# NEW APPROACHES FOR CONTROLLING BIOFILM FORMATION

By

ALLISON ROSE GUINTA

A thesis submitted to the

Graduate School-New Brunswick

Rutgers, The State University of New Jersey

in partial fulfillment of the requirements

for the degree of

Master of Science

Graduate Program in Microbiology and Molecular Genetics and the Graduate School of Biomedical Sciences

University of Medicine and Dentistry of New Jersey

written under the direction of

Dr. Michael Chikindas

and approved by

_____

_____

_____

New Brunswick, New Jersey

*[October 2010]*

ABSTRACT OF THE THESIS

NEW APPROACHES FOR CONTROLLING BIOFILM FORMATION

by ALLISON ROSE GUINTA

Thesis Director: Dr. Michael Chikindas

The following thesis is composed of two chapters represented by two manuscripts. The introduction chapter examines the challenges of biofilm-associated cells in the food industry and in medical applications and novel methods for biofilm prevention through altering genetic regulation. This chapter has been submitted for publication to *The International Journal of Microbiology*. The second chapter is research conducted during graduate studies that has been accepted for publication in *Biofilms: Formation, Development, and Properties* published by Nova Science Publishers, Inc. The ability of a novel salicylic acid based poly (anhydride ester) polymer (SA-PAE) to prevent biofilms in *Salmonella typhimurium* MAE52 was evaluated via cell enumeration studies, pH kinetics, and salicylic acid release kinetics. SA-PAE was found to prevent biofilm formation in *S. typhimurium* MAE52 through disruption of a bimodal nature in pH without significantly reducing cell population. Changes in genetic regulation, reflected in the disruption of the bimodal pH, resulted in biofilm prevention.

Dedication

*To Dad, Mom, and Paul: thank you for all of your support.*

Acknowledgements

I'd like to thank my advisor, Dr. Chikindas for giving me the opportunity to work on such an innovative and interesting project. Dr. Chikindas is an excellent mentor who genuinely cares for his students and is more than just an advisor for scientific and academic growth. He promotes more than an academic role within the laboratory; he maintains his group like a family. His encouragement fosters a balance between independent learning while maintaining availability for questions and constructive feedback. Moreover, Dr. Chikindas offered me developmental hints both as a scientific writer and professional. I hope that many more will get the opportunity to work with Dr. Chikindas and grow as much as I have during their graduate studies.

Special thanks to Dr. Montville for all of his helpful discussions during my graduate work. Dr. Montville was always available to provide helpful advice. His energy and sense of humor always made our interactions enjoyable as well as scientifically enriching.

I would also like to thank Dr. Nancy Woychik and Dr. Pavlo Takhistov for agreeing to serve on my committee and critiquing my dissertation.

I'd like to thank to Dr. Uhrich and Ashley Carbone for their help during my graduate student work both in scientific discussion and contribution to my work.

Thank you to the Center for Advanced Food Technology for funding my work (Grant 4-25539).

Thank you to all of my labmates for their help and friendship throughout my thesis work. Thank you for sharing the good times and being a part of the agony and ecstasy of graduate education. I'd also like to thank Katia for all her helpful discussions and, most of all, being a great friend during my graduate work. I'd especially like to thank Mohamed for always offering a helping hand and never giving up on me during this time, no matter how difficult I made it at times.

Last but not least, I'd like to thank my parents, Joseph and Josephine, and my brother, Paul. Thank you for all of your love, support and patience during these years.

Table of Contents

**Abstract of the thesis……...……...……..…..……. ii**

**Dedication…….……...…… iii**

**Acknowledgments…….iv**

**Table of contents……..…....vi**

Chapter I – Intelligent control of biofilms through targeted antimicrobials: a short review of a concept……..1

I.1. Abstract.……...……...…....2

I.2. Biofilms: a brief introduction….…….……..……2

I.3 Biofilms: A cause for concern in medical and food industries…….…3

I.4. Biofilms and persistent resistance to disinfection…….……...5

I.5. Prevention of biofilm formation through blocking initial attachment…….……7

I.6. Prevention of biofilm formation through development of active polymer coatings....9

I.7. Targeted release of active polymers for biofilm control and prevention……...…10

I.8. Novel approaches to biofilm treatment……..……12

I.9. Biofilm prevention through planktonic cell death: friend or foe?.....13

I.10. Prevention of biofilm without significant reduction of planktonic cell population.....14

I.11. The role of cyclic di-GMP signaling in biofilm formation……..16

I.12. Understanding the bacterial transition to biofilms……...17

I.13. Conclusion……19

I.14. Bibliography…….20

Chapter 2 – Slow release of salicylic acid from degrading poly(anhydride ester) polymer disrupts bimodal pH and prevents biofilm formation in *Salmonella typhimurium* MAE52 ……..……..26

II.1. Abstract…….27

II.2. Introduction……...27

II.3. Material and Methods……30

II.4. Results……...32

II.5. Conclusion…….35

II.6. Bibliography……..39

Suggestions for future studies……40

Appendix 1 Figure legend……..41

Appendix 2 Table of figures……..42

Curriculum Vitae……..….. 44

# Chapter 1: Intelligent control of biofilms through targeted antimicrobials: a short review of a concept.[1]

[1] This chapter is submitted for publication in *The International Journal of Microbiology*. References cited according to *The International Journal of Microbiology* specifications following the chapter.

**Abstract**

The growth of biofilms is a significant problem within the healthcare and food industries. The characteristic resistance of biofilm-associated communities of microorganisms confers persistent survival that is somewhat challenging to address. There are several approaches that are recognized in combating biofilms: i) physical and/or mechanical removal; ii) chemical removal; iii) use of antimicrobials in combination with (i) and/or (ii) to kill planktonic cells, and prevention of biofilm formation. Biofilms can be as much as a thousand times more resistant to these approaches than planktonic cells. For this reason, the use of novel methods to prevent biofilm formation is an advance in controlling biofilms instead of the conventional treatment with antimicrobials and disinfectants. In this review, different approaches to the prevention of biofilm formation are discussed and compared. The methods that focus on altering genetic regulation of biofilm formation offer promising targets which will ultimately lead to the prevention of planktonic cells' transition into biofilms, thus leaving them more susceptible to stress factors such as disinfection, etc. The prevention of biofilm formation can significantly reduce microbial contamination in both the medical and food industries.

**Biofilms: a brief introduction**

A biofilm is defined as a community of microorganisms networked within an exopolysaccharide (EPS) matrix with a distinct architecture. This EPS architecture serves both structural and protective functions. It forms channels that facilitate the transport of nutrients, enzymes, metabolites, and disposal of waste products within and outside of the biofilm matrix [6, 12, 56, 57]. This architecture is essential in terms of supporting the needs

of a multi-cellular biofilm, allowing for each individual cell's requirements to be met in order to sustain viability. In addition to the EPS, there are proteins, nucleic acids, peptidoglycan, lipids, phospholipids, and other cell components present in the matrix of biofilm communities [57, 65]. These components of the EPS and subsequent layers of cells can retard the penetration of antimicrobials, in addition to facilitating the transfer of nutrients in and waste products out of the biofilm structure [51, 55, 57]. As a result of these characteristics, the EPS matrix confers properties in biofilm-associated communities distinct from their planktonic counterparts.

**Biofilms: a cause for concern in medical and food industries**

In recent years, biofilms and the complex challenge of their eradication in medical and food industries have been studied in great depth. Biofilms are problematic in water and sewage treatment facilities, causing metal corrosion, increased contamination of products, decreased quality of water, and reduced efficacy of heat exchangers [8, 43, 65]. In many cases, biofilms irreversibly attach to processing equipment and can thrive in high flow systems [34]. The biofilm-associated cells within the biofilm structure permanently attach to processing surfaces and require abrasive removal techniques including scraping and/or harsh chemical disinfectants such as chlorine [43]. Moreover, biofilms existing within these facilities serve as reservoirs for contamination on production lines, potentially creating a health hazard to the community [14, 68].

Biofilms are often responsible for nosocomial infections and chronic illnesses. In many cases, biofilm-related infections are not resolved by antibiotics and persistently reoccur. Chronic respiratory illnesses associated with cystic fibrosis (CF) patients are a

major example of biofilm-driven infections [9]. *Pseudomonas aeruginosa*, a common biofilm-forming opportunistic pathogen, can lead to lung damage in both CF and immuno-compromised patients [52]. Other persistent illnesses such as chronic bacterial prostatitis, endocarditis, and otitis media are caused by biofilms [16]. Moreover, biofilms are heavily implicated in oral diseases such as dental carries, periodontitis, and denture stomatitis, a condition in which mucosal lesions are formed within the mouth [16, 39]. Furthermore, biofilms colonize medical devices causing recurring or persistent health problems. Biofilms also develop on urinary catheters, prosthetic heart valves, endotracheal tubes, surgical sutures, orthopedic devices, contact lenses, and dentures [13, 16, 55]. Persistent infections related to biofilm-populated medical devices lead to discomfort and inflammation which requires removal or replacement of the contaminated devices. In turn, removal of biofilm-infected medical devices endangers the patient's health and creates additional costs through medical treatments and procedures.

Furthermore, biofilms have been attributed to food-borne illnesses [23, 30]. Biofilms can cause premature biofouling in dairy and other processed foods as well as form on the surfaces of poultry and meat products [1, 28, 30, 53]. The presence of these pathogens can cause cross-contamination of processing equipment, leading to widespread production and post-production contamination that can reach the consumer [30]. Outbreaks related to the consumption of fresh produce such as spinach, onions, lettuce, tomatoes, etc. have been linked to surface colonization by pathogenic biofilm-associated bacteria [4, 7, 22]. The survival of pathogens on raw fruits and vegetables are directly related to the presence of biofilms on the produce surface. Biofilms "offer" the attachment, protection, and resistance qualities essential to ensure the survival of pathogens during harvest, transport, production, and distribution [4, 7, 22]. The ability to form biofilms is common for major foodborne

pathogens such as *Escherichia coli*, *Listeria monocytogenes*, *Salmonella* spp. and *Campylobacteria jejuni*, and remains a significant safety challenge within the food industry [7, 18, 40, 69].

The medical and food industries provide diverse niches for colonizing bacteria where biofilms can thrive. Ultimately, the "goal" of biofilm-associated microbial communities is to survive and prosper in the conquered environment, which can also be used as a foundation for future expansions. In general, the common problem for industries is the ability of biofilms to resist disinfection and stress factors. The challenge of removing biofilms formed within these settings has yet to be resolved, although many methods have been pursued.

**Biofilms and persistent resistance to disinfection**

The major challenge of biofilm removal is addressing their increased resistance to disinfection. Biofilms can be between 100-1000 times more resistant to antibiotics and disinfecting agents than planktonic cells [52, 55]. It has been well established in the literature that biofilm-associated cells have increased resistance to antimicrobials and disinfectants compared to free-living cells. Moreover, even though biofilms are frequently treated with antimicrobials, these methods do not often result in efficient or successful disinfection. As a consequence, biofilms unavoidably release planktonic cells that can cause a systemic infection.

*Salmonella* biofilms are exceedingly resistant to the commonly used disinfectant, triclosan, than planktonic cells [58]. Furthermore, cellulose biosynthesis genes (the genes responsible for biosynthesis of the major EPS component cellulose) are up-regulated in

*Salmonella typhimurium* biofilms. This indicates that a shielding effect provided by the EPS may enhance resistance in biofilms by restricting antimicrobial permeability. Although the EPS is a major factor in contributing to the resistance of biofilms, it is important to acknowledge that there are other factors that may influence resistance *Salmonella typhimurirum* biofilms. *Salmonella typhimurirum* biofilms up-regulate cellulose biosynthesis genes and *marA* and *acrA*, two genes involved in the production of broad-spectrum efflux pumps [58]. Furthermore, biofilms produce an increased amount of persister cells [34]. Persister cells exist as a minority population (approximately 1% of the population in stationary phase) within a cell culture with a slow growth phenotype [35]. Persister cells are a unique type of altruistic cell that down-regulate transcription of energy production and biosynthetic genes [35]. As a result of this phenotype, persister cells absorb compounds slowly and are able to survive in high concentrations of toxic substances. This phenomenon enhances tolerance by protecting persister cells from antibiotics that target actively dividing cells and the production of proteins to block antimicrobial target binding [35].

Biofilms are not effectively killed by common methods in food preservation such as heat, acidification, and exposure to ethanol compared to planktonic cells [28, 29, 50]. Kubota *et al.* [28] demonstrated resistance in three strains of food spoilage lactic acid bacteria treated with ethanol and acetic acid, two commonly used food preservatives. They report lactic acid bacteria biofilms as being resistant to 30% and 40% ethanol solutions whereas planktonic cells were beneath the detection limit. Additionally, biofilms are more resistant to 11% acetic acid solution as compared to planktonic cells [29]. Not only do biofilms display increased resistance to food preservatives, but also have increased production at suboptimum growth temperatures [45]. This presents a risk to the widely popular ready-to-eat and frozen foods.

As mentioned previously, biofilms are resistant to biocides such as triclosan, benzalkonium chloride and chlorhexidine gluconate, which are commonly used on surfaces [19, 50, 52, 58]. Smith and Hunter [52] performed a study on methicillin-resistant *Staphylococcus aureus* (MRSA) and *P. aeruginosa* comparing the effectiveness of these biocides in planktonic and biofilm-associated cells. MRSA biofilms were greater than 100 fold more resistant than planktonic cells and *P. aeruginosa* biofilms were not effectively reduced [52]. Resistance to biocides contributes to the problem of contamination of medical equipment and poses a subsequent health hazard to the patient.

Biofilms are also resistant to many antibiotics including ampicillin, tetracycline, penicillin, erythromycin, and chloramphenicol [21, 37]. Furthermore, *E. coli* biofilms were up to 50 times more resistant than planktonic cells to the antibiotics amikacin, ceftriaxone, and tobramycin [21]. Generally, the use of antibiotics to treat biofilm-related infections does not result in successful cures. Instead, persistent infections are a common outcome, causing significant discomfort and illness. Patients are forced to seek different courses of antibiotic treatments with little success [9].

**Prevention of biofilm formation through blocking initial attachment**

The prevention of biofilm formation is not a novel concept. The method of preventing bacterial adhesion to reduce infection through targeting a microbe's ability to attach to surfaces and host tissues has been reviewed in great detail by Ofek *et al.* [42]. Preventing bacterial adhesion can be addressed in a variety of ways, including the development of receptor blocks, inhibiting bacterial adhesion, and molecules that mediate

binding through the recognition of signal molecules on surfaces [3, 23, 42, 64]. Receptor blocks and/or inhibition of signal recognition molecules may alter ligand-receptor binding, preventing the bacterial recognition of a surface. Without the crucial step of surface binding, the first colonization of biofilms could not occur. Moreover, the use of polymer coatings may inhibit cell interaction with surfaces and release antimicrobials to prevent microbial attachment. The advantages and disadvantages of the aforementioned methods are discussed in subsequent sections of this review. The goal, of course, is to find and optimize the solution(s) that will most efficiently inhibit biofilms and prevent the selection of resistant mutants.

The blocking of initial bacterial attachment is an attractive approach because it can prevent initial biofilm colonization as well as the subsequent infection produced by biofilm-released planktonic cells. Cell recognition of surfaces is mediated by carbohydrate-receptor recognition between the cell and various surfaces [3, 42]. After a ligand's recognition of a receptor, initial adhesion is followed by complex colonization. This recognition can start a cascade leading to development of elaborate biofilm structures. Once a biofilm is established within the complex EPS architecture, resistance properties make removing the biofilm difficult to accomplish. Bacteria have genes coding for many types of adhesion factors that can interact with a variety of surfaces for primary attachment [42]. For this reason, treatment by one anti-adhesion method would not be sufficient. Moreover, expression of carbohydrates and cell surface ligands can be regulated differently depending on environmental conditions. This creates more variables within this mechanistic approach to block initial attachment, and, therefore, makes it more challenging [42].

Another approach to inhibit bacterial adhesion is through the use of "passive" polymer coatings [23, 60]. A "passive" polymer coating limits bacterial attachment to surfaces through altering the surface chemistry without the use of antimicrobial components. In this approach, the polymer coating interferes with bacterial adhesion through prevention of surface binding. For this reason, this method is rather attractive for use with medical devices. Although "passive" coatings may serve as a novel approach to address the biofilm problem on artificial medical devices, limited success has been achieved so far due to attachment variability between different strains [23].

**Prevention of biofilm formation through development of active polymer coatings**

The varied degree of success of "passive" polymer coatings to effectively prevent biofilm formation has lead to the development of active polymer coatings. An active polymer coating has a two-pronged attack, both altering surface chemistry of a device and offering the release of select antimicrobial substances that can be chemically linked to the polymer. These antimicrobial substances are then free to interact with the planktonic cell population which may result in a reduction or cessation of biofilms. Active polymer coatings can be designed to release a variety of antimicrobials such as antibiotics, bacteriocins, metal ions, or plant extracts [11, 15, 17, 25, 31, 36, 44, 49, 70, 72].

The use of active polymer coatings reduces biofilms through releasing antimicrobials, which act against both biofilm-associated cells and planktonic cells. DiTizio *et al.* [15] demonstrated the use of ciproflaxin-loaded liposomal hydrogels to prevent *P.aeruginosa* biofilms. Catheters coated with these hydrogels were able to resist bacterial colonization after exposure for seven days. A significantly lower bacterial presence was recovered from

the supernatant ($10^2$ CFU $mL^{-1}$) than the initial inoculum of $10^7$ CFU $mL^{-1}$. This indicates that planktonic cell death was the likely mechanism of biofilm prevention [15]. Similarly, Curtin and Donlan [11] studied the use of hydrogels coated with bacteriophage, viruses that infect bacteria, to prevent *Staphylococcus epidermidis* biofilm formation. There was an approximate 4.5 log reduction of initial *S. epidermidis* biofilms which was significantly lower ($P<0.0001$) than the control (approximately 7 log) after being treated with the bacteriophage hydrogel coating. Infection via phage successfully killed planktonic cells, significantly reducing the biofilm mass.

**Targeted release of active polymers for biofilm control and prevention**

Active release polymers offer a continuous release of antimicrobials. However, they do not deliver a targeted release which limits their efficacy. General (uncontrolled) release from an antimicrobial-loaded polymer provides short bursts of extremely high concentrations of the antimicrobial component which can be hundreds of times higher than the microbial MIC. This principle does not create sustained release that is targeted to the area requiring antimicrobial release. Targeted release polymers provide accessible antimicrobials available at critical time-points during biofilm development. For extensive review of other systems of antimicrobial delivery including microencapsulation spheres, films, coatings, and antimicrobial-loaded polymers see review written by Balasubramanian *et al.* [2]. The development of controlled release polymers results in the delivery of antimicrobials under specific parameters (i.e. via changes in temperature, ultrasound pulsation, pH, ion concentration, etc). Chemical linkages can be tuned to degrade under environmental conditions that would favor biofilm development.

Norris *et al.* [41] demonstrated the controlled release of ciproflaxin from poly(2-hydroxyethyl methacrylate) hydrogels using ultrasound to control *P.aeruginosa* biofilms. In this model, timed ultrasound pulses facilitate the release of ciproflaxin from a hydrogel, releasing approximately 800 times the planktonic cells' MIC. Biofilms were not significantly prevented by using ciproflaxin hydrogels without the use of ultrasound pulses. After the use of ultrasound pulses, distinct structural differences were observed in biofilms. Moreover, the biomass and average biofilm thickness significantly decreased ($P<0.05$) after this treatment. Control biomass measurements of 5 $\mu m^3/\mu m^2$ were reduced to <1 $\mu m^3/\mu m^2$ and the control average thickness decreased from 8 μm to <1 μm, respectively. Although biofilms were significantly reduced in this study, it is worth noting that ultrasound pulsation disrupts biofilms which may enhance the release of viable planktonic cells. Whereas biofilms naturally allow cells to detach from the matrix, a greater release of viable planktonic cells may promote systemic infection. A better strategy of targeted release is attained through polymeric design. Erdman *et al.*[17] describe controlled delivery of an antimicrobial through rising levels of pH. Degradation studies of salicylic acid poly(anhydride esters) show that the polymeric compound is stable in acidic conditions (pH of 3.5) for 90 days. Conversely, in basic conditions (pH of 10.0) there is a rapid degradation of the polymeric backbone resulting in total degradation after 38 hours [17]. In these conditions, the antimicrobial is released without increasing release of planktonic cells from the biofilm structure. Furthermore, the degradation parameters can be designed for applications (i.e. food packaging systems, coatings on medical devices, etc) to be released during specific conditions that are species-specific.

**Novel approaches to biofilm treatment**

Furthermore, other novel biofilm treatments have been developed to reduce biofilms through killing planktonic cells [38, 54, 61, 71]. Steczko *et al.* [54] demonstrated the efficacy of a catheter lock solution (CLS) against planktonic and biofilm-associated cells. The novel CLS had synergistic effects against *S. aureus*, *E. coli*, and *P. aeruginosa*. Moreover, this solution killed *E. coli* and *P. aeruginosa* planktonic cells in 0.5 and 2 hours, respectively. Furthermore, the CLS killed biofilms in one hour. This novel treatment has positive implications for both biofilm prevention and treatment; however, the authors acknowledge that the CLS may be inappropriate for use against resistant bacteria strains. Furthermore, the use of nanotechnology has become an effective weapon in the arsenal for biofilm reduction. Taylor and Webster [61] describe the use of $\gamma$-$Fe_2O_3$ superparamagnetic iron oxide nanoparticles. They report that generated hydroxyl radicals can depolymerize polysaccharides, cause breaks in DNA, and inactivate enzymes which could compromise the architecture of a biofilm's EPS matrix. They also describe that these nanoparticles can disrupt cell membranes, causing planktonic cell death.

Other studies have pursued biofilm prevention through the use of antimicrobial agents to kill planktonic cells. This method reduces or completely prevents biofilm formation through killing planktonic cells. Although it may seem like this is not a distinction that should be made, the use of high levels of antimicrobials may enhance biofilms' future resistance properties through selection of resistant mutants. Various studies have discussed prevention of biofilms through targeting planktonic cells and reported varied degrees of success ranging from a very modest reduction in viable biofilm communities to complete elimination of all bacterial life present [11, 15, 25, 31, 36, 38, 41, 43, 49, 54, 61, 63, 70-72].

**Biofilm prevention through planktonic cell death: friend or foe?**

Although the delivery of lethal antimicrobial doses can be accomplished in different ways, the mechanism of limiting bacterial colonization through planktonic cell death is essentially the same. It is important to emphasize that throughout the literature, the term "biofilm prevention" can be misleading. Some authors use this term to describe the prevention of planktonic cells transitioning into multicellular behavior without significantly affecting the planktonic cell population count. Conversely, other authors use the term "biofilm prevention" to illustrate the eradication of both biofilm-associated cells and planktonic cells. It may seem as if this is a distinction that should not be made. However, it should be noted that studies where planktonic cell numbers are significantly decreased do not offer insight into the intelligent control of biofilm formation. Moreover, they do not present a long-term solution to this challenge. Methods in which planktonic cells are killed are not suitable for novel treatments of existing biofilms due to the aforementioned selection advantage of resistant cells. Biofilm prevention treatments that do not significantly reduce the planktonic cell population effectively remove the threat of biofilm colonization and offer a high probability of disinfection. The intelligent control of biofilms lies in understanding the regulation of genes that promote the transition into biofilms.

In all the previous examples of biofilm prevention, the authors explore killing planktonic cells to reduce the formation of biofilms. Most methods result in a modest reduction of biofilm-associated cells, leaving some partial biofilms to survive with the same resistance properties and pathogenic characteristics of intact biofilms [11, 41, 60, 62, 70]. There were a few studies, however, that completely removed biofilms by drastically reducing planktonic cells population beneath the threshold necessary to form a successful biofilm [15,

37]. It should be noted that the presence of planktonic cells, no matter how seemingly insignificant, may result in re-colonization of biofilms if the right conditions are met.

**Prevention of biofilms without significant reduction of planktonic cells population**

The prevention of biofilms through altering planktonic cell transition is an ideal way to control the formation and accumulation of biofilm-associated cells. The exposure to biofilm-preventing stress factor(s) essentially turns off the "biofilm trigger" that promotes multi-cellular development. Recent studies have included the use of quorum sensing inhibitors, plant-derived non-steroidal anti-inflammatory drugs (NSAIDs), and molecules secreted by bacteria [5, 20, 26, 27, 48, 64]. Another study elucidated the use of poly(anhydride ester) polymers with chemically linked salicylic acid (SA-PAE), a plant-derived antimicrobial, built into the backbone [20]. These SA-PAE polymers are active-release substances that degrade, subsequently releasing salicylic acid (SA) as pH rises to alkaline conditions [17]. The authors observed biofilm prevention of *Salmonella typhimurium* MAE52, a Gram-negative rod that forms biofilms at the liquid-air interface, over the course of 48 hours. During these experiments, the planktonic cell population did not significantly deviate from the total biofilm population, indicating that released SA was responsible for a global regulatory change affecting the ability to transition into biofilms [20]. Other work studied the prevention of biofilms without significant planktonic cell death. For example, Janssens *et al.* [26] and Brackman *et al.* [5] each used quorum sensing inhibitors to reduce biofilm formation. Quorum sensing is prokaryotic cell-to-cell signaling using diffusible signal molecules. Quorum sensing has been linked to controlling bacterial swarming and the development of biofilm architecture [24, 62]. Each of the quoted research

groups noticed biofilm reduction without significantly reducing the planktonic cell population through targeting quorum sensing systems.

Another study observed biofilm inhibition through the use of a soluble capsular polysaccharide excreted by *E. coli* CFT073 [64]. When the sterilized supernatant of this organism was introduced into an *E. coli* culture (strain MG1655F'), planktonic viability was unaffected and no biofilms were formed [64]. Upon further investigation of this supernatant, K2 serotype group II capsule polysaccharide, involved in ABC-dependent export, was found to be the component responsible for reduction of biofilms in MG1655F' as well as other biofilm-forming bacteria (*E. coli, Klebsiella pneumoniae, P. aeruginosa, S. aureus, S. epidermidis,* and *Enterococcus faecalis*). Furthermore, 39 *E. coli* strains possessing this polysaccharide also inhibited biofilm formation without affecting growth rate. After the gene responsible for expression of K2 serotype group II capsule polysaccharide, *kpsD*, was mutated, biofilm inhibition was completely eliminated. Moreover, *kpsD* mutants displayed an increased biofilm phenotype indicating that strains carrying this polysaccharide may self-inhibit biofilm formation. In a similar study, Kolodkin-Gal *et al.* [27] observed *B. subtilis* biofilms lost their integrity after 6 days of incubation. Eight day old filter-sterilized supernatant was added to freshly inoculated *B.subtilis* culture, resulting in prevention of biofilms [27]. Upon examination of the supernatant, D-tyrosine, D-leucine, D-tryptophan, and D-methionine were found to be responsible for preventing nascent biofilms and disassembly of existing biofilms. Furthermore, *S. aureus* and *P. aeruginosa* cultures were unable to form biofilms in the presence of D-tyrosine and the D-amino acid mixture (D-tyrosine, D-leucine, D-tryptophan, and D-methionine) [27]. Incorporation of these secreted molecules in a targeted delivery system may have great potential in preventing biofilm formation in food packaging and on artificial medical devices.

**The role of c-di-GMP signaling in biofilm formation**

Within a biofilm community, there are several levels of messenger molecules. A common second messenger produced in response to quorum sensing is cyclic diguanylate (c-di-GMP). C-di-GMP is produced by diguanylate cyclase with GGDEF motifs [46, 59, 62]. In addition to the repression of motility and virulence factors, high concentrations of c-di-GMP stimulate biofilm formation through the production of EPS and curli fimbriae [10, 46, 47, 59, 62]. Low concentrations of c-di-GMP, supported by the degradative activity of phosphodiesterases, cleaves c-di-GMP, resulting in expression of virulence and motility while suppressing biofilm formation [47]. Furthermore, c-di-GMP increases the production of EPS through protein binding. For example, in *Acetobacter xylinus,* c-di-GMP increases cellulose production through binding the PelD protein, a c-di-GMP receptor in *P. aeruginosa* PA14 [33, 62]. Cellulose is the major EPS produced in *S. typhimurium* biofilms and has a similar c-di-GMP mechanism [6, 32, 33, 73]). Ueda and Wood [62] elucidated the regulatory cascade of c-di-GMP and biofilm formation. Their results indicate that after activation through quorum sensing, TpbA (tyrosine phosphatase related to biofilm formation) is produced, which inactivates the GGDEF protein, TpbB. The inactivation of TpbB then reduces the production of c-di-GMP thereby decreasing the expression of the *pel* operon and the production of EPS. As a biofilm starts developing, the "pioneer" cells that initially attach to the surface have high concentrations of c-di-GMP in a developing biofilm which results in surface-attached (sessile) cells. Cells moving towards the top of a developing biofilm have a lower concentration of c-di-GMP and are able to swim and coaggregate as colonization progresses [46, 47].

C-di-GMP signaling seems to regulate whether bacteria will exhibit acute infection (low c-di-GMP levels) or persistent infections characteristic of biofilms (high c-di-GMP levels) [10, 47]. Bacteria introduced into a favorable environment will produce low levels of c-di-GMP where they can maintain a virulent phenotype, producing an acute infection. If the environment is unfavorable, levels of c-di-GMP will rise, suppressing virulence genes and promoting biofilm formation. It may seem as if control of c-di-GMP signaling may not be an ideal target due to its expression of virulence genes when biofilm formation is inhibited. However, the swift treatment of antimicrobial-susceptible virulent bacteria is an easier challenge to address than a persistently-resistant biofilm-associated infection. Although biofilms are not inherently acute in nature, they serve as repositories that release virulent cells, producing severe infections. Due to the release of planktonic cells, infections will inevitably reemerge. Targeting c-di-GMP may be an effective way to prevent the initial colonization of biofilms, as well as the potential problem of a persistent repository of the infection. It is important to note that the subsequent expression of virulence genes would inevitably occur in the presence of low concentrations of c-di-GMP and patients would require immediate treatment. However, the planktonic cells are more susceptible to antibiotic treatment than fully mature biofilm-associated infections [21, 37, 52, 55].

It should be noted that there are multiple genes involved in c-di-GMP metabolism which are abundant throughout bacterial cells [47]. This makes targeting the c-di-GMP signaling system difficult. Romeling and Simm [47] report that Gram-positive bacteria have less c-di-GMP metabolizing enzymes which may be useful in prevention of *Staphylococcus* biofilms. Furthermore, c-di-GMP receptors can be blocked through the development of target molecules to impede effector proteins triggered by c-di-GMP signaling.

**Understanding the bacterial transition to biofilms**

A useful approach to understanding the mechanisms of biofilm cessation is the observation of physiological changes that occur during biofilm formation. These normal growth conditions can give an indication of what genes may be a potential target to investigate. The release of salicylic acid induces physiological effects reflected by pH changes in the biofilm-forming enteric pathogen *S. typhimurium* MAE52. In the absence of salicylic acid, S. *typhimurium* MAE52 had a bimodal nature in pH [20]. A "bimodal" pH nature is defined as an initial decrease in pH followed by a marked increase. These results confirmed a similar bimodal curve reported by Wilson *et al.* for *S. typhimurium* LT2 [66]. The bimodal pH of *Salmonella* can be explained through the source of carbon metabolized during the course of growth. When a culture is grown in fresh media, there is an excess of sugars that are the preferred nutritional source of bacteria. As the bacteria rapidly metabolize glucose, the pH becomes increasingly acidic due to the conversion of sugar into acetic and lactic acid metabolites. As glucose becomes depleted, a metabolic switch to the utilization of amino acids is necessary to sustain the microbial cells' requirements. The subsequent breakdown of amino acids creates an alkaline environment, explaining the existence of the bimodal pH curve observed during normal physiological conditions for *S. typhimurium* MAE52 [67]. After treatment with SA-PAE, the pH was acidic for the remainder of the culture incubation, indicating that pH may be an important physiological cue during biofilm formation. It is possible that the release of SA is affecting a global regulatory system that is influencing the disruption of the bimodal pH, and, subsequently, cessation of biofilms.

To prevent biofilm formation without inducing a selective pressure to resistant phenotypes, it is of the utmost importance to find global regulatory targets. The following

section discusses possible regulatory targets to stop the "conversion" of planktonic cells into biofilm-associated cells. Once appropriate targets are found, combinations of controlled release methods can be further explored.

## Conclusion

Biofilms remain a significant concern in the medical and food industries. The ubiquity of strains able to form biofilms with inherent resistance properties is a compelling force behind research for a solution better than over-saturation of disinfectants and antimicrobials. The development of intelligent-design strategies using compounds that prevent biofilm formation through altering genetic regulation are promising strategies. A targeted and controlled release of biofilm-inhibiting compounds is yet another step in combating biofilms. Combination approach(es) of biofilm prevention and stress factors (i.e., antibiotics, disinfectants, etc.) would hopefully solve the "biofilm problem" experienced in the medical and food industries as well as offer new insights into multicellular microbial communities.

**References**


1. Bagge, D., Hjelm, M., Johansen, C., Huber, I., Gram, L.,2001. Shewanella putrefaciens adhesion and biofilm formation on food processing surfaces. Applied and Environmental Microbiology 67, 2319-2325.

2. Balasubramanian, A., Rosenberg, L., Yam, K., Chikindas, M.,2009.Antimicrobial Packaging: Potential vs. Reality—A Review.Journal of Applied Packaging Research 3, 193-221.

3. Bavington, C., Page, C.,2005. Stopping bacterial adhesion: a novel approach to treating infections. Respiration; international review of thoracic diseases 72, 335-344.

4. Beuchat, L.R.,2002. Ecological factors influencing survival and growth of human pathogens on raw fruits and vegetables. Microbes and infection / Institut Pasteur 4, 413-423.

5. Brackman, G., Hillaert, U., Van Calenbergh, S., Nelis, H.J., Coenye, T.,2009. Use of quorum sensing inhibitors to interfere with biofilm formation and development in Burkholderia multivorans and Burkholderia cenocepacia. Research in microbiology 160, 144-151.

6. Branda, S.S., Vik, S., Friedman, L., Kolter, R.,2005. Biofilms: the matrix revisited. Trends in microbiology 13, 20-26.

7. Brandl, M.T.,2006. Fitness of human enteric pathogens on plants and implications for food safety. Annual Review of Phytopathology 44, 367-392.

8. Coetser, S.E., Cloete, T.E.,2005. Biofouling and biocorrosion in industrial water systems. Critical reviews in microbiology 31, 213-232.

9. Costerton, W., Veeh, R., Shirtliff, M., Pasmore, M., Post, C., Ehrlich, G.,2003. The application of biofilm science to the study and control of chronic bacterial infections. The Journal of clinical investigation 112, 1466-1477.

10. Cotter, P.A., Stibitz, S.,2007. c-di-GMP-mediated regulation of virulence and biofilm formation. Current opinion in microbiology 10, 17-23.

11. Curtin, J.J., Donlan, R.M.,2006. Using bacteriophages to reduce formation of catheter-associated biofilms by Staphylococcus epidermidis. Antimicrobial Agents and Chemotherapy 50, 1268-1275.

12. Cuthbertson, L., Mainprize, I.L., Naismith, J.H., Whitfield, C.,2009. Pivotal roles of the outer membrane polysaccharide export and polysaccharide copolymerase protein families in export of extracellular polysaccharides in gram-negative bacteria. Microbiology and molecular biology reviews : MMBR 73, 155-177.

13. de Carvalho, C.C.,2007. Biofilms: recent developments on an old battle. Recent patents on biotechnology 1, 49-57.

14. den Aantrekker, E.D., Boom, R.M., Zwietering, M.H., van Schothorst, M.,2003. Quantifying recontamination through factory environments--a review. International journal of food microbiology 80, 117-130.

15. DiTizio, V., Ferguson, G.W., Mittelman, M.W., Khoury, A.E., Bruce, A.W., DiCosmo, F.,1998. A liposomal hydrogel for the prevention of bacterial adhesion to catheters. Biomaterials 19, 1877-1884.

16. Donlan, R.M., Costerton, J.W.,2002. Biofilms: survival mechanisms of clinically relevant microorganisms. Clinical microbiology reviews 15, 167-193.

17. Erdmann, L., Uhrich, K.E.,2000. Synthesis and degradation characteristics of salicylic acid-derived poly(anhydride-esters). Biomaterials 21, 1941-1946.

18. Gandhi, M., Chikindas, M.L.,2007. Listeria: A foodborne pathogen that knows how to survive. International journal of food microbiology 113, 1-15.

19. Garcia-Castillo, M., Morosini, M.I., Galvez, M., Baquero, F., del Campo, R., Meseguer, M.A.,2008. Differences in biofilm development and antibiotic susceptibility among clinical Ureaplasma urealyticum and Ureaplasma parvum isolates. The Journal of antimicrobial chemotherapy 62, 1027-1030.

20. Guinta, A., Carbone, A., Rosenberg, L., Uhrich, K., Tabak, M., Chikindas, M., (n.d.). Slow release of salicylic acid from drading poly(anhydride ester) polymer disrupts bimodal pH and prevents biofilm formation in *Salmonella typhimurium* MAE52. Manuscript in preparation.

21. Harrison, J.J., Ceri, H., Roper, N.J., Badry, E.A., Sproule, K.M., Turner, R.J.,2005. Persister cells mediate tolerance to metal oxyanions in Escherichia coli. Microbiology (Reading, England) 151, 3181-3195.

22. Heaton, J.C., Jones, K.,2008. Microbial contamination of fruit and vegetables and the behaviour of enteropathogens in the phyllosphere: a review. Journal of applied microbiology 104, 613-626.

23. Hetrick, E.M., Schoenfisch, M.H.,2006. Reducing implant-related infections: active release strategies. Chemical Society Reviews 35, 780-789.

24. Hooshangi, S., Bentley, W.E.,2008. From unicellular properties to multicellular behavior: bacteria quorum sensing circuitry and applications. Current opinion in biotechnology 19, 550-555.

25. Jang, C.H., Park, H., Cho, Y.B., Choi, C.H., Park, I.Y.,2009. The use of piperacillin-tazobactam coated tympanostomy tubes against ciprofloxacin-resistant Pseudomonas biofilm formation: an in vitro study. International journal of pediatric

otorhinolaryngology 73, 295-299.

26. Janssens, J.C., Steenackers, H., Robijns, S., Gellens, E., Levin, J., Zhao, H., Hermans, K., De Coster, D., Verhoeven, T.L., Marchal, K., Vanderleyden, J., De Vos, D.E., De Keersmaecker, S.C.,2008. Brominated furanones inhibit biofilm formation by Salmonella enterica serovar Typhimurium. Applied and Environmental Microbiology 74, 6639-6648.

27. Kolodkin-Gal, I., Romero, D., Cae, S., Clardy, J., Kolter, R., Losick, R.,2010. D-Amino Acids Trigger Biofilm Disassembly. Science 328, 627-629.

28. Kubota, H., Senda, S., Nomura, N., Tokuda, H., Uchiyama, H.,2008. Biofilm formation by lactic acid bacteria and resistance to environmental stress. Journal of bioscience and bioengineering 106, 381-386.

29. Kubota, H., Senda, S., Tokuda, H., Uchiyama, H., Nomura, N.,2009. Stress resistance of biofilm and planktonic Lactobacillus plantarum subsp. plantarum JCM 1149. Food Microbiology 26, 592-597.

30. Kumar, C.G., Anand, S.K.,1998. Significance of microbial biofilms in food industry: a review. International journal of food microbiology 42, 9-27.

31. La Storia, A., Ercolini, D., Marinello, F., Mauriello, G.,2008. Characterization of bacteriocin-coated antimicrobial polyethylene films by atomic force microscopy. Journal of Food Science 73, T48-54.

32. Ledeboer, N.A., Jones, B.D.,2005. Exopolysaccharide sugars contribute to biofilm formation by Salmonella enterica serovar typhimurium on HEp-2 cells and chicken intestinal epithelium. Journal of Bacteriology 187, 3214-3226.

33. Lee, V.T., Matewish, J.M., Kessler, J.L., Hyodo, M., Hayakawa, Y., Lory, S.,2007. A cyclic-di-GMP receptor required for bacterial exopolysaccharide production. Molecular microbiology 65, 1474-1484.

34. Lehtola, M.J., Laxander, M., Miettinen, I.T., Hirvonen, A., Vartiainen, T., Martikainen, P.J.,2006. The effects of changing water flow velocity on the formation of biofilms and water quality in pilot distribution system consisting of copper or polyethylene pipes. Water research 40, 2151-2160.

35. Lewis, K.,2005. Persister cells and the riddle of biofilm survival. Biochemistry.Biokhimiia 70, 267-274.

36. Mauriello, G., Ercolini, D., La Storia, A., Casaburi, A., Villani, F.,2004. Development of polythene films for food packaging activated with an antilisterial bacteriocin from Lactobacillus curvatus 32Y. Journal of applied microbiology 97, 314-322.

37. May, T., Ito, A., Okabe, S.,2009. Induction of multidrug resistance mechanism in Escherichia coli biofilms by interplay between tetracycline and ampicillin resistance

genes. Antimicrobial Agents and Chemotherapy 53, 4628-4639.

38. McDowell, J.W., Paulson, D.S., Mitchell, J.A.,2004. A simulated-use evaluation of a strategy for preventing biofilm formation in dental unit waterlines. Journal of the American Dental Association (1939) 135, 799-805.

39. Monteiro, D.R., Gorup, L.F., Takamiya, A.S., Ruvollo-Filho, A.C., de Camargo, E.R., Barbosa, D.B.,2009. The growing importance of materials that prevent microbial adhesion: antimicrobial effect of medical devices containing silver. International journal of antimicrobial agents 34, 103-110.

40. Murphy, C., Carroll, C., Jordan, K.N.,2006. Environmental survival mechanisms of the foodborne pathogen Campylobacter jejuni. Journal of applied microbiology 100, 623-632.

41. Norris, P., Noble, M., Francolini, I., Vinogradov, A.M., Stewart, P.S., Ratner, B.D., Costerton, J.W., Stoodley, P.,2005. Ultrasonically controlled release of ciprofloxacin from self-assembled coatings on poly(2-hydroxyethyl methacrylate) hydrogels for Pseudomonas aeruginosa biofilm prevention. Antimicrobial Agents and Chemotherapy 49, 4272-4279.

42. Ofek, I., Hasty, D.L., Sharon, N.,2003. Anti-adhesion therapy of bacterial diseases: prospects and problems. FEMS immunology and medical microbiology 38, 181-191.

43. Palmer, J., Flint, S., Brooks, J.,2007. Bacterial cell attachment, the beginning of a biofilm. Journal of industrial microbiology & biotechnology 34, 577-588.

44. Pichette, C., Zhang, H., Davison, W., Sauve, S.,2007. Preventing biofilm development on DGT devices using metals and antibiotics. Talanta 72, 716-722.

45. Rode, T.M., Langsrud, S., Holck, A., Moretro, T.,2007. Different patterns of biofilm formation in Staphylococcus aureus under food-related stress conditions. International journal of food microbiology 116, 372-383.

46. Romling, U., Amikam, D.,2006. Cyclic di-GMP as a second messenger. Current opinion in microbiology 9, 218-228.

47. Romling, U., Simm, R.,2009. Prevailing concepts of c-di-GMP signaling. Contributions to microbiology 16, 161-181.

48. Rosenberg, L.E., Carbone, A.L., Romling, U., Uhrich, K.E., Chikindas, M.L.,2008. Salicylic acid-based poly(anhydride esters) for control of biofilm formation in Salmonella enterica serovar Typhimurium. Letters in applied microbiology 46, 593-599.

49. Ruggeri, V., Francolini, I., Donelli, G., Piozzi, A.,2007. Synthesis, characterization, and in vitro activity of antibiotic releasing polyurethanes to prevent bacterial

resistance. Journal of biomedical materials research.Part A 81, 287-298.

50. Scher, K., Romling, U., Yaron, S.,2005. Effect of heat, acidification, and chlorination on Salmonella enterica serovar typhimurium cells in a biofilm formed at the air-liquid interface. Applied and Environmental Microbiology 71, 1163-1168.

51. Simoes, M., Bennett, R.N., Rosa, E.A.,2009. Understanding antimicrobial activities of phytochemicals against multidrug resistant bacteria and biofilms. Natural product reports 26, 746-757.

52. Smith, K., Hunter, I.S.,2008. Efficacy of common hospital biocides with biofilms of multi-drug resistant clinical isolates. Journal of medical microbiology 57, 966-973.

53. Somers, E.B., Johnson, M.E., Wong, A.C.,2001. Biofilm formation and contamination of cheese by nonstarter lactic acid bacteria in the dairy environment. Journal of dairy science 84, 1926-1936.

54. Steczko, J., Ash, S.R., Nivens, D.E., Brewer, L., Winger, R.K.,2009. Microbial inactivation properties of a new antimicrobial/antithrombotic catheter lock solution (citrate/methylene blue/parabens). Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association 24, 1937-1945.

55. Stewart, P.S., Costerton, J.W.,2001. Antibiotic resistance of bacteria in biofilms. Lancet 358, 135-138.

56. Stoodley, P., Sauer, K., Davies, D.G., Costerton, J.W.,2002. Biofilms as complex differentiated communities. Annual Review of Microbiology 56, 187-209.

57. Sutherland, I.W.,2001. The biofilm matrix--an immobilized but dynamic microbial environment. Trends in microbiology 9, 222-227.

58. Tabak, M., Scher, K., Hartog, E., Romling, U., Matthews, K.R., Chikindas, M.L., Yaron, S.,2007. Effect of triclosan on Salmonella typhimurium at different growth stages and in biofilms. FEMS microbiology letters 267, 200-206.

59. Tamayo, R., Pratt, J.T., Camilli, A.,2007. Roles of cyclic diguanylate in the regulation of bacterial pathogenesis. Annual Review of Microbiology 61, 131-148.

60. Tamilvanan, S., Venkateshan, N., Ludwig, A.,2008. The potential of lipid- and polymer-based drug delivery carriers for eradicating biofilm consortia on device-related nosocomial infections. Journal of controlled release : official journal of the Controlled Release Society 128, 2-22.

61. Taylor, E.N., Webster, T.J.,2009. The use of superparamagnetic nanoparticles for prosthetic biofilm prevention. International journal of nanomedicine 4, 145-152.

62. Ueda, A., Wood, T.K.,2009. Connecting quorum sensing, c-di-GMP, pel polysaccharide, and biofilm formation in Pseudomonas aeruginosa through tyrosine

phosphatase TpbA (PA3885). PLoS pathogens 5, e1000483.

63. Valappil, S.P., Ready, D., Abou Neel, E.A., Pickup, D.M., O'Dell, L.A., Chrzanowski, W., Pratten, J., Newport, R.J., Smith, M.E., Wilson, M., Knowles, J.C.,2009. Controlled delivery of antimicrobial gallium ions from phosphate-based glasses. Acta biomaterialia 5, 1198-1210.

64. Valle, J., Da Re, S., Henry, N., Fontaine, T., Balestrino, D., Latour-Lambert, P., Ghigo, J.M.,2006. Broad-spectrum biofilm inhibition by a secreted bacterial polysaccharide. Proceedings of the National Academy of Sciences of the United States of America 103, 12558-12563.

65. Vu, B., Chen, M., Crawford, R.J., Ivanova, E.P.,2009. Bacterial extracellular polysaccharides involved in biofilm formation. Molecules (Basel, Switzerland) 14, 2535-2554.

66. Wilson, P.D., Wilson, D.R., Brocklehurst, T.F., Coleman, H.P., Mitchell, G., Waspe, C.R., Jukes, S.A., Robins, M.M.,2003. Batch growth of Salmonella typhimurium LT2: stoichiometry and factors leading to cessation of growth. International journal of food microbiology 89, 195-203.

67. Wolfe, A.J.,2005. The acetate switch. Microbiology and molecular biology reviews : MMBR 69, 12-50.

68. Wong, A.C.,1998. Biofilms in food processing environments. Journal of dairy science 81, 2765-2770.

69. Wood, T.K.,2009. Insights on Escherichia coli biofilm formation and inhibition from whole-transcriptome profiling. Environmental microbiology 11, 1-15.

70. Xu, Q., Czernuszka, J.T.,2008. Controlled release of amoxicillin from hydroxyapatite-coated poly(lactic-co-glycolic acid) microspheres. Journal of controlled release : official journal of the Controlled Release Society 127, 146-153.

71. Yanti, Rukayadi, Y., Lee, K.H., Hwang, J.K.,2009. Activity of panduratin A isolated from Kaempferia pandurata Roxb. against multi-species oral biofilms in vitro. Journal of oral science 51, 87-95.

72. Zhang, L.F., Yang de, J., Chen, H.C., Sun, R., Xu, L., Xiong, Z.C., Govender, T., Xiong, C.D.,2008. An ionically crosslinked hydrogel containing vancomycin coating on a porous scaffold for drug delivery and cell culture. International journal of pharmaceutics 353, 74-87.

73. Zogaj, X., Nimtz, M., Rohde, M., Bokranz, W., Romling, U.,2001. The multicellular morphotypes of Salmonella typhimurium and Escherichia coli produce cellulose as the second component of the extracellular matrix. Molecular microbiology 39, 1452-1463.

# Chapter 2: Slow release of salicylic acid from degrading poly(anhydride ester) polymer disrupts bimodal pH and prevents biofilm formation in *Salmonella typhimurium* MAE52[2]

[2] This chapter is accepted for publication as a chapter in *Biofilms: Formation, Development, and Properties* published by Nova Science Publishers, Inc.. References cited according to Nova Science Publishers, Inc. specifications following the chapter.

**Abstract**

The effect of a slow-released natural antimicrobial, salicylic acid (SA), was tested on biofilm formation in *Salmonella typhimurium* MAE52. Glass coverslips coated with poly(anhydride) ester with salicylic acid built into the polymer backbone (SA-PAE) were used to study the release of SA during polymer degradation. *S. typhimurium* MAE52 was found to follow bimodal pH kinetics when cultured in initially-neutral BHI medium (pH 7.2), with the formation of biofilms occurring after 12 hours of incubation. Continuous release of SA from SA-PAE coverslips resulted in a disruption of the pH profile and prevention of biofilm formation. The controlled release of SA over time influenced cellular functions (i.e. metabolism), reflected in the disruption of the bimodal pH. While future research is necessary to elucidate how cellular regulation is affected during exposure to salicylic acid, the delivery of salicylic acid through a degradable polymer shows great potential in the prevention of biofilm formation.

**Introduction**

Biofilms are complex populations of microorganisms. These communities of cells are organized through an exopolysaccharide matrix made of complex carbohydrate-rich polymers and other macromolecules such as DNA, RNA, and proteins (Sutherland, 2001; Branda et al., 2005). Biofilms aggregate as multicellular communities on virtually any surface. The complex architecture of biofilm-associated matrices provides enhanced resistance to multiple stress factors and allows for the influx of nutrients, water, and small signaling molecules that in turn provide effective communication and signaling between the cells (Watnik & Kolter, 2000; Tarver, 2009).

Biofilms continue to be a persistent problem in medicine through the contamination of medical devices including prosthetic joints, artificial heart valves, and contact lenses. These medical device-attached biofilms may serve as a repository for pathogens ultimately leading to chronic infections such as endocarditis, dental caries, cystic fibrosis pneumonia, periodontitis, and bladder infections (Marsh, 2004; Behlau & Gilmore, 2008). Moreover, biofilms are a cause in concern within the food industry. Biofilms are responsible for the biofouling of dairy and meat products (Kumar & Anand, 1998) and the contamination of processing equipment. The presence of biofilms within food processing facilities results in post-production contamination, tainted food reaching the consumer, and a significantly shorter product shelf life (Kumar & Anand, 1998).

Typically, biofilms have an increased resistance to disinfectants and antibiotics, making their eradication from these environments challenging. The intrinsic properties of biofilms allow for the use of different mechanistic approaches to counteract antimicrobials. Biofilm communities show reduced diffusion rates, increased efflux and enzymatic degradation of antimicrobials (Nikaido, 1994; Kumar & Schweizer, 2005). Furthermore, a sturdy EPS matrix hinders the penetration of antimicrobials to the internal biofilm microorganisms, accounting for the increased resistance in these communities (Mah & O'Toole, 2001*)*. In addition, cells incorporated within biofilms have resistance to specific antimicrobial substances that are lost when planktonic cells are removed from the biofilm matrix. According to Tabak et al. (2007), *Salmonella* cells incorporated within biofilms have a higher resistance to the antimicrobial triclosan than planktonic cells removed from the biofilm matrix. A controlled delivery of antimicrobials to a developing biofilm has potential to prevent planktonic cells from transitioning into a resistant biofilm form.

The development of degradable polymers with therapeutic agents built into the polymer has the potential to deliver a drug as it is continuously degraded (Erdmann & Uhrich, 2000). The natural antimicrobial, salicylic acid (SA), is built into a poly(anhydride)ester backbone which yields free salicylic acid as it is degraded, allowing this antimicrobial to interact with a microorganism continuously over time (Erdmann & Uhrich, 2000). This time-controlled, slow release of an antimicrobial has the potential to target production of biofilms as they form. The steady release of salicylic acid has the potential to deliver a sufficient amount of stress to allow for efficient control of non-desired microorganisms.

Salicylic acid, the active component of aspirin, is also referred to as a nonsteroidal anti-inflammatory drug (NSAID). NSAIDs are attractive to use as they are analgesic, antipyretic, and anti-inflammatory in nature. Salicylic acid is a plant metabolite that has an integral role in plant defense against pathogens and has demonstrated antimicrobial activity against microbial pathogens such as *Heliobacter pylori* (Wang et al., 2003; Halim et al., 2007). Moreover, salicylic acid is capable of preventing the attachment of *Staphylococcus epidermidis* biofilms to polymeric cathedras and of attenuating the virulence of *Staphylococcus aureus* (Polonio et al., 2001; Kupferwasser et al., 2003).

Wilson et al. (2003) reported that *Salmonella* has a bimodal pH which increases as cells reach the end of log phase and subsequently drops during stationary phase. Previously, it was shown that salicylic acid released from poly(anyhydride)ester polymer into the microbial environment prevents biofilm formation in *Salmonella typhimurium* MAE52 (Rosenberg et al.,2008). In this chapter we investigate the physiological changes associated

with biofilm formation in *S. typhimurium* MAE52 in the presence of a time-controlled release of salicylic acid and the resultant changes in pH and the kinetics of salicylic acid release.

## Materials and Methods

### Poly(anhydride-ester) synthesis and formation of polymer-coated glass coverslips

Poly[1,6-bis(o-carboxyphenoxy)-hexanoate] was prepared using previously described methods (Prudencio, Schmeltzer and Uhrich, 2005; Rosenberg et al., 2008).

### Preparation of salicylic acid polymer-coated glass coverslips for microbiological assay

Coated and uncoated glass coverslips were sterilized under the Fotodyne, Inc. (New Berlin, WI, USA) ultraviolet (UV) light for 2 minutes. Coverslips were subsequently transferred into sterile 24 well plates using sterile forceps.

### Culture Preparation

*Salmonella* serovar *typhimurium* MAE52 (Scher et al. 2005) was streaked onto Brain Heart Infusion (BHI) agar (Becton Dickinson & Co., Sparks, MD) and incubated at 37°C for 24 hours. The following day a single colony was transferred into 4.5mL of BHI broth (BD) and incubated at 37°C overnight. The $OD_{600}$ of the overnight culture was recorded using a BioRad Smart Spec 3000 spectrophotometer prior to inoculation. Sterile Falcon 24-well plates (Becton Dickinson Labware; Becton, Dickinson and Company, Franklin Labs, NJ, USA) were used to grow biofilms. Two hundred μL of *S. typhimurium* MAE52 overnight

culture diluted to $10^5$ CFU $ml^{-1}$ and 1.8 mL of BHI broth with an initial pH of 7.2-7.3 were added to the experimental wells. For controls, 2 ml of BHI broth were used in the designated wells. The inoculated 24-well plates were incubated at 37 °C with gentle agitation as described previously (Tabak et al., 2007).

**Plate Count**

Each experiment was performed twice in triplicate, with 500 μL samples collected at predetermined hourly time points (0, 5, 8, 12, 24 and 48 h). To break biofilms for the cell enumeration, the biofilms were placed into 4.5 ml of saline containing approximately 15-20 sterile 3 mm glass beads (Fisher Scientific; Pittsburgh, PA, USA) and vortexed for 2 minutes. Subsequently, the samples were serially diluted in BHI broth to obtain a countable range, and 100 μL were plated in triplicate.

**Sample Preparation for pH and Salicylate Measurement**

At each time point, the supernatants were collected and the cells were removed by centrifugation (Hermle Z400K; LabNet, Woodbridge, NJ, USA) at 1500 *g* (25°C). Then, 0.45 μm syringe filters (Nalge Nunc International, Rochester, NY, USA) were used to sterilize the supernatant. Salicylate concentrations and pH measurements were subsequently determined.

**Quantification of Salicylate Concentration**

The Immunalysis Salicylates Direct ELISA Kit (Immunalysis Corp.; Pomona, CA, USA) protocol was followed with a few modifications. The Salicylates ELISA kit-supplied salicylate standard was diluted using a 50% ethanol (v/v) solution to a range of 0-100 μg $ml^{-1}$ for the standard curve measurement. The supplied salicylic acid standard was diluted ten times while experimental samples were diluted to fit into a target absorbance range. Standards and samples were diluted using BHI broth (pH 7.2).

**Statistical Analysis**

Each experiment was performed at least twice in triplicate. Student's *t*-test was used to calculate the difference between replicates to determine significance between time-points. P values of $\leq 0.05$ were regarded as significant.

**Results**

**Disruption of a bimodal pH prevents biofilm formation by *Salmonella typhimurium* MAE52**

In order to determine the influence of a slowly-released antimicrobial on biofilm formation, *S. typhimurium* MAE52 cells were grown in the presence and absence of salicylic acid-based poly(anhydride ester) (SA-PAE) polymer fixed on glass coverslips. In addition, the effect of the control-released salicylic acid (SA) on environmental pH during biofilm formation was studied. The growth of *S. typhimurium* MAE52 and pH changes were

elucidated in BHI broth at an initial pH of 7.2. In the absence of SA-PAE, visible biofilms were formed at the liquid-air interface after 12 hours of incubation, during the early stationary growth phase. The pH kinetics were recorded for 48 hours of incubation and revealed their bimodal nature (Fig. 1A). Under these conditions, a "bimodal nature" is defined as an initial decrease in pH followed by a subsequent increase to a value higher than the initial. During 12 hours of incubation, the medium pH decreased significantly ($P<0.01$) from 7.2 to its lowest point of 5.6. Subsequently, the pH started to rise until it reached its highest point of 8.6 at 48 hours of incubation. There was no significant pH change for the remainder of the experiment (Fig. 1B).

In the presence of the slowly-released SA, there was also a significant ($P<0.01$) decrease in pH to 5.2 after 12 hours of incubation, and there was no significant change in the pH for the remaining time of the experiment (Fig. 1B). Therefore, the bimodal nature of the pH was disrupted in the presence of released SA and there was no biofilm formation, despite the presence of a sufficient number of cells in the environment (as compared to Fig 1A).

**SA release from SA-PAE coverslips is pH dependant**

Previously, Erdmann & Uhrich (2000) reported the complete degradation of the SA-PAE polymer in alkaline (pH 10.0) and neutral (pH 7.0) phosphate buffer saline after 38 hours and 90 days of exposure, respectively. In acidic (pH 3.5) phosphate buffer saline, SA-PAE remained stable with no significant SA release over 90 days of exposure (Erdmann & Uhrich, 2000). The SA-PAE polymer was tested in sterile (control) and S. *typhimurium* MAE52-inoculated BHI borth at acidic (6.2), neutral (7.2), and alkaline (8.5) pH's, all of which allowed for microbial growth. The presence of SA was evaluated at pre-determined time points using the Immunalysis Salicylates Direct ELISA. There was no instantaneous SA

release observed in either the presence or absence of *Salmonella* cells. Alkaline BHI broth stimulated SA release, with 87.7 μg $mL^{-1}$ of free SA observed after 5 hours of incubation. In neutral and acidic BHI, however, SA release was not observed until 8 (6.3 μg $mL^{-1}$) and 24 (4.7 μg $mL^{-1}$) hours of incubation, respectively, suggesting a greater level of polymer stability in these conditions. Throughout the predetermined time-points, alkaline BHI stimulated a significantly greater release of SA than acidic and neutral conditions (Fig. 2A; $P<0.05$ or $P<0.01$ indicated by * and **, respectively), confirming Erdmann & Uhrich's previously reported results for the phosphate buffer system.

A similar trend in the dependency of SA release on initial pH was observed in the presence of Salmonella cells (Fig. 2B). In this case, acidification of the BHI broth caused a significantly lower ($P<0.01$) release of SA compared to neutral and alkaline conditions at all observed time-points. In non-inoculated BHI broth (pH 6.2) there was a detectable amount of SA observed only after 24 and 48 hours of incubation (4.7 μg $mL^{-1}$ and 15.9 μg $mL^{-1}$, respectively). Conversely, acidic BHI inoculated with MAE52 cells had a significantly greater ($P<0.01$) SA release observed at the same time points (179.9 μg $mL^{-1}$ and 384.0 μg $mL^{-1}$, respectively).

The presence of released SA (6.3 μg $mL^{-1}$) in neutral pH (7.2), non-inoculated BHI broth was first observed after 8 hours of incubation. Conversely, in the same medium, a ten-fold higher presence of SA (62.5 μg $mL^{-1}$) was detected in the supernatant of MAE52 culture after only 5 hours of incubation. Furthermore, in the presence of *S. typhimurium* MAE52 cells a significantly greater release of SA ($P < 0.05$) into a neutral pH BHI broth was observed at all time-points as compared to the non-inoculated control. The highest concentration of SA in the supernatant was detected at 48 hours of incubation in both the

control and inoculated BHI broth samples (122.6 μg $mL^{-1}$ and 681.2 μg $mL^{-1}$, respectively). When SA-PAE was exposed to BHI broth with an initial pH of 8.5, the greatest amount of SA release was observed both in the presence and absence of MAE52 cells. The control and inoculated samples had an initial release of SA of 87.7μg $mL^{-1}$ and 293.2μg $mL^{-1}$, respectively, at 5 hours of incubation. The greatest amount of SA released was seen in inoculated alkaline BHI at 48 hours of incubation (1356.6 μg $mL^{-1}$) with the control having a significantly lower ($P<0.01$) SA release (853.0μg $mL^{-1}$). In all initial pH conditions tested, BHI broth inoculated with MAE52 cells had a significantly higher ($P<0.01$) release of SA than in their respective control samples (Figs. 2A and 2B).

**Conclusion**

To the best of our knowledge, this is the first study showing that the addition of control-released salicylic acid (SA) prevents biofilm formation by Salmonella while disrupting the bimodal nature of pH kinetics. During cultivation in BHI broth with no added antimicrobial, the *S. typhimurium* growth medium reached its lowest pH value of 5.6 after 12 hours of incubation. Subsequently, biofilm formation was observed with a concurrent increase of pH to 8.6 at 48 hours of incubation. A decrease in the medium's glucose concentration was detected along with a pH shift to acidic levels (data not shown). The major metabolite produced by *Salmonella* is acetic acid; it has been found to be the main contributing factor to pH lowering in the course of the microorganism's growth in liquid culture (Wilson et al., 2003). With glucose depletion, *Salmonella* switches to an amino acid metabolism which results in a pH increase (Wilson et al., 2003; Wolfe, 2005). Previously, Wilson et al. (2003) reported *Salmonella typhimurium* LT2 displayed a bimodal pH corresponding to rapid glucose metabolism followed by the switch to an amino acid

metabolism. These findings indicate that biofilm formation can be attributed to the availability of nutrients, which is reflected in a bimodal behavior of pH.

After addition of SA-PAE to inoculated BHI broth, a controlled release of SA into the microbial environment was observed. In the presence of SA, the pH reached its lowest value by hour 12 of incubation and did not change significantly ($P<0.05$) over 48 hours of total incubation time. The amount of SA present in the supernatant detected at each time-point was added to uninoculated BHI broth to see if SA was solely responsible for the disruption of the bimodal pH. The addition of SA did not significantly reduce the pH to values seen in the presence of *S. typhimurium* cells (data not shown). These results suggest that SA induced metabolic changes in the cells, reflected in disruption of the bimodal pH.

We determined the minimal inhibitory concentration (MIC) of SA for MAE52 cells grown in BHI broth as 337 $\mu g\ mL^{-1}$. Controlled release of SA from SA-PAE at pH 7.2 did not significantly reduce the cells' viability, although the final concentration of SA at 48 hours of incubation was approximately 2 folds higher than the determined MIC. The slow release of SA influenced cellular metabolism which resulted in prevention of biofilm formation; however, it was not responsible for significant cell death. Previously, Chi-Zhang et al. (2002) reported similar results in *L. monocytogenes*, where low concentrations of an antimicrobial peptide, nisin, efficiently controlled microbial growth in comparison to 5-fold higher concentrations slowly released into the microbial environment. Similarly, our *Salmonella* cells gained tolerance to the continuous release of SA while at the same time became incapable of biofilm formation.

SA-PAE polymer degrades rapidly in alkaline pH and remains stable in acidic and neutral pH phosphate buffer (Edmann & Uhrich, 2000). To investigate if the SA-PAE would

have the same kinetics of SA release in BHI media, SA-PAE coverslips were tested at acidic, neutral, and alkaline pH levels. Unlike the findings in phosphate buffer, when placed in BHI broth the SA-PAE polymer degraded in all environmental pH conditions; however, alkaline BHI promoted a more efficient release ($P<0.01$) of SA in comparison to acidic and neutral BHI (Fig. 2A). To study the effect of cellular metabolism on SA release, SA-PAE coverslips were tested in inoculated BHI broth. In acidic, neutral, and basic pHs, SA release into the culture medium was significantly ($P<0.05$) higher than into uninoculated medium. The higher concentrations of SA in the supernatant can be attributed to the action of cellular metabolic products capable of enhancing the breakdown of the SA-PAE polymer.

Based on the changes observed in microbial physiology, we speculate that cells may consume SA. However, there are no data published on the effect that bacterial metabolism has on environmentally-present SA. The slow, controlled release of SA may have affected the regulation of certain gene(s,) making cells incapable of biofilm formation. These gene(s) may become future targets for a specific antimicrobial design aimed at the prevention of biofilm formation.

**References**

Behlau, I; Gilmore, MS. Microbial biofilms in ophthalmology and infectious disease. *Archives of Ophthalmology*, 2008, 126, 1572-1581.

Branda, SS; Vik, S; Friedman, L; Kolter, R. Biofilms: the matrix revisited. *Trends in Microbiology*, 2005, 13, 20-26.

Chi-Zhang, Y; Yam, KL; Chikindas,ML. Effective control of Listeria monocytogenes by combination of nisin formulated and slowly released into a broth system. *International Journal of Food Microbiology*, 2004, 90, 15-22.

Erdmann, L; Uhrich, KE. Synthesis and degradation characteristics of salicylic acid-derived poly(anhydride-esters). *Biomaterials*, 2000, 21,1941-1946.

Halim, VA; Eschen-Lippold, L; Altmann, S,;Birschwilks, M; Scheel, D ; Rosahl, S. Salicylic acid is important for basal defense of Solanum tuberosum against Phytophthora infestans. *Molecular plant-microbe interactions: MPMI*, 2007, 20, 1346-1352.

Kumar, A; Schweizer, HP. Bacterial resistance to antibiotics: active efflux and reduced uptake. *Advanced Drug Delivery Reviews*, 2005, 57, 1486-1513.

Kumar, CG ; Anand, SK. Significance of microbial biofilms in food industry: a review. *International journal of food microbiology*, 1998, 42, 9-27.

Kupferwasser, LI; Yeaman, MR; Nast, CC; Kupferwasser, D; Xiong, YQ; Palma, M; Cheung, AL ; Bayer, AS. Salicylic acid attenuates virulence in endovascular infections by targeting global regulatory pathways in Staphylococcus aureus. *The Journal of clinical investigation*, 2003, 112, 222-233.

Mah, TF; O'Toole, GA. Mechanisms of biofilm resistance to antimicrobial agents. *Trends in microbiology*, 2001, 9, 34-39.

Marsh, PD. Dental plaque as a microbial biofilm. *Caries research*, 2004, 38, 204-211.

Nikaido, H. Prevention of drug access to bacterial targets: permeability barriers and active efflux. *Science (New York, N.Y.)*, 1994, 264, 382-388.

Polonio, RE; Mermel, LA; Paquette, GE; Sperry, JF. Eradication of biofilm-forming Staphylococcus epidermidis (RP62A) by a combination of sodium salicylate and vancomycin. *Antimicrobial Agents and Chemotherapy*, 2001, 45, 3262-3266.

Prudencio, A;Schmeltzer, RC ; Uhrich, KE. Effect of Linker Structure on Salicylic Acid-Derived Poly(anhydride−esters).*Macromolecules*, 2005, 38, 6895-6901.

Rosenberg, LE;Carbone, AL; Romling, U; Uhrich, KE ; Chikindas, ML. Salicylic acid-based poly(anhydride esters) for control of biofilm formation in Salmonella enterica serovar Typhimurium. *Letters in applied microbiology*, 2008, 46, 593-599.

Scher, K; Romling, U ; Yaron, S. Effect of heat, acidification, and chlorination on Salmonella enterica serovar typhimurium cells in a biofilm formed at the air-liquid interface.*Applied and Environmental Microbiology*, 2005, 71, 1163-1168.

Sutherland, IW. The biofilm matrix- an immobilized but dynamic microbial environment. *Trends in microbiology*, 2001, 9, 222-227.

Tabak, M; Scher, K; Hartog, E; Romling, U; Matthews, KR; Chikindas, ML ; Yaron, S. Effect of triclosan on Salmonella typhimurium at different growth stages and in biofilms.*FEMS microbiology letters*, 2007, 267, 200-206.

Tarver, T. Biofilms A Threat to Food Safety. *Food Technology*, 2009, 2, 46.

Watnick, P; Kolter, R. Biofilm, city of microbes. *Journal of Bacteriology*, 2000, 182, 2675-2679.

Wilson, PD; Wilson, DR; , Brocklehurst, TF; Coleman, HP;, Mitchell, G; Waspe, CR;, Jukes, SA ; Robins, MM. Batch growth of Salmonella typhimurium LT2: stoichiometry and factors leading to cessation of growth. *International journal of food microbiology*, 2003, 89, 195-203.

Zhang, XP; Wang, WH; Tian, Y; Gao, W ; Li, J. Aspirin increases susceptibility of Helicobacter pylori to metronidazole by augmenting endocellular concentrations of antimicrobials. *World journal of gastroenterology: WJG*, 2009, 15, 919-926.

**Suggestions for future studies**

The previous manuscript, investigated the effect of salicylic acid released from a poly(anhydride ester) polymer on biofilm formation in Salmonella typhimurium MAE52 in an in vitro study. This research can be expanded to investigate the changes of mRNA during key time-points to elucidate the actual changes of genetic regulation occurring during the disruption of bimodal pH and cessation of biofilm formation. Moreover, the investigation of the physiological components that causes the manifestation of the bimodal pH in S. typhimurium MAE52 (i.e. glucose, amino acid, and acetic acid concentrations). In addition to this, examination of other important biofilm-forming species (*Escherichia coli*, *Pseudomonas aeruginosa*, *Listeria monocytogenes, Staphylococcu aureus*, etc.) should be investigated to see if the effect of the drug-delivery system is the same and/or the species displays similar physiological aberrations (i.e. bimodal pH during biofilm formation). Furthermore, more practical applications can be investigated including simulations of medical device coatings such as cathedras and/or application of coatings into a food packaging system.

## Appendix 1 Figure legend

**Fig.1. Controlled release of salicylic acid disrupts biofilm formation and bimodal pH kinetics of *S. typhimurium* MAE52 grown at initial pH of 7.2**.

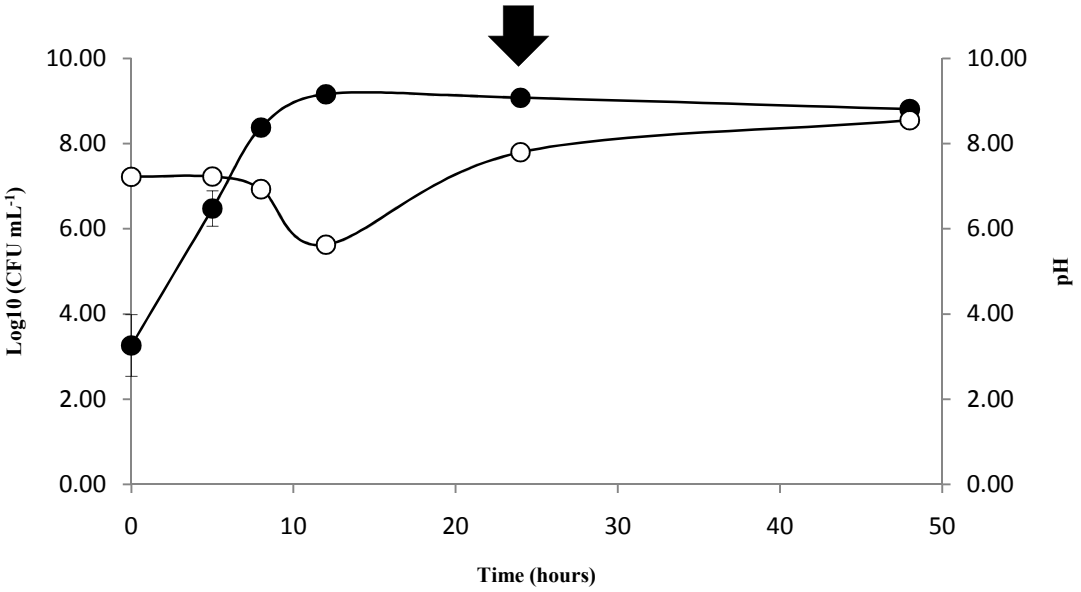
Closed circles indicate cell numbers (CFU $mL^{-1}$) and open circles indicate pH value. **Fig. 1A** represents the control: growth of *S. typhimurium* MAE52 and pH changes with no antimicrobial present in BHI broth (initial pH of 7.2). Time at which biofilm formation started is indicated with an arrow. **Fig.1B** illustrates the growth of *S.typhimurium* MAE52 and disruption of bimodal pH in the presence of released salicylic acid (SA).

**Fig.2. Increased release rate of salicylic acid from SA-PAE is dependant on an increase of medium pH.**

The amount of SA released (μg $mL^{-1}$) in BHI broths with initial pH values of 6.2 (acidic), 7.2 (neutral), and 8.5 (alkaline) are indicated by white, gray, and striped bars, respectively. **Fig. 2A** represents the amount of SA released in non-inoculated (sterile) BHI medium after 0, 5, 8, 12, 24, and 48 hours of incubation. SA release at alkaline pH was significantly higher ($P<0.05$ indicated by * and $P<0.01$ indicated by **, respectively) than in acidic and neutral media through time-points 5 - 48. There was no significant SA release observed in the media with neutral and acidic initial pH until after 8 and 24 hours of incubation, respectively. **Fig.2B** represents the amount of SA released in BHI medium inoculated with *S. typhimurium* MAE52 after 0, 5, 8, 12, 24, and 48 hours of incubation. After 5 hours of incubation, significant release of salicylic acid (** indicates $P<0.01$) was detected in acidic, neutral, and alkaline BHI broths for all time-points. SA release was significantly higher ($P<0.01$) in BHI broth inoculated with *S. typhimurium* MAE52 (Fig 2B) than in non-inoculated medium (Fig 2A).

## Appendix 2 Table of figures

A.




B.

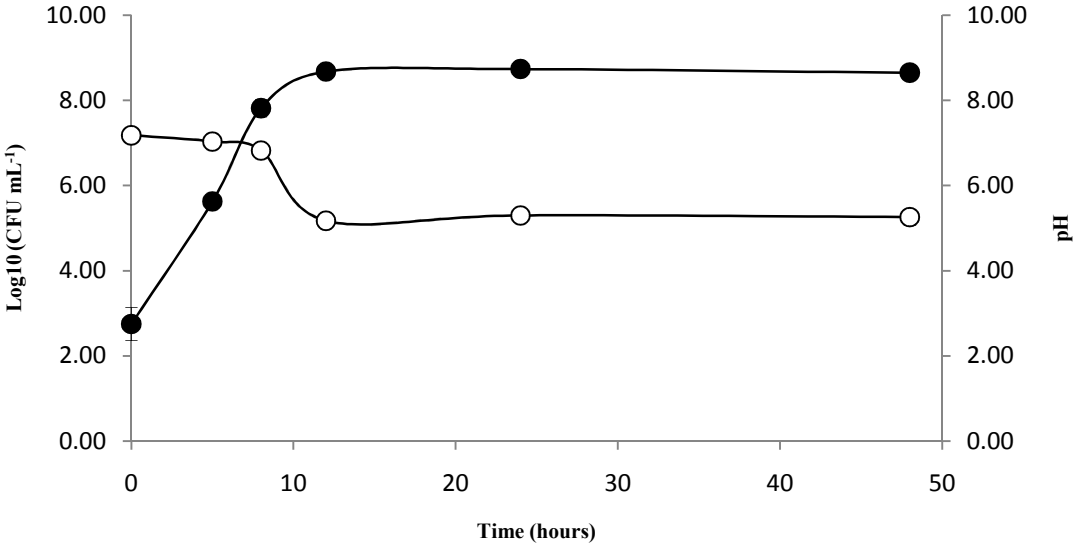



Fig 1

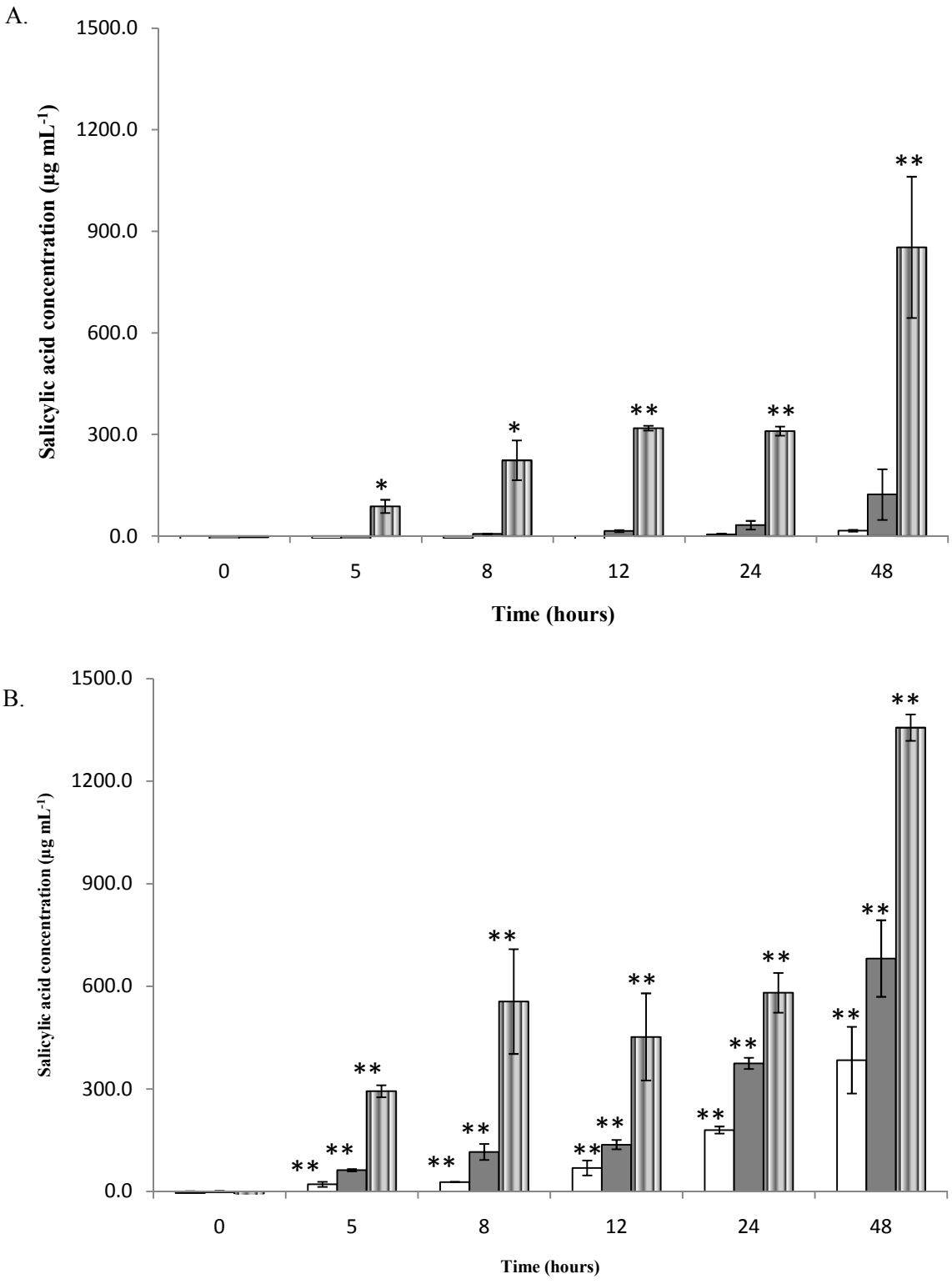

A.
1500.0
1200.0
900.0
600.0
300.0
0.0
Salicylic acid concentration (µg mL-1)
0
5
8
12
24
48
Time (hours)
B.
1500.0
1200.0
900.0
600.0
300.0
0.0
Salicylic acid concentration (µg mL-1)
0
5
8
12
24
48
Time (hours)

Fig 2

**Curriculum Vitae**

ALLISON ROSE GUINTA

Education

| | |
|---|---|
| September 2007 – September 2010 | Rutgers University, Microbiology and Molecular Genetics, M.S., |
| September 2002 – May 2006 | Seton Hall University, Biology, B.S., Magna Cum Laude |

Positions Held

| | |
|---|---|
| May 2010 – present | R&D Microbiologist, Johnson & Johnson |
| January 2010 – May 2010 | UR Co-op, GlaxoSmithKline |
| January 2008 – December 2009 | Teaching Assistant, Rutgers University |
| July 2006 – December 2008 | Microbiologist, Celsis Laboratory Group |

Publications

- "Salicylic acid delivery through degradation of poly(anhydride ester) polymer prevents biofilm production in *Salmonella typhimurium* MAE52 through disruption of bimodal pH." Accepted for publication. *Biofilms: Formation, Development, and Properties*.

- "Intelligent control of biofilms through controlled delivery of antimicrobials." Submitted for publication. *The International Journal of Microbiology*.