A PRELIMINARY INVESTIGATION OF *ESCHERICHIA COLI* K12 BIOFILM INHIBITION ON AN ANTIMICROBIAL POLYSILOXANE COATING USING WHOLE TRANSCRIPTOME PROFILING

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MASTER OF SCIENCE

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ABSTRACT

Whole transcriptome profiling was examined in *E. coli* K12 when cultured on the surface of a pure polysiloxane coating (Sil) and a polysiloxane coating containing a tethered quaternary ammonium compound (QSil) shown to inhibit biofilm formation. An optimized protocol was developed for isolating high quality RNA from the surface of these coatings prepared in multi-well plates. DNA microarray data obtained from the Sil and QSil coatings revealed that 222 genes were differentially expressed between these two surfaces by a factor of at least 2-fold and with a 90% level of confidence. Several genes of the *lsr* operon, which encode the various components of the AI-2 based quorum sensing system, were repressed on the QSil coating surface. The QSil coatings ability to effectively interfere with the AI-2 based quorum sensing system was most likely the primary factor that contributed to the impairment of *E. coli* K12 biofilm formation on that surface.

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INTRODUCTION

In recent years, there has been a fundamental paradigm shift in the way microbiologists view the interactions and existence of bacteria residing in natural settings. What was once thought to be a life of complete solitude has now evolved into an understanding that bacteria cohabitate and communicate with one another in complex multi-cellular communities known as biofilms. The establishment of such communities enables the occupants to harness and maximize the oftentimes limited resources found in their immediate surroundings. Furthermore, the biofilm matrix that is produced, composed primarily of proteins, polysaccharides, and nucleic acids, serves as a protective barrier to shield the resident microorganisms from adverse environmental conditions.

Although biofilms are quite beneficial for bacteria, they are in most instances a nuisance and cause for concern for humans. This is especially true in the context of healthcare and food processing settings where the development of microbial biofilms on surfaces located in these environments can serve as persistent source of infection and disease. To complicate matters further, biofilms are incredibly difficult to eradicate using conventional cleaning and sterilization techniques once they have been established. One of the most promising strategies for mitigating biofilm development on these surfaces has been to apply an antimicrobial or anti-infective coating to thwart bacterial attachment and growth. Several classes of antimicrobial coatings have been developed to date with the most effective technologies employing silver salts or quaternary ammonium compounds (QACs).

Although effective antimicrobial coating technologies have indeed been developed, the mechanisms or modes of activity are in most instances, poorly understood. Understanding how specific coating technologies interact and mitigate the attachment, growth or biofilm formation

of microorganisms can potentially lead to the design and development of advanced materials that offer superior performance, such as long lasting, broad-spectrum antimicrobial activity. One potential approach to elucidate the mechanistic aspects of a particular coating technology is to examine how direct contact and interaction of a microorganism with the coating surface influences its gene expression. Biofilm formation has been shown to occur through a coordinated series of molecular events that is tightly controlled and regulated. By monitoring how a coating surface modulates gene expression, valuable insight can be obtained regarding the cellular processes that are most crucial for biofilm development and maturation. This information can then be leveraged to generate multi-functional or smart coatings where the surface chemistry has been designed to specifically interfere with these key cellular processes to prevent biofilm from developing across a broad-spectrum of microorganisms.

The long term goal of this research project is to elucidate the genetic determinants of microbial biofilm inhibition on contact-active antimicrobial coatings being developed at NDSU. The information garnered from these studies can potentially lead to the design of advanced and more effective coating technologies in the future. In the present study, a preliminary investigation of gene expression using whole transcriptome profiling (i.e., DNA microarrays) was conducted with *E. coli* K12 cultured on the surface of an antimicrobial polysiloxane coating containing a covalently attached QAC. The results of this study will enable us to determine the feasibility of using this type of approach to gain a mechanistic understanding of how antimicrobial coating technologies mitigate bacterial biofilm formation.

OBJECTIVES

The major objectives of this research project were:

1) Optimize *E. coli* K12 biofilm growth in multi-well plates.
2) Determine if *E. coli* K12 biofilm inhibition occurs on the QSil coating surface.
3) Optimize the process for isolating high quality RNA from *E. coli* K12 cultured on the surface of coatings prepared in multi-well plates.
4) Assess differential gene expression in *E. coli* K12 recovered from the surface of the QSil and Sil coating using a DNA microarray.
5) Identify differentially expressed gene sets from the preliminary DNA microarray data and postulate how their differentially expression on the QSil coating may have resulted in *E. coli* K12 biofilm inhibition.

LITERATURE REVIEW

Bacterial Biofilms

It is now well accepted that most microorganisms reside in their particular environmental niche as a part of a multi-cellular community known as a biofilm. In general terms, a biofilm is defined as a consortium of surface-associated microorganisms that are encased in an extracellular polymeric substance (EPS) matrix that they themselves generate (16). In reality, this definition is a dramatic oversimplification of a highly dynamic and complex microbial mode of life.

To begin, microbial biofilms are often composed of more than just one organism and can include a combination of fungi, microalgae, viruses, protozoa and bacteria, the latter of which tend to predominant in most natural and industrial settings (51). Bacterial biofilms are typically composed of multiple species, although certain environmental settings can foster the development of a single species biofilm, such as those created artificially in the laboratory or encountered in the human body in the context of an implanted medical device (63). Even within a single species bacterial biofilm, the population of individual cells is often highly heterogeneous with regards to key phenotypic characteristics, such as growth and metabolic rates and gene and protein expression profiles (24). This phenotypic heterogeneity enables the residents of these biofilm communities to setup and maintain intricate relationships to maximize the utilization of oftentimes limited resources and adequately adapt to the ever changing environmental conditions (15). In fact, biofilms provide an ideal medium through which intra and inter-species communication pathways can be established to regulate, control and maintain the precise microbial interactions required to thrive in their particular environment niche. This bacterial communication process, termed as quorum sensing, is considered to be an essential process for the occupants of the biofilm, as it is used to coordinate the parallel expression of key factors vital

to their ultimate survival, including those related to virulence, resistance to antimicrobial agents or environmental stress and translocation of sub-populations to new environmental locations (22, 79, 82). Furthermore, the relatively high population densities encountered in biofilm communities provides fertile ground for the exchange of genetic traits through a variety of horizontal gene transfer mechanisms (32).

Taking all of these elements into consideration, a more accurate and concise definition of a biofilm would be as follows; a consortium of surface-associated microorganisms encased in a self-produced EPS matrix, which are phenotypically diverse and highly adaptable to their environment.

Biofilm Development

The processes that govern the development of microbial biofilms have largely been investigated through the use of bacteria. As a result, the term "biofilm" will be used in the remaining sections of this document to describe bacterial-based biofilms. Furthermore, although surfaces are usually required for the establishment of biofilms, they can also develop at liquid-air interfaces in the form of microbial mats or pellicles. For sake of simplicity and relevance to the research described in this document, the process of biofilm formation will be focused solely on surfaces.

The prevailing model of biofilm development has been categorized into five distinct or discrete stages; 1) preconditioning of the surface by biomacromolecules 2) primary cell adhesion (reversible) 3) secondary cell adhesion (irreversible) 4) biofilm maturation and 5) biofilm dispersal. It should be pointed out, however, that this developmental model has been generalized and the specific mechanisms and processes involved in each stage is highly dependent upon the species of bacteria and the environment in which the bacteria are located.

Immediately upon immersion in an aqueous environment, most materials adsorb a surface layer of dissolved organic matter (i.e., proteins, polysaccharides, nucleic acids) (52). This preconditioning film can significantly alter the physicochemical properties of a surface and plays an important role in the subsequent attachment of bacteria and other microorganisms (20). For instance, some surfaces can exhibit a high overall net negative charge which can serve as a rather formidable electrostatic barrier to a colonizing bacterium. The adsorption of a preconditioning film can oftentimes neutralize these electrostatically charged materials to a level where bacteria are able to effectively make contact with the surface and explore this environment for suitability of colonization (29).

Once a surface has become appropriately preconditioned, bacteria can approach and make contact by processes such as gravitational diffusion, Brownian motion and flagellar-mediated locomotion (65). The colonizing bacterium's ability to attach to a surface is governed by a number of factors, such as the electrostatic forces indicated above, Van der Waals forces, hydrobphobic/hydrophilic interactions and steric hindrance considerations (4, 26). If these conditions are conducive to colonization, the bacterium undergoes the process of primary, reversible attachment through mechanisms such as simple physical adsorption, hydrogen and/or covalent bonding and hydrophobic interactions between membrane-associated proteins and the materials surface (18, 85). The transition from this initial, reversible attachment to a more permanent, irreversible attachment is often a gradual event and can occur through a number of different processes. In most cases, irreversible attachment is mediated by the expression of specific cell surface organelles and appendages, such as pili, fimbriae and exopolysaccharides, which serve to anchor or "lock" the bacteria onto the surface of the substrate (26).

Shortly after establishing a permanent, stable attachment to a surface, the individual bacteria begin to form small cellular aggregates or "microcolonies" through a combination of cell division, planktonic cell recruitment and surface-associated motility (24, 63). For example, several species of bacteria are known to undergo the process of pili-mediated twitching to transverse across relatively large distances and make contact with neighboring cells (20). These cellular aggregates then establish a strong cohesive bonding network by exuding a diverse array of extracellular adhesive polymers that also serve to reinforce the adhesion of the developing community to the substrates surface. From this point, the maturation phase of the biofilm begins to unfold through the expansion and development of the microcolonies into an extensive and complex three dimensional structure that is primarily composed of EPS (up to 90% of the total organic load) (28). Bacterial microcolonies are heterogeneously distributed through the EPS matrix and separated by vast networks of interstitial voids that allow water to freely diffuse throughout the biofilm architecture for distribution of nutrients and removal of metabolic waste products (27, 80). Depending on the environmental conditions, a fully "mature" biofilm can require as little as 12 hours to several weeks to be established and can reach an overall thickness of several centimeters.

The final stage of biofilm development is often characterized as the dissemination or dispersal of bacterial cells back into the surrounding environment (43). In most instances, individual cells or colonies are liberated from the outer-most layers of the biofilm through controlled processes such as swarming, clumping and surface dispersal (35). It is also been shown that cells can be mechanically removed as a consequence of hydrodynamic flow conditions that serve to either erode, slough or abrade the periphery of the biofilm (43). Bacteria

that are successfully released back into the environment are then free to explore and establish biofilms on new surfaces (26, 51).

Gene Expression in Biofilms

The previous section provided a general overview of the developmental phases that constitute the life cycle of a surface-associated biofilm. In this regard, the transition of bacteria from a planktonic existence to a biofilm mode of life is largely driven or accompanied by the expression and repression of specific sets of genes. A wide variety of bacterial species have been used to investigate the genetic determinants of biofilm formation, including both Gram-negative and Gram-positive strains. The results of these investigations have revealed that differential gene expression during biofilm development is highly dependent upon the species of bacteria and environmental/experimental conditions being studied. As one might expect, this has led to substantial degree of disparity in the published literature regarding the identification of genetic factors that are universally required for biofilm development. Taking this into consideration, and for simplicity and relevance to this research project, differential gene expression during biofilm development will be discussed primarily in the context of *E. coli.*

The Gram-negative bacterium *E. coli* is one of the most widely used microorganisms to investigate the genetic basis of microbial biofilm formation in bacteria. The utilization of this microorganism as a model biofilm system is primarily attributed to the abundance of information that is freely available to the general scientific community, including whole genome sequencing and annotation data. Several recent studies comparing the genetic expression profiles of planktonic vs. biofilm-associated cells (i.e., whole-transcriptome profiling) have clearly demonstrated that there are a considerable number of genes, up to 10% of the genome, involved in *E. coli* biofilm formation (9, 23, 70, 72). The co-ordinate expression of these biofilm-specific

genes or gene sets is highly dependent on the temporal nature of the biofilm developmental process. In fact, a recent DNA microarray investigation revealed that 32% of the *E. coli* genome was differentially expressed by more than 2.5 fold when comparing 4 hr vs. 24 hr biofilms (23). Interestingly, 27% of the genome was induced while only 5% of the genome was repressed in this study. The genes deemed most essential to the temporal development of *E. coli* biofilms include those related to the processes of quorum sensing, stress response, motility, transport, flagella, fimbriae, colanic acid production and sulfur/tryptophan metabolism.

The results of the Domka et al. study (23) indicate that a vast majority of these temporally regulated genes show the highest degree of expression in the later stages of biofilm development (i.e., maturation), including the uptake and export of the quorum sensing signal molecule autoinducer 2 (AI-2) (e.g., *lsrA* and *tqsA*), colanic acid biosynthesis (e.g., *wcaB*), flagellar biosynthesis (e.g., *fliLMQR* and *flgBCEFH*), fimbriae biosynthesis (e.g., *ydeQRS*, *fimZ*, *ppdD* and *sfmF*), phosphate transport (e.g., *phoU* and *phnCJMK*) and generation of heat-shock promoter transcriptional regulator proteins (e.g., *htgA*). In contrast, a number of biofilm-specific genes have been shown to be important in the early stages of biofilm development (i.e., highly expressed), but are subsequently repressed as the biofilm matures. Examples include certain genes associated with fimbriae biosynthesis (e.g., *fimA*), tryptophan synthesis (e.g., *tnaLBA*), dihydroxyacetone-phosphate synthesis (e.g., *gatZ-ABCDR*) and transcriptional regulators of cold-shock proteins (e.g., *cspA* and *cspB*). Interestingly, the authors provide compelling evidence which suggests that genes essential to the processes of fimbriae and flagella biosynthesis are expressed, and hence important, throughout all stages of biofilm development. These results appear to shift the current paradigm of biofilm development where flagella were

previously thought to only be important in the initial stages of cell attachment and early colonization, as concluded through investigations with *Pseudomonas aeruginosa* (71).

The overall importance or impact of temporally regulated genes in the process of biofilm formation (indentified in the studies detailed above) may not always be obvious or clearly understood. In an attempt to elucidate their overall importance/impact on *E. coli* biofilm formation, comparisons have been made between isogenic mutants that are deficient in one or more of the differentially expressed genes to the wild type strain. Niba et al. employed 3985 single-gene mutants of *E. coli* (i.e., Keio collection) to identify specific genes required for biofilm formation (59). The authors of this study identified 110 mutants that showed a reduction in biofilm formation compared to the wild type strain, including mutants deficient in genes for cell surface structures and the cell membrane. The results of this study confirmed previous data generated from DNA microarray investigations of differential gene expression where type 1 fimbraie, flagella and curli are required for *E. coli* biofilm development (68, 83). However, none of the mutants deficient in colanic acid synthesis showed a significant reduction in biofilm formation in this study, even though several genes related to this process (i.e., *wca* operon, *galU*) have been reported to be important in generating the three dimensional architecture of the biofilm (17, 23).

It is important to reiterate that a variety of environmental factors can influence the expression and/or function of biofilm-associated genes, such as temperature, growth medium composition, pH, osmolarity, oxygenation and the presence of antimicrobial agents (26). As one might suspect, the temporal nature of biofilm development is then highly dependent on the specific combination of these environmental variables (i.e., local microenvironment) of the colonizing microorganism(s). In fact, the wide variation in environmental/experimental

conditions employed by investigators to assess the role or function of specific genes during biofilm development can often result in confounding or contradictory conclusions. For example, the role of the *rpoS* gene product (RpoS), the master regulator of gene expression during stationary phase growth, in *E. coli* biofilm formation has been investigated by several research groups. Adams et al. reported a 50% decrease in biofilm cell density for an *rpoS* mutant when cultured in a glucose-limited medium while Schembri and colleagues also observed inhibition of biofilm growth in a glucose-limited minimal medium, with no detectable biofilm evident after 48 hrs of culture (1, 72). In contrast, a 3 to 5 fold increase in biofilm formation was observed when a nutrient rich medium was used (LB broth) and no difference in biofilm growth was detected when colony forming antigen (CFA) medium was used over the period of 24 hours, as compared to the wild type strains (14, 41). This example of contradictory results published in the scientific literature illustrates the difficulty of constructing a universal paradigm for gene expression during biofilm development and highlights the caution one must exercise when comparing results and conclusions among different studies employing differing experimental setups or culture conditions.

The vast majority of studies investigating the genetic determinants of biofilm development have focused on the impact of variation in growth conditions (e.g., nutrient medium) when cultured on a single type of surface, predominantly polystyrene. However, the surface being presented to the microorganism for attachment and colonization can also serve as an "environmental variable". In this regard, the physico-chemical properties of the material or substratum to be colonized can have a profound influence on the biofilm developmental process by modulating the response of two-component regulatory systems utilized by bacteria to switch from a planktonic to sessile means of existence. For example, the *cpx*, *rcs* and *env/omp*

regulatory systems of *E. coli* can modulate the expression of specific genes, such as those required for production of flagella, to promote optimal cell attachment and biofilm formation (38, 62, 64). These systems are sensitive, to varying degrees, to changes in the local microenvironment (e.g., osmolarity, pH) when transitioning from the bulk fluid phase to the substratum surface. Therefore, the substrate or material properties (e.g., surface energy, roughness, chemical composition) can drastically alter these microenvironments leading to differential gene expression, and subsequently, altered phenotypes (e.g., matrix production/composition, thickness, antimicrobial resistance) when investigating biofilm formation on different surfaces. To illustrate this point, a recent report by Shemesh and colleagues showed that *Streptococcus mutans* biofilm formation was more confluent and thick on the surface of hydroxyapatite (HA) when compared to titanium and a zirconia/silica dental composite (74). Global gene expression analysis, using DNA microarrays, also revealed that several genes related to stress response and the production of cell wall-associated proteins were differentially expressed on HA, when compared to the other two materials. Furthermore, the authors observed a significantly higher level of autoinducer-2 (AI-2) production on the HA surface, suggesting that the material surface can affect quorum-sensing regulatory networks that have been shown to play a pivotal role in the early development and maturation of bacterial biofilms (7, 15).

Methods for Gene Expression Profiling in Biofilms

A variety of molecular biology techniques have been employed to elucidate the genetic determinants of biofilm formation (highlighted in the previous section). For example, the expression of individual genes or gene sets has been precisely monitored by using qRT-PCR and gene-targeted *gfp* fusions (40, 66). Isogenic mutant strains have also been harnessed to

investigate the phenotypic impact of single gene deletions (59). However, the most commonly used approach has been to conduct whole-transcriptome profiling with DNA microarrays. This methodology has been effectively utilized as an efficient and powerful conduit to survey differential gene expression profiles related to variations in environmental conditions and differences among genetic strains (3, 83).

In most cases, global gene expression profiling using DNA microarrays is often partnered with one or more of the other techniques indicated above to confirm microarray results (e.g., qRT-PCR) and provide additional information related to sub-population heterogeneity (e.g., *gfp* gene fusions). Interestingly, a relatively new technique coined "laser capture micro-dissection" has been used to isolate and collect different regions of a biofilm to investigate the influence of spatial heterogeneity on the expression of specific genes using qRT-PCR (66). This approach may also be amenable with DNA microarrays to examine differences in global gene expression among the sub-populations of cells residing at different locations in the biofilm (e.g., bulk liquid-biofilm interface vs. biofilm-substratum interface).

Several different commercially available DNA microarray platforms are currently available, with in situ-synthesized high-density arrays from Affymetrix, Roche NimbleGen and Agilent being the most widely utilized to date (58). Each of these microarray platforms have different attributes related to the length of the DNA probes, number of possible features and the solid support used to construct the arrays. Affymetrix arrays (GeneChips) are synthesized directly onto the surface of a quartz wafer using a photolithographic mask to obtain $>10^6$ features comprised of 20-25 bp probes. Due to this relatively short length, multiple probes for each target are included to enhance the accuracy, specificity and sensitivity of the microarray chips. In comparison to the Affymetrix platform, the microarray technology offered by Roche NimbleGen

is generated through a mask-less photolithographic process to synthesize arrays onto the surface of quartz wafers while Agilent chips are prepared on glass slides using an ink-jet printing technique. Both Agilent and Roche Nimblegen chips can also contain $>10^6$ features, but each feature is composed of 45-60 bp probes to increase sensitivity. Furthermore, the Agilent and Roche NimbleGen array chips can be purchased in a variety of formats (e.g., 1 X 385,000 features, 4 X 72,000) and are amenable to multi-color hybridizations while the Affymetrix arrays are only available in one format and limited to the use of one hybridization label.

In order to adequately monitor gene expression during biofilm development, sufficient amounts of high quality RNA must be isolated. Many techniques are available and include a number of chemical, enzymatic and mechanical isolation methods. In most cases, surface-associated biofilms are first removed using a scraping or swabbing technique and collected into a perservative solution to stabilize and/or protect the integrity of the RNA (e.g., Qiagen RNAlater, 5% phenol:95% ethanol) (30, 36). The recovered biofilms are then typically pelleted via centrifugation, to remove the preservative solution, and either immediately subjected to RNA extraction protocols or frozen and stored at -80°C until further processing. RNA extraction using chemical processes is typically achieved with methods such as hot phenol:chloroform and Trizol LS (Invitrogen) (10, 81). Enzymatic-based RNA extractions are most often carried out with lysozyme or zymolase (30). Mechanical extraction techniques include those based on physical shearing or pulverization of the cells with homogenizers (e.g., BIO 101 FastPrep System) or mini-bead beater systems (e.g., BioSpec Mini-BeadBeater 8) and physically grinding the cells with a mortar and pestle (9, 61, 70). Once the RNA has been successfully liberated from the cells, it is usually treated with DNase to degrade the genomic DNA and purified with a

commercially available kit (e.g., Qiagen RNeasy) to obtain high quality RNA that can then be used for downstream processing (i.e., cDNA synthesis) (30, 70).

Antimicrobial Coatings for Prevention of Biomaterial-Associated Biofilms

Biofilms can be found in almost any environment residing in the world around us, provided that sufficient moisture is present. These include some of the harshest and most extreme conditions encountered in nature, such as deep-sea hydrothermal vents rising from the ocean floors to glacial ice formations located in Antarctica. Biofilms can also take up residency in relatively mundane environments encountered in our everyday lives, such as sinks, bathtubs and floor drains. Due to ubiquitous nature of biofilms, they often play a key role in a number of beneficial processes including microbial fuel cells, water purification, sewage treatment, nitrogen fixation in crops and bioremediation of environmental contaminants (48, 69, 73, 75, 86). However, biofilms have largely gained notoriety for their adverse or detrimental effects related to the fouling of abiotic surfaces, such as ship hulls, heat exchangers, water or oil pipe lines, food processing equipment and invasive medical devices (25, 57, 60, 78). Once established on these surfaces, biofilms are extremely difficult to remove without causing significant operational delays and increasing overall costs (15). In the context of food processing equipment and medical devices, biofilms can serve as a reservoir for microorganisms that cause infection and disease (16).

One of the most widely used strategies to mitigate or prevent the formation of surface-associated biofilms is to apply a protective antimicrobial coating. A multitude of technologies have been developed in this regard and include 1) active-release coatings that leach out antimicrobial compounds, 2) passive coatings that prevent microbial attachment or adhesion through the generation of “non-stick” surface chemistries and 3) contact-active coatings that

either inhibit the growth or kill microorganisms upon their initial contact with the coating surface (5, 31, 37, 49). Although all three of these general classes of coatings have been shown to be effective, the latter two have gained favor in recent years as they are considered to be more environmentally-friendly and less likely to succumb to the development of resistant strains. Contact-active coatings are typically composed of antimicrobial agents that are covalently bonded or "tethered" to the polymer chains that comprise the bulk material of the coating (45). This tethering of the active compound serves to prevent it from leaching into the environment, enabling the generation of permanent or long-lasting antimicrobial surfaces (49). Researchers within the Center for Nanoscale Science and Engineering (CNSE) at NDSU have been actively engaged in the development of contact-active coatings for variety of applications, including the prevention of marine fouling on ship hulls and microbial contamination on invasive medical devices (46, 56). The primary technology being developed is based on the covalent attachment of QACs to silicone elastomer-based coatings (56). Quaternary ammonium compounds are well known for exhibiting contact-activity when immobilized to a surface through their direct interaction and disruption of essential processes and functions of the microbial cell wall. A wide variety of QAC based coating technologies have been investigated to date and several compositions have been shown to possess broad-spectrum activity towards bacteria, microalgae and fungi (54, 55).

An interesting phenomenon has been observed for several QAC based coating compositions when evaluating their antimicrobial activity towards the Gram-negative marine bacterium, *Cellulophaga lytica*. Specifically, results of high-throughput screening assays have shown that these coatings can effectively prevent the generation of the biofilm EPS matrix without adversely affecting cell attachment and growth of *C. lytica*, as compared to a silicone

control coating without tethered QAC (unpublished data). These results appear to be surprisingly similar to other non-toxic surfaces that are effective in preventing the formation of microbial biofilms, such as those containing immobilized furanones (2). These materials may also be similar to other contact-active technologies that prevent the growth of microorganisms, but do not adversely affect cellular viability (34, 44). Since the EPS matrix is known to play a pivotal role in the recalcitrant nature of biofilms (i.e., resistance to antibiotics/disinfectants and aggressive adhesion of the microbial community to surfaces), materials such as these developed within CNSE at NDSU may dramatically improve the efficacy of conventional sterilization and cleaning protocols used to prevent microbial contamination of surfaces. Furthermore, since the QAC based coatings do not appear to adversely affect cell attachment and growth, they may be less likely to foster the development of resistant strains (83).

MATERIALS AND METHODS

Coating Preparation

The coatings for bacterial biofilm growth and RNA recovery studies were prepared in 24-well plates as described by Majumdar et al. (56). The QSil formulation was based on 49,000 g $mole^{-1}$ silanol terminated polydimethyl siloxane (PDMS) with the C18 QAC, octadecyldimethyl(3-trimethoxysilylpropyl) ammoniumchloride, covalently attached to the PDMS backbone. The Sil coating substituted the non-quaternary ammonium C18 compound, n-octadecyl trimethoxysilane, for the C18 QAC. For each coating solution, 0.25 ml was dispensed into each well of a 24-well plate and allowed to cure at room temperature for 24 hours, followed by an additional 24 hours of curing at 50°C. The 24-well plates containing cured coatings were immersed in a re-circulating water tank for 21 days to leach out any toxic impurities that may remain from the coating preparation process (i.e., solvent, catalyst, non-tethered QAC).

Bacterial Strain

E. coli K-12 strain AJW678 was generously provided by Dr. Alan Wolfe, Loyola University of Chicago. Cultures were maintained weekly on Luria-Bertani (LB; 10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl, pH 7.0) agar at 4°C from cryo-preserved stocks (LB broth containing 20% glycerol) stored at -80°C. For biofilm growth and recovery experiments, broth cultures were prepared by transferring a single colony from LB agar into 10 ml of LB broth and incubating at 37°C for 24 hours with shaking (150 rpm). Cells were then pelleted via centrifugation at 4000*g* for 10 minutes and re-suspended in 1X phosphate buffered saline (PBS). The process of centrifugation and re-suspension was repeated two more times and the final suspension was used to inoculate growth medium used for biofilm cultivation.

Biofilm Growth Conditions

Prior to culturing *E. coli* K12 on the coatings prepared in 24-well plates for RNA recovery and gene expression studies, an experiment was conducted to determine the optimal conditions for biofilm growth. The culture resuspended in 1XPBS, detailed above, was used to inoculate M63 minimal media (M63; 2g/l $(NH_4)_2SO_4$; 13.6g/l KH_2PO_4; 0.5 g/l $FeSO_4$ $7H_2O$; 0.12g/l $MgSO_4$; 2g/l dextrose) and tryptic soy broth (TSB; 17g/l casein peptone; 2.5g/l dipotassium hydrogen phosphate, 2.5g/l glucose; 5g/l NaCl, 3g/l soya peptone, pH 7.3) to obtain final concentrations of 10^6 cells.ml^{-1}, 10^7 cells.ml^{-1} and 10^8 cells.ml^{-1}. 1.0 ml of each growth medium inoculated with the desired concentration of cells was dispensed into the bottom three rows of a single column of two blank polystyrene 24-well plates. The top row received fresh growth medium only (i.e., no *E. coli* K12 added) and served as an assay control. The plates were transferred to a 37°C incubator for either 24 or 72 hrs and then quantified for biofilm growth using the crystal violet colorimetric assay described below.

A high-throughput bacterial biofilm screening assay was used to culture *E. coli* biofilms on the QSil and Sil coatings prepared in 24-well plates (46, 76, 77). Based on the results obtained for the optimal growth condition study, the following protocol was carried out. 1.0 ml of 10^7 cells.ml^{-1} of *E. coli* in TSB was dispensed into each well of a 24-well plate containing either the QSil or Sil coating. The inoculated plates were covered with lids and then transferred to a 37°C incubator for 72 hours to facilitate cell attachment and biofilm growth. A reservoir of water was placed in the incubator to prevent evaporation of TSB during the 72 hours of incubation. The plates were then removed from the incubator and used for biofilm growth and viability assays as well as RNA isolation, as described in the protocols below.

Biofilm Characterization

Alcian Blue and Crystal Violet

After the appropriate period of incubation (24 or 72 hours), the 24-well plates were removed from the incubator and rinsed three times with 1.0 ml of deionized water. The plates were then inverted, tapped gently against a paper towel and dried for 1hr at ambient laboratory conditions. 0.5 ml of either an alcian blue (AB) or crystal violet (CV) solution (0.3% wt/v in water) was added to each well and for 15 minutes at ambient laboratory conditions. The plates were then inverted over a plastic container to remove the excess AB/CV dye, rinsed three times with 1.0 ml of deionized water and then inverted and tapped firmly against a paper towel. After 1 hr of drying at ambient laboratory conditions, the plates were placed on the deck of an automated liquid handling robot and then an extraction template was applied (46). Using the robotic system, 0.5 ml of 33% glacial acetic acid was added to each well for 15 minutes and then 0.15 ml of the resulting eluates were transferred to a 96-well plate and measured for absorbance (600 nm) using a multi-well plate spectrophotometer. The average absorbance of the three replicate samples, minus the absorbance value of the assay control well, was reported and considered to be directly proportional to the amount of biofilm attached to the coating surface. Error bars represent ± one standard deviation of the mean AB/CV absorbance value.

ATP Bioluminescence

The 24-well plates were removed from the 37°C incubator after 72 hours of incubation and rinsed three times with 1.0 ml of 1XPBS. After the final rinse, 0.25 ml of 1XPBS and 0.25 ml of BacTiter-Glo reagent (Promega, Madison, WI) were added to each well. The plates were then placed on an orbital shaker (150 rpm) for 5 minutes and then 0.150 ml of each solution was transferred to an opaque white 96-well plate and immediately measured for luminescence using a

multi-well plate spectrophotometer (sensitivity of 25-100). The average luminescence value (RLU; relative luminescence units) of the three replicate samples, minus the RFU value of the assay control well, was reported and considered to be an indicator of viability of the bacteria residing in the biofilm or attached directly to the coating surface. Error bars represent ± one standard deviation of the mean RFU value.

Viable Plate Count

After 72 hours of incubation, the 24-well plates were removed from the 37° incubator and rinsed three times with 1.0 ml of 1XPBS. Nylon flocked dry swabs (Copan Diagnostics Inc., Murrieta, CA) were then used to recover the bacterial cells/biofilms from each well of the three replicate samples of the Sil and QSil coatings and then immediately transferred to a glass culture tube containing 10 ml of Letheen Broth (5g/l NaCl; 0.7g/l lecithin; 5 ml of Tween 80 (polysorbate 80); pH of 7.0 at 25°C) used to neutralize quaternary ammonium compounds. The tubes of Letheen Broth containing the recovered cells/biofilm on the nylon flocked swabs (representing the 10^0 sample) were vortexed vigorously for 1 minute and then serially diluted 1:10 in fresh Letheen Broth. Each dilution tube was vortexed for 30 seconds and the appropriate amount of each dilution was then plated out onto LB agar using a drop plate technique where five replicate drops (0.01 ml) of four dilutions were dispensed onto one agar plate (one dilution per quadrant). The plates were inverted and placed in a 37°C incubator overnight and then examined the next day for viable colonies. The number of colony forming units (CFUs) for each dilution was reported as the mean value of five replicate drops that contained between 3 - 30 countable CFUs. Error bars represent ± one standard deviation of the mean.

Fluorescence Microscopy

The 24-well plates were removed from the 37°C incubator after 72 hours of incubation and rinsed three times with 1.0 ml of 1XPBS. After the final rinse, the plates were inverted, tapped gently against a paper towel and 0.5 ml of 2.5% w/v of formaldehyde in 1XPBS was added to each well to fix the bacterial cells/biofilm to the coating surfaces. The plates were transferred to a 4°C incubator for 1.5 hrs and then rinsed with 1.0 ml of 50:50 1XPBS:deionized water followed by a second rinse with 25:75 1XPBS:deionized water and a final rinse of deionized water only. After rinsing, 0.5 ml of Syto 9 nucleic acid dye (Promega, Madison, WI) was added to each well and the plates were transferred to a dark cabinet (ambient laboratory temperature) and incubated for 10 minutes. The Syto 9 dye solution was removed from the plates, rinsed once with deionized water and then placed on the stage of an Olympus fluorescence microscope to capture fluorescent images using a 40x long working distance objective and a FITC filter cube.

RNA Isolation

Hot Phenol:Chloroform Extraction

Two different techniques were used to extract RNA from the biofilms using the hot-phenol:chloroform method (13). The first technique was based on the extraction of RNA directly from the biofilms attached to the coating surfaces in multi-well plates. Specifically, the plates were rinsed one time with 1X PBS and then 0.8 ml of hot lysis buffer (2% SDS, 200 mM NaOAC, 20 mM EDTA heated in boiling water bath) was immediately added to each well of the plate. The plates were then transferred to a heating block (95°C) for 3 minutes and 0.5 ml of hot phenol (60°C) was then added to each well and incubated for 1 minute. The entire volume from each well of a single coating plate was pooled in a 50 ml conical tube and centrifuged at 10,000*g*

for 10 minutes (10°C). The supernatant was collected from each tube (cell pellet discarded) and split equally between two new 50 ml conical tubes (~10 ml per tube). A 10 ml volume of 5:1 hot phenol:chloroform (8.4 ml:1.6 ml) was added to each tube, mixed thoroughly by inversion and centrifuged at 10,000*g* for 10 minutes (10°C). The aqueous phase (top layer) of each tube was collected with a Pasteur pipette and transferred to a new 50 ml conical tube. This extraction procedure was repeated two more times using a 1:1 ratio (5 ml: 5 ml) of hot phenol:chloroform and then a full 10 ml volume of chloroform (no hot phenol). After centrifugation, the supernatant in chloroform was transferred to a new 50 ml tube and 10 ml of isopropanol was added. The tubes were then centrifuged at 10,000*g* for 120 minutes (4°C) to precipitate the extracts. The supernatant was discarded and then 5.0 ml of 70% ethanol was added to each tube and centrifuged at 10,000*g* for 5 minutes (4°C). Finally, the 70% ethanol was discarded and the tubes were immediately placed on ice to facilitate drying of the RNA pellets.

The second technique was based on the utilization of nylon flocked dry swabs (Copan Diagnostics Inc., Murrieta, CA) to recover the biofilms from the coatings surfaces prior to lysing the cells. Specifically, the plates were rinsed one time with 1X PBS and then the first column of each plate (i.e., 4 wells) was swabbed with one nylon flocked dry swab (after removing 1X PBS with a pipette) and the swab was transferred immediately to a 50 ml conical tube containing 10 ml of 1X PBS and 1 ml of stop solution (5% phenol: 95% ethanol). Each well was swabbed for approximately 10 seconds. This procedure was then repeated for columns 2 through 6 of each plate with all swabs containing recovered biofilm from a single plate pooled in the same 50 ml tube. The tubes were then vortexed vigorously for 30 seconds to liberate the recovered biofilms from the swabs into the recovery solution. The swabs were then removed from each tube with a sterile forceps and the tubes were centrifuged at 4500 rpm for 15 minutes to pellet the biofilms.

The supernatants were discarded and the tubes were immediately placed on ice. Next, 0.480 ml of hot lysis buffer was added to each tube, aspirated gently to reconstitute the pellets and the entire volumes transferred to sterile 1.5 ml microcentrifuge tubes. The microcentrifuge tubes were capped and placed in a boiling water bath for 4-5 minutes or until the solutions became clear and subsequently centrifuged at 10,000*g* for 10 minutes. The supernatants were then transferred to new microcentrifuge tubes and 0.5 ml of phenol and 0.1 ml of chloroform (both preheated to 60°C) was added, followed by gentle mixing of the tube contents by inverting several times until a cloudy, white mixture was obtained. The tubes were centrifuged at 10,000*g* for 10 minutes and the aqueous phase (top layer) of was transferred to a new microcentrifuge tube. This extraction procedure was repeated two more times using a 1:1 ratio (0.3 ml: 0.3 ml) of room temperature phenol:chloroform and then a full 0.6 ml volume of room temperature chloroform (no phenol). After centrifugation, the supernatants in chloroform were transferred to new microcentrifuge tubes and 0.6 ml of isopropanol was added. The tubes were then centrifuged at 10,000*g* for 120 minutes (4°C) to precipitate the extracts. The supernatants were discarded and then 1.0 ml of 70% ethanol was added to each tube and centrifuged at 10,000*g* for 10 minutes (4°C). Finally, the 70% ethanol was discarded and the tubes were immediately placed on ice to facilitate drying of the RNA pellets.

RNA Purification

The purification of RNA extracted from the recovered biofilms was carried out using an RNeasy® mini kit (Qiagen, Valencia, CA). 0.1 ml of RNase-free water was added to each RNA pellet and gently reconstituted by aspiration with a pipette. Then, 0.35 ml of buffer RLT (with 1% v/v β-mercaptoethanol) and 0.25 ml of 100% ethanol were added to the reconstituted precipitates and mixed thoroughly by inverting the tubes several times. The entire volume for

each tube was transferred to a mini spin column and centrifuged at 10,000*g* for 30 seconds. The flow through content of the collection tubes was then discarded and 0.35 ml of buffer RW1was added to the mini spin columns and centrifuged at 10,000*g* for 30 seconds. The flow through content was discarded again and an on-column DNA digest was carried out by adding 0.08 ml of DNase I/RDD buffer solution (0.01 ml/0.07 ml) to each mini spin column. After 15 minutes of the DNase treatment at ambient laboratory conditions, 0.350 ml of buffer RW1 was added to each mini spin column and centrifuged at 10,000*g* for 30 seconds. The flow through content was discarded and 0.5 ml of buffer RPE was to each mini spin column and centrifuged at 10,000*g* for 30 seconds. The flow through was discarded again and another 0.5 ml volume of buffer RPE was added to each mini spin column and centrifuged at 10,000*g* for 2 minutes. The mini spin columns were then transferred to new collection tubes and centrifuged again at 10,000*g* for 1 minute. The mini spin columns were transferred to 2.0 ml collection tubes with caps and 0.03 ml of RNase-free water was added to each mini spin column and let sit at ambient laboratory conditions for 30 seconds. The mini spin columns were centrifuged at 10,000*g* for 1 minute and the mini spin columns were subsequently discarded. The total volume of the product recovered in each collection tube was measured with an electronic pipette. A subsequent off-column DNA digest was performed for each sample by first bringing the recovery product volumes up to 0.88 ml (in the 2.0 ml collection tubes), then adding 0.01 ml of RDD buffer and 0.025 ml of DNase I and incubating for 15 minutes at ambient laboratory conditions. Next, 0.35 ml of buffer RLT (with 1% v/v β-mercaptoethanol) and 0.25 ml of 100% ethanol were added to each collection tube, mixed gently by inversion and the entire volume of each tube transferred to a new mini spin column. The mini spin columns were then processed using the same protocol described in the previous paragraph for the on-column DNA digest, except that the RW1 washing step was

omitted. The volume of the final products recovered in RNase-free water was measured with an electronic pipette and the concentrations of RNA were determined using a Nanodrop 2000 micro-volume spectrophotometer (Thermo Scientific). The quality and integrity of the RNA samples were characterized using an Agilent 2100 Bioanalyzer per the manufacturer's instructions. The samples were subsequently stored at -80°C until further use.

Mini-bead Beater Extraction

In addition to the hot phenol:chloroform extraction method, a mechanical cell disruption technique was investigated for its ability to isolate high quality RNA from recovered biofilms and was based on a previously published procedure using a mini-bead beater system from BioSpec (i.e., Mini-BeadBeater-16) (70). Specifically, the plates were rinsed one time with 1X PBS and subsequently swab recovered into a 50 ml conical tube as described in the hot phenol:chloroform section above. However, the volume of recovery solution (10:1 ratio of 1X PBS to stop solution) was reduced from 10.0 ml to 2.0 ml. The 2.0 ml final recovery volume from each sample was transferred to a mini-bead beater tube and centrifuged at 10,000*g* for 5 minutes. The supernatants were discarded and 1.0 ml of RLT solution from the RNeasy mini-kit and 0.2 ml of zirconia/silica beads (0.1 mm diameter) were added to each mini-bead beater tube. The mini-bead beater tubes were then transferred to the Mini-BeadBeater-16 apparatus and treated for 30 seconds by turning on the system power (default settings which are not adjustable). The mini-bead beater tubes were then removed and placed on the bench for ~ 1 minute to allow zirconia beads to settle out to bottom of the tubes. The supernatants were then transferred to sterile 1.5 ml microcentrifuge tubes and the zirconia beads and mini-bead beater tubes were discarded. Next, 0.250 ml of 100% ethanol was added to each microcentrifuge tube, containing 0.350 ml of RLT extracts from mini-bead beater treatments, and mixed thoroughly by inverting

the tubes several times. The entire volume from each microcentrifuge tube was then transferred to a mini spin column and purified with both an on-column and off-column DNase treatment, as described in the previous section.

cDNA Synthesis

The purified RNA recovered from the Sil and QSil coatings, isolated by using a combination of the mini-bead beater extraction method and pooling of replicate plate samples onto one mini spin column, was reverse transcribed to obtain sufficient amounts of cDNA for global gene expression profiling using DNA microarray analysis. The appropriate volume of each sample to obtain 10 μg of RNA was added to a 0.2 ml microcentrifuge tube. Each sample then received 0.003 ml of a random hexamer primer solution (3.3 μg/μl) and the final volume was brought to 0.012 ml by adding RNase-free water. The tubes were immediately transferred to a thermal cycler unit and incubated at 70°C for 10 minutes followed by an additional 10 minutes of incubation at 4 °C. The tubes were placed on ice and 0.006 ml of 5x buffer, 0.006 ml of 5x amino-allyl dUTP/dNTPs, 0.003 ml of DTT, 0.001 ml of RNase out inhibitor and 0.002 ml of superscript III were added to each sample. The tubes were then transferred to the thermal cycler and incubated at 42°C for 2 hours. Following the 2 hours of incubation, 0.010 ml of 1M NaOH and 0.010 ml of 0.5M EDTA was added to each sample and mixed thoroughly by aspiration with a pipette. The tubes were then transferred to the thermal cycler and incubated at 65°C for 15 minutes. Next, 0.3 ml of RNase-free water and 0.025 ml of 1M TRIS buffer (pH 7.4) was added to each sample and the entire volume was subsequently transferred to Micron 30 concentrator column and centrifuged at 12,000*g* for 7 minutes. The flow through was discarded and each sample received another 0.45 ml of RNase-free water and centrifuged again at12,000*g* for 7 minutes. This 0.45 ml water rinsing step was repeated 3 more times. After the final water

rinse, each column was inverted and placed into a new collection tube and centrifuged at 1,000*g* for 3 minutes to recover the reverse transcribed cDNA. The final volume and concentration of cDNA for each sample was measured with a Nanodrop 2000 micro-volume spectrophotometer and stored at -20°C until further use.

cDNA Labeling for Microarray Analysis

The labeling of cDNA for DNA microarray analysis was carried out using an Alex Fluor 555 labeling dye (Invitrogen, Carlsbad CA). Only one dye was required as each sample was hybridized to its own quadrant of a 4-plex Nimblegen DNA microarray chip (non-competitive hybridization) (58). The cDNA pellets were first reconstituted in 0.005 ml of nuclease-free water by aspiration with a pipette, incubated at 42°C for 5 minutes and then 0.003 ml of 1M $NaHCO_3$ was added to each sample. Next, 0.002 ml of DMSO was added to a vial of labeling dye (one vial prepared for each sample) and mixed thoroughly by vortexing until the dye was completely dissolved. The entire volume of each cDNA sample was then transferred to a vial of labeling dye reconstituted in DMSO and vortexed for 15 seconds. The vials were wrapped in tinfoil and incubated in a dark drawer for 1 hour. Each vial then received 0.035 ml of 0.1M sodium acetate and 0.050 ml of nuclease-free water to achieve a final volume of ~0.1 ml. Five hundred microliters of PB buffer was added to each vial and the entire volume for each sample was transferred to a QIAquick spin column and centrifuged at 17,900*g* for 1 minute. The flow through was discarded and 0.75 ml of PE buffer was added to each column and centrifuged at 17,900*g* for 1 minute. The flow through was discarded again and the columns were centrifuged for an additional 60 seconds to ensure all PE buffer was removed. The columns were then transferred to clean 1.5 ml microcentrifuge tubes and 0.03 ml of 0.1M EB buffer was added to each sample. The samples were incubated at ambient laboratory conditions for 1 minute and

then centrifuged at 13,000*g* for 30 seconds. The 0.1M EB buffer elution step was repeated again and the columns were discarded. The recovered samples were then transferred to a vacuum centrifuge for 2 hours to evaporate off the EB buffer and the dried samples were subsequently stored at -20°C until further use.

DNA Microarray Hybridization and Scanning

The hybridization protocol was carried out as instructed in the NimbleChip Arrays User's Guide (Chapter 4. Hybridization and Washing). The Alexa Fluor 555 labeled cDNA samples were re-hydrated by adding 0.025 ml of nuclease-free water and the concentrations were measured using a Nanodrop 2000 micro-volume spectrophotometer. The samples were subsequently dried using a vacuum centrifuge and 0.0033 ml of tracking control solution was added to reconstitute the samples. Each sample then received 0.0087 ml of a hybridization solution master mix (0.0295 ml of 2x hybridization buffer; 0.0118 ml of hybridization component A and 0.0012 ml of alignment oligo) and mixed well by vortexing. The samples were then incubated at 95°C for 5 minutes and then transferred to the hybridization station (Nimblegen Hybridization Station 4) pre-heated to 42°C. Next, an X4 mixer was applied to a 4-plex Nimblegen microarray chip (*E. coli* K12; 071112_Ecoli_K12_EXP_X4) using a precision mixer alignment tool (PMAT) and 0.008 ml of each sample (Sil and QSil) was loaded onto one quadrant of the chip. The other two quadrants on the 4-plex chip were not used in this study. The cover was then placed over the vent holes and fill ports and the hybridization station was activated by turning on the mix mode and selecting the B mode option. The samples were then allowed to hybridize to the Nimblegen microarray chip for 24 hours at 42°C. The microarray chip and mixer were removed from the hybridization station and placed into the disassemble array tool (DAT). The entire assembly was then transferred into a container with wash I solution

and the mixer was carefully removed from the microarray by peeling it up and over the chip. The microarray chip was removed from the DAT, placed into a slide rack in located in the reservoir containing wash I solution and washed vigorously by continual shaking/agitation for 2 minutes. The slide rack containing the microarray chip was then transferred to a new reservoir containing wash II solution and agitated for 1 minute. The microarray slide was removed from the slide rack and immersed in wash III for 15 seconds (no agitation) and subsequently dried for 2 minutes using a Galaxy Mini Microcentrifuge (VWR, Radnor, PA). The microarray chip was then transferred to the slide carriage of an Axon GenePix 4300A microarray scanner (Molecular Devices, Sunnyvale CA) and scanned as instructed in the NimbleChip Arrays User's Guide (Chapter 5. One-Color Array Scanning). The slide was placed in a protective sleeve and stored in a desiccator in a dark cabinet until further use.

Gene Expression Analysis

Gene expression analysis of the Nimblegen DNA microarray results was carried out with an ArrayStar® software package (DNASTAR, Inc., Madison, WI). The microarray data signal intensities were first processed using an RMA method (Robust Multichip Analysis) for background corrections and quantile normalization (39). A moderated t-test was applied to the pair-wise comparison of each gene, between the Sil and QSil coating, and a Benjamini and Hochberg FDR multiple testing correction was applied at a significance level of $p < 0.01$. The utilization of the FDR multiple testing correction estimates that 1% of differentially expressed genes will be due to chance. Expression ratios were calculated for a select number of genes (Table 1) by dividing the processed, linear fluorescence value obtained from the QSil coating by the processed, linear fluorescence value obtained from the Sil coating for each gene. Thus, an expression ratio >1 indicated that the gene expression on the QSil coating was higher than the

gene expression on the Sil coating. In contrast, an expression ratio <1 indicated that the gene expression on the QSil coating was lower than the gene expression on the Sil coating.

RESULTS AND DISCUSSION

Characterization of *E. coli* K12 Biofilm Growth

One of the first items that needed to be established for this research project was the set of culture conditions that would promote adequate biofilm growth of the *E. coli* K12 strain in multi-well plates. Figure 1 shows the results of the biofilm growth optimization experiment after staining with CV, a cationic dye that binds to negatively charged biomacromolecules associated with both microbial cells and the EPS matrix (Li and Burmole). Based on the visual inspection of the plates, it is clear that the nutrient-rich TSB medium facilitated substantially more biofilm growth than the nutrient-limited M63 minimal medium, regardless of the initial cell inoculum concentration or duration of incubation at 37°C. It is also evident that the amount of biofilm growth obtained in the TSB medium was greater after 72 hrs of incubation as opposed to 24 hrs of incubation. The variation in initial cell inoculum concentration appeared to have less of an impact on biofilm formation than growth medium composition or duration of incubation as all three cell concentrations resulted in a similar amount of biofilm growth. This latter observation was consistent with the results of a recent report that investigated the effect of environmental factors on the biofilm growth of *E. coli* K12 using the same strain employed in this study (strain AJW6780) (67). Based on the results of this optimization experiment, the culture conditions consisting of a 10^7 cells.ml^{-1} initial inoculum concentration, TSB growth medium and 72 hour duration of incubation at 37°C was selected for coating surface studies.

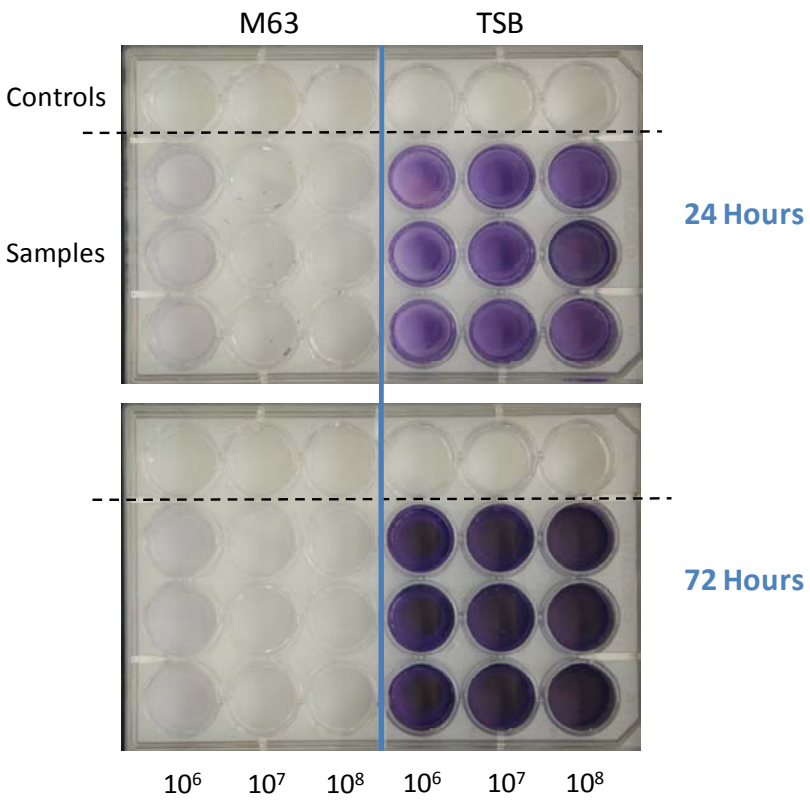


Figure 1. Optimization of *E. coli* K12 biofilm growth conditions. Images of *E. coli* K12 biofilm growth after CV staining. The plates were incubated at 37°C and the culture conditions were varied with respect to growth medium composition (M63 and TSB), initial cell inoculum concentration (10^6 cells.ml^{-1}, 10^7 cells.ml^{-1} and 10^8 cells.ml^{-1}) and duration of growth (24 hrs and 72 hrs).

Once the optimal culture conditions for promoting biofilm formation in multi-well plates had been established, an experiment was conducted to determine if *E. coli* K12 exhibited a similar growth behavior as the marine bacterium, *C. lytica*, when cultured on the Sil and QSil coating surfaces. Previous analysis of these two coatings with *C. lytica* showed that the QSil coating, containing a chemically tethered QAC, was able to achieve a substantial reduction in biofilm growth when compared to the pure/unmodified silicone coating (Sil) (unpublished data). Furthermore, the evaluation of cellular viability (i.e., tetrazolium salt respiratory assay) revealed

that there was no reduction in the number of *C. lytica* cells attached to the QSil coating surface. These results suggested that the QSil surface was able to prevent *C. lytica* biofilm formation (i.e., EPS matrix) without adversely effecting cell attachment and proliferation. Figure 2 shows the biofilm formation on the Sil and QSil coating surface for both *E. coli* and *C. lytica*. These results clearly show that a substantial reduction in biofilm formation was achieved for both bacteria on the surface of the QSil coating.

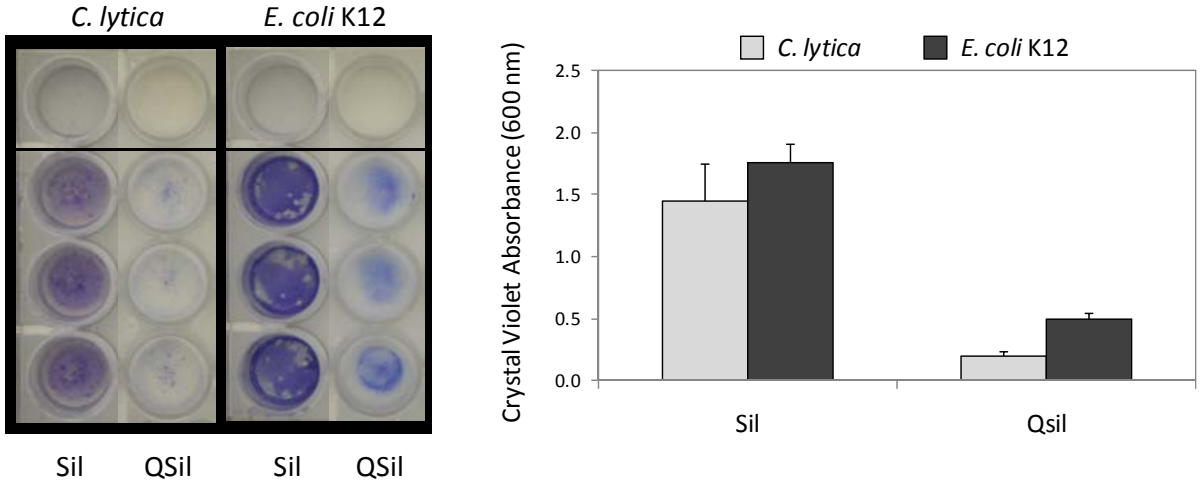


Figure 2. *C. lytica* and *E. coli* K12 biofilm growth on the Sil and QSil coatings. Images of biofilm growth after CV staining (left). Crystal violet absorbance values (right).

Figure 3 displays the results of a subsequent biofilm growth experiment carried out with *E. coli* K12 on the Sil and QSil coating surfaces. In addition to the CV assay, an alcian blue (AB) colorimetric and ATP bioluminescence assay were also employed to characterize biofilm growth and cellular viability, respectively. As with the first experiment, a substantial reduction in biofilm growth was once again observed on the QSil coating surface using the CV assay. This result was confirmed by the AB assay which specifically binds to anionic polysaccharides associated with the microbial cell wall and the EPS matrix (12, 71). However, the results of the

ATP bioluminescence assay revealed that no reduction in viable cells attached to the coating surface occurred during the 72 hours of incubation. The results of this experiment with *E. coli* K12 were in good agreement with the previous data collected for *C. lytica* as both bacteria showed a substantial reduction in biofilm growth without adversely effecting cell attachment and proliferation.

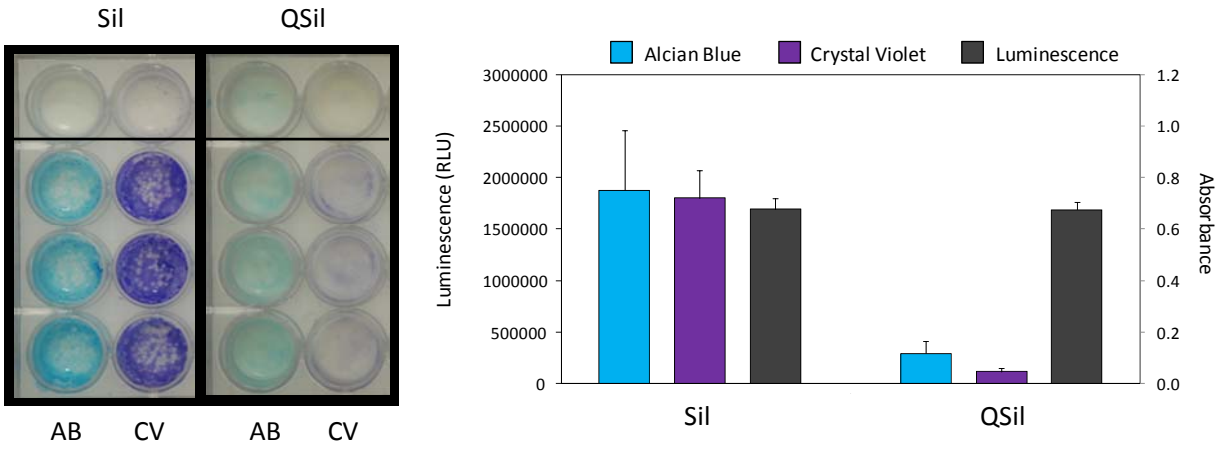


Figure 3. *E. coli* K12 biofilm growth on Sil and QSil coatings. Images of biofilm growth after AB and CV staining (left). AB absorbance, CV absorbance and ATP luminescence values (right).

Two additional characterization techniques were employed to support the results obtained with the CV, AB and ATP bioluminescence screening assays. The first technique was based on the utilization of the nucleic acid dye, Syto 9, and fluorescence microscopy (Note: fluorescence microscopy was carried out by CNSE Research Specialist Justin Daniels, under my direction and supervision). Figure 4 shows a representative fluorescence image of *E. coli* K12 cultured on the Sil and QSil coating surfaces prepared in multi-well plates (72 hours of incubation). When examining the image of the Sil coating surface, both the EPS matrix and attached cells can clearly be discerned. In contrast, the image of the QSil coating revealed that only cells and cell

clusters (i.e., microcoloines) were attached to the surface while no EPS matrix was evident. Thus, these results provide further evidence that the cells where able to attach and proliferate on the surface of the QSil coating but were prevented from generating the biofilm EPS matrix.

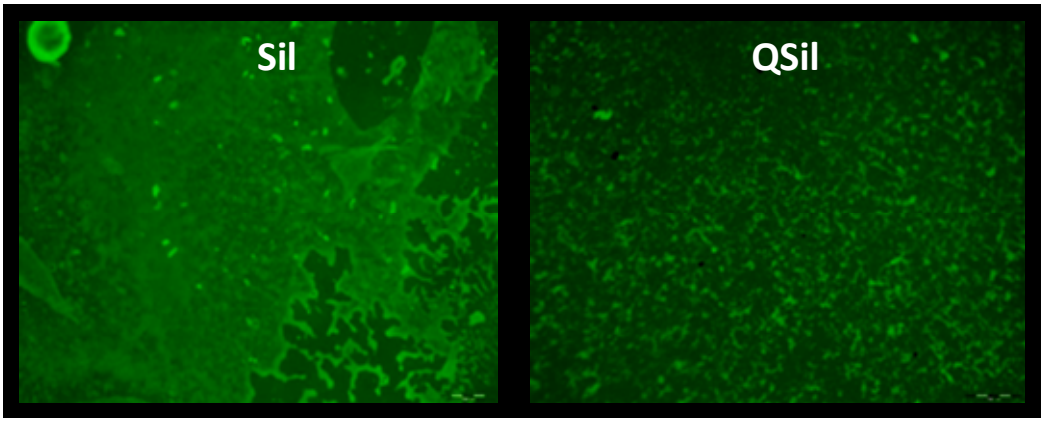


Figure 4. Fluorescence images of *E. coli* K12 biofilm growth on the Sil and QSil coatings. Images were captured after Syto 9 staining (72 hours of incubation at 37°C) using a 40x long working distance objective (400x total magnification).

The second technique used to support and confirm the results obtained with the CV, AB and ATP bioluminescence assays was based on viable plate counting of the cells/biofilm recovered from the coating surfaces using nylon flocked swabs. As shown in Figure 5, only a slight reduction in viable cell counts (0.17 $\log_{10}$ reduction) was observed for the QSil coating when compared to the Sil coating. It is highly unlikely that this relatively small reduction in viable cells compromised the ability of the attached bacterial community to generate the EPS matrix over the 72 hour duration of incubation on the QSil coating surface. In fact, other groups that have investigated the anti-biofilm activity of QAC-based coatings typically report a 1-2 $\log_{10}$ reduction in viable plate counts that coincides with a substantial reduction in biofilm formation (49). In this regard, the inhibition of biofilm formation observed on these surfaces was

determined to be a direct result of the inhibition of cell attachment and proliferation. The results from the viable plate counting in this study, along with the ATP bioluminescence and fluorescence microscopy analyses, suggest that a different mechanism (i.e., not related to cell density/quorum sensing) was responsible for preventing *E. coli* K12 biofilm formation and that it was related to a surface associated (i.e., direct contact) phenomenon with the novel QSil coating.

It is also important to point out that the 0.17 $\log_{10}$ reduction in viable cells may have also been attributed to an insufficient recovery of the cells/biofilm from the positively charged QSil surface, rather than an inhibition of cell attachment and cell proliferation. This argument is supported by other reports in the published literature that have also employed a swab or scraping technique to recover bacteria from positively charged surfaces to conduct viable plate counts (33). In this regard, it has been postulated that the overall net negative charge of the bacterial cell wall can facilitate a strong electrostatic interaction with positively charged surfaces. This interaction can then potentially impede the ability of conventional recovery techniques to adequately remove the cells attached to these types of surfaces. As a result, a slight to modest reduction in viable cells recovered from positively charged surfaces, as observed in this study for *E. coli* K12 on the QSil coating, could be misinterpreted as a bacteriostatic or bactericidal effect. When considering the ATP bioluminescence and fluorescence microscopy results, it is most likely that the 0.17 $\log_{10}$ reduction in viable cells recovered from the QSil coating was indeed an artifact of the swab recovery and plate counting technique rather than a true reduction in the number of viable cells.

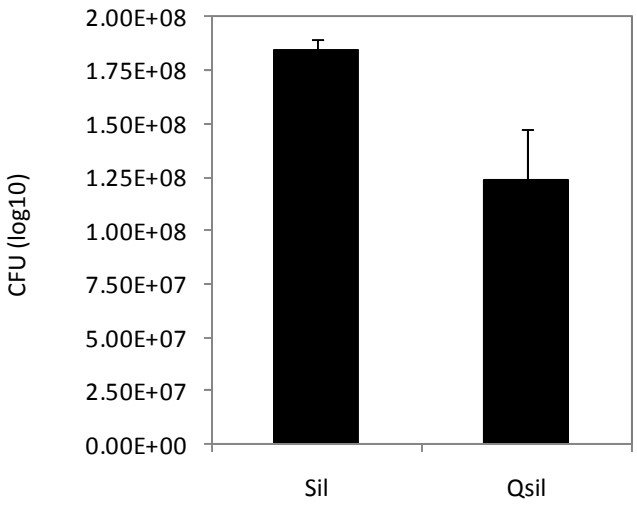


Figure 5. Viable plate counts of *E. coli* K12 recovered from the Sil and QSil coatings. Cells were recovered from the coating surfaces using nylon flocked swabs (72 hours of incubation at 37°C).

It is important to reiterate that the overall objective of the biofilm growth experiments, discussed above, was to determine if the phenomenon of *C. lytica* biofilm growth inhibition on silicone coatings containing chemically tethered QACs, without adversely effecting cell attachment and proliferation, also occurred when using *E. coli* K12. The rationale for this comparison was based on two factors; 1) the abundance of information available in the public domain for *E. coli* K12 regarding the environmental and genetic factors that govern biofilm formation and 2) the entire *E. coli* K12 genome has been sequenced and annotated and commercially available DNA microarrays chips are readily available. As widely accessible information on *C. lytica* biofilm development is limited and no DNA microarray chip is commercially available for this bacterium, *E. coli* K12 was considered to be a more suitable model for elucidating how the QSil coatings effectively prevent the generation of the biofilm matrix without adversely effecting cell viability.

RNA Extraction and Purification Techniques

Although DNA microarrays are an extremely powerful technology that can be used to carry out gene expression profiling, caution must be exercised when planning and executing these experiments to minimize the chances that erroneous data and conclusions are made. One of the most important steps to ensure that robust and accurate gene expression levels are obtained is to isolate high quality RNA from the samples of interest. One of the most widely used methods to isolate RNA is the hot phenol:chloroform extraction technique. This method was initially used in this study in an attempt to isolate high quality RNA from *E. coli* K12 cultured on the surface of a polysiloxane coating that did not contain a tethered QAC for antimicrobial activity (i.e., Sil coating).

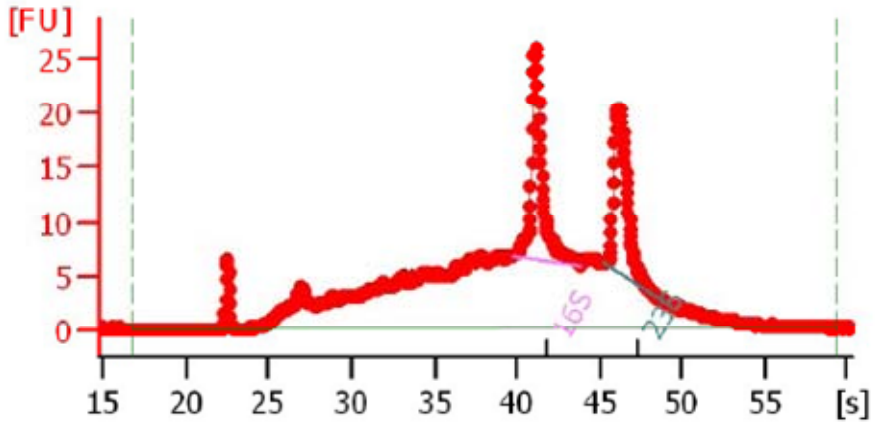
The first experiment was conducted by adding hot lysis buffer directly to the wells of the plate that contained the Sil coating and then the entire lysate from each well was subsequently transferred to a conical tube for phenol:chloroform extraction and purification. This RNA isolation protocol resulted in the recovery of 0.03 ml of RNA at a concentration of 1.4 μg/μl (40.6 μg of total RNA). Although this experiment resulted in a good yield of RNA from 1 Sil coating plate, this technique was not used in subsequent experiments as it was realized that any cells that may have attached to the sides of the wells, rather than to the surface of the coating, would have also been lysed and contributed to the total amount of RNA recovered.

The next experiment employed a swabbing technique, prior to lysing the cells, to remove only those cells that were attached to the coating surface. With this technique, the biofilms recovered from each well of an entire coating plate were pooled in a 50 ml conical tube and then treated with the hot lysis buffer. As one might expect, this modification to the protocol resulted in a lower amount of RNA recovered from 1 Sil coating plate (0.03 ml of 0.34 μg/μl RNA or

10.2 µg of total RNA) as the cells attached to the side of the wells were excluded from the lysis buffer treatment. However, the quality or integrity of the recovered RNA, as determined with an Agilent 2100 Bioanalyzer system, was determined to be inadequate for downstream applications such as DNA microarray analysis. In this regard, the isolated RNA was shown to possess an RNA integrity number (RIN) of 6.2 and an rRNA ratio (23s/16s) of 0.9 (Figure 6). The isolated RNA should exhibit a RIN and rRNA ratio of at least 7.0 and 1.6, respectively, for subsequent processing and analysis. It is important to point out that the RIN value is derived from the electrophoretic trace of the RNA sample subjected to a microfluidic-based electrophoretic separation, including the presence and absence of degradation products, and is used to determine the overall quality and integrity of the RNA. In this regard, a RIN value of 10 would indicate that the RNA sample is completely intact while a RIN value of 1 would indicate that the RNA sample is completely degraded. With regards to the rRNA ratio, this value is calculated by dividing the area of the 23s chromatographic peak (a component of the large 50s ribosomal subunit) by the area of the 16s chromatographic peak (a component of the small 30s ribosomal subunit). As the 23s component is ~3000 bp long and the 16s component is ~1500 bp long, an rRNA ratio of 2.0 would indicate completely intact and high quality |RNA. Although the RIN value and rRNA ratio are both useful for determining the quality of RNA, it has been suggested that the RIN is a more important and accurate indicator of RNA integrity as rRNA ratios often exhibit a high degree of variability (42).

It is also evident when examining the chromatogram provided in Figure 6 that a substantial amount of RNA degradation had occurred, as shown by the relatively severe degree of curvature of the baseline away from the x-axis between 25 and 55 seconds. If the RNA had

not been significantly degraded, the baseline would be almost completely flat or close to 0 FU on the y-axis throughout the duration of the analysis (60 seconds).



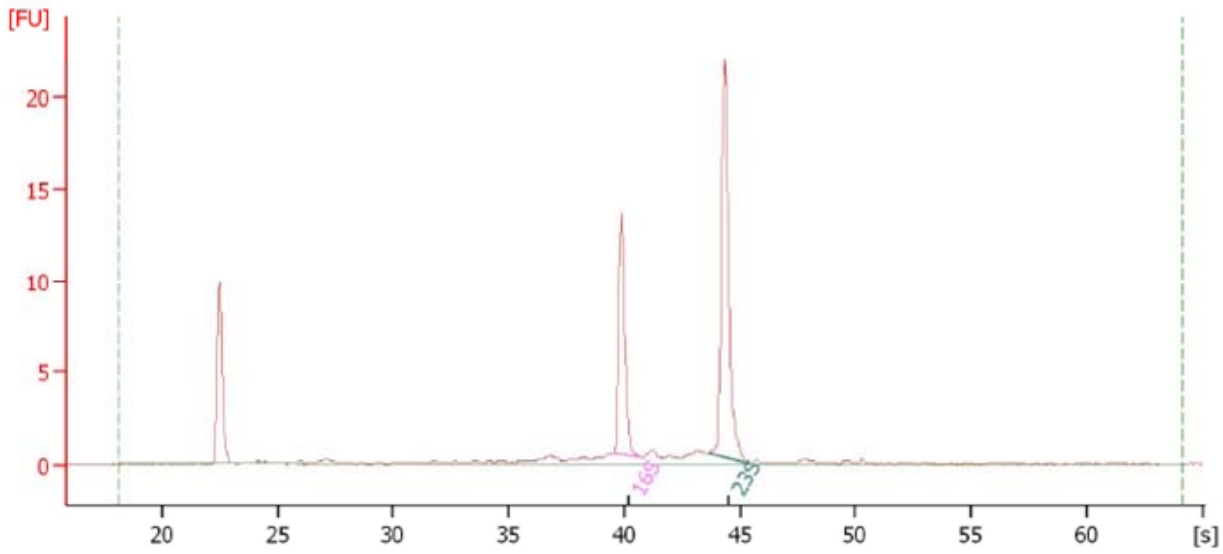
rRNA Ratio [23s/16s]:	0.9
RNA Integrity Number (RIN):	6.2
RNA Concentration:	340 ng/µl

Figure 6. Bioanalyzer analysis of total RNA isolated with hot phenol-chloroform.

A second attempt was made to use the swab recovery and hot chloroform:phenol extraction technique to recover sufficient amounts of high quality RNA. For this experiment, new reagents, solutions and supplies (i.e., pipette tips, tubes etc.) were used to account for the possibility that nuclease contamination was the cause of the RNA degradation observed in the first experiment. The results from the Agilent Bioanalyzer analysis revealed that 3.6 µg of total RNA was recovered from one plate (0.03 ml of 0.12 µg/µl RNA) and was shown to possess a RIN value of 6.4 and an rRNA ratio (23s/16s) of 0.8. As with the first experiment employing the swabbing technique, the integrity of the RNA was shown to be of poor quality and not suitable for downstream processing. Since both experiments exhibited a similar chromatographic profile (i.e., severe curvature of the baseline away from the x-axis), nuclease contamination of the

reagents and supplies was most likely not the primary cause of the RNA degradation. It is possible that nuclease contamination could have occurred as some other critical point when carrying out the hot phenol:chloroform extraction technique. However, attempts to determine the actual cause of this degradation would be quite difficult to investigate as this extraction technique consists of a lengthy multi-step process which requires a considerable amount of handling and manipulation of the samples.

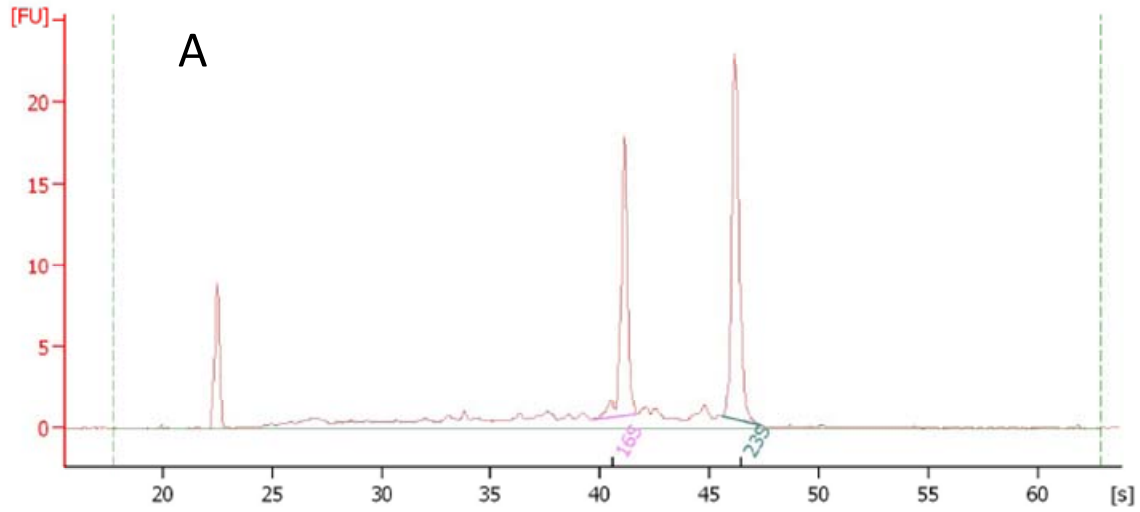
A survey of the published literature revealed an alternative method to the hot phenol:chloroform technique for extracting RNA from bacterial biofilms for use in DNA microarray studies. This alternative methodology relies on the mechanical shearing and pulverization of the recovered cells using zirconia/silica mini beads. This technique is considerably less time intensive and requires significantly less handling of the cells before carrying out the RNA purification steps using the RNeasy® mini kit. As can be seen in Figure 7, the first experiment carried out with the mini-bead beater extraction technique dramatically improved the quality and integrity of the RNA recovered from the Sil coating surface (RIN of 9.4 and rRNA ratio of 1.8). Not only was the RIN value and rRNA ratio much higher than the previous two hot phenol:chloroform trials, but the baseline was much more flat or closer to the x-axis of the chromatogram (< 1 FU) from 25 – 55 seconds. As a result, the mini-bead beater mechanical RNA extraction technique was selected for all subsequent experiments in this study, including the DNA microarray analysis of differentially expressed genes between the Sil and QSil coating surfaces.



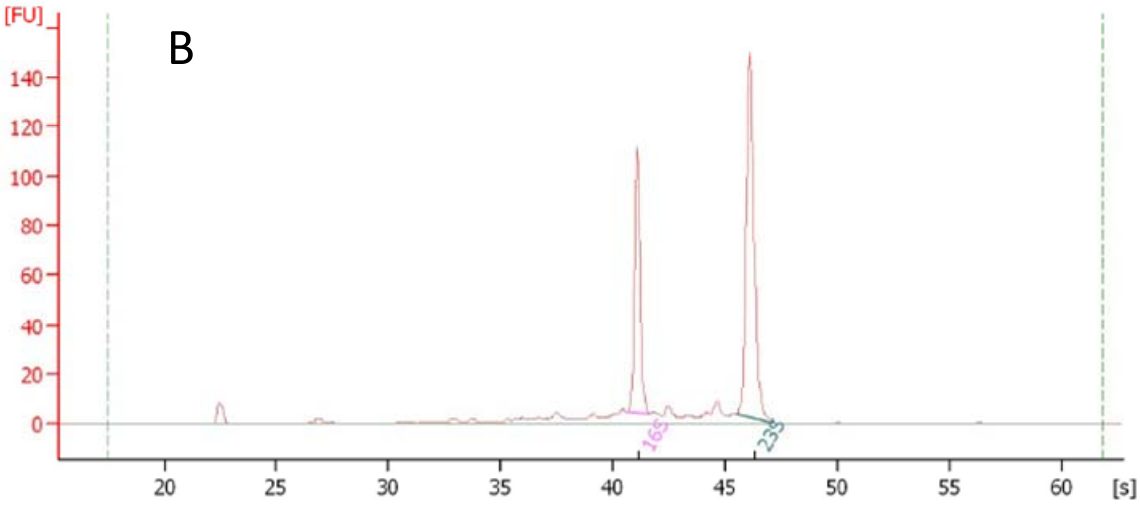
rRNA Ratio [23s/16s]:	1.8
RNA Integrity Number (RIN):	9.4
RNA Concentration:	43 ng/µl

Figure 7. Bioanalyzer analysis of total RNA isolated with mini-bead beater method.

Although the integrity of the RNA was substantially better for the mini-bead beater extraction technique, as compared to the hot phenol:chloroform method, a lower amount of total RNA was recovered. In fact, only 1-2µg (0.03 ml of 0.042 µg/µl) and 2-5 µg (0.03 ml of 0.161 µg/µl) of total RNA (nano-drop measurements) was recovered from an entire plate of the QSil and Sil coating, respectively (Figure 8). This relatively low amount of RNA may not be sufficient for carrying out cDNA synthesis, Alex-Fluor dye labeling and microarray analysis as 2 µg of labeled cDNA is required for loading of the Nimblegen microarray chips. Furthermore, a sufficient amount of unlabeled cDNA from each sample needs to be retained for qPCR confirmation of DNA microarray expression data for selected genes.



rRNA Ratio [23s/16s]:	1.3	RNA Concentration [Bioanalyzer]:	85 ng/µl
RNA Integrity Number (RIN):	8.3	RNA Concentration (Nanodrop):	42 ng/µl



rRNA Ratio [23s/16s]:	1.6	RNA Concentration [Bioanalyzer]:	371 ng/µl
RNA Integrity Number (RIN):	9.5	RNA Concentration (Nanodrop):	161 ng/µl

Figure 8. Bioanalyzer analysis of total RNA isolated from one plate of QSil and Sil. The biofilms were recovered with nylon flocked swabs and the RNA was extracted with the mini-bead beater method for both (A) QSil and (B) Sil.

It is also important to note that the concentration of RNA determined for the samples discussed above was different when measured with both the Agilent Bioanalyzer and Nanodrop systems. In this regard, the concentration of RNA obtained from the Agilent Bioanalyzer was approximately 2 to 2.5 times higher that obtained with the Nanodrop system (Figure 8). The Bioanalyzer system is based on the combination of micro-fluidic capillary electrophoresis and fluorescence spectroscopy to separate and detect the samples, respectively, and provides a measurement of both the quality and quantity of the RNA. The Nanodrop system is based on an absorbance measurement captured with a specialized UV-Vis spectrophotometer to provide the concentration of the RNA only. Although the reason for the difference in RNA concentrations between the two systems is unknown, it is more likely that the Nanodrop system provides a more accurate measurement of RNA concentration than the Bioanalyzer system as it is specifically designed to only make this measurement. In contrast, the Bioanalyzer system was designed to primarily provide information regarding the quality and integrity of the RNA.

Several options were contemplated for generating samples with higher concentrations of RNA while maintaining a high degree of integrity. The first option considered was to try and concentrate the RNA samples using a vacuum centrifuge. However, this option did not seem to be very practical as the starting volume was only 0.030 ml and the vacuum centrifugation process itself could potentially degrade the sample as it is carried out at room temperature. The second option considered was to split the cells recovered from one plate into two mini-bead beater tubes to potentially achieve a more efficient mechanical treatment of the cells. In this regard, it is possible that the cells and biofilm EPS matrix collected from one entire coating plate may not be sufficiently disrupted to liberate all of the RNA when placed in a single mini-bead beater tube.

To investigate this possibility, biofilms were recovered from two plates of the Sil coating where the first plate was processed as one individual sample and the second plate processed as two separate samples by splitting the recovered biofilm equally into two mini-bead beater tubes. Each sample was purified using a separate RNeasy® mini spin column and subjected to both an on-column and off-column DNA digest. The final RNA concentrations were determined using the Nanodrop 2000 micro-volume spectrophotometer (Agilent Bioanalyzer was unavailable at the time this experiment was conducted). The total amount of RNA recovered for the sample collected from a single plate was 4.0 μg while the plate split into two separate samples yielded 5.0 μg (sample 1 = 1.8 μg; sample 2 = 3.2 μg). The results of this experiment showed that approximately 20% more RNA was indeed recovered from a single Sil coating plate when the cells/biofilm sample was split equally between two mini-bead beater tubes. Although 20% more RNA was obtained, the amount was not deemed substantial enough to warrant changing the method and processing twice the samples for each experiment. Furthermore, the two split samples would still need to be concentrated down and pooled together to obtain one composite sample of sufficient RNA concentration.

The third option considered was to simply collect RNA samples obtained from replicate plates onto one RNeasy® mini spin column to generate one composite sample. This option seemed feasible as one mini spin column can easily accommodate up to 45 μg of sample, or approximately 13 and 30 plates of Sil and QSil, respectively. In this regard, the next experiment carried out was to determine if two mini-bead beater tube RNA extractions from two Sil plates could be concentrated into one sample using a single RNeasy mini spin column. The results of the Nanodrop measurement and Agilent Bioanalyzer analysis of the concentrated sample showed that 10.4 μg of total RNA was obtained (0.03 ml of 0.347 μg/μl RNA) and that the integrity of

the RNA was of sufficient quality for downstream processing (RIN = 9.2 and rRNA ratio = 1.6). As expected, this was approximately twice the amount recovered from a single Sil coating plate using one mini spin column (Nanodrop measurement of Figure 8B).

An attempt was also made to concentrate the cells/biofilm recovered from four Sil coating plates onto a single mini spin column (data not reported). In contrast to the two plate experiment, only 5 µg of total RNA was recovered, which was considerably less than would be expected (20 µg) based on RNA yields from previous experiments. It is possible that the substantial amount of cellular debris and biofilm EPS matrix remaining from the mini-bead beater treatments may have impeded the ability of the mini spin column to proficiently purify the RNA. Nevertheless, the results of this study clearly demonstrate that samples recovered from two replicate coating plates can be effectively concentrated onto one mini spin column to obtain a pooled sample of sufficient RNA concentration for downstream processing and analysis.

It was also shown that carrying out an on-column DNA digest only resulted in less RNA loss than carrying out both and on-column and off-column DNA digest treatment. In this regard, 75 ng/µl of RNA was recovered after the on-column DNA digest only while 46 ng/µl of RNA was recovered after both the on-column and off-column treatments. As the RNA was also of sufficient quality and integrity after the on-column treatment only (RIN = 9.0), the second off-column treatment could potentially be omitted, thereby enabling more RNA to be utilized for downstream processing. However, this would need to be verified in future experiments to ensure that the genomic DNA has been adequately removed after the single on-column treatment.

Nimblegen DNA Microarray Analysis

The long term goal of this research project is to gain a fundamental understanding of how novel contact-active antimicrobial coatings prevent or minimize the microbial colonization of

surfaces. One of the most interesting technologies developed to date is based on the covalent attachment of QACs to silicone elastomers. Several of these coating compositions have been shown to effectively prevent the development of bacterial biofilms without adversely affecting cell attachment, growth and viability (unpublished data). These results are somewhat unique as the vast majority of studies in the published literature investigating the antimicrobial efficacy of covalently bound QACs report a contact kill-based mechanism of activity (49). In this regard, immobilized QACs are thought to interact with the bacterial cell wall in a way that compromises its structural integrity ultimately leading to lysis and death of the cell. If this phenomenon had occurred on the surface of the QSil coating investigated in the present study, a dramatic reduction in *E. coli* K12 cell attachment, growth or viability would have been observed with both the high-throughput screening assays and fluorescence microscopy. These disparate results suggest that some other mode of contact activity was responsible for the QSil coatings ability to impede *E. coli* K12 biofilm development.

In attempt to gain some level of understanding how the QSil coating may be preventing the development of the biofilm phenotype, whole-transcriptome profiling of *E. coli* K12 using a DNA microarray was carried out. The primary objectives of this experiment were to assess the feasibility of using ss cDNA with the Nimblegen microarray platform and to determine if any differential gene expression occurred between the QSil and Sil coatings after 72 hours of incubation. If a set of genes was differentially expressed, it would then be examined in more detail to determine if its' up-regulation/repression had been shown in previous studies to impact the process of biofilm formation. The successful identification of gene sets that were both differentially expressed between the two coatings and previously shown to be involved in

biofilm formation would provide preliminary evidence that the QSil coating was capable of altering gene expression through direct contact of the bacteria with the surface of the coating.

The evaluation of differential gene expression for *E. coli* K12 biofilms cultured on Sil and QSil coatings was carried out by 1) recovering biofilms from the coating surfaces prepared in multi-well plates using nylon flocked swabs, 2) extracting the RNA from the recovered biofilm samples using the mini-bead beater extraction technique, 3) purifying and pooling the extracted RNA samples recovered for each coating surface using an RNeasy® mini spin column, 4) synthesizing ss cDNA from the total RNA isolated from each coating surface, 5) labeling the ss cDNA samples with Alexa-Fluor 555 dye, 6) hybridizing the Alexa-Fluor 555 labeled ss cDNA samples to a 4-plex Nimblegen DNA microarray chip, 7) scanning for fluorescence using a GenePix 4300A microarray scanner and 8) analyzing the gene expression data using an ArrayStar® software package.

Figure 9 shows the scatter plot views generated from the ArrayStar® software package that provides a visual comparison of the *E. coli* K12 gene expression levels between the Sil and QSil coatings. A strong linear correlation ($R^2 = 0.9412$) was observed between the two normalized data sets where only 222 of the 4,254 genes represented on the microarray chip (approximately 5.2% of the *E. coli* K12 genome) were identified as differentially expressed at least 2-fold and at a 90% confidence level. Of the 222 differentially expressed genes, 42 were shown to be repressed on the QSil surface while 180 genes were shown to be up-regulated. Furthermore, 14 genes were differentially expressed by 4-fold and 1 gene was determined to be 8-fold differentially expressed.

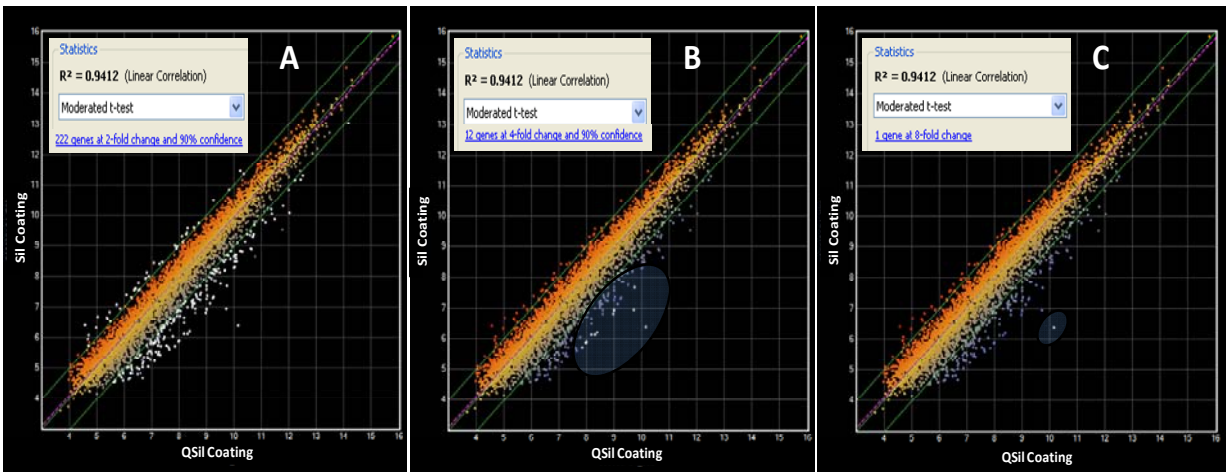


Figure 9. ArrayStar® scatter plot views of the differentially expressed genes. (A) 2-fold, (B) 4-fold and (C) 8-fold with a 90% degree of confidence. Differentially expressed genes are represented as white colored dots with the 4-fold and 8-fold genes highlighted in panels B and C, respectively.

A list of selected genes from the 222 that were identified as at least 2-fold differentially expressed and at a 90% level of confidence is provided in Table 1.

Table 1. List of differentially expressed genes of interest.

Open Reading Frame	Gene	Protein Description or Function	Expression Ratio	Moderated t-test *P* value
Quorum Sensing				
b1513	*lsrA*	Autoinducer-2-transporting ATPase	0.44	0.0416
b1516	*lsrB*	Periplasmic autoinducer-2 binding protein	0.45	0.0395
b1515	*lsrD*	Autoinducer-2-transporting membrane channel protein	0.48	0.0439
b1518	*lsrG*	Autoinducer-2 modifying protein	0.49	0.0333
b1601	*tqsA*	Exporter of autoinducer-2	0.50	0.0431
Transport				
b1487	*ddpA*	D-ala-D-ala transporter subunit; periplasmic-binding component	0.42	0.0565
b1486	*ddpB*	D-ala-D-ala transporter subunit; membrane component	0.40	0.0509
b1485	*ddpC*	D-ala-D-ala transporter subunit; membrane component	0.41	0.0418
b1484	*ddpD*	D-ala-D-ala transporter subunit; ATP-binding component	0.44	0.0719
b1483	*ddpF*	D-ala-D-ala transporter subunit; ATP-binding component	0.46	0.0560

The expression of four genes associated with the autoinducer 2 (AI-2) quorum sensing system encoded by the *lsr* operon, namely, *lsrA, lsrB, lsrD* and *lsrG*, were shown to be repressed on the QSil coating by 2.3-fold, 2.2-fold, 2.1-fold and 2.0-fold, respectively. The *lsrA, lsrB* and *lsrD* genes encode proteins that comprise the ATP-binding cassette transporter which is responsible for shuttling exogenous AI-2 into the interior of the cell. The gene *lsrG,* in concert with *lsrF*, modifies the AI-2 signaling molecule after its internalization (84). An additional gene involved in AI-2 based quorum sensing in *E. coli*, *tqsA*, was also shown to be repressed on the QSil coating (2-fold). The functional component of *tqsA*, or transport quorum sensing A, serves to export the AI-2 signaling molecule out of the cell (19). These results indicate that the entire AI-2-based quorum sensing transport system was effectively down-regulated in the cells recovered from the QSil coating surface. In a sense, the QSil coating appears to jam or interfere

with the process of shuttling AI-2 in and out of the cell, effectively preventing the colonizing bacterium from sensing and communicating with other bacteria residing in its local environment.

This modulation of the AI-2 quorum sensing machinery would be expected have important ramifications with respect to biofilm formation as AI-2-based quorum sensing has been shown to play a pivotal role in controlling this process in *E. coli*. In this regard, Gonzalez Barrios and colleagues showed that the addition of exogenous AI-2 to *E. coli* K12 cultured in LB medium resulted in a 4-6 fold increase in biofilm formation (6). The biofilms generated in the presence of AI-2 were more thick and compact than those generated in the absence of the signaling molecule. Furthermore, it was demonstrated that an *E. coli* K12 *lsr* mutant was incapable of forming a biofilm altogether, regardless of whether or not AI-2 was added to the culture medium. Thus, the repression of several genes of the *lsr* operon would be expected to reduce the uptake of AI-2 and subsequently prevent or minimize biofilm development on the QSil coating surface.

Interestingly, five genes associated with the *ddp* operon encoding the subunits of a D-alanyl-D-alanine (D-ala-D-ala) transporter, namely, *ddpA*, *ddpB*, *ddpC*, *ddpD* and *ddpF*, were also shown to be repressed on the QSil coating by 2.4-fold, 2.5-fold, 2.5-fold, 2.3-fold and 2.2-fold, respectively. The dipeptide D-ala-D-ala is used to synthesize a muramyl peptapeptide precursor that is subsequently utilized to synthesize peptidoglycan (47). Loo and colleagues showed that several transposon mutants of the oral bacterium *Streptococcus gordonii*, which rendered them deficient in peptidoglycan biosynthesis, showed reduced biofilm formation as compared to the parent strain (53). The authors of this study suggested that disruptions in genes involved in the process of peptidoglycan biosynthesis adversely affect the ability of bacteria to respond to environmental changes, such as extracellular osmolarity, which is important during

biofilm growth. Thus, the repression of the D-ala-D-ala transporter on the QSil coating would be expected to reduce the uptake of the D-ala-D-ala dipeptide, potentially reducing the biosynthesis of peptidoglycan and accordingly, limiting the development of the biofilm. However, future studies will need to be carried out to determine the plausibility of this effect.

CONCLUSIONS AND FUTURE RESEARCH

Whole transcriptome profiling has the potential to make a significant impact in the area of materials science. This is particularly true with respect to the development of new coatings and materials that are being designed to resist the attachment and colonization of harmful microorganisms. By understanding how bacteria and other microbes interact and respond to surfaces, materials scientists may be able to leverage this information towards the design of advanced, mutli-functional materials that interfere with key cellular processes known to be crucial to the establishment of microbial communities, such as biofilms.

The results of this research project have provided convincing preliminary evidence that whole transcriptome profiling using DNA microarrays can be effectively employed to elucidate the genetic determinants of *E. coli* K12 biofilm inhibition when attempting to colonize the surface of contact-active antimicrobial coatings. In this regard, an efficient and effective protocol was developed to isolate high quality RNA from *E. coli* K12 cultured on the surface of coatings prepared in multi-well plates. The RNA samples recovered from these coatings were able to be transcribed into ss cDNA, labeled with an Alexa-Fluor dye and successfully hybridized to a Nimblegen DNA microarray chip for the evaluation of differential gene expression. An extensive examination of a preliminary set of microarray data revealed that several genes associated with AI-2 based quorum sensing (i.e., *lsr* operon) and peptidoglycan biosynthesis (i.e., *ddp* operon) in *E. coli* K12 were differentially expressed on the QSil coating and may have contributed to the inhibition of biofilm observed on this surface. These results suggest that the QSil coating prevents *E. coli* biofilm formation and maturation by modulating gene expression through direct contact with the colonizing bacteria and that this interaction is not

detrimental to the general metabolic processes and physiology of the cells (as demonstrated with the biofilm characterization assays).

It is important to note that the results and conclusions of this research project are based on one data set obtained from a single DNA microarray experiment. In this regard, only one quadrant was used on the 4-plex microarray chip for both the Sil and QSil Alexa-Fluor 555 labeled ss cDNA samples (i.e., one technical replicate for each sample). Future studies will need to be conducted with a sufficient amount of biological and technical replicates, typically three of each, to ensure that the general trends in differential gene expression between the QSil and Sil coating observed in this study are indeed a real and reproducible phenomenon. Furthermore, a subset of the key genes identified from these repeat trials will need to be verified by qPCR to provide conclusive evidence of their relative expression levels.

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