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Development of an Escherichia coli Biofilm Model on Transwell®

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Escherichia coli biofilm, reported to be produced in the human intestine causing a significant health risk, was successfully grown on transwell®. This biofilm layer was identified by crystal violet staining and prepared for the *in vitro E. coli* biofilm system which can be used to screen for inhibitors. The biofilm formation did not show a change in transepithelial electrical resistance values. Furthermore, rhodamine 123 staining showed that the dye did not pass through the membrane once biofilm was formed.

Key Words : *Escherichia coli*, biofilm, rhodamine 123, crystal violet, transepithelial electrical resistance

Introduction

Microorganisms are often organized in masses which can be arranged in the planktonic state or in a state called biofilm [Marti, 2011]. Biofilm is formed when microorganisms attached on surfaces secrete extracellular polymers. Bacterial biofilm are embedded in a matrix and are organized in microcolonies in which bacteria are developed into organized communities [Costerton et al., 1999].

Biofilms are of great importance in regard to the development of infectious diseases [Folwaczny and Hickel, 2003]. The usage of synthetic material for medical technology comprises a risk of infection because bacteria can sessile on the surface of the device and build biofilms [Stickler et al., 1998]. In hospitals, infections related to microbial biofilm are partly attributable to the usage of implants or any other medical device which include endoscopes, artificial cardiac valve, articular implants or venous catheter [Khoury et al., 1992; Marrie et al., 1982]. In recent years, certain microbes have been found to colonize in the human body, form biofilms and cause antibiotic resistance which can cause mortality in patients as in the case of *Pseudomonas aeruginosa* infection in cystic fibrosis lungs [Sriramulu et al., 2005].

E. coli have been reported to produce biofilms in the human intestine causing a significant risk to health [Houdt and Michiels, 2005]. Among the various strains, *E. coli* O157:H7 is recognized as an enteric pathogen and has been characterized in several laboratories as causing self-limiting diarrhea, hemorrhagic colitis, hemolytic uremic syndrome and thrombotic thrombocytopenic purpura in children and others as susceptible groups of individuals. *E. coli* O157:H7 has been reported to form biofilm on stainless steel and various kinds of surfaces [Houdt and Michiels, 2005]. Like most bacterial biofilms, *E. coli* biofilm produces exopolysaccharides which can provide a physical barrier to protect the cells against environmental stress [Ryu and Beuchat, 2005].

Because of the important health implications these mi-

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crobial biofilms have, it is crucial that means to remove or inhibit these biofilms are developed. For this, the nature of biofilm need to be understood after which an experimental model set up for a screening system to be established. One of the significant findings on the mechanism of biofilm formation is called quorum sensing. Quorum sensing describes a cell-to-cell communication procedure of microorganisms which sense the density of surrounding cells by usage of extra cellular signals. At sufficient population densities the concentration of the extra cellular signal reaches a certain threshold to initiate gene activation giving rise to phenotypes as bioluminescence or biofilm formation [Zhang et al., 1993].

Herein we report the results of an initial study for the characterization of the biofilm of *E. coli* grown on Transwell®. The aim was to successfully grow the biofilm on a membrane like surface and to characterize the nature of the biofilm by physicochemical and biological means.

Materials and Methods

Bacterial strains and culture conditions

The *E. coli* W3110 and Agrobacterium radiobacter have been purchased from the Korean Culture Center while the *E. coli* JM109 has been kindly provided by Professor Jin Hwa Lee(Dongseo University, Busan).

E. coli(W3110 and JM109) was cultured in LB media at 37℃ with shaking and the *Agrobacterium radiobacter* was grown on M8 medium at 30℃. Difco™ LB Broth (Bactec and Dickson Company, U.S.A) was used for the diffusion culture. M8 media [1g tryptone (Bacto™, USA), 2.7g Sodium phosphate, Dibasic, Anhydrous (Yakuri, Japan), 3g potassium phosphate monobasic (ReagentPlus™, ≥99.0%, Sigma, USA), 0.5g sodium chloride (Shinyo, Japan), 120.4mg magnesium sulfate anhydrous (reagent grade, ≥97%, Sigma-Aldrich, USA), 6mg ammonium iron(III) citrate (reagent grade, Sigma-Aldrich, USA), 0.75mg boric acid (Kanto, Japan), 0.5mg cobalt(II) sulfate heptahydrate (〉 99%, Sigma-Aldrich, Switzerland), 0.125mg zinc chloride (reagent grade, ≥98%, Sigma-Aldrich, USA), 0.075mg manganese(II) chloride tetrahydrate (ReagentPlus®, Sigma-Aldrich, USA), 0.075mg sodium molybdate (98+%, Sigma-Aldrich, USA), 0.05mg nickel(II) chloride (Sigma-Aldrich, USA), 0.025mg copper(II) chloride dehydrate (reagent grade, Sigma-Aldrich, China) in 100ml distilled water] was used for cell growth and for forming the biofilm.

Biofilm formation on Transwell®

Transwell® (12mm Diameter Inserts, 3.0mm pore size, Tissue culture treated, Polyester membrane from Corning Inc., NY, U.S.A) was used for forming the *E. coli* and *A. radiobacter* biofilm. The outer (① in Fig. 1) and inner (② in Fig. 1) container were filled with 1.3 ml and 0.3 ml M8 media, respectively. The inner container was innoculated with 50㎕ of the *E. coli* over night culture. The control well did not contain any bacterial culture. The wells were cultured for 10 days at 37℃ and biofilm growth was determined at the membrane of the transwell (blue area in Fig. 1).

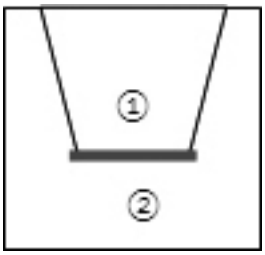

Figure 1. Illustration of the trans-section of the biofilm growing Transwell®

Measurement of TEER value

The transepithelial electrical resistance (TEER) was measured by reading the values of an EVOM voltohmmeter device (WPI, Sarasota, FL) and corrected by subtracting the background due to the blank transwell® inserts and medi-

um as described in Lin *et al.*, (2005). Contamination problems with the electrode was cleared with 4% NaOCl for 3 min and rinsing with H_2O. *Agrobacterium radiobacter*, a strain known to exhibit quorum sensing but not forming a biofilm was used for comparison.

Crystal Violet staining

Crystal Violet (Sigma, China) was used to verify the formation of biofilm on the transwell®. Distilled water (Milli-Q, millipore, 18mΩ) was used as solvent and staining was done as previously described (O'Toole, 2011). The stained biofilm was compared in color darkness as well as under an electric microscope.

Rhodamine staining

Rhodamine solution (Rhodamin 123 powder 10 mg dissolved in ethanol 5 ml) was prepared and used to stain the bacteria bioflm as described by Johnson *et al.*, (1980). Prior to the staining, a blank experiment on transwell® without the biofilm was conducted where the eluting rhodamine was analyzed by UV spectrophotometer (UV-1650PC, Shimadzu, Japan) over a 300 minute time interval. NaCl solution (0.85%, Shinyo, Japan) was used for washing out the planktonic bacteria to rinse the biofilm as well as for refilling and washing throughout the experiment. Sampling of the eluent, after 2 ml of rhodamine was added to the biofilm, was done after 30 min, 60 min, 120 min, 180 min, 240 min, and 300 min by taking 100 μl samples, refilling with the same volume of normal saline and measuring the absorbance at 500 nm.

Results

Bacterial growth of *E. coli* JM109 and *E. coli* W3110

The strains *E. coli* JM109 and *E. coli* W3110 showed identical growth behavior and were considered the same strain throughout the study even though they were obtained by different sources. Thus some results were obtained of the former and some of the latter strain.

Biofilm formation of Transwell®

As shown in Fig. 2, a turbid growth due to the growth of planktonic cells was identified compared to the control. The left shows the control without any microbe and the right the *E. coli* W3110 biofilm.

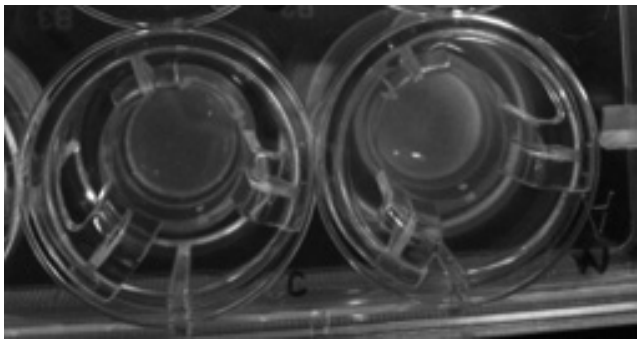

Figure 2. *E. coli* biofilm growth on transwell. Left is control without any microbe and right shows the *E. coli* W3110 biofilm.

Measurement of TEER value

Transwell® inserts were inoculated from an overnight planktonic culture and the electrical resistance between the outer and inner inlet was measured at given time intervals. Fig. 3 shows that no difference in TEER value was observed between the control and the *E. coli* biofilm. Moreover, the *A. radiobacter* showed similar TEER values as well. However, results of staining and microscopic observation (Fig. 4, 5) reveal that biofilm did form.

Crystal Violet staining and microscopical observation

The biofilm that has been formed was stained with crystal violet. Compared to the control, darker purple coloration was observed for the biofilm (Fig. 4).

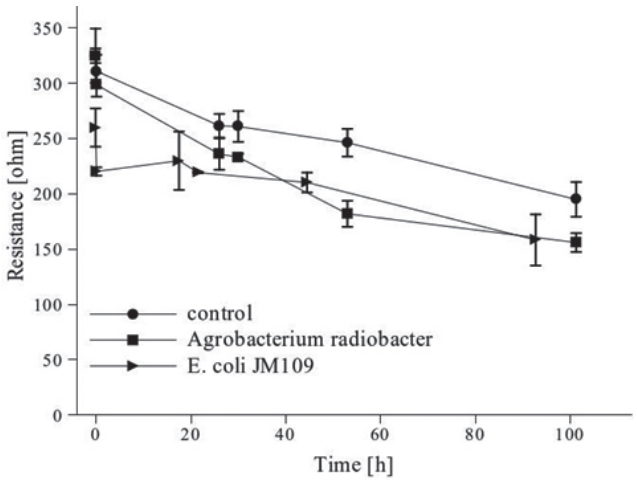

Figure 3. Transwell® inserts were 1:10 inoculated from an overnight planktonic culture and the electrical resistance between the outer and inner inlet was measured at given time intervals. Data points represent the mean ± sd of 3 independent samples.

When these biofilm were carefully removed and observed under a microscope, a tighter growth was observed after 10 days of biofilm growth on transwell® membranes and crystal violet staining (Fig. 5).

Rhodamine staining

Prior to biofilm growth, rhodamine 123 solution was passed through the transwell® and eluent outside the well collected every 30 minutes and measured by UV. As shown in Fig. 6, a steady increase in OD of rhodamine 123 measurement was observed over 300 minutes. However, when rhodamine 123 was passed through the transwell® with biofilm formation, a steady OD without a significant increase over time was observed.

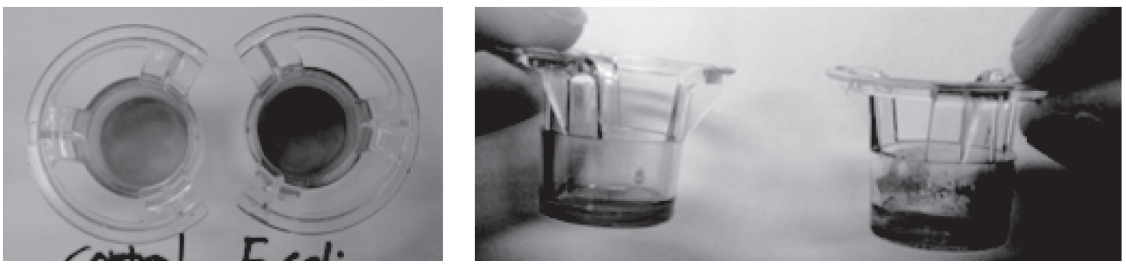

Figure 4. Crystal violet staining of the E.coli biofilm in transwell®; (A) left is control and right is the *E. coli* biofilm stained to a darker violet color; (B) The side view of the biofilm formation is shown.

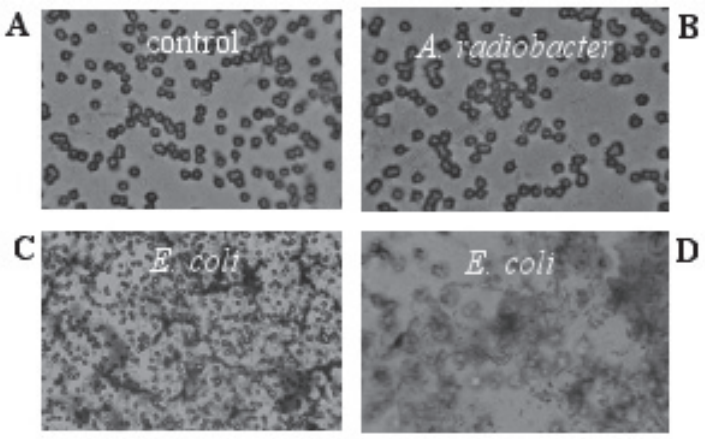

Figure 5. Microscopical observation of the biofilm; After 10 days of biofilm growth transwell® membranes were stained by crystal violet and analyzed by microscopic examination. A. control, without cell growth (100 x enlargement): B. A.radiobacter (100 x enlargement): C. E.coli JM109 (40 x enlargement): D. E.coli JM109 (100 x enlargement)

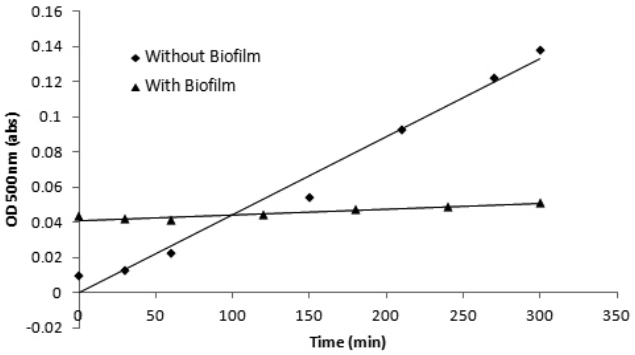

Figure 6. OD change over time of rhodamine blank (square) and rhodamine measurement after biofilm formation (triangle). The y axis is OD at 500 nm and x axis is time in minutes.

Discussions

Biofilm normally forms on the side of the vessels as a ring. However, making use of the transwell insert system brought about a successful formation of the biofilm on the surface of the membrane insert. A turbid growth due to the growth of planktonic cells was identified compared to the control as shown in Figure 2. When this biofilm was subject to transepithelial electrical resistance measurement, no difference in TEER value was observed between the control and the *E. coli* biofilm. Moreover, the *A. radiobacter* showed similar TEER values as well. Transepithelial electrical resistance measurement is usually done to determine the formation of tight junctions in cell culture studies. High TEER value implies the formation of a tight junction that is an impermeable junction on the inner part of the well. Thus, at first sight, one could doubt the formation of biofilm of the *E. coli* and *A. radiobacter*. However, as shown in the results of staining and microscopic observation (Figure 4, 5), formation of biofilm was confirmed. Yet the nature of the biofilm seems to not be able to alter the electrical resistance. Obviously, the biofilm cells neither form tight junctions nor ion transport through the biofilm that is inhibited altering the electrical resistance. Further studies are under way to verify the nature of the microbial contact within the biofilm.

When the biofilm that has been formed was stained with crystal violet, darker purple coloration was observed compared to the control (Figure 4). Moreover, when these biofilm were carefully removed and observed under a microscope, a tighter growth was observed for the *E. coli* strains (Figure 5). No bacterial cells were observed for the control and the *A. radiobacter* samples. Only the membrane pores are visible on the photographs. The *A. radiobacter* biofilm is usually very loose. Probably the biofilm on the membrane has been incidentally removed during the washing step. However, for both *E. coli* membranes, clustered rod like bacterial cells could have been observed that clearly indicate the formation of *E. coli* biofilms.

Rhodamine 123 is known to bind to the mitochondria of cells to result in staining. The fact that a steady OD was observed upon elution of rhodamine 123 indicates that rhodamine 123 did not elute through the membrane once the biofilm formed. It is possible that all the rhodamine 123 added was bound to the cells of the biofilm. Another possibility is that the biofilm formation did not allow passage of the rhodamine 123 dye and remained adsorbed onto the biofilm. Further studies are under way to determine the whereabouts of the rhodamine 123 dye.

In conclusion, *E. coli* biofilm was successfully formed on transwell® as shown by the crystal violet staining. Although the biofilm cells did not form tight junctions enough to bring about a change in the TEER values, rhodamine 123 was not detected in the eluent outside the transwell®. Although further studies are still necessary to fully characterize the *E. coli* biofilms, the significance of this study is in the initial setup of an in vitro biofilm layer which can be utilized for a screening system of biofilm inhibitors.

Acknowledgement

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