Report as of FY2009 for 2009OH89B: "MONITORING THE ROLE OF BIOFILM BIOPOLYMERS AGAINST DISINFECTANTS IN WATER DISTRIBUTION SYSTEMS"

Publications

- Articles in Refereed Scientific Journals:
  - Z. Xue, Y. Seo, W. Panmanee, and D. J. Hassett, Impact of the Pseudomonas aeruginosa Exopolysaccharide Alginate on Bacterial Inactivation Kinetics by Model Disinfectants, Applied Environmental Microbiology (In Review).
- Conference Proceedings:
  - Z. Xue, Y. Seo, Susceptibility of biofilm to disinfectants in the presence of disinfectant-demanding substrate, 240th American chemical society national meeting, Boston, USA, August, 2010 (accepted for oral presentation).
  - Z. Xue, Y. Seo, Role of Extracellular Polymeric Substances of Biofilm on Bacteria Inactivation, 239th American chemical society national meeting, San Francisco, USA, March, 2010.
  - Z. Xue, Y. Seo, The Role of Extracellular Polymeric Substances of Biofilm on Bacteria Inactivation, AWWA WQTC, 2009.

Report Follows

Monitoring the Role of Biofilm Biopolymers against Disinfectants in Water Distribution Systems

Youngwoo (Young) Seo

1. PROBLEM AND RESEARCH OBJECTIVES

Biofilm formations in water distribution systems are ubiquitous. Reports from many water utilities in the US including utilities in Ohio have shown that biofilms survive in water distribution systems despite the continuing presence of disinfectants (Tuovinen and Hsu, 1982; LeChevallier et al. 1996) is great concern about the resistance of biofilms against disinfectants, the inactivation kinetics of biofilms are not well understood, especially compared to the inactivation kinetics of suspended microbial cultures (AWWA, 2007). There is not enough information for water utilities to assess and optimize disinfectant dosage to control biofilms in water distribution systems (AWWA, 2005).

One reason for this could be the complexity of biofilm EPS (Momba et al. 2000; Stewart, 2002; Stewart et al 2002). More than 80% of biofilm is comprised of EPS (Characklis and Marshall, 1990), and it is believed that these structures provide protective barriers for microorganisms (Sibille 1998; Hughes et al. 1998). However, there is still a significant knowledge gap, especially concerning the reaction kinetics of EPS with disinfectants. To date, the role of extracelluar polymeric substances (EPS) as a protective barrier against disinfectants has not been quantitatively analyzed, even though the simultaneous interaction between disinfectants and EPS is known to lead to the transport limitation of disinfectants into biofilms. Previous studies mostly focused on retarded or limited transport of disinfectants without considering reactive sites and reaction kinetics of biofilm EPS (Stewart, 2002; Stewart et al. 2002).

The principal research objective in this proposal is to monitor the role of both cell-bond EPS and biofilm EPS as protective barriers against disinfectants in water distribution systems. Physical transports of a model disinfectant and its reaction kinetics in biofilm were quantitatively studied using molecular probes and a chlorine sensitive microsensor. The reaction and disinfection kinetics of EPS was elucidated by 1) characterizing EPS components and their reaction kinetics with a model disinfectant; 2) quantifying EPS and viability of biofilm with fluorescently labeled molecular probes under a model disinfectant; 3) monitoring the transport limitation of a model disinfectant in biofilm. This study provides fundamental and effective biofilm control strategies in water distribution systems supporting research, education and local water utilities.

2. STATEMENT OF RESULTS OR BENEFITS

Water utilities in Ohio have experienced bacteria growth and biofilm formation in water distribution systems (Tuovinen and Hsu, 1982; Craun and Calderon, 2001), even under the presence of residual chlorine. However, the inactivation of biofilm in

drinking water distribution system is not well understood. One reason for our lack of understanding is strongly correlated to the complexity of biofilm EPS and their role in protecting biofilms from disinfectants. In this proposed study, this knowledge gap will be addressed. The results from the proposed study will enable local water utilities to incorporate biofilm control strategies since the results will aid in the development of effective biofilm control methods with disinfectants

3. MATERIALS/METHODOLOGY

Preparation of Buffer Solutions and Disinfectants

All disinfection experiments were conducted with chlorine demand-free (CDF) buffer (pH=7). CDF buffer was prepared by dissolving 0.54 g of Na_2HPO_4 and 0.88 g of KH_2PO_4 per liter in deionized water. The prepared buffer solution was pre-reacted with chlorine by adding sodium hypochlorite solution and allowed to stand at room temperature for one week, followed by UV light exposure for 48 hours to achieve dechlorination. When the chlorine concentration was lower than 0.01 mg/l, the buffer solution was considered to be chlorine demand free (Engelbrecht et al., 1980).

Chlorine stock solutions were prepared with Clorox bleach (The Clorox Co., Oakland, CA) and concentration were determined by the N, N-diethyl-*p*-phenylenediamine (PDP) method (Engelbrecht et al., 1980). The chlorine stock solution was diluted to 0.5 mg/l with CDF buffer solution immediately preceding the inactivation experiments. Stock chloramine solution was prepared immediately before each experiment by combining solutions of sodium hypochlorite and ammonium chloride in a 4:1 ratio (chlorine-to-ammonia-nitrogen mass ratio). To obtain the highest monochloramine yield and minimize ammonia volatilization, both solutions were pre-adjusted to a pH of 8.3. Stock chloramine solutions were diluted to a target concentration of 2 mg/l with the CDF buffer. Stock chlorine dioxide solutions were prepared from sodium chlorite ($NaClO_2$, Selective Micro Technologies, Beverly, MA, USA) (Jang et al., 2006). For inactivation tests, the ClO_2 stock solution was diluted to 0.5 mg/l before each test (Aieta et al., 1986). The concentration of all three disinfectants was selected based on residual disinfectant concentration in water distribution systems (USEPA, 1999) and measured using a DR/2700 spectrophotometer (HACH Company, Loveland, CO, USA).

Batch Experiments of planktonic cells

In this study, three *P. aeruginosa* strains were employed. The first was wild-type strain PAO1 and a well-characterized DNA sequenced strain. Two isogenic mutants of strain PAO1 were also used, (i) *algT(U)* encoding the alternative extracytoplasmic sigma factor AlgT(U) and (ii), *mucA*, encoding a cytoplasmic membrane-bound anti-sigma factor that produces copious quantities of the exopolysaccharide alginate. With these strains in hand, we examined how differences in the relative amount of EPS affected the efficacy of three common disinfectants (chlorine, chloramines, and

chlorine dioxide).

All batch experiments were performed in 250 ml amber glass bottles (Fisher Scientific, Itasca, IL) at room temperature for planktonic cells. Three amber-glass bottles were used as parallel reactors. The first bottle, containing bacterial suspension and the CDF buffer solution without disinfectant, served as a control reactor. The other two bottles contained only bacterial suspension and disinfectant solution. Experiment setup is shown in Fig 1. Microbial inactivation tests with disinfectants were performed and disinfectant decay and bacteria survival were measured simultaneously. Enumerations of viable microbial cells were performed using the heterotrophic plate count method. Serial dilutions were conducted in CDF buffer solution containing $Na_2S_2O_3$ (1 mmol/l final concentration) to quench residual disinfectants, followed by spreading 0.1 ml aliquots onto R2A media plates (Difco Laboratories, Detroit, MI). All plates were incubated at 37°C for 24 hours prior to enumeration of colony forming units (CFU).

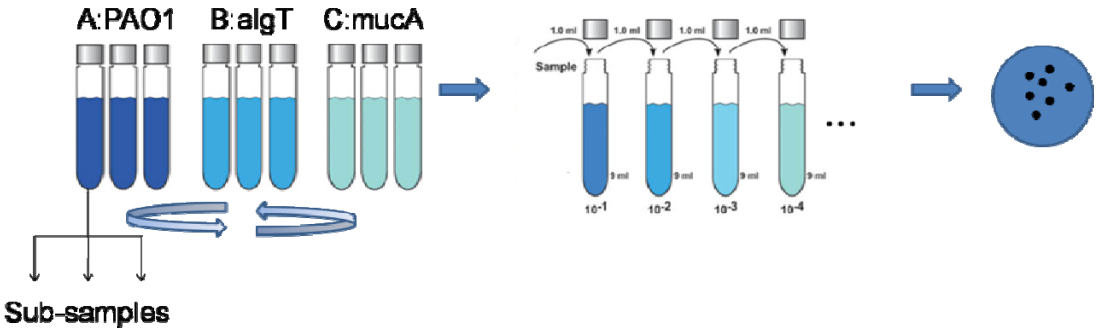

Figure 1: Batch experiment setup

Biofilm experiment

Two carboys were used as medium feeding and chlorine supply reservoirs respectively. A 0.02 strength LB broth was used as a medium to create nutrient limiting growth conditions mimicking low-carbon environment as in drinking water distribution systems. All feeds to reactors were delivered using a multichannel peristaltic pump (ISMATEC, Glattbrugg, Switzerland) and silicone tubing (Masterflex, Vernon Hills, IL). The flow cell system is shown in Fig. 2. Flow cells, tubing and solutions were sterilized at the start of each experiment. Operation and sampling of the flow cells followed aseptic technique throughout the experiments.

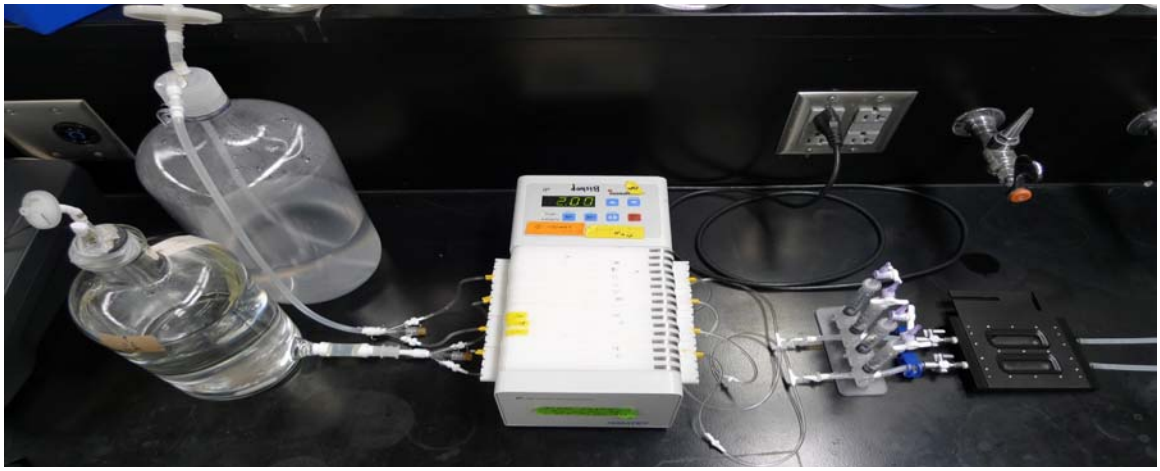

Figure 2: flow cell system setup

Biofilms were grown in continuous-culture flow cells (channel dimensions, 1.6 by 12.7 by 47.5 mm; flow rate, 0.2 ml/min) at room temperature. The flow cell contained a standard glass microscope slide on one side and a glass cover slip on the other side. Flow rate of the flow cells simulated laminar flow with an average flow velocity of 0.16 mm/s throughout each experiment. Under this flow condition, a residence time that improved biofilm formation was achieved. Channels were inoculated with bacterial suspension and incubated statically for 1 h at room temperature for initial bacterial attachment. After 1 hour, flow rate was gradually increased to 0.2 ml/min. For each experiment, the two channels in one flow cell were operated in parallel under identical conditions, the only exception being that one channel received chlorine and the other served as a non-chlorinated control.

Bacteria Cell Staining

The LIVE/DEAD Bacterial Viability Kit (*Bac*Light, InVitrogen) was applied to estimate both viable and total counts of bacteria in disinfectant treated samples. The *Bac*Light LIVE/DEAD stain is composed of two nucleic acid-binding stains: SYTO 9E and propidium iodide (PI). SYTO 9E penetrates all bacterial membranes and stains the cells green, while PI only penetrates cells with damaged membranes, while the combination of the two stains stoichiometrically produces red fluorescing cells. Total (red and green) and viable (green) cells can hence be counted simultaneously (Boulos et al., 1999). Stained solution was filtered through black polycarbonate filters for fluorescence microscopic imaging. Fluorescent images were observed at 480/500 nm for SYTO 9 and 488/617 nm for PI, respectively. For fluorescent stained cell counting, an Olympus fluorescent microscope with an 100X oil immersion objective and a TCS SP5 multi-photon laser scanning confocal microscope (Leica Microsystems) were used. Images were processed by CellCounter (Heracle Software), CellAnalyst (AssaySoft, Inc.) and COMSTAT.

EPS Extraction

All extraction procedures were performed on three separate 50 ml samples from an initial 200 ml bacterial culture. The modified EDTA extraction method described by Brown and Lester (1980) was employed (Brown et al., 1980). In this method, 100 ml of 2% EDTA (tetrasodium salt) was added to 100 ml of each culture suspension and shaken for 3 h at 4°C. High speed votex was applied both at the initial mixing of EDTA and culture suspension and after 3 h interaction. The samples were then centrifuged at 14,000 x g for 20 min at 4°C. The supernatant was analyzed to quantify EPS composition.

EPS Characterization

Total protein and polysaccharide was measured using standard colorimetric techniques. Protein concentrations were determined using the modified Lowry Protein Assay Kit (Pierce Biotechnology, Rockford, IL) with bovine serum albumin (BSA) as standard. Polysaccharide concentration was measured using the phenol-sulfuric acid method using glucose as standard (Jost Wingender, 1999).

Principle Findings and Significance

Both chlorine and chlorine dioxide are very effective disinfectants. However, these two disinfectants were heavily consumed during the initial period of inactivation. Limited by such short inactivation times and high reactivity, no significant disinfectant residual differences were observed. Chloramine was found to be a slow-acting disinfectant for the three strains. Thirty minutes of chloramine exposure were required to achieve 99% inactivation for the three strains in this study. The *mucA* mutant consumed the greatest amount of chloramine, while the *algT(U)* mutant had comparably high levels of residual chloramine. For chloramine inactivation, disinfectant residual is in inverse proportion to cell bound EPS amount. In other words, the more EPS bound to bacterial cells the more disinfectants were consumed. The result of the chloramine consumption study indicated that cell-bound EPS interacted with disinfectant during the inactivation process and part of the disinfectant consumption could be attributed to cell bound EPS. The transport limitation of disinfectant in biofilm was also monitored by microelectrode.

The results in this study indicate that the higher EPS production yields higher survival ratio and viability rate, which was confirmed by both heterogeneity plate counting and Live/Dead staining results. The mucA22 mutant with higher EPS production had a proportionally greater survival ratio and viability rate and more variation in protein and polysaccharide functional groups by interaction with disinfectants. The mucA22 mutant also had a prolonged lag time when interacting with the less reactive disinfectant chloramine.

For low disinfectants concentrations as used in water distribution system, key factor of chlorination bactericidal is not extensive membrane damage but functional

group deformation in bacteria membrane, which lead to membrane permeabilization. The acidic polysaccharide alginate, representative component in EPS of P. aeruginosa, has strong deformation after inactivation, which confirms that cell-bound EPS have high reactivity with the disinfectant used in this study. The combined results support that cell bound EPS consume disinfectant, retard bacterial membrane permeabilization, and thus decrease the susceptibility of bacteria.

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