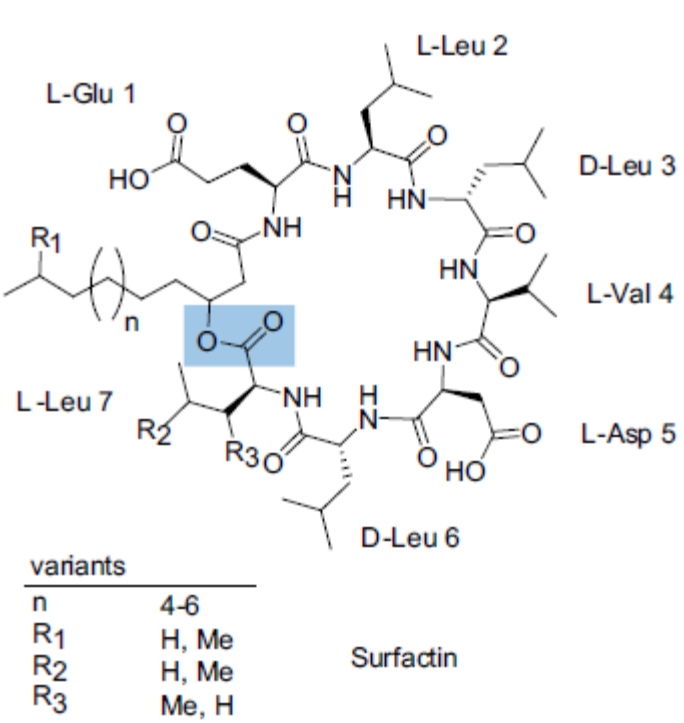


Figure 1-3. Structure of surfactin. The figure shows the structure of surfactin and lists the main variants of surfactin. The light blue box shows the lactone bond. (Reproduced with permission from Reference [Kraas et al., 2010]; License Number: 4213851138555)

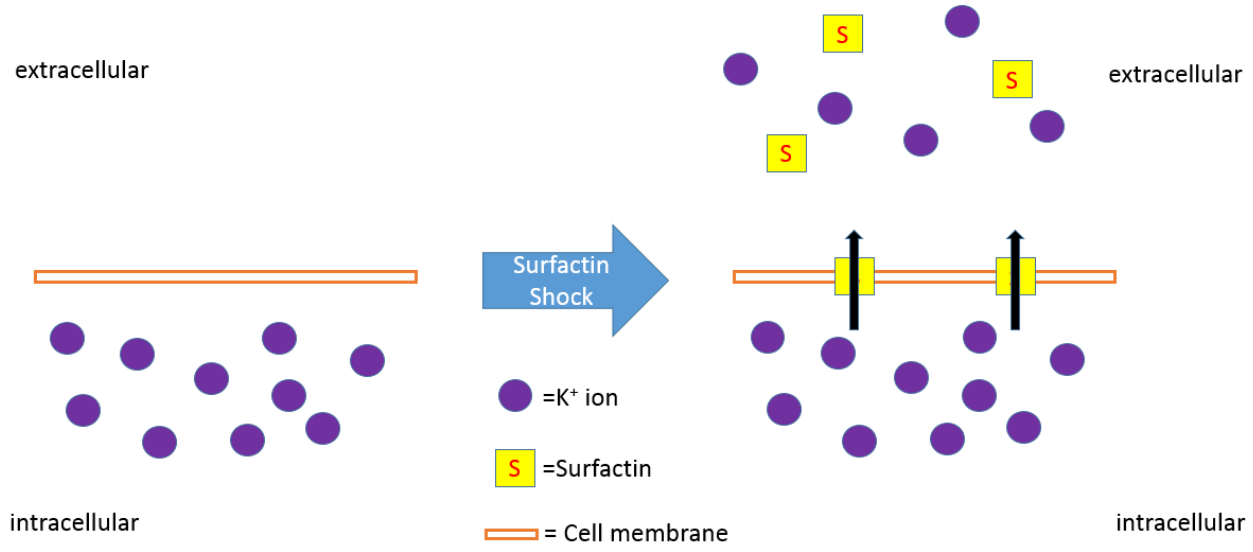


Figure 1-4. Surfactin cause potassium leakage across the cell membrane. The cell membrane is semipermeable, and intracellular potassium concentration is higher than extracellular potassium concentration. When surfactin exist in the environment, it will attach to the lipid membrane and open channels for potassium leakage.

CHAPTER 2
MATERIALS AND METHODS

2.1 Materials

Strain. Strains are listed in Table 2-1. In most experiments, the wild-type strain NCBI 3610 was used. This strain was brought from the American Type Culture Collection (ATCC), and the part number is ATCC 6051.

The srfAA::erm mutant ZK3858 (Branda et al., 2001) was used in mutant experiments and compared with WT. ZK3858 is an isogenic srfA mutant of NCBI 3610. This strain was a gift from the Kolter laboratory, Department of Microbiology & Immunobiology, Harvard Medical School.

Medium. For long-time planktonic growth, cells were cultured in LB medium. LB medium contained 5 g/L yeast extract, 10 g/L Tryptone and 10 g/L NaCl. LB medium was sterilized using an autoclave.

For the biofilm formation in experiments, MSgg medium (Prindle et al., 2011) was used. Every 100 mL MSgg medium contained 0.5 mmol potassium phosphate buffer (pH 7.0), 10 mmol MOPS buffer (pH 7.0), 0.2 mmol $MgCl_2$, 70 µmol $CaCl_2$, 10 µmol $FeCl_3$, 5 µmol $MnCl_2$, 0.2 µmol thiamine HCl, 0.1 µmol $ZnCl_2$, 0.5 mL glycerol and 0.5 g monosodium glutamate. In KCl shock experiment, MSgg (KCl shock) medium contained extra 300 mM KCl. In NaCl shock experiment, MSgg (NaCl shock) medium contained extra 300 mM NaCl. MSgg medium was filter sterilized.

Important stocks. Thioflavin T (ThT) and 3,3'-dipropylthiadicarbocyanine iodide ($DiSC_3(5)$) were used to report the change of biofilm membrane potential. ThT (ICN biomed Inc.) was solved in DMSO, and the stock concentration was 10 mM. $DiSC_3(5)$ (Thermo Fisher Inc.) was dissolved in deionized water, and the stock concentration was

10 mM. Unless specifically described, ThT was used at 40 µM, and $DiSC_3(5)$ was used at 10 µM.

Asante Potassium Green-4 (APG-4) was used to report the change of extracellular potassium concentration. APG-4 (TEFLabs Inc.; salt form) was dissolved in deionized water and the stock concentration was 2 mM. APG-4 was used at 2 µM.

Surfactin (Sigma Inc.) was dissolved in DMSO, and the stock concentration was 20 mM.

Erythromycin (ACROS Organics Inc.) was dissolved in EtOH, and the stock concentration was 20 mg/mL. The working concentration of erythromycin was 20 µg/mL.

Microfluidic device. The microfluidic device used for this project was designed and made in the Hagen laboratory (Son et al., 2014). The device has three inlet channels and one outlet channel. The outlet channel was also used for loading cells. A 3 mm x 3 mm chamber, 4 mm x 4 mm chamber, 5 mm x 5 mm chamber were located on the corresponding channel. The depth of the chambers is 20 µm. The microfluidic device was used to observe membrane potential oscillation of *Bacillus subtilis* biofilm.

ibidi slides. A commercial microfluidic channel, ibidi µ-Slide III 3in1 (catalog number: 80311) was used in KCl shock and surfactin shock experiments. It has three inlets, one large main chamber, and one outlet. The depth of the chamber is 0.4 mm. The inlets can connect to syringes with different medium. Pushing different syringe allows switching medium in the main chamber.

24 well plates. Falcon 24 Well Polystyrene Clear Flat Bottom Untreated Cell Culture Plates were used to test the effect of surfactin on *Bacillus subtilis* biofilm formation.

2.2 Growth Conditions

To get the cell culture for experiments, 1 µL cells from -80°C glycerol stock was inoculated into 3 mL LB medium and incubated in a shaker (37 °C, 180 rpm) for 15 h.

For microfluidic experiments, overnight cell culture was diluted 15 folds, and then centrifuged at 3000 rpm (LWS-815, LW Scientific Inc.) for 2 min. The supernatant was removed and the cell pellet was re-suspend in the MSgg medium. The MSgg medium containing cells was loaded into the microfluidic device. The cells in the microfluidic device were incubated at 37 °C for 1.5 h, no flow, and then in flowing medium at 37 °C for another 2.5 h, with flow a rate at 0.02 mL/h. Before imaging, the temperature was set to 30 °C until the experiment finished.

For ibidi slides experiments, overnight cell culture was diluted 6 folds, and then centrifuged at 3000rpm (LWS-815, LW Scientific Inc.) for 2 min. The supernatant was removed and the cell pellet was re-suspend in the MSgg medium and then loaded into an ibidi slide. The MSgg medium containing cells were loaded into the ibidi slide. The cells in the slide were incubated at 37 °C for 1 h, no flow, and then in flowing medium at 37 °C for another 1.5 h, with a flowing rate at 0.12 mL/h. Before imaging, the temperature was set to 30 °C and kept until the experiment finished.

For 24 well plate experiments, each well had 1.5 mL MSgg medium containing the corresponding concentration of surfactin (0-20 µM) and 1 µL corresponding cell culture. The plate was incubated at 30 °C for the whole experiment.

2.3 Data Collection

To collect phase contrast and fluorescence images, a Nikon eclipse TE2000U microscope and Photometrics CoolSNAP HQ2 camera were used. In most of the experiments, 20 X objectives were used to image the biofilms. The image size is 1040 x

1392 pixels. Every 10 min in biofilm experiments and every 5 min in background tests, one phase contrast image, and one corresponding fluorescence image were taken. The exposure time for phase contrast image was 200 ms; the exposure time for fluorescence image was 5000 ms.

2.4 Data Analysis

Custom scripts of MATLAB (MathWorks) were used for data analysis. To ensure the objective can match in all images of a timeline, images were registered. Unless specifically described, the mean fluorescence value of a 5 x 5 pixels region on a biofilm was measured to represent membrane potential of this region at one time point. All the values of a timeline were collected together to generate a membrane potential curve.

Table 2-1. List of strains used in this thesis

Strain	Genotype	Source
Wild Type	*Bacillus subtilis* NCBI 3610	ATCC
ZK3858	srfAA::erm	Branda et al., 2001.

CHAPTER 3
RESULTS

3.1 ThT and $DiSC_3(5)$ Were Verified to Report The Change of Membrane Potential Reliably

This project focused on the membrane potential of *Bacillus subtilis* biofilm. Directly measuring the membrane potential of *Bacillus subtilis* biofilm is difficult. Indirectly but reliably reporting the membrane potential of *Bacillus subtilis* biofilm is more realistic.K^+

The positively charged dyes ThT and $DiSC_3(5)$ had already been used to report membrane potential in previous studies (Kralj et al., 2011; Strahl et al., 2010); however, it is preferable first to demonstrate their ability to report the change of biofilm membrane potential in our lab.

To reach this goal, KCl shock was used to change the membrane potential of *Bacillus subtilis* biofilm manually. The Goldman equation for the membrane potential is

$$\boldsymbol{V} = \frac{\boldsymbol{RT}}{\boldsymbol{F}} \ln\left(\frac{\boldsymbol{P_K}[\boldsymbol{K^+}]_{\boldsymbol{out}} + \boldsymbol{P_{Na}}[\boldsymbol{Na^+}]_{\boldsymbol{out}} + \boldsymbol{P_{Cl}}[\boldsymbol{Cl^-}]_{\boldsymbol{out}}}{\boldsymbol{P_K}[\boldsymbol{K^+}]_{\boldsymbol{in}} + \boldsymbol{P_{Na}}[\boldsymbol{Na^+}]_{\boldsymbol{in}} + \boldsymbol{P_{Cl}}[\boldsymbol{Cl^-}]_{\boldsymbol{in}}}\right) \quad (3\text{-}1)$$

(Jackson, M. B., 2006), where ***V*** is the equilibrium potential; ***R*** is the universal gas constant (8.314 $JK^{-1}mol^{-1}$); ***T*** is the temperature in Kelvin; ***F*** is Faraday's constant (96485 C/mol); $\boldsymbol{P_K}$ is the permeability (ions/sec) for the potassium ion, $\boldsymbol{P_{Na}}$ and $\boldsymbol{P_{Cl}}$ are similar; $\boldsymbol{[K^+]_{out}}$ is the extracellular potassium concentration, $\boldsymbol{[Na^+]_{out}}$ and $\boldsymbol{[Cl^-]_{out}}$ are similar; $\boldsymbol{[K^+]_{in}}$ is the intracellular potassium concentration, $\boldsymbol{[Na^+]_{in}}$ and $\boldsymbol{[Cl^-]_{in}}$ are similar. From the Goldman equation, when a high concentration of potassium exists in the extracellular environment, the membrane potential should be more positive. When the inner membrane is more positive, the positively charged fluorescent dyes will

accumulate less within the inner membrane and so the level of fluorescence will be lower.

When MSgg (KCl shock) medium (containing extra 300mM KCl) flows, biofilm should have more positive membrane potential compared to the potential when normal MSgg medium flows. Moreover, when MSgg medium flows again, the biofilm membrane potential should recover to the previous level. A reliable reporter should report these changes.

For $DiSC_3(5)$, the background fluorescence of MSgg medium and MSgg (KCl shock) medium was relatively stable, as shown in Figure 3-1A. The fluorescence of biofilm decreased when MSgg (KCl shock) medium flowed and recovered when MSgg medium flowed again, as shown in Figure 3-1B. $DiSC_3(5)$ successfully reported the change of membrane potential.

For ThT, the background fluorescence of MSgg medium and MSgg (KCl shock) medium was relatively stable, as shown in Figure 3-2A. The fluorescence of biofilm decreased when MSgg (KCl shock) medium flowed and recovered when MSgg medium flowed again, as shown in Figure 3-2B. ThT successfully reported the change of membrane potential.

In KCl shock experiments, ThT and $DiSC_3(5)$ both were demonstrated to be able to report the change of membrane potential. The experiments using these dyes to report membrane potential should give us reliable membrane potential data.

3.2 Surfactin Can Make The Membrane Potential of *Bacillus subtilis* Biofilm More Negative

Surfactin was not previously reported to influence the membrane potential of *Bacillus subtilis* biofilm. However surfactin was reported to cause potassium leakage

across the lipid bilayer membrane, which will increase potassium flux rate through the cell membrane (Sheppard et al., 1991). The charged ions moving across the cell membrane can change the membrane potential (Prindle et al., 2015). We hypothesize that a higher potassium flux rate would lead to more negative membrane potential, so that surfactin would decrease the membrane potential of *Bacillus subtilis*.

The cell membrane is semipermeable, and the intracellular potassium concentration is higher than the extracellular potassium concentration. Surfactin can cause potassium leakage across the membrane, increasing the $\boldsymbol{P_K}$ that appears in the Goldman equation given earlier. This will cause the membrane potential to become more negative. When the inner membrane is more negative, there will be more positive charged fluorescent dyes concentrated within the inner membrane and so have a higher level of corresponding fluorescence. Therefore if an area within the biofilm shows brighter fluorescence, the membrane potential of this area is more negative.

To test this hypothesis, a surfactin shock experiment that was similar to a KCl shock experiment was designed. By this hypothesis, when MSgg (surfactin shock) medium (containing extra surfactin) flows, the membrane potential of *Bacillus subtilis* biofilm should be more negative compared to the potential when MSgg medium flows. When MSgg medium flows again, the biofilm membrane potential should recover to the normal level.

When ThT dye was chosen as a reporter, the background fluorescence of MSgg medium and MSgg (surfactin shock) medium was relatively stable, as shown in Figure 3-3A. As expected, the fluorescence of biofilm increased when MSgg (surfactin shock) medium flowed and recovered when MSgg medium flowed again, as shown in Figure 3-

3B and Figure 3-3C. Thus, surfactin was demonstrated to be able to make the membrane potential of *Bacillus subtilis* biofilm more negative.

During the surfactin shock, the fluorescence increased at the beginning and then dropped back. This activity fits the fact that surfactin can cause potassium leakage. Initially, surfactin caused higher P_K and so, from the Goldman equation, caused a more negative membrane potential. After several minutes, higher potassium flux caused lower intracellular potassium concentration ($[K^+]_{in}$) and higher extracellular potassium concentration ($[K^+]_{out}$). Thus the membrane potential later became more positive.

Moreover, it seems that the membrane potential decreased more when high concentration surfactin shock (20μM surfactin) medium flowed (Figure 3-3B) compared to that when low concentration surfactin shock (2μM surfactin) medium flowed (Figure 3-3C). This indicated that the effect of surfactin might be concentration related. This can be a good point for modeling and future work.

3.3 Surfactin Can Cause Potassium Leakage in *Bacillus subtilis* Biofilm

Asante Potassium Green-4 (APG-4) is an established reporter to measure the concentration of potassium, and the salt form of APG-4 cannot permeate the cell membrane (Prindle et al., 2015).

To test the specificity of APG-4, NaCl shock and KCl shock were used. As shown in Figure 3-4A, the fluorescence of APG-4 decreased slightly when MSgg (NaCl shock) medium flowed; and the fluorescence of APG-4 increased when MSgg (KCl shock) medium flowed. This showed that APG-4 could select potassium specifically.

To verify that surfactin can cause potassium leakage in biofilm, APG-4 was used as the reporter in surfactin shock experiments. The background fluorescence of MSgg medium and MSgg (surfactin shock) medium was relatively stable, as shown in Figure

3-4B. As expected, the fluorescence of APG-4 increased when MSgg (surfactin shock) medium flowed and recovered when MSgg medium flowed again, as shown in Figure 3-4C. Thus, surfactin was demonstrated to be able to cause potassium leakage in *Bacillus subtilis* biofilm. Moreover, the fluorescence of APG-4 increased more when high concentration surfactin shock (20μM surfactin) medium flowed compared to that when low concentration surfactin shock (2μM surfactin) medium flowed (Figure 3-4C). This again indicated that the effect of surfactin might be concentration related.

3.4 The Membrane Potential Oscillation of *Bacillus subtilis* Biofilm Was Observed in Our Lab

We attempted to duplicate the observation of the membrane potential oscillation reported in 2015 (Prindle et al., 2015).

In our microfluidic device, the biofilm had expanded much during long time culture. The movement of the cells that were chosen to show the membrane potential cannot be ignored. To collect reliable data, a custom function of MATLAB was created to track specific areas located on the edge of biofilm in registered images, as shown in Figure 3-5A. Here, 3 areas located on the different positions of biofilm were chosen. Each area equals to 25 x 20 pixels. The mean value of the fluorescence in this area represents the membrane potential of this area.

$DiSC_3(5)$ was used as a reporter. Each line in Figure 3-5B represent the mean value of the fluorescence of corresponding area. The fluorescence showed oscillation behavior (Figure 3-5B). Thus, the membrane potential oscillation of *Bacillus subtilis* biofilm was successfully repeated in our microfluidic device.

3.5 High Concentration of Surfactin Can Eventually Make The Membrane Potential of *Bacillus subtilis* Biofilm More Positive

To further investigate the effect of surfactin on the membrane potential oscillation of *Bacillus subtilis* biofilm, a long time surfactin shock experiment was designed. ThT was chosen as a reporter in this experiment.

As shown in Figure 3-6, in the first 420 minutes, the fluorescence showed oscillation behavior when MSgg medium flowed. Then, MSgg (surfactin shock) medium (containing 20μM surfactin) flowed until the end of the experiment. At the beginning of surfactin shock, the fluorescence increased and then dropped back but held an overall higher level than the overall fluorescence when MSgg medium flowed. This result fits previous data that surfactin can make the membrane potential of *Bacillus subtilis* biofilm more negative. However, an interesting phenomenon happened after the 560 minute time point. The fluorescence decreased and then held a relatively lower level than the overall fluorescence when MSgg medium flowed. This result showed that in continuous high concentration surfactin shock, the membrane potential of *Bacillus subtilis* biofilm will eventually become more positive. This activity also could be explained by the fact that surfactin can cause potassium leakage. As mentioned above, after shock, the intracellular potassium concentration became lower. Eventually, a new balance with a lower potassium flux rate between inside and outside cell membrane established and made the more positive membrane potential.

3.6 Examination of The srfAA Mutant

The Kolter laboratory provided a sample of the ZK3858 mutant, which is an isogenic srfA mutant of *Bacillus subtilis* NCBI 3610. This mutant cannot produce surfactin.

To compare ZK3858 with WT, and observe the influence of surfactin on biofilm formation, ZK3858 and WT were grown in MSgg medium containing the different concentration of surfactin on 24 well plate.

As shown in Figure 3-7, WT formed biofilm in the normal MSgg medium (containing no extra surfactin). ZK3858 could not form biofilm in the MSgg medium (containing no extra surfactin) while it formed biofilm in MSgg medium containing 2μM surfactin. This result was in agreement with previous studies and demonstrated that surfactin is very important in *Bacillus subtilis* biofilm formation.

Moreover, in MSgg medium containing high concentration surfactin, ZK3858 did not form a visible biofilm, while WT formed smaller biofilm than the biofilm in MSgg medium containing no extra surfactin and MSgg medium containing 2μM surfactin within 24h. This result may indicate that when surfactin concentration is too high, surfactin may prohibit *Bacillus subtilis* biofilm formation.

Comparing WT grown in MSgg medium containing no extra surfactin and MSgg medium containing 2μM surfactin, *Bacillus subtilis* formed biofilm earlier or faster in MSgg medium containing 2μM surfactin. This result may indicate that low extra surfactin concentration may promote the formation of *Bacillus subtilis* biofilm. This suggests that surfactin possibly has a dual function in *Bacillus subtilis* biofilm formation.

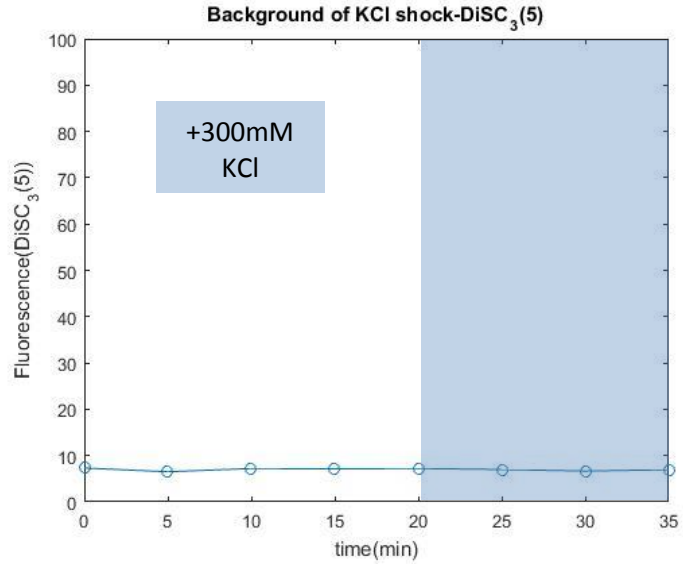
3.7 Low Concentration of Surfactin Can Hold The Membrane Potential of srfAA Mutant More Negative

A normal MSgg medium shock experiment was designed to investigate the effect of surfactin on the srfAA mutant. ThT was chosen as a reporter.

As shown above, the srfAA mutant ZK3858 could not form biofilm in the normal MSgg medium while it formed biofilm in MSgg medium containing 2μM surfactin. So the

cells were cultured with MSgg (low surfactin concentration) medium (containing 2μM surfactin) in the device. As shown in Figure 3-8, the overall fluorescence when MSgg (low surfactin concentration) medium (containing 2μM surfactin) flowed is higher than that when normal MSgg medium flowed. This data showed that the low concentration could help the srfAA mutant to hold a more negative membrane potential. This might be because the srfAA mutant cannot produce surfactin, and so lacks an important way to export potassium. In this way, the membrane potential of the srfAA mutant was more positive when normal MSgg medium flowed.

A



B

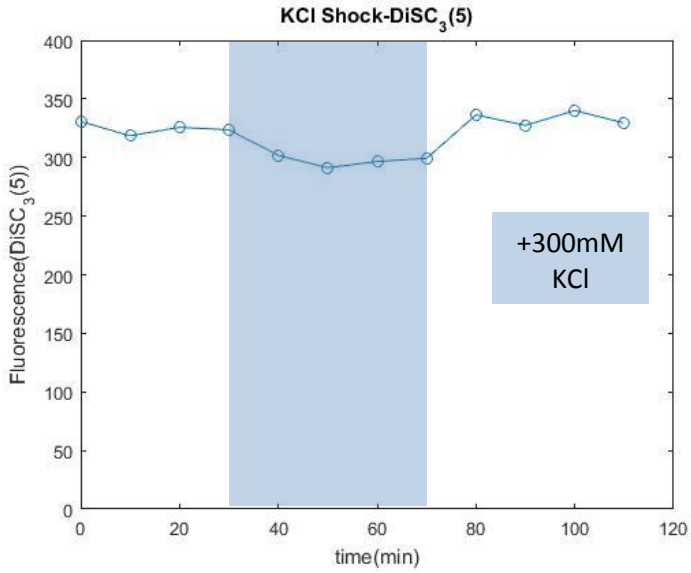
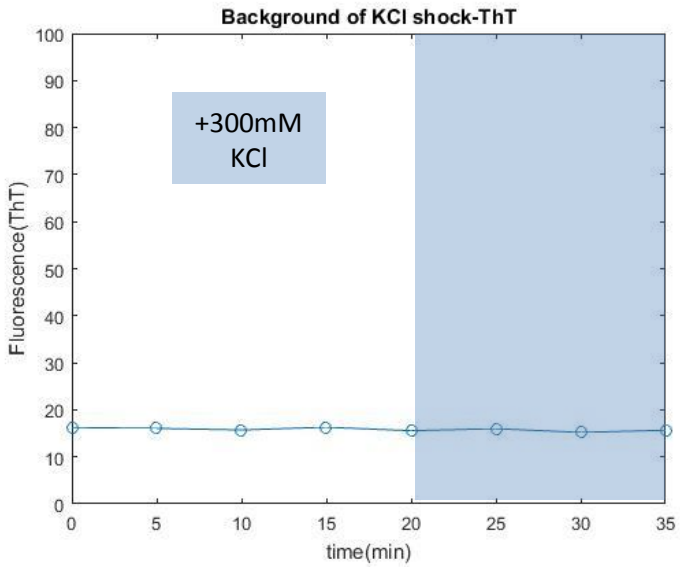


Figure 3-1. $DiSC_3(5)$ can report the change of biofilm membrane potential. A) The background fluorescence of $DiSC_3(5)$ is relatively stable in the absence of biofilm. B) The fluorescence of biofilm decreased when MSgg (KCl shock) medium flowed and recovered when MSgg medium flowed again.

A



B

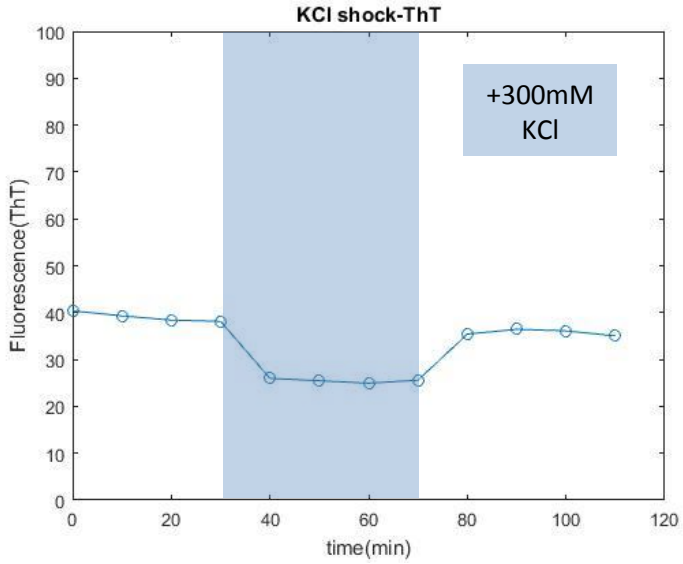


Figure 3-2. ThT can report the change of biofilm membrane potential. A) The background fluorescence of ThT is relatively stable in the absence of biofilm. B) The fluorescence of biofilm decreased when MSgg (KCl shock) medium flowed and recovered when MSgg medium flowed again.

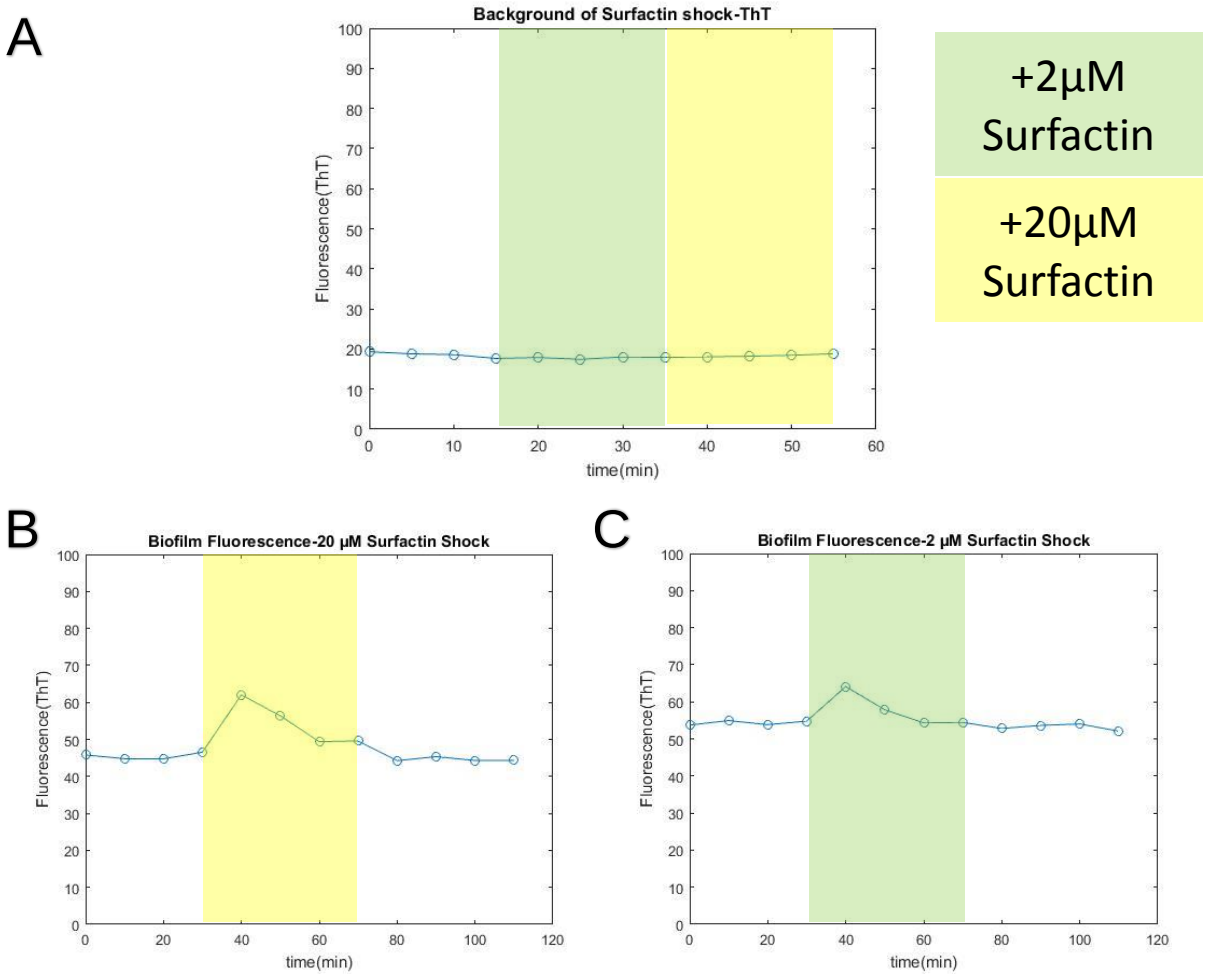


Figure 3-3. Surfactin can increase membrane potential of *Bacillus subtilis* biofilm. A) The background fluorescence of ThT is relatively stable in the absence of biofilm. B) The fluorescence of biofilm increased when MSgg (20μM surfactin shock) medium flowed and recovered when MSgg medium flowed again. C) The fluorescence of biofilm increased when MSgg (2μM surfactin shock) medium flowed and recovered when MSgg medium flowed again.

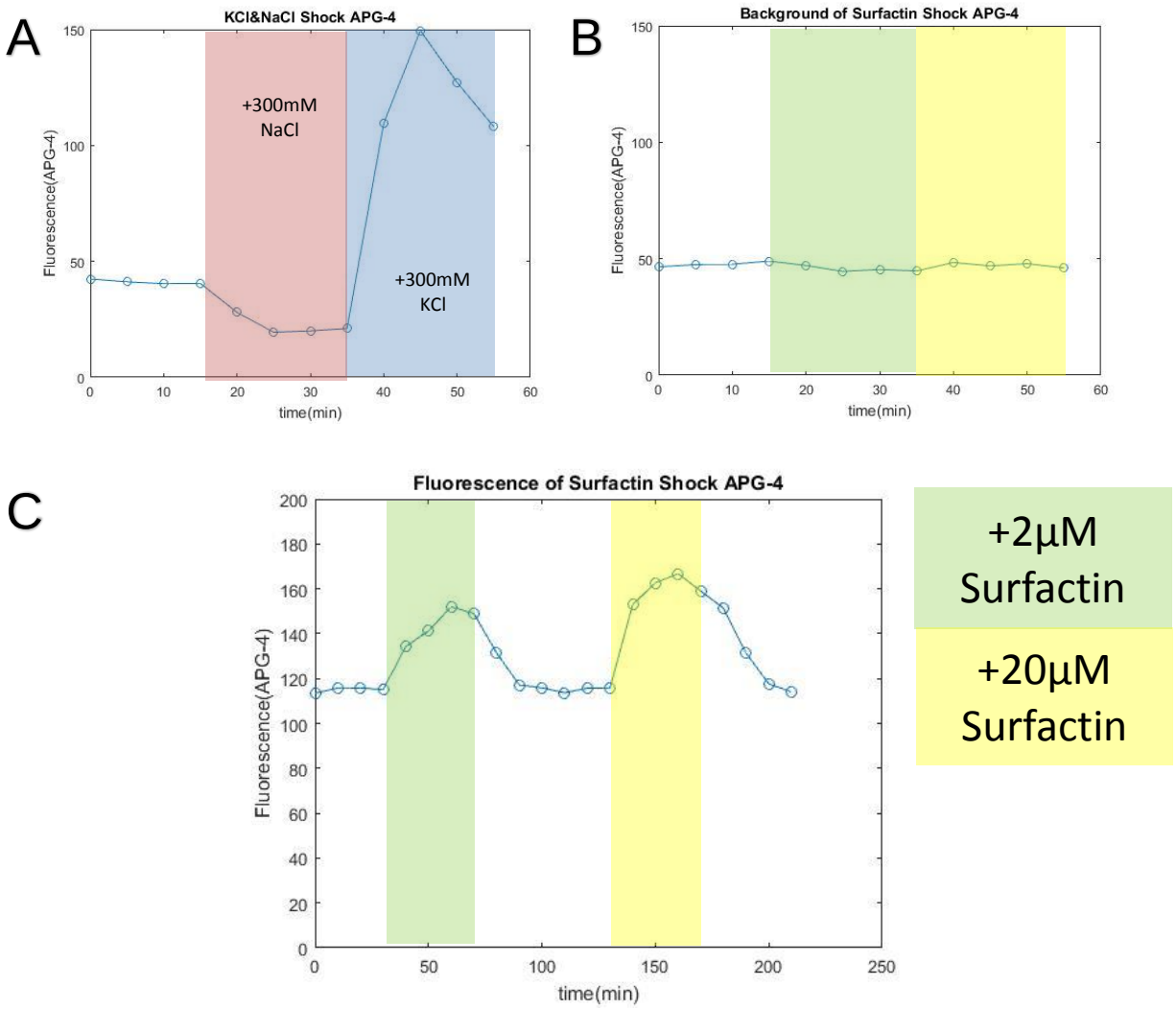


Figure 3-4. Surfactin can cause potassium leakage across the cell membrane of *Bacillus subtilis* biofilm. A) APG-4 can select potassium specifically, B) The background fluorescence of APG-4 is relatively stable. The change of ThT fluorescence was not likely caused by itself nor experimental conditions, C) The fluorescence of biofilm increased when MSgg (2μM surfactin shock) medium and MSgg (20μM surfactin shock) medium flowed and recovered when MSgg medium flowed again.

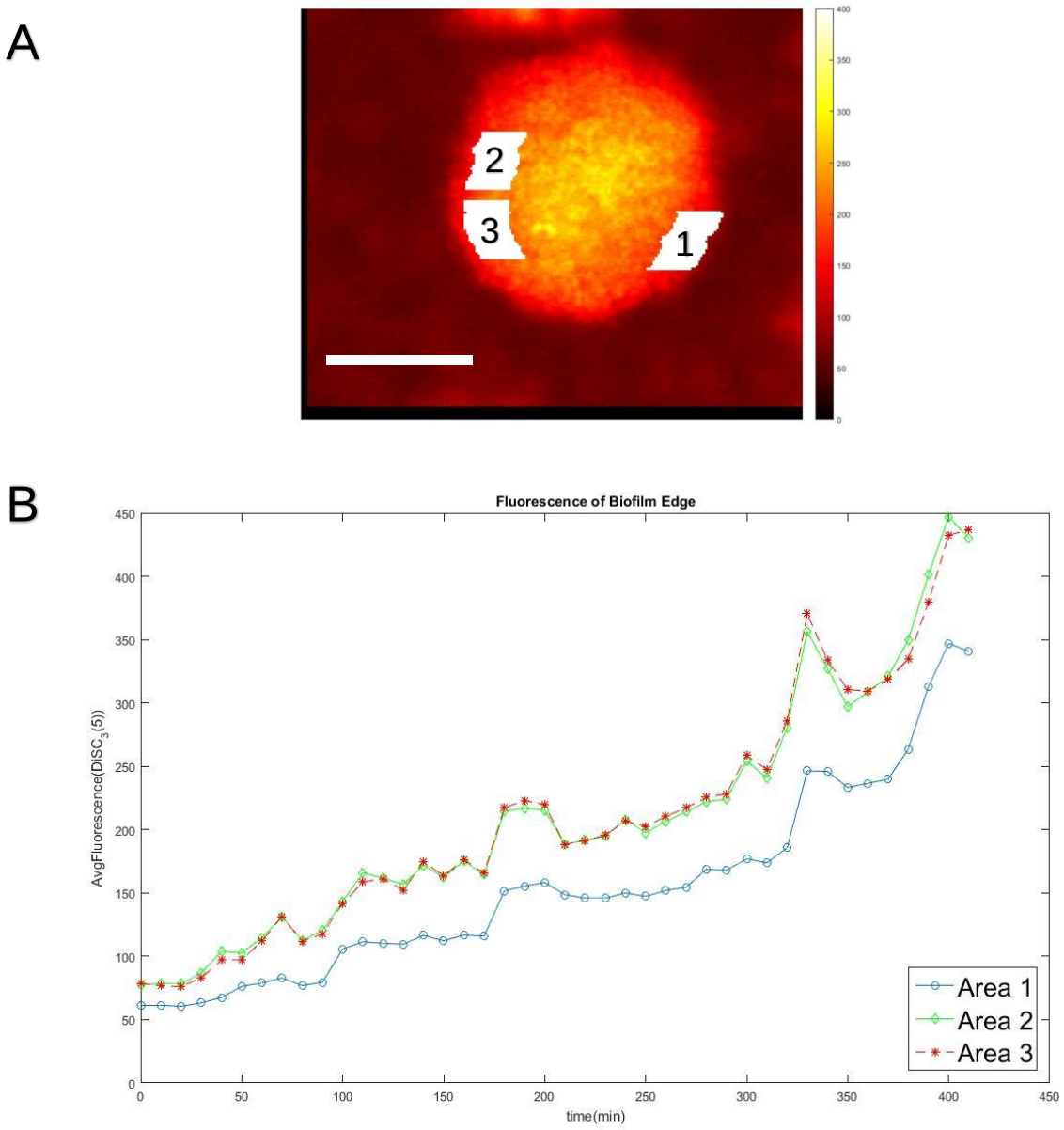


Figure 3-5. The membrane potential oscillation of *Bacillus subtilis* biofilm. A) The white areas on the biofilm were tracked in all time points; the scale bar is 20 μM, B) Each line showed the fluorescence of corresponding area on the biofilm. The fluorescence of the white areas showed oscillation behavior.

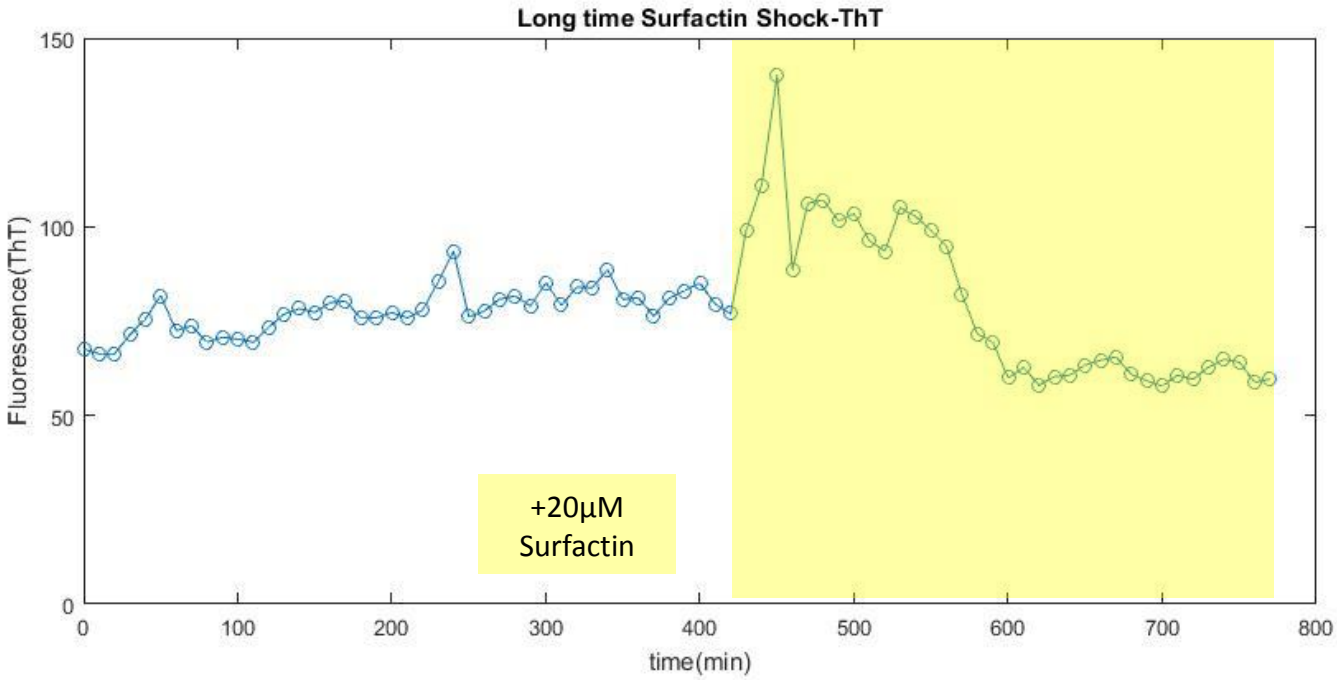


Figure 3-6. Long time surfactin shock on biofilm. The fluorescence of biofilm increased in the beginning when MSgg (surfactin shock) medium (containing 20μM surfactin) flowed. Then the fluorescence dropped back but held a higher level until 560 min. After 560 min, the fluorescence decreased and kept a lower level.

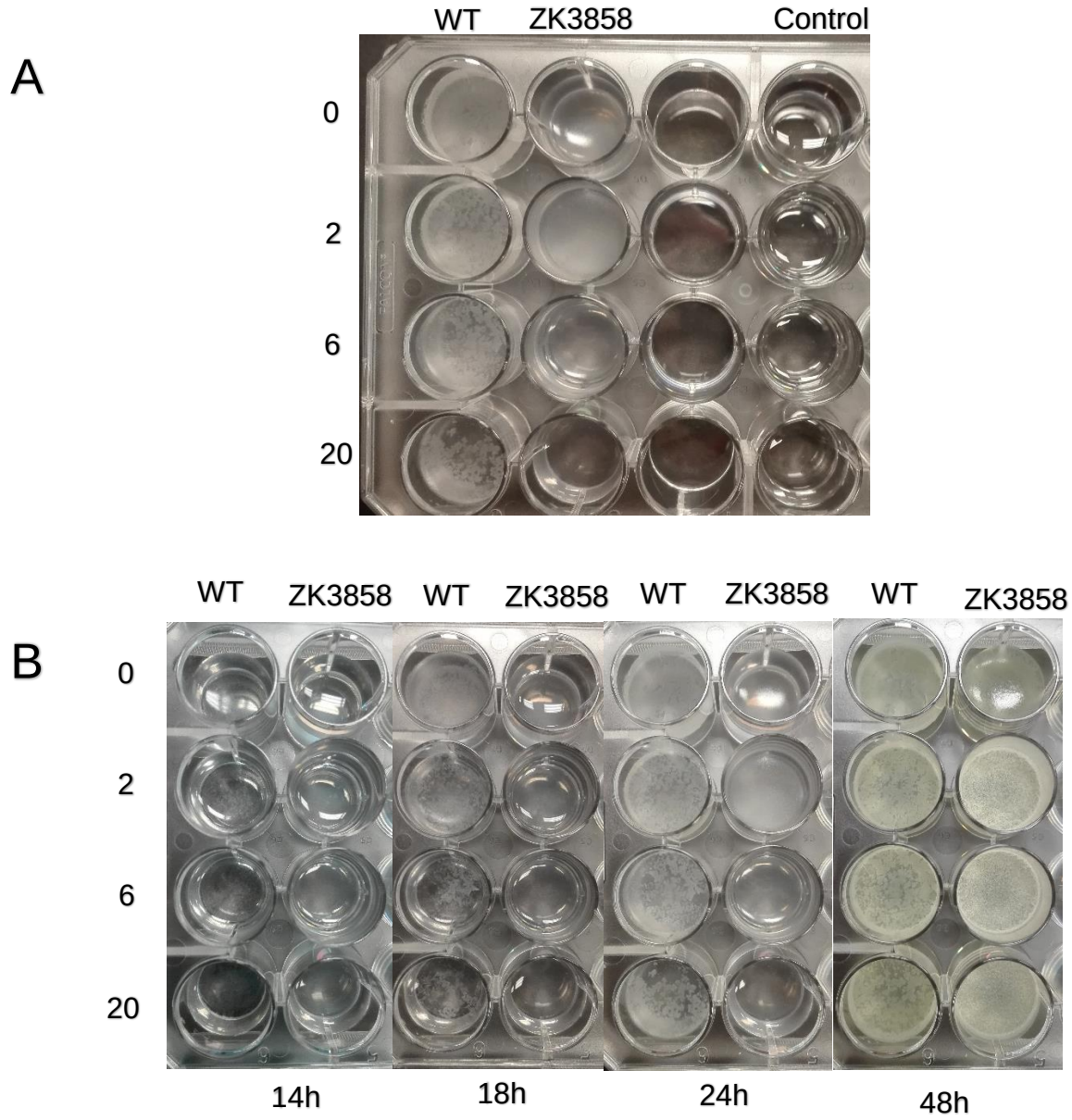


Figure 3-7. srfAA mutant examination. A) WT and srfAA mutant were grown in MSgg medium containing different concentrations of surfactin in 24 well plate. The numbers at left give the concentration of surfactin in micromolar units. B) The WT and mutant growth conditions of different time points.

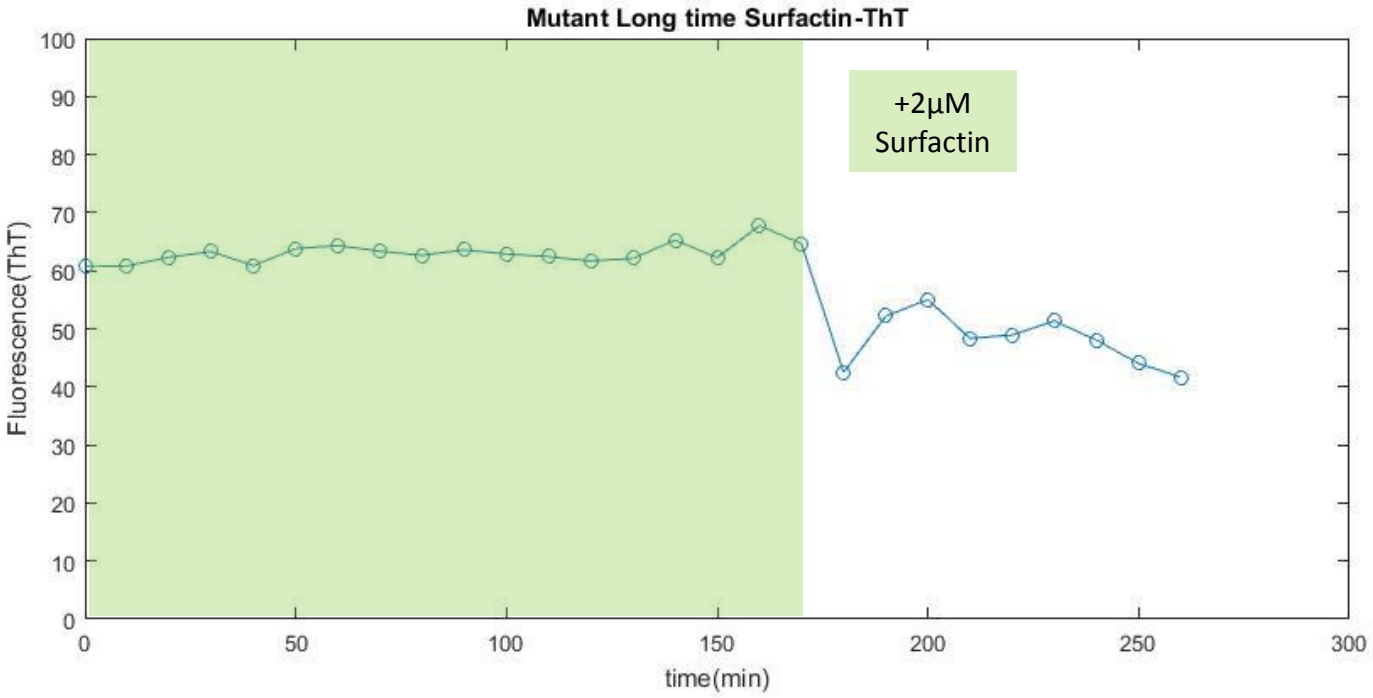


Figure 3-8. Low concentration surfactin can hold the membrane potential of srfAA mutant more negative. The fluorescence of srfAA mutant decreased when normal MSgg medium flowed. This showed the low concentration surfactin held the membrane potential of srfAA mutant more negative.

CHAPTER 4
DISCUSSION

4.1 Remarks on This Project

In this project, fluorescent dyes were used to report the membrane potential of *Bacillus subtilis* biofilm. The dyes are positively charged and can enter cell membranes. The inner membrane of *Bacillus subtilis* biofilm is at a negative electrical potential with respect to the extracellular space and therefore will retain dyes. When the membrane has more negative electrical potential, it will contain a higher concentration of dyes and so have brighter fluorescence. Here, we successfully verified that ThT and $DiSC_3(5)$ could report the membrane potential of *Bacillus subtilis* biofilm in our laboratory.

To test the influence of surfactin on the membrane potential of *Bacillus subtilis* biofilm, extra surfactin was added to the MSgg medium. The result demonstrated that surfactin could decrease the membrane potential of *Bacillus subtilis* biofilm.

Moreover, APG-4 was used to verify that surfactin can cause potassium leakage across the cell membrane. This result demonstrated that surfactin renders the membrane potential of *Bacillus subtilis* biofilm more negative by causing potassium leakage.

The data of different surfactin concentrations in these two experiments indicated that the effect of surfactin is concentration-related. This can be a good point for future work.

The membrane potential oscillation of *Bacillus subtilis* biofilm was first reported in 2015 (Prindle et al., 2015). We successfully repeated it in the microfluidic device designed and made by our laboratory (Son et al., 2014). Meanwhile, the phenomenon

was also repeated in the commercial microfluidic channel, ibidi µ-Slide III 3in1, which has much larger space than our microfluidic device.

To test the influence of surfactin on *Bacillus subtilis* biofilm formation, WT and srfAA mutant ZK3858 were cultured in MSgg medium containing different concentrations of surfactin. In agreement with the previous study, the result successfully demonstrated that surfactin is very important in *Bacillus subtilis* biofilm formation. Surprisingly we found that low extra surfactin concentration may promote the formation of *Bacillus subtilis* biofilm while high extra surfactin concentration may prohibit *Bacillus subtilis* biofilm formation. This indicated that surfactin might play a dual role in *Bacillus subtilis* biofilm formation.

The effect of surfactin on the membrane potential of *Bacillus subtilis* biofilm at long times was tested by flowing with MSgg (surfactin shock) medium (containing 20µM surfactin). The data surprisingly showed that the high concentration surfactin eventually made the membrane potential of *Bacillus subtilis* biofilm more positive. The data on the effect of low concentration surfactin on the membrane potential of the srfAA mutant at long times showed that low concentration of surfactin can help the mutant to hold a more negative potential. Taking into account the growth conditions with different surfactin concentrations, these results indicate that biofilm formation is related to the overall membrane potential. The more negative membrane potential can promote the biofilm formation of *Bacillus subtilis*; while the more positive membrane potential can prohibit it. If this indication can be verified, then we could further investigate the mechanism behind it, e.g. what genetic events related to biofilm formation are involved

in different membrane potentials or whether the different membrane potential will affect protein activities that can affect biofilm formation.

4.2 Future Work

To further understand the membrane potential in biofilm, there are many things to do.

First, a method to report biofilm membrane potential directly is still needed. Using fluorescent dyes to report membrane potential has many limitations. Fluorescent dye is only a reporter, and it cannot directly indicate the value of the membrane potential. Fluorescent dyes also have frequency limitations. When it comes to high-frequency changes, the fluorescent may not be considered to be reliable. Fluorescent dyes are also subject to photo bleaching, which may make the results less precise. Moreover, fluorescent dyes are extra components, which may have an unknown influence on the biofilm. Thus, finding a way to detect membrane potential of biofilm directly seems to be very important. Microelectrode arrays (MEA) have good potential to do this. An MEA is a platform that contains an array of small-size electrodes. The MEA method is widely used in stimulating and recording bioelectricity in cell and tissue cultures (Stett et al., 2003). Using MEA could directly detect the voltage of biofilm and record the change of membrane potential. More importantly, MEA method will not have the disadvantages of fluorescent dye and can record reliable real-time data of membrane potential.

Second, further investigating the effect of surfactin is very important, especially the relationship between surfactin effect and surfactin concentration. In this project, the results indicate that low extra surfactin concentration may promote the formation of *Bacillus subtilis* biofilm while high extra surfactin concentration may prohibit the formation of *Bacillus subtilis* biofilm. Meanwhile, high extra surfactin concentration

seems to increase membrane potential of *Bacillus subtilis* biofilm more than low surfactin concentration. These results indicated that the effect of surfactin might be correlated with its concentration. Further investigation of the relationship between surfactin effect and surfactin concentration can help us to understand more about the role that surfactin plays in formation and membrane potential of *Bacillus subtilis* biofilm. With more data, a model for the relationship between surfactin and the membrane potential could be generated. Considering that surfactin is a quorum sensing factor in *Bacillus subtilis,* the model might help us to find a feedback loop in the surfactin quorum sensing system.

Third, understanding possible genetic mechanisms corresponding to these processes is another direction for understanding the relationship between biofilm formation and biofilm membrane potential. There are many genetic activities related to the biofilm formation, such as *eps*A-O operon activities in *Bacillus subtilis* biofilm formation. Regulation of these genes is an essential part of biofilm formation. Further studies about how membrane potential can influence gene regulation could lead us to understand more about the relationship between biofilm formation and biofilm membrane potential. With these understandings, all the related genes could be linked together to generate a model for the genetic network.

Moreover, further understanding about the surfactin effect could be a key to deciphering the secret of communication among different bacteria species. The previous study showed that nystatin from *Streptomyces noursei* could partly replace the function of surfactin and induced the formation of *Bacillus subtilis* biofilm (López et al., 2009). Investigating how nystatin induce the formation of the formation of *Bacillus*

subtilis biofilm could help us to learn more about communication between these two bacteria and provide insight into chemical communication among the Gram-positive bacteria.

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BIOGRAPHICAL SKETCH

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