Deciphering the Biofilm Eradication Mechanism of Thymol against *Cryptococcus neoformans* Using Multiomics Approach

**Title:** Deciphering the Biofilm Eradication Mechanism of Thymol against *Cryptococcus neoformans*

**Abstract:**

**Keywords:** *C. neoformans*, biofilm, metabolomics, proteomics, NMR, *ERG11*
Background: *Listeria monocytogenes* is an ubiquitous Gram-positive foodborne pathogen, and the agent of listeriosis. Its mode of growth in biofilm represents a source of resistance and contamination at all stages of the food-processing chain. These stable structures are composed of sessile bacterial communities surrounded by a self-secreted extracellular polymeric matrix, by which microorganisms aggregate and interact with each other. The aim of current study is to explore in vitro antibiofilm efficacy of molecules of various origins against *L. monocytogenes*. **Methods:** Four strains of *L. monocytogenes* were used in the present study. These strains were isolated from pork slaughterhouses and cutting facilities after sanitation procedures. Antibiofilm, dose-dependent activity was screened in a microtiter plate biofilm assay to determine active concentrations of tomatidine (plant compound), zinc chloride and EDTA (chemical compounds), and staphylococci exoproducts (bacterial compounds). Inhibition of *L. monocytogenes* biofilm formation was also evidenced using a microfluidic system (BioFlux 200) and confocal microscopy. Additional experiments were subsequently carried out in order to target the mode of action of these different antibiofilm molecules, such as bactericidal or bacteriostatic effects, autoaggregation, and bacterial motility. **Results:** All molecules were effective to inhibit *L. monocytogenes* biofilm formation under static (microtiter plates) and dynamic (microfluidic system) conditions. Active antibiofilm concentrations were found to be 144 µM for tomatidine, 1000 µM and 100 µM respectively for zinc chloride and EDTA and, 50% V/V for staphylococci preparations. Further experiments indicated that these molecules were affecting an early stage of biofilms’ development (adhesion phase) but failed to disperse mature biofilms. This adhesion defect seems to be mainly due to an autoaggregation and motility disruption. **Conclusions:** Our study highlights the critical role of motility for *L. monocytogenes* initial surface attachment in the steps leading to biofilm formation. Overall, our results indicate that flagellum-mediated motility is a promising molecular target to develop new strategies against *L. monocytogenes* colonisation and biofilm formation in the food processing environment.
Staphylococcus aureus is notorious for its ability to become resistant to antibiotics and biofilms play a critical role in antibiotic tolerance. S. aureus is also capable of secreting several exotoxins associated with the pathogenesis of sepsis and pneumonia. Thus, the objectives of the study were to examine S. aureus biofilm formation in vitro, and the effects of herring oil and its main components, omega fatty acids (cis-4,7,10,13,16,19-docosahexaenoic acid (DHA) and cis-5,8,11,14,17-eicosapentaenoic acid (EPA)), on virulence factor production and transcriptional changes in S. aureus. Herring oil decreased biofilm formation by two S. aureus strains. GC-MS analysis revealed the presence of several polyunsaturated fatty acids in herring oil, and of these, two omega-3 fatty acids, DHA and EPA, significantly inhibited S. aureus biofilm formation. In addition, herring oil, DHA, and EPA at 20 μg/ml significantly decreased the hemolytic effect of S. aureus on human red blood cells, and when pre-treated to S. aureus, the bacterium was more easily killed by human whole blood. Transcriptional analysis showed that herring oil, DHA, and EPA repressed the expression of the α-hemolysin hla gene. Furthermore, in a C. elegans nematode model, all three prolonged nematode survival in the presence of S. aureus. These findings suggest that herring oil, DHA, and EPA are potentially useful for controlling persistent S. aureus infection.
Abstract:

**Background:** Currently, treatment options for fungal infections, such as oral candidiasis, present limitations due to the low availability of antifungal drugs and the emergence of drug resistant strains. The use of probiotics is considered promising because they have antimicrobial activity as well as immunomodulatory action, but its clinical application in immunocompromised patients is critical due to the possibility of bacteremia caused by bacteria of the genus *Lactobacillus*. In this context, the use of *Lactobacillus* supernatant extract with its bioactive substances for the control of oral candidiasis becomes innovative and safe for hospitalized and immunocompromised patients. Therefore, the objective of this study was to verify the antifungal action of the crude extract of the culture supernatant of *Lactobacillus paracasei* strain 28.4 on *Candida albicans* SC5314.

**Methods:** First, the supernatant of the *Lactobacillus* culture was extracted with ethyl acetate and purified. The minimum inhibitory concentration (MIC) of the crude extract of the culture supernatant of *L. paracasei* was determined and tested on the filamentation and in biofilm of *C. albicans* by colony-forming unit (CFU) count and quantification of the total biomass. **Results:** The crude extract of the culture supernatant of *L. paracasei* exhibited a MIC value of 15 mg/mL against *C. albicans*. *C. albicans* filamentation was completely inhibited *(p=0.0001)* at the concentrations of 15 mg/mL and 30 mg/mL of the crude extract of the supernatant. In addition, the crude extract of the *Lactobacillus* supernatant significantly reduced the biomass *(p=0.0001)* and the CFU count *(p=0.0001)* of the *C. albicans* biofilm. **Conclusions:** The crude extract of the culture supernatant of *L. paracasei* have antifungal activity against *C. albicans*. *L. paracasei* strain 28.4 produce bioactive substances that caused a significant reduction in biofilms of *C. albicans*. 

**Title:** Inhibitory Effect of Probiotic *Lactobacillus Paracasei* Supernatant Extract on *Candida albicans* Filamentation and Biofilm

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Biofilm formation is a serious problem in medical and industrial settings due to the increased resistance of these communities to killing compared to free-living bacteria. This has prompted the search for agents that can inhibit both bacterial growth and biofilm formation. In this study, N-halamine rechargeable nanoparticles (NPs) were synthesized by co-polymerization of the monomer methacrylamide and the cross-linker monomer N,N-methylenebisacrylamide, and were subsequently loaded with Cl\(^+\), using bleach. The chlorinated NPs demonstrated remarkable stability and durability to organic reagents and to repetitive bacterial loading cycles. The antibacterial mechanism of the P(MAA-MBAA)-Cl NPs involved generation of reactive oxygen species (ROS) only upon exposure to organic media, but not upon suspension in water, revealing that the mode of action is target-specific. Further, a unique and specific interaction of the chlorinated NPs with *Staphylococcus aureus* bacteria but not with human cells was discovered, whereby these microorganisms were all specifically targeted and marked for destruction. Finally, in collaboration with Netafim Ltd. irrigation drippers containing the P(MAA-MBAA)-Cl were incubated in the field and were shown to prevent fouling on them for 5 months compared with the control, hence providing the drippers with 'self-cleaning' and 'self-sterilizing' properties. Further, the NPs offer recharging to the surface, thus providing long-lasting protection that does not exist in the products available today. In summary, our findings underscore the potential of developing sustainable P(MAA-MBAA)-Cl NPs-based devices for inhibiting bacterial colonization and growth.
Abstract: Bacteria in the oral microbiome communicate with each other affecting behavior within the bacterial community. Very little is known about how bacteria in the oral flora may inhibit virulence of pathogenic species and molecular interactions between species in this community. The aim of this study is to identify effective probiotics against *Porphyromonas gingivalis*, a keystone pathogen for the establishment of periodontal disease, & target metabolic pathways of interactions between these organisms. Multiple virulence factors of *Pg* have been identified like adhesion molecules (fimbriae), a capsule & proteolytic enzymes (arg&lys gingipains (ggp)), but these factors can function only when *Pg* can form robust biofilms. The means by which commensals maintain health and prevent *Pg* growth in the oral cavity is a new area of exploration. We hypothesized that the treatment of the oral cavity with bacteria associated with health would have probiotic effects preventing oral disease. A screen of 300 oral commensal bacterial species (*Streptococcus* & *Actinomyces* spp) isolated from healthy adults & children, revealed 60% of strains completely inhibit growth of *Pg*. To identify probiotic inhibitor molecules, HPLC fractions of supernatants of inhibitory strains were tested for probiotic activity against growth of several subspecies of *Pg* strains & a ggp mutants by agar overlay assays & we saw similar inhibitory activity in all as with *Pg*. The inhibitory fractions did not show activity against other oral commensals (*F. nucleatum & P. intermedia*) suggesting that the activity may be *Pg* specific & ggp may not be the target of these inhibitors. Some *Streptococcus* strains only produced inhibitory activity in co-culture with *Pg*, suggesting communication between the organisms. Identification of *Pg* genes that render it sensitive to inhibitors is currently underway using a saturated *Pg* Tn-seq library to be challenged by coculture with the inhibitory metabolites of probiotic strains to identify *Pg* Tn-mutants defective in inhibitor target genes or pathways. The results of this study will increase our understanding of molecular mechanisms of probiotics & will allow us to determine the *in vivo* role of probiotics in preventing periodontal disease.
Abstract:

**Background:** Current methods for measuring bacteria viability in biofilms have challenges beyond what is encountered for planktonic cultures. The majority of live bacteria in biofilms are sessile, and current viability assays based on metabolism or membrane integrity often provide unclear results. Membrane voltage probes partition across the cell membrane in proportion to the membrane voltage strength and have been used as indicators of bacteria viability. In this work, we evaluate whether bis(1,3-dibutylbarbituric acid) trimethine oxonol (DiBAC4(3)), an anionic membrane voltage probe, can differentiate dead from sessile bacteria using fluorescence lifetime microscopy (FLIM). FLIM is very sensitive to fluorophore local environment and much less susceptible to photobleaching than affects intensity measurements. Here we use a stationary phase (SP) planktonic culture of *Streptococcus mutans* in buffer as a starting model system. The SP is our sessile component which is compared against heat killed *S. mutans*. We extract the FLIM variables for individual bacteria in both conditions and use statistical visualization and machine learning to evaluate the effectiveness of this approach to quantify bacteria viability.

**Methods:** *S. mutans* (UA159, ATCC) were grown overnight in Todd Hewitt Broth (THB) at 37 °C, 5% CO2 to SP and resuspended in phosphate buffered saline (PBS). For the dead control, the SP was exposed to 65 °C for 1 h. *S. mutans* (SP or dead) were incubated with 1 μM DiBAC4(3) at 25 °C for 20 min, washed, and resuspended in PBS. 5 μl of culture was sealed between a microscope slide and coverslip. Fluorescence lifetime decay images of *S. mutans* incubated with DiBAC4(3) were collected using a two-photon laser operating at 900 nm, 1 mW and time correlated single photon counting. The fluorescence lifetime response exhibited a double exponential decay. The effectiveness of fluorescence intensity, lifetime variables (t_m, t_1, t_2, p_1) and phasor values (G,S) to differentiate SP and dead bacteria was evaluated using statistical visualization and machine learning. Each data point is the average pixel value over an individual bacteria object.

**Results:** Notch box plots of selected variables reveal that the median value for intensity is significantly different for individual SP and dead bacteria. The same is true for phasor variable G. For both variables, the distributions for SP and dead overlap, leading to possible uncertainty in assigning viability to any individual. Machine learning based on a random forest model using multiple variables provided a much more robust approach to classifying the viability of individual bacteria. **Conclusions:** We show that FLIM response of a membrane voltage probe has merit to distinguish heat killed from SP *S. mutans* in planktonic culture. This foundational study paves the way for improved methods for evaluating the effectiveness of antibiotics and antimicrobials for biofilms.
Abstract:

**Background:** Biofilm is a dynamic, heterogeneous cluster of cells encased in an extracellular matrix. Biofilm cells are encountered in almost all wet surfaces and cause serious problems in the food sector, medical devices etc. Biofilms are resistant to stress conditions as they form a layer of polymeric matrix (extracellular polymeric substances) around them which is difficult to penetrate for destroying the microbial cell. It has been reported that enzymes isolated from microbes can degrade the extracellular matrix of biofilm. Enzymes use environmental friendly approach for complex chemical transformations. They are highly selective biocatalyst which reacts with the specific components in the biofilm. The property of enzymes makes it a suitable candidate for biofilm removal, but free enzymes cannot be easily isolated from the reaction mixture, further, poor stability and reusability limit its applications. Therefore, enzymes need to be immobilized for a better performance. Magnetic CLEA (cross-linked enzyme aggregate) is a new technique for immobilization of enzyme which improves its stability, easy isolation from the reaction mixture, and it can be reused. In the present study, magnetic CLEA of pectinase (MCP) was prepared which is used for biofilm removal of gram-positive and gram-negative bacteria. **Methods:** Preparation of magnetic CLEA of pectinase MCP was prepared by incubating pectinase enzyme, with amino-functionalized magnetic nanoparticles. The mixture is then incubated at 30°C for 15 minutes at 150rpm in a shaking incubator. Then, ethanol and glutaraldehyde were added for precipitation and cross-linking. m-CLEA was separated using a magnet and used for further studies. Biofilm preparation and degradation *Escherichia coli* and *Staphylococcus aureus* were grown in a flask containing Luria broth medium and incubated at 37°C, 120 rpm for 24 hours. 200 µl of the microbial suspension containing $2.5 \times 10^6$ cells of *E. coli* and *S. aureus* were inoculated separately in 96 well plate and incubated without shaking at 27°C for 48 hours as performed earlier by Pitts et.al 2003. Different concentration of enzymes and MCP were added to the wells. Well without any treatment was taken as blank. The absorbance was taken at 595nm by microtiter plate reader. **Results:** MCP was prepared which shows thermal stability at 70°C and no loss of enzyme activity was observed in magnetic CLEA until the sixth cycle. The percentage degradation of *E.coli* biofilm by free pectinase and MCP at the concentration of 200U was observed to be 61.8% and 51.52% respectively. Similarly, percentage degradation of biofilm of *S. aureus* by free pectinase and MCP was observed to be 52.46% and 40.14% respectively. **Conclusions:** MCP is successful in degrading biofilm of *E. coli* and *S.aureus* up to most extent, therefore it can be helpful in removing biofilms in storage tanks in industries, medical devices, etc and can be commercialized.
**Abstract:**

**Background:** Biofilms have raised significant public health concerns and economic losses. For example, pathogenic biofilms are believed to result in hospital acquired infections and foodborne diseases. Compared with conventional disinfection strategies, photocatalysis holds promise for a biofilm control because of broad spectrum effectiveness under ambient conditions, low cost, easy operation and maintenance. We develop graphitic carbon nitride (g-C₃N₄), an emerging visible-light-responsive photocatalyst, for biofilm inhibition and eradication, and understand the mechanism of photocatalytic biofilm control.

**Methods:** g-C₃N₄ powder was synthesized through thermal polycondensation of melamine, cyanuric acid, and barbituric acid and g-C₃N₄ coupons were fabricated via hydraulic press of the powder. *Staphylococcus epidermidis* (*S. epidermidis*), a Gram-positive bacterium, was selected as a model pathogen to grow biofilms on the coupons. Biofilms were grown in the dark and under visible light irradiation to understand biofilm inhibition by photocatalysis, and mature biofilms were also explored for eradication under visible light exposure. Optical coherent topography (OCT), confocal laser scanning microscopy (CLSM), and scanning electron microscopy (SEM) were used to characterize the thickness, coverage, morphology, and viability of the biofilms. Atomic force microscopy (AFM) was used to characterize the mechanical properties of the biofilms. In addition, a florescent lectin, polysaccharide intercellular adhesin (PIA), was utilized to evaluate the presence and content of extracellular polymeric substances (EPS) of the biofilms. Reactive oxygen species (ROS) generated were quantified using radical probes to explore biofilm control mechanisms.

**Results:** OCT and CLSM suggested that g-C₃N₄ coupons inhibited biofilm development and eradicated mature biofilms under the irradiation of white LED light. Compared to mature biofilms developed in the dark, photocatalysis reduced the EPS content based on SEM and PIA staining. In addition, biofilms after photocatalysis became stiffer, which could also be explained with the loss of EPS. H₂O₂ and ·O₂ were identified as the main ROS that control biofilm development and eradication, not only inactivated bacterial cells but also weakened chemical bonds within the EPS matrix. A mechanism of ROS diffusion into and inactivation of the biofilms was proposed based on a biofilm eradication kinetics study.

**Conclusions:** g-C₃N₄ holds promise for antibiofilm applications including both biofilm inhibition and eradication under visible light irradiation. Photocatalysis controls biofilms via simultaneous bacterial inactivation and EPS decomposition under ambient conditions, and it can potentially use indoor visible lighting to reduce the cost, energy consumption, and chemical footprint for sustainable antimicrobial applications.
Abstract:
Quorum sensing (QS) is a crucial mechanism involved in pathogenesis of numerous bacterial infections. Emergence of multi drug resistance is increasing every day and hence it is essential to develop novel strategies against these super bugs. LasR, a transcriptional regulator that plays a vital role in regulation of QS and pathogenesis of *Pseudomonas aeruginosa*. The present study reports a novel urea tailed Mannich base (1-(phenyl (o-tolylamino) methyl) urea with enhanced quorum sensing inhibition. The synthetic compound revealed prolific interactions with LasR quorum sensing receptor and exhibit LasR mediated antagonistic activities in *P. aeruginosa*. In-vitro LasR-based inhibitory activities were further confirmed by biofilm and pyocyanin inhibition assays. In-silico and structure activity relationship studies confirm that the urea moiety present in the Mannich base plays a vital role in antagonizing the LasR receptor by forming a discrete H-bond with Tyr47 residue in the active site and also the presence of carbonyl group in the Mannich base is a discerning advantage. Overall, the findings will be useful in the development of urea-based drugs against *P. aeruginosa* infections in the future.
Background: Periprosthetic joint infections (PJI) and chronic pain remain challenging post-arthroplasty complications, significantly affecting patients’ quality of life. While the prevalence rate of PJI is low, the mortality rate associated with these infections is estimated at 25%. Post-arthroplasty chronic pain is more common, and is often addressed with systemic opioids, which have serious side effects. We propose the local delivery of analgesics from ultra-high molecular weight polyethylene (UHMWPE) bearing surfaces to address post-arthroplasty pain more effectively with minimal side effects. Given that various analgesics were shown to possess antimicrobial activity against Staphylococci – species commonly causing PJI – we hypothesize here that an analgesic-loaded UHMWPE can also provide antibacterial properties to mitigate biofilm formation relevant to multimodal therapy for preventing PJI after an arthroplasty. Methods: Minimum inhibitory concentrations (MIC) of bupivacaine, lidocaine, and ropivacaine were determined using the micro-dilution method. Bupivacaine was incorporated into medical grade UHMWPE at 10, 15, and 20 wt% via phase-separated compression molding. Drug elution studies were conducted in deionized water for up to 3 days. The agar diffusion test was used for the preliminary assessment of antimicrobial properties of analgesic-loaded UHMWPE against methicillin-sensitive S. aureus (MSSA). Anticolonizing activity of the tested materials was initially assessed using crystal violet (CV) assay. Further, the dynamics of bacteria attachment over the 48-hour period was evaluated. The obtained biofilms were additionally visualized using scanning electron microscopy (SEM). Results: Bupivacaine was shown to possess the most pronounced antimicrobial activity against MSSA, and thus, it was chosen for incorporation into UHMWPE matrix to render anticolonization properties. The obtained drug release kinetics suggested that the effective daily dose of bupivacaine delivered from the UHMWPE matrix (30 – 300 mg) falls above its MIC value for at least 3 days. The agar diffusion test confirmed that bupivacaine-loaded UHMWPEs possesses dose-dependent antibacterial properties against MSSA. The CV assay showed antibiofilm activity of the anesthetic-loaded samples, which significantly increases with the increase of bupivacaine content (p < 0.05, n = 5). Further, we showed that drug-loaded UHMWPEs can significantly mitigate bacterial adhesion and biofilm development, which was additionally supported by the SEM images. Conclusions: To the best of our knowledge, this is the first study showing that bupivacaine-loaded UHMWPE possess anticolonizing activity. Therefore, anesthetic-loaded UHMWPE bearing surface is a good example of a multifunctional approach addressing post-operative pain and providing antibacterial prophylaxis after an arthroplasty.
Abstract: Biofilms are also associated with several device-related infections, given their capability of adhering and growing in abiotic surfaces (such as on catheters, bone and joint prosthetics, intrauterine devices, among others). Formation of biofilms increases the bacterial population survival despite the individual bacterial genotypes present in the population (resistant or sensitive to the drug), and in some cases antibiotics can even prompt the survival of bacterial biofilms due to drug tolerance, a phenomenon that is not yet fully understood. The project aims at developing an in vitro screening assay for anti-biofilm compounds against Staphylococcus aureus and Staphylococcus epidermidis. The assay will be developed using high content technology and can be used to determine compound activity and classify them as biofilm-formation inhibitors or biofilm disruption agents (or both). Once developed, the anti-biofilm high content assay will be used for compound triage from Brazilian pharmaceutical companies synthetic libraries. Also, the prospected hit compounds will be characterized and evaluated for human cells cytotoxicity, mechanism of action and resistance induction.
**Session:** Poster Session 4  
**Date & Time:** Thursday, October 11, 2018, 4:00 pm - 6:00 pm  
**Poster Board #:** 013  
**Topic:** Antibiofilm Strategies  
**Abstract Title:** Novel Immunotherapeutics to Treat Pathogenic Periodontal Biofilms *In vivo*  

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**Background:** Periodontitis is a chronic biofilm-mediated disease caused by dysbiosis of the oral community from a healthy to pathogenic state by pathogens such as *Porphyromonas gingivalis (Pg)*, which over time can cause systemic disease and bone loss. *Pg* itself cannot establish an independent biofilm *in vivo*, but instead requires an established biofilm community with specific partners e.g. *Streptococcus gordonii (Sg)*. Biofilms universally contain extracellular DNA that is stabilized by the ubiquitous DNABII family of proteins. Antibodies directed against DNABII proteins sequester free DNABII proteins which causes catastrophic collapse of the biofilm. We have found that antibodies directed against one member of the DNABII family have high avidity to other members of the DNABII family. Indeed, *Sg* comprises a single highly conserved DNABII protein, HU*Sg*, that, similar to most tested DNABII family members, is recognized by an extensive panel of DNABII antisera assayed to date. In contrast, *Pg* expresses an antigenically unique DNABII protein, HUβ*Pg* that is only recognized by antibodies raised against it. **Methods:** Western analysis, immunofluorescence and confocal laser scanning microscopy was used to characterize the roles of DNABII proteins in mixed species biofilms of *Pg* and *Sg in vitro* where *Pg* is added to established *Sg* biofilms to mimic *Pg* entry into an extant biofilm in the oral cavity. Furthermore we introduce a novel rat model to study periodontal disease, using a dual species biofilm of induced osteolytic infection. *Pg*, *Sg*, and *Pg*-*Sg* dual species biofilms (*Pg* added to extant *Sg* biofilms) were grown on the heads of titanium implant screws (sterile screws served as negative controls) and surgically implanted into the maxilla alveolar bone. **Results:** We demonstrated that both extracellular HUβ*Pg* and HU*Sg* are increased in dual species biofilms compared to mono-species biofilms *in vitro*. Strikingly, HUβ*Pg* is more abundant than HU*Sg* (>3 fold), even when *Pg* represents only 5% of the biofilm population. Addition of HUβ*Pg* antibodies to *Pg*-*Sg* biofilms resulted in a significant reduction of *Pg* within the dual species biofilm with minimal disturbance of the underlying *Sg* biofilm. *In vivo*, 14 days post implantation, micro computed tomography was used to measure bone loss surrounding the screws, where only rats that harbored *Pg* as both mono and dual species biofilms showed bone loss. qPCR from DNA extracted from the screws as well as the tissue and bone surrounding the screws revealed that *Pg* and *Sg* were present 14 days post screw implantation. **Conclusions:** The *in vitro* outcomes suggest that HUβ*Pg* antibodies could be used for selective treatment to target *Pg* for elimination, with minimal disturbance of healthy commensals (*Sg*). Our *in vivo* studies validated our animal model for the native sequential events that lead to *Pg* entry into an extant *Sg* biofilm and a means to test our selective anti-*Pg* therapeutic *in vivo*. 
Interactions of Gold Nanoparticles, Chitosan and their Combination with Bacterial Biofilms

Abstract:

Biofilms are currently the most emerging issue regarding food safety. The most significant is the ability of microorganisms to adhere and grow up on foods and surface which they are in contact with. Biofilm formation usually occurs when microorganisms are not completely removed from the surface. Biofilm matrix provides higher protection against negative impact of external conditions in comparison with planktonic growth. Cells in biofilm have more than thousand times higher resistance against antimicrobial substances. To avert biofilm formation, it is necessary to apply different preventive and control strategies. This study was aimed on selection and testing of gold nanoparticles, chitosan and their combination to effectively suppress the adhesion of microbial cells, and thus eliminate the biofilm formation. Staphylococcus aureus 816, 1241; Listeria monocytogenes 149, 164; Escherichia coli 683/17, 693/17 and Salmonella spp. S13, S59 strains isolated from food processing facilities were used for testing. The ability of planktonic cells to adhere and create biofilm were studied after treatment with gold nanoparticles, chitosan and their combination. Biofilm development after pre-cultivation and its eradication were also tested. Biofilm of S. aureus 816, 1241; L. monocytogenes 149, 164 and E. coli 683/17, 693/17 were cultured at 37°C in tryptone soya broth. Salmonella spp. S13, S59 were cultivated at 25°C in the brain heart infusion broth. Cultivation was performed in a pre-sterilized, polystyrene, 96-well, flat-bottomed microtiter plate. Optical density and absorbance were measured spectrophotometrically. Optical cell density was assased at 620 nm. The biofilm quantity after crystal violet staining and metabolic activity of cells were observed at 595 nm. From the performed analyzes, minimum inhibitory concentration (MIC80) and minimum biofilm eradication concentration (MBEC80) of gold nanoparticles, chitosan and their combination were established. We determined the MIC80 of gold nanoparticles to planktonic cultures of S. aureus 816, 1241, and of chitosan to S. aureus 1241; L. monocytogenes 149, 164 and Salmonella spp. S13, S59 strains. The MBEC80 of gold nanoparticles modified by chitosan were defined for planktonic S. aureus 816 and Salmonella spp. S13, S59 strains. The MBEC80 of gold nanoparticles was not yet established to any of the tested strains. The MBEC80 of chitosan was determined for S. aureus 816, 1241 and L. monocytogenes 149, 164, and of modified gold nanoparticles to S. aureus 1241. Our results indicate higher effects of chitosan than gold nanoparticles on planktonic cultures and biofilms. No effect was observed for gold nanoparticles modified by chitosan. This study has been supported by GACR project 17-15936S and specific university research (MSMT No 21-SVV/2018).
Background: The formation of biofilms by bacterial pathogens presents a major obstacle in the treatment of a wide variety of infectious diseases, as biofilm formation confers increased resistance to harsh environmental conditions, host immune effectors, and antibiotic therapies relative to the planktonic phenotype. *Salmonella enterica* serovar Typhi, the etiological agent of Typhoid fever, is able to establish asymptomatic chronic infection in the human gallbladder by forming biofilms that are recalcitrant to conventional antibiotic therapy. These chronic carriers serve as the major reservoir for the bacteria and aid in typhoidal transmission via fecal shedding. The identification of novel strategies to inhibit *S.* Typhi biofilms is therefore of the utmost importance towards the prevention of chronic *S.* Typhi infections.

Methods: In order to identify compounds capable of inhibiting *S.* Typhi biofilms, we screened 90 kinase inhibitor derivatives for their ability to inhibit biofilm formation in the related *S.* enterica serovar Typhimurium, resulting in the identification of the small molecule T315. EC\(_{50}\) concentrations for inhibition of both *S.* Typhimurium and *S.* Typhi biofilms by T315 were determined, and inhibitory activity against additional Gram-negative biofilms was tested. T315 anti-biofilm activity against *S.* Typhimurium and *S.* Typhi was further examined by testing the compound in the presence of sub-MIC doses of ciprofloxacin. In an effort to identify the bacterial target(s) of T315, biotinylated T315 probes were synthesized and used to pull down putative targets from *S.* Typhimurium lysate. Results: Biofilm inhibitor T315 EC\(_{50}\) values were 21.0 and 7.4\(\mu\)M for *S.* Typhi and *S.* Typhimurium, respectively. T315 also exhibited anti-biofilm activity against the nosocomial pathogen *Acinetobacter baumannii* but did not reduce biofilm formation in *Pseudomonas aeruginosa*, suggesting some level of specificity. Additionally, exposure of *Salmonella* to a combination of T315 and sub-MIC doses of ciprofloxacin resulted in an augmented reduction in biofilm formation. Proteomic analysis of pull-down fractions identified the flavin mononucleotide-dependent NADH:quinone oxidoreductase WrbA as a bacterial target of T315. Using a ΔwrbA mutant of *S.* Typhimurium, we showed that this protein is involved in *S.* Typhimurium biofilm formation and that it is required for maximum T315 anti-biofilm activity. Conclusions: We describe for the small molecule T315 a novel application as a *S. enterica* biofilm inhibitor and demonstrate that the use of such anti-biofilm compounds in combination with sub-MIC doses of antibiotics is a promising strategy to combat biofilm-mediated infections such as the chronic carriage of typhoidal *Salmonella*.
**Title:** Developing a Small Molecule Inhibitor of The *Pseudomonas aeruginosa* biofilm Exopolysaccharide Deacetylase Pela

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**Background:** *Pseudomonas aeruginosa* is an opportunistic Gram-negative pathogen and a leading cause of pulmonary and nosocomial infections. The success of *P. aeruginosa* as a pathogen stems, in part, from its ability to grow in a biofilm. One of the exopolysaccharides utilized by *P. aeruginosa* to form a biofilm is Pel, a β-(1,4)-linked polymer composed of partially deacetylated N-acetylglucosamine and N-acetylgalactosamine. This cationic polysaccharide binds to eDNA in the base of the biofilm forming a structural core. Pel is crucial for maintaining cell-to-cell interactions, and providing protection against aminoglycoside antibiotics. Each of the proteins encoded by the pel operon, pelABCDEFG, are required for the biosynthesis and export of the polysaccharide. During biosynthesis Pel is chemically modified by PelA; a multi-domain periplasmic protein that contains a carbohydrate esterase family 4 (CE4) deacetylase domain. De-N-acetylation of the hexosamine sugars in Pel imparts the positive charge to the polymer, and mutation of deacetylase catalytic residues abrogates biofilm formation. This suggests that de-N-acetylation is an important process in Pel biosynthesis, and a potential therapeutic target to prevent Pel-dependent biofilm formation.

**Methods:** To identify inhibitors of PelA deacetylase activity, a high-throughput targeted enzyme assay screen of 68,666 compounds was completed. This screen identified 56 compounds that were classified as hits capable of significant inhibitory activity in vitro. These compounds have been assessed for biofilm inhibition using a secondary ex vivo biofilm assay. **Results:** Four compounds were found to prevent biofilm formation in a Pel overexpression strain. One of the compounds was able to inhibit biofilm formation in several Pel-dependent strains in a dose-response manner. A small library synthesized to explore structure-activity relationships (SAR) of this compound identified an analog that inhibits Pel-dependent biofilm formation and has low cytotoxicity to human lung fibroblast cells. **Conclusions:** The high-throughput screen and subsequent SAR study has identified a low cytotoxic compound able to inhibit Pel-biofilms. Continued SAR will identify the pharmacophore and allow further optimization. Steady-state enzyme kinetics are in progress to determine the mode of inhibition. Structure determination of PelA will advance our understanding of the mode of inhibition and aid in the development of second generation inhibitors.
Membrane Integrity Based Viability Staining with DNA-binding Propidium Iodide Underestimates Viability of Sessile Bacterial Cells on Glass

Background: Intact membrane impermeable DNA-binding stain propidium iodide (PI) is widely used for bacterial viability staining in combination with membrane-permeable DNA-binding counterstains. Although some criticism to the method has arisen concerning for example counterstain fluorescence intensity differences for viable and dead cells and energy transfer during co-staining, the principle of such co-staining has proven to be quick, reliable, commercially available, and widely used for planktonic cultures. PI-based viability staining has also been used for sessile bacteria in biofilms where staining offers a valuable tool as culture-based methods for viability assessment in biofilms, especially in multispecies or environmental settings, are limited.

Methods: Monolayer aggregates of 24 h old Staphylococcus epidermidis DSM-20044 or Escherichia coli MG1655 biofilms on glass in phosphate buffered saline (PBS) were either rinsed, stained with 30 µM PI and 5 µM Syto 9 in situ or cells were harvested via sonication, stained and filtered. Samples were incubated in dark for 15 minutes prior to epifluorescence microscopy. Staining with 25 µg/ml fluorescein diacetate and plate counts were used as controls. Results: In situ stained 24 h biofilms consist of 75.69±18.44% to 96.35±5.3% PI-positive red cells for S. epidermidis and E. coli respectively even though 68% the cells of either species in these aggregates are metabolically active when stained with fluorescein diacetate (FDA) while 99% or more planktonic cells above the surface stain green when co-stained with PI and Syto 9. Higher biofilm viability estimates with 19.56±8.93% to 43.50±5.30% PI-positive red cells for S. epidermidis and E. coli respectively were achieved after harvesting adherent cells via sonication probably due to extracellular matrix being removed during the process but the results still underestimated viability compared to 82% of harvested E. coli and 89% of S. epidermidis being cultivable. Confocal laser microscopy reveals that this false dead layer of red cells consists of cells that have green interiors under red coating layer which hints at extracellular DNA being stained outside intact membranes. Conclusions: In this study we show that PI-based viability staining significantly overestimates dead cell counts in 24 h E. coli and S. epidermidis biofilms formed on glass in PBS. We conclude that extracellular DNA that is needed for biofilm formation and attachment to surfaces and its possible impact on PI-based viability staining outcome must be considered and controlled for to avoid significant overestimation of dead cells in biofilms. This finding has critical impact on estimation of biofilm viability especially in antimicrobial surface efficiency testing as well as assessment of biofilm viability in general.
**Background:** Aquatic biofilm can function as a refuge microcosm enabling pathogens to survive austere conditions and evade control measures. Biofilm of water systems such as drinking water distribution networks and cooling towers typically predominate as surface-associated structures. Alternately, estuarine biofilm can exist as loose, untethered migratory masses that deposit upon sediment beds, resuspend into water columns, and travel with tidal currents to reach distant sites. In this study, estuarine suspended sediment biofilm parameters (architecture, diffusivity, and disinfectant reactivity) were examined to develop strategies for effective control of microbes of complex waters to address recent national and global regulations for ship ballast management. **Methods:** Samples of estuarine water with suspended sediment were collected from a recreational beach of a Chesapeake Bay tributary (Magothy River). Suspended sediment biofilm and extracellular polymeric substance (EPS) structures were characterized by brightfield and polarized light microscopy. Intact suspended sediment and sample dispersed by physical and chemical treatments were compared to elucidate biofilm properties. Dispersion was quantified by increases in microbe-size particles (MSP), heterotrophic plate count (HPC) bacteria (R2A medium), UV-absorbing substances (UVAS), and turbidity. Rates of solute diffusion and disinfectant reactivity by intact and dispersed biofilm were derived by uptake of a cationic compound (methylene blue) and consumption of free available chlorine (FAC), respectively. **Results:** Suspended sediment consisted primarily of floccular biofilm masses harboring large numbers of MSP and HPC bacteria. EPS association with suspended sediment was suggested by birefringence of MSP and diffuse material within and at the periphery of the floccular masses. Effective dispersion of suspended sediment was demonstrated by microscopic examination and significant release of MSP, HPC bacteria, and soluble UVAS. Rates of methylene blue uptake and FAC consumption were much greater with dispersed suspended sediment than with intact suspended sediment. Dye uptake displayed first-order kinetics before reaching equilibrium. FAC consumption appeared as first-order kinetics initially (first min) followed by a mixed-order reaction period prior to equilibrium. **Conclusions:** Estuarine suspended sediment as biofilm masses harboring large numbers of microorganisms can be recalcitrant to disinfectant treatment and present challenges to microbial control operations. Strategies that combine dispersion with disinfection could provide the basis for more effective biofilm treatment. In addition to supporting efforts to meet recent ship ballast microbial control regulations, the model biofilm system and findings of this study could be applied to control other complex water systems.
Session: Poster Session 4
Date & Time: Thursday, October 11, 2018, 4:00 pm - 6:00 pm
Poster Board #: 020
Topic: Antibiofilm Strategies
Abstract: Delivering Antibiotics Locally to Biofilms: Targeted Drug Delivery vs. Local Synthesis from Non-toxic Prodrugs
Title: R. Walter, S. M. Nielsen, P. O. Andersen, L. Hansen, H. V. Quang, R. Christiansen, J. Kjems, A. Zelikin, R. L. Meyer;
Aarhus University, Aarhus, DENMARK.

Abstract:

**Background:** Antibiotic treatment of biofilm infections often fail because the dose that can be delivered without adverse side effects is not high enough to eradicate the biofilm. We explore two different approaches to delivering a high local dose of antibiotics at the site of infection: Targeted drug delivery and local drug synthesis from nontherapeutic pro-drugs. Targeted drug delivery relies on drug encapsulation and accumulation in the biofilm, followed by burst release of the drug. Local drug synthesis, however, uses immobilized enzymes to convert less toxic prodrugs to the active drug at the site of infection. This approach was not previously available for antibiotics, but our development of a novel synthesis method for generating glucuronide-prodrugs of two fluoroquinolones have opened the door for using prodrug therapy in microbiology. **Methods:** For targeted drug delivery, we encapsulated vancomycin and rifampicin in temperature-sensitive 100 nm liposomes decorated with aptamers that bind specifically to *Staphylococcus aureus* biofilms. Particle accumulation, drug release, and kill efficiency was studied on *S. aureus* biofilms. Local drug synthesis was achieved by immobilizing the enzyme catalyst (β-glucuronidase) in a polyelectrolyte layer-by-layer coating on titanium surfaces representing an implant. Drug release was achieved by supplying prodrugs of moxifloxacin in solution, and the effect on bacterial growth, survival, and biofilm formation was studied on *S. aureus*. **Results:** Drug-loaded aptamer-targeted liposomes accumulated in *S. aureus* biofilms and resulted in eradication of biofilms in vitro, while non-targeted liposomes were less effective. Although this result is promising, one could argue that the released drug will diffuse out of the biofilm in vivo, resulting in short exposure. We therefore investigated the effect of continued drug synthesis at the implant surface, using prodrug therapy. The embedded enzyme quickly converted the prodrug to moxifloxacin, which inhibited bacterial growth near the implant surface and prevented biofilm formation. The antimicrobial effect of prodrug therapy on pre-formed biofilms was comparable to the effect of the active drug at the same concentration. Advantages of prodrug therapy can therefore be expected if prodrugs can be administered in higher doses than conventional drugs due to their lower toxicity. **Conclusions:** Targeted drug delivery and pro-drug therapy are both able to expose biofilms to high local antibiotic concentrations. Drug release is better controlled in prodrug therapy, but it requires the placement of a catalyst near the biofilm unless it is already embedded in the implant. Future developments of long-term stable catalysts may therefore determine which of these strategies that is better suited for treatment in vivo. This work paves the way for advances in the use of locally induced antimicrobial treatment.
Abstract: The increased incidence of Staphylococcus Aureus (S. aureus) biofilm infections that are resistant to various antimicrobial drugs necessitates the development of novel treatment strategies. It has been shown that hyperthermia treatment to increase the temperature up to 45°C could enhance the antibiotic susceptibility of S. aureus. The question is how heat can be applied topically without risking thermal burns in the host tissue. We previously validated a magnetic nanoparticle thermotherapy platform that can target and substantially reduce the viability of bacterial pathogen using both in vitro and in vivo mouse model of S. aureus infection. The principle of this method is to induce a localized increase in temperature in bacteria by targeted activation of magnetic nanoparticles (MNPs) with externally applied energy source of high frequency alternating magnetic field (AMF). In this study, we have examined whether MNP/AMF hyperthermia treatment can be synergistic with conventional antibiotic therapy on S. aureus biofilm. 

Methods: Varying concentrations (1-3 mg/mL) of MNPs (100 nm, super paramagnetic nanoparticles, Micromod Inc.) were added to the wells of biofilm formed by S. aureus (2x10^8 CFU, ATCC 6538) and incubated for 2 hour. Then, the samples were treated with an AMF for 6 min duration at a field strength of 30 kA/m and at a frequency of 2.1 MHz. Following the application of MNP/AMF, biofilms were treated with antibiotics (ciprofloxacin and vancomycin) for 24 hours, at MIC determined from the planktonic phase of S. aureus. 

Results: The MIC values of ciprofloxacin and vancomycin for planktonic phase of S. aureus (2x10^8 CFU) were measured to be 16 μg/mL. However, biofilm phase of S. aureus exhibited ~100-fold higher resistance to both antibiotics than those seen in planktonic culture. The application of AMF to the S. aureus biofilm resulted in increase of ambient temperature in the solution via MNP concentration dependent manner (from 23°C to 39°C for 1 mg/mL, to 44°C for 2 mg/mL, and to 48°C for 3 mg/mL MNPs over 6 min exposure of AMF), which was associated with a concomitant reduction in bacterial CFU number (by 1 log reduction for 2 mg/mL and 2 log reduction for 3 mg/mL MNPs). The pretreatment of MNP/AMF hyperthermia to the biofilm phase of S. aureus has significantly augmented the antibiotic susceptibility of S. aureus biofilm, up to 5 log reduction in CFU number for ciprofloxacin and vancomycin at 16 μg/mL, while antibiotics alone did not alter CFU numbers. This was associated with increased uptake of antibiotics to the bacterial cells and generation of reactive oxygen species. 

Conclusion: Our study validates that non-invasive MNP/AMF hyperthermia can be successfully applied to eradicate biofilm infections. Importantly, the combined use of MNP/AMF hyperthermia with antibiotic treatment can synergistically improve treatment efficacy compared to either therapeutic approach alone.
Abstract:

**Background:** Post-operative *Propionibacterium acnes* (P. acnes) and Methicillin-resistant *Staphylococcus aureus* (MRSA) surgical site infections (SSIs) continuously prove to be hard to prevent and treat. These infections can be attributed to inadequate skin prep techniques that fail to eradicate bacteria at the site of injury. The current methods of sterilizing a patient’s body include the application of an antiseptic compound that can include Chloraprep, Duraprep, or Betadine. However, it has been shown that P. acnes and MRSA may be resistant to preparations using these compounds as strains of Chlorhexidine-resistant pathogens have been emerging in hospital environments. This presents a problem as incisions made through skin (ie: scalpel or sutures) can inoculate the incision site with bacteria; allowing biofilm formation and infection. This study validated the efficacy of a silver carboxylate antimicrobial skin prep penetrance into the dermis and the pilosebaceous glands of porcine skin and as an effective antimicrobial agent against P. acnes and MRSA. **Methods:** Kirby Bauer assay was performed with 10mm Whatman filter paper as a vehicle. MRSA was plated at a concentration of $1 \times 10^9$ CFU/mL. P. acnes was plated at a concentration of $1 \times 10^7$ CFU/mL. Zones of inhibition for MRSA were visualized at the 24, 48, and 72-hour time points. P. acnes visualization was offset by an additional 24 hours. With IACUC approval, the axillary regions of 12 Yorkshire and Yucatan pigs were treated on 3 sites with Chlorhexidine alone, Silver carboxylate alone, and silver carboxylate coating followed by Chlorhexidine. The pig skin was excised, fixed in 10% neutral buffered saline, and stained with Sirius fast-red/fast-green. Penetrance of silver hybrid skin prep and Chlorhexidine was measured via ImageJ. **Results:** The Silver Carboxylate Skin Prep is effective at completely inhibiting MRSA and P. acnes growth up to 72 and 96 hours respectively. Higher concentrations of the skin prep showed the ability to eliminate all bacteria in 24 hours. Additionally, Chlorhexidine was found to have no penetration into the pilosebaceous glands of the pig skin whereas the silver carboxylate skin prep was shown to penetrate the pilosebaceous glands and deposit antimicrobial silver. **Conclusion:** The Silver Carboxylate Skin Prep may be more effective in a clinical setting against P. acnes and MRSA than currently utilized surgical site preps due to its high bactericidal activity and ability to penetrate deeper into the skin.
Next Science It is widely documented that biofilm is present in the oral cavity as dental plaque. Not only is dental plaque difficult to remove using physical means such as brushing and flossing, but microorganisms that promote diseases such as gingivitis can be found on soft tissue surfaces in the mouth. As physical removal techniques are commonly not used on soft tissues, it is suggested that oral rinse solutions be used to control the microorganisms present thereby delaying the formation of dental plaque and development of gingivitis. Next Science has developed a mouthwash using antibiofilm technologies that aim at removing this dental plaque and reducing prevalence of gingivitis. *In-vitro* tests were conducted on biofilms produced in a drip-flow reactor from spit collected samples. The Next Science product was tested alongside Listerine® and Perioguard® with the Next Science formulation showing equivalent efficacy to Listerine against *Candida albicans* with a 5-log reduction in biofilm. The Next Science product showed superior efficacy to Listerine® against both subgingival (2.8 log reduction) and supragingival (1.5 log reduction) bacterial biofilms. The Next Science mouthwash and Listerine® both outperformed Perioguard® against all microorganism types. Clinical studies were completed with 200 patients demonstrating active gingivitis. Plaque scores, gingivitis indices and the composition of oral microbial flora were determined following 6 and 12 weeks of product use. A significant reduction in plaque (p<0.05) was demonstrated though the reduction in plaque did not show an improvement in the gingival index (p>0.35). The DNA analysis for composition of microbial flora is ongoing. These data suggest that the formation of plaque may play a role in development of gingivitis though that role may be minor.
Numerous bacteria communicates using small, diffusible signalling molecules to adapt to environmental challenges. A fascinating bacterial communication system is quorum sensing (QS), a system used to regulate gene expression, and thereby coordinate actions in a cell density-dependent manner. Bacteria constantly produce signaling molecules, whose concentration increase proportionally with cell density: when a specific cell density is reached, termed “quorum”, a certain concentration of the signaling molecule is reached and will result in a population-wide changes in behavior. These changes, in bacterial pathogens, relate to the expression of virulence factors and the formation of biofilms. Interfering with bacterial communication is an appealing strategy in our fight against multi-antibiotics resistant strains and bacterial biofilms. We have identified enzymes, called lactonases that can efficiently degrade bacterial signalling molecules, and interfere with bacterial communication. These enzymes, isolated from hyperthermophilic organisms, are extremely stable and resistant to harsh chemical processes. We have engineered these enzymes to further increase their stability and efficiency against specific signalling molecules, and demonstrated that they effectively inhibit biofilm formation and virulence factors production. We will show their effects on complex microbial communities and highlight that the availability of highly stable enzymatic quenchers opens up new fields of study, including biofilm and biofouling studies in complex community contexts, and in vivo studies in animal and plant models.
Background: Biofilm formation in central venous catheter is one of the main causes of bloodstream infection of hospitalized patients. Currently, this represents an important global public health problem, leading to death of 25 to 38% of patients, where the yeasts of the genus *Candida* spp are the most frequently isolated. Such infections exhibit an increased tolerance for antifungal agents, biocides and immunological variations, making it difficult to treat with conventional therapeutic agents rendering remediation even impossible. In biomedical area, atmospheric plasmas have shown great potential in several applications, however, one of the main researches focuses on microbial inactivation, both in the form of biofilms and planktonic cells of bacteria or fungi. It has been shown that plasma efficacy is due to reactive species such as reactive oxygen species (ROS) and reactive nitrogen species (RNS).

Methods: This study aims to evaluate the antimicrobial action of a plasma jet generated by a surfatron source using different gas compositions on silicone samples contaminated with clinical *Candida parapsilosis* biofilm. The strain was obtained in a previous study duly approved by the Ethics Committee (CEP/ UNITAU No. 542/11). The samples were treated with plasma of 4 SLM of Ar + 6 SLM of air at 100W of power (group 1), 4 SLM of Ar + 9 SLM of Ar plus water vapor at 50W (group 2) and 4 SLM of Ar + 9 SLM of Ar plus water vapor at 150W (group 3). Treatments were performed in the post-discharge region (30 mm) during 10 min. The characterization of the plasmas/substrate surface was performed by optical emission spectroscopy (OES) and IR camera, while for the treated samples was used colony forming units (CFU) counting and scanning electron microscopy (SEM).

Results: Temperature monitoring showed that no substantial substrate heating occurred during plasma treatment (maximum of 36.2 °C). Results show that all investigated plasma compositions were able to significantly reduce the contamination of the samples. The obtained reductions were 88.14%, 93.81% and 90.21% for groups A, B and C, respectively. The analysis of variance (ANOVA) and Tukey's test were used to compare the values obtained in the groups treated with their respective control groups, all were statistically significant. SEM micrographs evidenced morphological alterations of the cells for all treated groups, i.e. the cells are lysed or have completely lost the structure, forming a "mass".

Conclusions: In this work we used the surfatron technique for the generation of highly reactive plasma jets that allowed an efficient inactivation of clinical *C. parapsilosis* biofilms on silicon.
**Background:** Due to high-level tolerance to antimicrobial agents, microbial biofilms are a leading cause of chronic infections in humans and persistent biofouling in industries. Due to the protection of extracellular matrix, mature biofilm remains challenging to eradicate. Thus, the need to develop better biofilm control strategies is urgent. Recently, we engineered surfaces with dynamic changes in topography using a tert-butyl acrylate (tBA) based shape memory polymer (SMP) and demonstrated up to 99.9% removal of established *Pseudomonas aeruginosa* PAO1 biofilm upon triggered shape recovery. **Methods:** *P. aeruginosa* was cultured for 48 h to form biofilm on tBA based SMP that was stretched by 50% using a manual stretcher. Shape recovery was triggered at 40°C for 10 min. The biofilm cells detached by shape recovery or sonication (control using unstretched substrate) were compared for antibiotic susceptibility, the level of intracellular adenosine triphosphate (ATP), and *rrnB* gene expression. RNA-seq and quantitative PCR (qPCR) were used to study the effects of shape recovery triggered dispersion at the genome-wide scale. **Results:** The results show that shape recovery triggered dispersion caused physiological changes in *P. aeruginosa* and the detached cells were sensitized to bactericidal antibiotics. Shape recovery in the presence of 50 µg/mL tobramycin reduced biofilm cell count by more than 3 logs (2,479 fold) compared to the untreated control. The observed effects were attributed to the disruption of biofilm structure and increase in cellular activities as evidenced by an 11.8-fold increase in intracellular level of adenosine triphosphate (ATP), and 4.1-fold increase in expression of the *rrnB* gene in detached cells. The results suggest that, unlike other dispersion methods based on strong physical forces, shape recovery triggered biofilm dispersion does not kill bacterial cells but render them to enter a more active physiological stage (but not with fully resumed growth) and opens the door for antibiotic attack. **Conclusions:** The results of antibiotic susceptibility, intracellular ATP level, *rrnB* expression level, and the genomic-wide gene expression analyses suggest that the shape recovery triggered dispersion rendered bacterial cells to leave the physiological stage of biofilm growth and entered a more active and drug susceptible stage. These results indicate that the biofilm cells may actively sense physical changes in the substratum and more effective control strategies can be developed to eradicate biofilm cells with combined physical (dynamic surface) and chemical (antibiotics) factors.
Abstract:

Background: Persisters are multidrug tolerant cells that present within antibiotic sensitive population. Large populations of persisters are common in biofilms, which lead to stress conditions and can result in relapses of bacterial infections. In contrast to antibiotic resistant bacteria, the tolerance arises from transient phenotypic variants rather than genetic mutations. Cells which express persister gene stochastically can switch to persister states. Although previous study identified many interesting genes, molecular mechanisms of bacterial persistence remain unclear because of their redundancy. In order to identify novel persister genes, we developed a marker for E.coli persisters and performed transcriptome analysis of isolated persisters. Objective: The objective is to reveal molecular mechanisms of persister formation via lactate dehydrogenase (ldhA) and aldehyde dehydrogenase (aldB) which were identified from our transcriptome data. Methods: Transcriptome data suggested expression of anaerobic respiration and fermentation genes. We constructed overexpression and knockdown strains derived from E.coli MG1655. Persisters were measured by determining the number of CFUs after exposure to ofloxacin. CRISPR interference was used for gene knockdown. To visualize ldhA expression, YFP was cloned into pSC101 vector that contain ldhA promoter. The YFP fluorescence of reporter strain was analyzed in microfluidic devise. Conclusions: ldhA overexpression increased persister population 1000 times, and knockdown decrease the population 10 times. Stochastic expression of ldhA may influence bacterial metabolic activity because ldhA use central metabolite pyruvate and NADH. Time-lapse microscopy images of ldhA reporter strain showed the most of cells did not express ldhA, but few (~1%) cells highly expressed ldhA. These cells stop dividing and tolerated lethal concentration of ampicillin. Interestingly, although ldhA was expressed transiently, the cells showed dormant phenotype over 1 hour. These results suggest that stochastic expression of ldhA trigger persister formation.
The 2-aminoimidazole (2-AI) class of compounds can inhibit the formation of bacterial biofilms, disperse existing biofilms, and even resensitize multidrug resistant bacteria to antibiotics. These compounds are active against both Gram-positive and Gram-negative bacteria, making them a powerful weapon against biofilms and antibiotic resistance. We have found that 2-AI compounds interact with bacterial response regulators, the transcriptional regulatory component of canonical two-component systems. Two-component systems allow the cell to detect and respond to changes in its environment, and often trigger quorum sensing, virulence factors, and initiation of biofilm formation. As such, response regulators have been highly sought after as therapeutic targets, but have yet to be successfully exploited. Here, we explore the interaction between response regulators and 2-AI compounds. BfmR from Acinetobacter baumannii and QseB from Francisella tularensis/novicida are well known for regulating biofilm formation. We demonstrate that BfmR and QseB are cellular targets of 2-AI compounds, and that 2-AI compounds are potent inhibitors of biofilms in A. baumannii and F. tularensis/novicida. We propose a model for how 2-AI compounds inhibit response regulator activity, based on a combination of structural biology and molecular modeling. Understanding the mechanism of action between 2-AI compounds and response regulators will lead to the development of more potent, specific, and broad-range compounds.
Abstract:

Background: In clinical settings, antibiotics are widely applied and problems caused by MRSA biofilm formation have been frequently reported. However, antibiotic pressure on biofilm formation of MRSA, as well as the mechanism, remains unclear. Materials/methods: Twelve clinical MRSA isolates were subjected to MIC determination on 12 commonly used antibiotics, including ampicillin, penicillin G, metropenem, streptomycin, kanamycin, gentamycin, erythromycin, ciprofloxacin, tetracycline, oxytetracycline, trimethoprim and vancomycin. Viability and biomass were determined during biofilm formation at 8, 16, 24 and 48 h under 10 concentrations (from 4 to 1/128 MIC with 1/2 dilution) of the 12 antibiotics, by MTT assay and crystal violet assay. The biofilm samples of 2 MRSA isolates under specific concentrations of antibiotics were further analyzed by RNA sequencing (in triplicate). Expression of genes related to two-component system (TCS) and biofilm formation were selected to be quantified by Q-PCR. Results: Dramatical increase was obtained under 1/4 MIC of ampicillin and streptomycin for biomass, and 1/4 MIC of ampicillin, ciprofloxacin and kanamycin for viability. Five hour biofilm samples of 2 MRSA isolates under these specific sublethal concentration of antibiotics were subjected to transcriptomics study. Based on bioinformatics analyses, 24 up-regulated and 27 down-regulated genes were identified in all sublethal concentration of antibiotics treated samples. Differentially expressed genes were enriched to TCS which plays important role in the response of microbes to adverse environment. Combined with Q-PCR results, tagA, lytR, arlR, hssR, clfB, and altA genes were hypothesized to be critical during the biofilm formation under sublethal concentration of antibiotics environment. Conclusions: Dramatical increase in biofilm formation for biomass or viability was obtained under sublethal concentration of antibiotics environment. Transcriptomics study revealed tagA, lytR, arlR, hssR, clfB, and altA genes may play important roles.
The drug resistance of *Helicobacter pylori* (*H. pylori*) is gradually becoming a serious problem. Biofilm formation is an important factor that leads to multidrug resistance in bacteria. The ability of *H. pylori* to form biofilms on the gastric mucosa has been known. However, there are few studies on the regulation mechanisms of *H. pylori* biofilm formation and multidrug resistance. Guanosine 3'-diphosphate 5'-triphosphate and guanosine 3',5'-bispyrophosphate [(p)pGpp] are global regulatory factors and are synthesized in *H. pylori* by the bifunctional enzyme SpoT. It has been reported that (p)pGpp is involved in the biofilm formation and multidrug resistance of various bacteria. However, whether SpoT is involved in *H. pylori* biofilm formation and multi-drug resistance remains unknown. Based on the successful construction of *spoT* mutant strain (ΔspoT) and complemented-strain (spoT*), the difference in biofilm formation between ΔspoT strain and wild-type strain was compared by confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM). The difference of MIC (minimum inhibitory concentration) between ΔspoT strain and wild-type strain was compared. We found that SpoT also plays an important role in *H. pylori* biofilm formation and multidrug resistance. Therefore, it is necessary to carry out some further studies regarding its regulatory mechanism. Considering that efflux pumps are of great importance in the biofilm formation and multidrug resistance of bacteria, we tried to find if efflux pumps controlled by SpoT participate in these activities. Then, we found that *Hp1174* (glucose/galactose transporter, gluP), an efflux pump of the MFS (Major Facilitator Super) family, is highly expressed in biofilm-forming and multi-drug resistance (MDR) *H. pylori* and is upregulated by SpoT. Through further research, we determined that gluP involved in *H. pylori* biofilm formation and multidrug resistance. Furthermore, the average expression level of gluP in clinical MDR strain was considerably higher than that in clinical drug-sensitive strain. Taken together, our results revealed a novel molecular mechanism of *H. pylori* tolerance to multidrug.
**Background:** Bacterial and fungal biofilms in wounds significantly increase morbidity and mortality, and the increasing prevalence of drug resistant strains has prompted the need for new antimicrobial compounds. Kn2-7 is a scorpion venom-derived peptide with broad-spectrum activity against multiple bacterial strains at <10 µg/mL *in vitro*. In this study, antifungal activity, protease stability, and mammalian cell toxicity of Kn2-7 and its D-isoform (dKn2-7) are described. **Methods:** Planktonic fungal susceptibility: Seven *Candida albicans* strains were exposed to Kn2-7 and dKn2-7 at concentrations up to 100 µg/mL. Optical density measurements at 600 nm and the colony forming unit (CFU) assay were used to determine the minimum inhibitory and fungicidal concentrations (MIC and MFC, respectively). Fungal biofilm susceptibility: Five *C. albicans* strains formed biofilms, which were exposed to the peptides at concentrations up to 1000 µg/mL for 24 hours. The XTT assay was then used to determine metabolic activity within the biofilms. **Protease stability:** Peptides were exposed to purified trypsin in phosphate buffered saline for 4 and 24 hours. The concentration of peptide in solution was determined using LC-MS. **Fungal time kill assay:** Planktonic *C. albicans* was exposed to dKn2-7 up to 50 µg/mL and the CFU assay was used to determine fungal viability at various time points up to 24 hours. **Hemolysis assay:** Human erythrocytes were exposed to peptides up to 400 µg/mL and percent hemolysis was measured using supernatant absorbance at 490 nm. **Results:** In six of the *C. albicans* strains, Kn2-7 and dKn2-7 exhibited MICs of 12.5-100 µg/mL and 6.25-25 µg/mL, respectively, and MFCs of 25-100 µg/mL and 12.5-50 µg/mL, respectively; one strain exhibited MICs of 100 µg/mL and MFCs of >100 µg/mL for both peptides. Biofilm IC₅₀ values were between 62.5-125 µg/mL in all five strains for dKn2-7 and 500-1000 µg/mL in four strains for Kn2-7. In the presence of purified trypsin, dKn2-7 exhibited significantly higher stability than Kn2-7. dKn2-7 was thus selected for further study due to its greater antifungal efficacy and stability. **Conclusions:** The D-form of the Kn2-7 peptide is a potent, stable, broad-spectrum antimicrobial compound capable of killing planktonic *C. albicans* and significantly affecting biofilm viability at <125 µg/mL. Cytotoxicity testing of dKn2-7 against mammalian cells is currently ongoing.
**Background:** The specific environment in which microbial biofilms develop, are challenged with antimicrobials, and recover following removal of that challenge is a critical factor affecting their antimicrobial tolerance. The objective of this study was to evaluate the impact of minimal and clinically relevant nutritional conditions on the interaction of aztreonam and silver NPs against *P. aeruginosa* PAO1 biofilms. **Methods:** MBEC assays were used for *P. aeruginosa* PAO1 biofilm formation, antimicrobial challenge, and biofilm recovery following antimicrobial challenge. Aztreonam (Azt) and citrate-capped, 10 nm-diameter silver NPs (Ag-NPs) were the antimicrobial agents selected for testing. The nutrient environments tested were modified Whiteley’s medium (MWM), a nutrient-rich synthetic sputum medium, and defined minimal media (DMM). Biofilm biomass was evaluated using crystal violet staining and biofilm viability was evaluated using an ATP assay and planktonic growth following biofilm recovery. **Results:** Combinations of Ag-NP and Azt against *P. aeruginosa* PAO1 biofilms developed, challenged and recovered in MWM or DMM were tested. For testing in MWM, biofilms were not inhibited even at the highest combined concentration tested (4096 µg/ml Azt and 5 µg/ml Ag-NP). For testing in DMM, biofilms were inhibited at combined concentrations as low as 2 µg/ml Azt and 0.625 µg/ml Ag-NP. The synergistic interaction between Azt and Ag-NPs against *P. aeruginosa* biofilms in DMM was not observed with MWM. The impact of nutrient environment on PAO1 biofilm development, antimicrobial challenge with Azt (concentrations ranging from 4 - 4096 µg/ml) and subsequent recovery were evaluated. Biofilms formed in MWM or DMM produced similar amounts of biomass (OD595nm = 0.2-0.4 after 20h). Biofilms formed, challenged with Azt and recovered in MWM had about 400% more biomass than the untreated control following recovery from antibiotic challenge, up to 64 µg/ml Azt. Further, biofilm recovery following challenge with Azt concentrations ≥128 µg/ml resulted in decreasing amounts of biomass to a minimum of about 50% less than the untreated control. Biofilms formed in DMM or MWM, and challenged and recovered in DMM had biomass amounts about equivalent to the untreated control, following recovery from antibiotic challenge up to 128 µg/ml Azt. Above 128 µg/ml Azt, biofilm recovery resulted in increasing biomass to a maximum of 670% more than the untreated control following recovery from challenge with increasing concentrations of Azt. The only condition resulting in biofilm inhibition were biofilms formed in DMM, but challenged and recovered in MWM, with challenge at 4096 µg/ml Azt. **Conclusions:** The use of MWM and/or DMM during *P. aeruginosa* biofilm formation, antibiotic challenge and recovery resulted in distinctive biofilm recoveries in response to challenge by increasing aztreonam concentrations.
**Poster Session 4**

**Date & Time:** Thursday, October 11, 2018, 4:00 pm - 6:00 pm

**Poster Board #:** 034

**Topic:** Biofilm Antimicrobial Tolerance

**Abstract**

**Title:** Role of Extracellular Vesicles from *Staphylococcus epidermidis* on Bacterial Growth and Biofilm Formation Under Antimicrobial Selective Pressure Conditions

**Author Block:** M. Zaborowska, C. Taulé Flores, F. Vazirisani, P. Thomsen, M. Trobos;
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**Background:** Implanted medical devices provide surfaces for bacterial attachment and biofilm formation, which may lead to implant-associated infection. It is a feared complication commonly caused by staphylococci, often requiring implant removal and long-term use of antimicrobial agents. In addition, it is known that bacteria secrete extracellular vesicles (EVs), however fundamental understanding on their secretion and function in staphylococci is lacking. The aims of this study were to study (i) the effect of sub-inhibitory concentrations of antibiotics on the formation and secretion of EVs, and (ii) if EVs play a role in antimicrobial tolerance and biofilm formation.

**Methods:** The secretion of EVs under sub-inhibitory concentrations of gentamicin (GEN; 0, 0.03 and 0.06 µg/mL) was investigated in a clinical *Staphylococcus epidermidis* strain (CCUG 64523; MIC_{GEN}=0.094 µg/mL) isolated from a patient with implant-related osteomyelitis. The secreted EVs (5, 60 µg/mL) were then added to the same strain to investigate their potential protective effect under selective pressure (0, 0.03, 0.06 and 0.12 µg/mL GEN). Bacterial growth was measured with optical density over 18 h, thereafter viability of planktonic bacteria was measured with LIVE/DEAD staining. Adhered bacterial cells to polystyrene were evaluated using the microtiter plate assay. In a similar experiment, EVs derived from a strong-biofilm producing GEN resistant *S. epidermidis* strain (ATCC 35984) were cultured with the non-biofilm producing GEN susceptible clinical strain (CCUG 64523) under the selective pressure of GEN (0, 0.03, 0.12, 0.5 µg/mL).

**Results:** The mean size of EVs was the same for the different culture conditions. In the presence of GEN, the number of EVs per CFU was higher and contained significantly more proteins than control (0 µg/mL GEN). In the presence and absence of GEN, addition of 60 µg/mL EVs significantly decreased the growth of *S. epidermidis* compared to un-stimulated control (no EVs). Stimulation with 5 µg/mL EVs reduced adhesion (27-82%) compared to unstimulated control in the absence and presence of GEN (0, 0.03 µg/mL), whereas 60 µg/mL EVs reduced adhesion in the presence of GEN. Addition of EVs derived from a biofilm producing strain to the clinical strain significantly increased the total growth compared to unstimulated control, by increasing the growth rate and decreasing the generation time by 5 min. The EVs significantly decreased bacterial adherence by more than 90% compared to control (no EVs).

**Conclusions:** Sub-inhibitory concentrations of GEN altered the production and content of EVs, which in turn affected cell growth and adherence to polystyrene, both under normal and pressure conditions. These findings could have potential clinical implications, with consequences for staphylococcal survival and biofilm formation on implants, in the presence of sub-inhibitory therapeutic doses.
Session: **Poster Session 4**
Date & Time: Thursday, October 11, 2018, 4:00 pm - 6:00 pm
Poster Board #: 035
Topic: Biofilm Antimicrobial Tolerance
Abstract Title: Examining the Correlation between Antibiotic Susceptibility and Biofilm Formation of Group B Streptococcus Isolates of Indian Origin
Author Block: **S. Verma**, P. Yadav, A. Johri
1Central University of Haryana, Mahendragarh, INDIA, 2Jawaharlal Nehru University, Delhi, INDIA.

**Abstract:**

*Streptococcus agalactiae* (Group B *streptococcus*), is a common commensal organism that colonizes the gastrointestinal and genitourinary tract and causes severe infection in neonates, adults, and immune-compromised patients. Many microorganisms such as *s. aureus* and *p. aeruginosa*, have ability to form biofilm which is important in the infection establishment and generate antibiotic resistance by decreasing the antibiotic penetration rate and mediating bacterial gene expression. The mechanism of biofilm formation in *S. agalactiae* and its association with antibiotic resistance have not been investigated yet. This study purposed to examining the biofilm formation among *S. agalactiae* isolates from diverse sources and the antibiotic susceptibilities of *S. agalactiae* strains. As GBS can switch from planktonic stage to biofilm formation which is an important for the establishment of infection as well as providing resistance to antibiotics. In the present work, we determined, in vitro resistance of GBS isolates to different antibiotics for examining any changes in antibiotic resistance pattern with its correlation to biofilm formation. These outcomes may throw light on the knowledge of mechanism by which biofilm formation in *S. agalactiae* is contributing to antibiotic resistance. If this would be the possible outcomes, the involved virulence factors could establish new therapeutic and precautionary goals against this important human pathogen.
Abstract:

**Background:** *Pseudomonas aeruginosa* is an important biofilm forming, opportunistic, bacterial pathogen that causes approximately 51,000 nosocomial infections per year including periprosthetic joint infections (PJI). Orthopedic surgeons routinely place antibiotic loaded CaSO₄ beads into PJI surgical sites to allow for local antibiotic therapy. Variant like antibiotic tolerant or resistant colony generation has previously been shown as a response to tobramycin loaded CaSO₄ beads which could lead to recurrent or persistent infections. The aim of this study was to further characterize the variant colonies produced in response to a tobramycin loaded CaSO₄ bead. **Methods:** In this study, variant colonies were generated by placing a tobramycin loaded CaSO₄ bead into an agar plate containing a pre-grown lawn biofilm of *P. aeruginosa*. After generation, colonies were isolated and cultured, before being plated for minimum inhibitory concentration (MIC) testing. Growth curves were also generated for the isolates to look for the presence of a growth defect which could explain their survival. Finally, generation of variant colonies was repeated with a biofilm lawn that had been exposed to an efflux pump inhibitor (CCCP) to examine efflux pumps as a possible mechanism for the variants’ antibiotic tolerance. In addition to variant colony characterization, the antibiotic concentration throughout the agar over time was also characterized. Tobramycin loaded CaSO₄ beads were placed in sterile agar plates and punches were taken from the agar at varying radii and time points. The punches were melted and used in a Kirby Baeur test to approximate the tobramycin concentration. **Results:** Three phenotypes were identified from the variant colonies which survived the tobramycin bone CaSO₄ therapy - classically resistant colonies, and two novel variants, viable but non culturable colonies (VBNCs), and a tolerant phenotype which we call “phoenix” colonies. Phoenix colonies are variants which grow up within the zone of inhibition of the tobramycin bead while there are still high levels of antibiotic present, but upon subculturing, return to wild-type susceptibility. They show no growth defects indicating they are not persister cells, are morphologically similar to wild-type colonies aside from a pigmentation change from green to white, and are not efflux pump driven. While variant colonies were produced in response to the tobramycin beads, there was also a consistent zone of killing around the bead from which nothing could be cultured. **Conclusions:** These findings suggest that resistant colonies, VBNCs, and phoenix colonies may be generated in biofilms in response to antibiotic therapies, but by achieving high enough local antibiotic concentrations, we may be able to kill classically resistant mutants, phoenix colonies, VBNCs, and persister cells thus reducing the incidence of recurrent or persistent infection.
**Abstract:***

**Background:** Photocatalytic reactions hold promise in biofilms control for healthcare, food, and environmental applications. Graphitic carbon nitride (g-C₃N₄) has proved to be an effective photocatalyst, because of its visible-light-response, low cost in fabrication, stability, and biocompatibility. We select g-C₃N₄ for biofilm control, and aim to understand how the mechanical properties of biofilms change when subject to photoreactions. Elucidating the mechanism is the key to the rational design of effective photocatalytic materials for antimicrobial applications. The atomic force microscope (AFM) is a powerful characterization tool that provides simultaneous information about sample surface topography and probe-sample interacting forces with high spatial resolution and sensitivity. This unique capability has helped us to highlight the mechanical response of biofilms to photocatalysis in our study.

**Methods:** *Staphylococcus epidermidis* biofilms were grown over g-C₃N₄ coupons in a multi-well cell culture plate at 37 °C for 72 h in the dark. For assessing the change of biofilm morphology, viability, and mechanical properties, the biofilms were next exposed to visible white LED light for 6 h. A control sample of bacterial cells with minimal extracellular polymeric substances (EPS) was prepared by washing repeatedly the suspended cells with phosphate buffered saline (PBS) solution. We used two basic modes of AFM operation to get knowledge of surface morphology and tip-sample force interactions, namely, tapping mode and static force spectroscopy (SFS). From SFS measurements we derived the elastic moduli of the biofilms by using the Derjaguin-Muller-Toporov (DMT) theory.

**Results:** Topographical exploration with tapping mode AFM evidenced substantial morphological changes occurring in the biofilms when exposed to light on g-C₃N₄ coupons. The biofilms lost the integrated structure after photoreactions, and only single cells or small clusters of cells were able to be imaged. Results from SFS data revealed that the biofilms exposed to light experience an increase in their (Young’s) elastic modulus, which we ascribe to a reduction in the amount of EPS. We have confirmed this hypothesis with the aid of a control sample of bacterial cells with minimal EPS (see methods section).

**Conclusions:** g-C₃N₄ has shown to be an efficient photocatalyst for biofilm eradication by degrading EPS, which is critical for maintaining a cohesive structure of the biofilms. This exploratory study sheds important light into the mechanical changes experienced by the biofilms upon the imposition of oxidative processes. As a future step, we plan to consider the viscoelastic nature of the biofilms in the mechanical characterization using more sophisticated mechanical models, and identify key mechanical properties that control biofilm eradication.
Background: *Pseudomonas aeruginosa* evolves during chronic pulmonary infections of Cystic Fibrosis (CF) patients, forming adapted variants that are selected for. Mucoid and rugose small-colony variants (RSCVs) are isolated from CF sputum samples. The emergence of these variants is associated with increased treatment difficulties and a worsening patient outcome. RSCV and mucoid variants overproduce different exopolysaccharides in the biofilm extracellular polymeric substance (EPS). Mucoid variants overproduce alginate, whereas RSCVs overproduce Psl and Pel. Changes to the mechanical properties of their biofilms, due to the overproduction of the EPS exopolysaccharides and associated advantages in an infection are not well understood. Methods: Here, we analyze *P. aeruginosa* RSCV (PAO1Δ*wspF*) and mucoid (PAO1Δ*mucA*) biofilms compared to their isogenic wildtype parent (PAO1). Colony biofilms were grown on sterile filter discs on *Pseudomonas* isolation agar, with the biofilm transferred to a new plate every 24h. The mechanical properties of the colony-biofilms were measured at 2-d, 4-d and 6-d using mechanical indentation and flat spinning disk rheometry on a TA instruments Discovery Hybrid HR2 rheometer. Results: We identified that the mechanical properties of wildtype biofilms undergo extensive temporal changes. Initially, wildtype biofilms are relatively stiff and transition to become more fluid-like on 4-d before returning to a stiff phenotype on 6-d. The mechanical properties of RSCV and mucoid biofilms showed a gradual progression to more elastic-solid behavior. Theoretical mucociliary and cough clearance indices have been determined for sputum which correlate sputum viscoelasticity to predicted clearance by either mechanism from the lung. The viscoelasticity of bacterial biofilms has not been considered regarding their mechanical clearance from the lung during infection. The mechanical properties of *P. aeruginosa* biofilms determined here predict that mucoid biofilms are more resistant to both mechanisms of clearance, whereas wildtype and RSCV biofilms would show reduced clearance by cough at later timepoints when biofilms are highly elastic. Conclusion: Our results suggest that adaptation to evolve phenotypes with different mechanical properties may allow *P. aeruginosa* biofilms to insure against mucociliary and cough clearance from the lung. This may be a mechanism for facilitating persistence within an infection. We propose that the mechanical properties of a biofilm be included in the virulence factors that these communities possess.
Extracellular RNA Contributes to Robust Biofilm Organization

Abstract:

Background: Biofilms are complex surface-bound microbial communities. microbes within biofilms are embedded in a self-produced extracellular matrix (ECM) composed of proteins, polysaccharides, and/or DNA that protects them from various stressors. Thus, biofilm-forming bacteria often cause various human chronic infectious diseases. Knowledge of how these communities develop is important for their eradication; however, the mechanistic basis for biofilm formation remains poorly understood at the molecular level. Identifying ECM components contributing to biofilm structural integrity can provide insight into the process of biofilm development that can lead to the development of strategies for their inhibition. In this study, we explored the presence of extracellular RNA (eRNA) in bacterial biofilms and analyzed its roles in biofilm development.

Methods: Several clinically isolated strains of Staphylococcus aureus and Staphylococcus epidermidis were used. ECM components were extracted from the biofilms with 1.5 M NaCl and were subsequently analyzed by electrophoresis. Localization of eRNA in the biofilms was analyzed by confocal laser scanning microscopy (CLSM). To identify eRNA in the ECM, RNA-seq was performed. Physiological role of eRNA in the biofilm ECM was clarified by investigating the effects of RNase A on biofilm formation and dispersal. Molecular interaction between eRNA and polysaccharides was examined by surface plasmon resonance (SPR) analysis. Results: Nucleic acids were detected in the ECM of S. aureus and S. epidermidis. The nucleic acids were degraded by RNase A but neither by dispersin B, proteinase K, nor DNase I, indicating the presence of eRNA in the ECM. The molecular size of the eRNA was estimated 20 to 100 nucleotides by denaturing polyacrylamide gel electrophoresis. RNase A inhibited biofilm formation and dispersed pre-formed biofilms, representing the importance of eRNA in the structural integrity of the biofilms. The results of time-course experiments suggested the requirement for eRNA at various stages of biofilm formation. RNA-seq revealed that eRNA originated from bacteria were tRNA and rRNA. CLSM visualized colocalization of extracellular polysaccharides with eRNA in the biofilm. SPR analysis showed direct interaction between synthesized RNA with an abundant sequence in the ECM and purified polysaccharides. Conclusions: Our findings provide evidence of a novel function for RNA that has important implications for understanding biofilm physiology and the treatment of biofilm-associated problems.
**Abstract:**

Biofilms consist of a consortium of cells affixed to a surface, embedded in a matrix of proteins, polysaccharides and DNA. This lifestyle differs vastly from that of free floating, planktonic counterparts, as spatial and temporal architecture can directly alter the behavior, properties and composition of a biofilm. Biofilms present unique challenges to medical treatment and industrial biofouling remediation. Such obstacles have only worsened as antimicrobial resistance increases. Phage therapy recently resurfaced as a candidate to supplement traditional antibiotics and biofilm removal, yet we are only beginning to understand the ramifications of phage exposure on microbial ecosystems and community assembly. *E. coli* biofilms resist bacteriophage infection by secretion of a polymeric protein, curli, forming a diffusion barrier on the surface. However, the implications of phage tolerance in this system extend beyond survival or death of biofilm-dwelling cells. Community composition often changes as a result of biofilm formation and can promote or obstruct multi-species coexistence. Coexistence or co-occurrence of multiple species, strains or subpopulations is often evaluated by “invasibility” or the ability of an organism to increase in abundance when it is less numerous. Given that phages are trapped by the curli mesh of mature *E. coli* biofilm matrix, we speculated that this may influence the ability of other cells to subsequently colonize the biofilm-liquid interface. Under control conditions in the absence of phage, invading cells do not penetrate into the interior of mature biofilms, but they can become established along a biofilm’s outer periphery. However, when resident biofilms are pretreated with phage, a protective barrier is created by the phages themselves: resident cells remain uninfected due to their embedding within the curli mesh, whereas potential invaders land on the phage coated matrix, become infected and die. On the other hand, if the invasion occurs prior to phage application and ample time is given for acquisition or production of curli, the invaders are likewise protected, and rates of invasion are comparable to control conditions. Large scale phage protection via matrix production and biofilm formation thus has the potential to drastically alter community assembly and coexistence of bacteria within the same family of phage susceptibility.
Background: Our long-term interest is to model how planktonic cells can introduce plasmids into a biofilm community. Plasmid transfer begins with interaction of planktonic cells with the biofilm. The composition of the matrix may influence interaction of planktonic cells with the biofilm. The goal of the current study was to develop a technique to compare the interaction of 1 micrometer negatively charged glyoxylate beads with bacterial biofilms with and without bacterial amyloids over a 20-minute time period. Methods: Bead movement was tracked in a 20 micrometer thick biofilm over 20 minutes using laser-scanning confocal microscopy. Software was developed to track the bead movement over time, calculate the velocity of movement, follow the tracks, determine bounding boxes to measure total area covered by a bead and determine the density around each bead. This information was used to plot bead velocities, determine average velocities, variance and weighted velocities (density-dependence) as well as determine the stability of the interactions over twenty minutes and the amount of area covered by each bead. Bead movement was compared between *Salmonella enterica* serotype Typhimurium, an isogenic amyloid curli mutant and *Enterococcus faecalis* biofilms. Results: Beads in low density regions of *Salmonella* biofilms appeared to have reduced movement as compared to low density regions of *Salmonella* biofilms appeared to have reduced movement as compared to low density *E. faecalis* biofilms. Consistent with the visual observations, curli containing *Salmonella* biofilms had average bead velocities ranging from 0.2-1.1 nm/sec, *E. faecalis* biofilms from 21-64 nm/sec and *Salmonella* curli mutants from 15-91 nm/sec. The weighted velocity of the beads (density dependence) was 0.001 to 0.06 in *Salmonella*, 0.04-0.13 in *Salmonella* curli mutants and 0.1-0.65 for *E. faecalis* biofilms. In general, curli containing biofilms tended to have more beads with stable interactions (trajectories that could be followed for all 20 minutes) than biofilms lacking curli. However, in biofilms lacking curli, beads with longer trajectories had a greater range of movement (larger bounding box). Ongoing analysis is being done to compare overall stability of interactions and range of movement of the beads. Preliminary data suggests that *E. coli* and isogenic curli mutant biofilms behave much the same as the respective *Salmonella* biofilms. The technique is currently being used to characterize *Staphylococcus aureus* and *Streptococcus mutans* biofilms under amyloid-inducing and non-inducing conditions. Conclusions: The presence of curli conferred rigidity to *S. Typhimurium* biofilm. This rigidity was visually evident in low density areas; it reduced average biofilm movement speeds, increased bead contact time and decreased density dependence of the bead movement when compared to *E. faecalis* biofilms lacking amyloids and isogenic curli mutant *Salmonella* biofilms.
For our research, we define electrochemically active biofilms (EAB) as biofilms that exchange electrons with electrodes. The biofilms grown on electrodes allow us to quantify electron transfer rates and identify electron transfer processes. The limitations in electron transfer processes need to be determined to maximize utilization of these biofilms, especially in scaling up. We have demonstrated that the current density decreased with the increased electrode size. We hypothesize that the loss of current density is attributed to substrate transport limitations and microscale heterogeneity in biofilms. The heterogeneity of local conditions on biofilm electrodes at low substrate environments are expected to decrease the current density of large electrodes. Microelectrodes can be used to measure depth profiles in biofilms and determine electron donors/acceptor limitations as well as measure the redox and pH gradients. Here we present how chemical and electrochemical gradients in biofilms are influenced by electron donor/acceptor availability. Acetate and formate microbiosensors along with pH and redox microelectrodes were used to record depth profiles and stationary measurements in electrochemically active biofilms to understand the role of electron donor/acceptor limitations and electrochemical gradients in scaling up. Therefore, we developed new redox and pH microelectrodes with built-in reference electrodes. We found that 1) the biofilms can grow thicker in the absence of electron acceptor or electron donor because the bottom part of the biofilms is conductive, 2) the redox potential gradients depends on the electrode potentials, and 3) pH decreases towards the bottom of the biofilm. When we tested the effect of electron donor and acceptor concentrations on current density of anodes and cathodes at different sizes, we found that scaling up is controlled by the mass transfer. Finally, we found almost identical microbial communities on electrodes at different size demonstrating that the microbial community structure was not the main limitation controlling scale up.
Chlorate Addition Enhances Perchlorate Reduction and Inhibits Sulfate Reduction in a Perchlorate-reducing Membrane Biofilm Reactor

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Microbial degradation is a promising strategy to remediate perchlorate, reducing it to innocuous chloride. However, perchlorate is typically found in the µg/L range, and therefore exerts a weak selective pressure for perchlorate-reducing bacteria (PRB). Besides, nitrate can inhibit perchlorate reduction, so low nitrate levels are needed for perchlorate reduction to occur. Low nitrate allows the proliferation of sulfate-reducing bacteria (SRB). Chlorate, an intermediate in the perchlorate reduction pathway, may select for PRB and inhibit SRB. We tested this hypothesis with two hydrogen-based membrane biofilm reactors (H₂-MBfRs) in three phases. First, Reactors A (control) and B (experimental) were supplied 600 µg/L perchlorate, 5 mg-N/L nitrate, 80 mg/L sulfate, and 8.4 mg/L oxygen during 25 days. Then, we continuously added chlorate (5 mg/L) to Reactor B for 30 days. In the last phase, we operated both reactors without chlorate for 15 days. In both H₂-MBfRs, nitrate was completely reduced after 5 days, and perchlorate was reduced by 95-100% after 10 days. When sulfate removal averaged 10% on day 20 in both H₂-MBfRs, perchlorate reduction decreased to 77-95%.

However, during chlorate addition in Reactor B, sulfate reduction decreased to below 5% and perchlorate reduction increased to >95%. Once we removed chlorate, Reactor B quickly resumed with high sulfate reduction rates and lower perchlorate removals (<95%). Results of quantitative polymerase chain reaction (qPCR) from the last phase showed lower proportion of SRB in reactor B (0.063%±0.007) compared to Reactor A (0.95%±0.19). Sequencing results of 16 rRNA results coincided with qPCR results of Reactor A, with an increase of SRB (Desulfovibrionales) and a decrease of denitrifying bacteria (DNB) and PRB (represented by Rhodocyclales and Burkholderiales) along the three stages. However, in Reactor B, sequencing results showed an increase of SRB (41% of Desulfovibrionales) during chlorate addition (phase 2) and a decrease of SRB after stopping chlorate addition (phase 3). This might indicate that SRB can be involved in chlorate reduction. Also, the increased of SRB, and decreased DNB and PRB in Reactor A does not agree with the constant reduction rates of nitrate, sulfate and perchlorate. This could show that SRB could also be involved in nitrate and perchlorate reduction. Our results suggest that chlorate addition diminishes sulfate reduction, but enriches for SRB. The mechanisms and the capability of SRB to reduce chlorate are still being investigating.
Pseudomonas aeruginosa, a Gram-negative bacterium, is a ubiquitous opportunistic pathogen able to form biofilms that aid its survival in a variety of environments. In previous work, we demonstrated that the specific interaction between the bacterioferritin BfrB, the main iron storage protein in P. aeruginosa, and its associated ferredoxin Bfd is required to establish a dynamic equilibrium between Fe³⁺ compartmentalized in BfrB and cytosolic free iron (Fe²⁺) that buffers the concentration of usable intracellular iron. In the present study we investigated the implications of perturbing the BfrB:Bfd interaction on the formation and integrity of P. aeruginosa biofilms at the air-liquid interface (pellicles). We compared the establishment and stability of pellicles formed by the wild type strain with mutant strains where the BfrB:Bfd interaction was blocked by in frame deletion of the bfd gene and in a variant with the bfrB allele encoding BfrB (L68A/E81A). The effects of the mutations were evaluated by the time-dependent microscopic inspection of the pellicles, and by quantitative determination of (i) total biofilm mass, (ii) concentration of iron and pyoverdine in the spent culture media, (iii) production of quorum sensing molecules and rhamnolipids. Our results reveal that inhibiting BfrB:Bfd interaction triggers an iron starvation response that weakened the pellicle structure in the late stage, causing an early cell detachment and concomitant biofilm dissolution. We show that when cytosolic levels of free iron decreases there is an exacerbated production of pyoverdine, quorum sensing molecules, rhamnolipids and an increased bacterial motility. These results suggest that a rational deregulation of iron homeostasis may be a promising strategy to avoid the persistence of P. aeruginosa biofilms.
Abstract:

**Background:** The Microtitter assay is one of the most widely used methods to assess biofilm formation. Though, high throughput this assay is notoriously irreproducible in its outcome from experiment to experiment, and even from well to well. Since the assay constitute one of the pillars of biofilm research, we decided to examine the wells of a microtitter tray directly during growth, treatment and the steps involved in Crystal Violet (CV) measurements. **Methods:** We used an inverted Zeiss LSM 880 confocal laser scanning microscope (CLSM) to visualize and quantify biomass directly in the wells of the microtitter tray. We applied both GFP-tagged *Pseudomonas aeruginosa*, PAO1, and Live/dead stains to assess structure and compression of biomass build up. With the use of CLSM we were able to image the development of whole well biofilms in real-time. All observations were compared with CFU counts and CV assays. **Results:** We were intrigued to see a development of a distinctly structured architecture of the biomass in the wells over time. We were able to capture 3D images of all biomass throughout the entirety of the microtitter wells. These structures were highly variable from well to well, which may introduce variable outcomes. Even though there was an accumulation of biomass from 24 towards 72 hours, the number of variable cells in this biomass declined significant over the same period. We saw large variations in biomass, depending of the chosen method of removing the supernatant prior CV staining (etc. pipetting or throwing the fluid out, washed or unwashed wells). Remaining biomass was highly inconsistent, even with the same method performed by the same person. It was clear that CV measurements did not reflect living biomass, biomass found in the wells before staining or provided any consistent picture of the state of biomass grown in the wells. **Conclusions:** This study was conducted in an effort to expand our knowledge of a seemingly trivial and widely used method of assessing biofilm formation and treatment. We found that most steps involved in CV assay causes high deviation due to divergence in the structure of biomass between the individual wells and how these steps effect the biomass. Additionally we found that over time, the biomass of a microtitter well would develop into complex structures by increasing biomass while the viable fraction of the biomass decreases. The highly structured microenvironment of biomass in these microtitter wells needs to be taken into account when designing and analyzing experiments using this model.
Abstract:

**Background:** *Pseudomonas aeruginosa* is the major pathogen responsible for lung infections and respiratory failure in cystic fibrosis (CF) patients. The multidrug-resistant nature of these infections is facilitated by the viscous sputum of the CF patient, which contains high concentrations of mucin, DNA, and amino acids. *P. aeruginosa* exists in CF sputum as a suspended mucoid biofilm with a thick extracellular matrix. The evaluation of *P. aeruginosa* antibiotic susceptibility using sputum from CF patients is impractical due to several factors, including patient-to-patient variability, history of antibiotic treatment, and changes imposed by sterilization. Artificial Sputum Medium (ASM) has emerged as an *in vitro* model that can produce suspended mucoid biofilms like those found in CF sputum. This study uses ASM to develop mature, suspended *P. aeruginosa* biofilms for antibiotic efficacy testing.

**Method:** ASM was inoculated with *P. aeruginosa* BAA-47 at a final concentration of approximately 5x10^5 CFU/mL. Inoculated ASM was aliquoted into a 24-well plate and incubated for 6 days, with the daily addition of fresh media to counter evaporation and provide additional nutrients. The 6-day old biofilms were treated with gentamicin (10, 30, 50, and 100 µg/mL; n=4) or tobramycin (10, 20, 40, 80 µg/mL; n=4) for 24 hours. Untreated controls (n=4) were included for each antibiotic. After the designated treatment period, antibiotics were neutralized, and biofilms were disrupted via sonication. The resulting suspension was spot-plated or spread-plated to enumerate bacterial survivors, expressed as colony-forming units (CFU)/mL.

**Results:** Visible aggregates of *P. aeruginosa* were formed in ASM that were found to be antibiotic resistant. Both gentamicin and tobramycin showed dose-response in terms of efficacy but failed to eradicate *P. aeruginosa* biofilms at the highest tested concentrations. Biofilms treated with 100 µg/mL gentamicin (25x the MIC value) survived with over 10^4 CFU/mL, approximately a 5-log reduction from the untreated control at 10^6 CFU/mL. Biofilms treated with 80 µg/mL tobramycin (80x the MIC value) also survived with over 10^4 CFU/mL. **Conclusion:** The ASM model produces suspended, antibiotic-resistant biofilms resembling those found in the CF lung. This new model is clinically relevant due to its sensitivity and low detection limit, proving new opportunities to evaluate antibiotics against chronic CF infections.
**Abstract:**

The killing of bacteria by antibiotics in biofilms is known to be reduced by 100-1000 times relative to planktonic bacteria. This makes such infections difficult to treat. We suggest that a biofilm should be regarded as an independent compartment with distinct pharmacokinetics. To elucidate this, we have measured the penetration of the tobramycin into seaweed alginate beads which serve as a model of the extracellular polysaccharide matrix in *P. aeruginosa* biofilm. We find that, rather than a normal first order saturation curve, the concentration of tobramycin in the alginate beads follows a power-law as a function of the external concentration. The power-law appears to be a consequence of binding to a multitude of different binding sites. In a diffusion model these results are shown to produce pronounced retardation of the penetration of tobramycin into the biofilm. This filtering of the free tobramycin concentration inside biofilm beads is expected to aid in augmenting the survival probability of bacteria residing in the biofilm. PLOS ONE, 11, 4, e0153616, 2016.
Introduction: Presence of bacteria is essential for hosts’ function. The main form of bacterial survival is a biofilm. Growth of bacterial biofilms is a complex process, which involves three generalized phases: adhesion, biofilm maturation, and dispersion. *Lactobacilli* spp. are critical components of gut and reproductive health. Despite the fact that biofilm growth of *LB* has been described extensively, the temporal characteristics of biofilm maturation have not been validated in real time mode yet. Here we are describing two methods of label-free quantification of biofilm growth of *L. Plantarum*: analyses of video-tracing and electrical impedance-based technologies. Additionally, we are comparing real-time growth of *L. Plantarum, Lactobacillus crispatus, Lactobacillus gasseri, Lactobacillus jensenii.*

**Material and Methods:** Biofilm growth was studied in 1) Biofermenter system (Lous Pasteur, France) with video-recording and subsequent analyses, using IMARIS software (Bitplane, South Windsor, Connecticut, United States) and in 2) real-time cell analyzer: xCELLigence (ACEA Biosciences, San Diego, CA), based on impedance measurement. **Results:** The dynamic of biofilm growth of *L. Plantarum* was similar in both systems with the exception of detachment phases. Four *LB* species differed in the duration of attachment phases, demonstrating negative cell index, while growth phases were similar. **Conclusion:** Temporal dynamic of biofilm growth is in agreement with published physiological and pathophysiological data and points out that real-time detection of this phase is an important tool in understanding growth of microbial communities.
Session: Poster Session 4
Date & Time: Thursday, October 11, 2018, 4:00 pm - 6:00 pm
Poster Board #: 049
Topic: Biofilm: From Nature to Models
Abstract: A Novel environment-specific Oxylipind-dependent Quorum-sensing System Promotes Biofilm Formation
Title: Formation *in vivo* by *Pseudomonas aeruginosa*
Block: Southern Research, Birmingham, AL.

Oxygenated unsaturated fatty acids, known as oxylipins, are signaling molecules commonly used for cell-to-cell communication in eukaryotes. However, a role for oxylipins in mediating such communication in prokaryotes was not previously known until the recent description of the novel Oxylipin-Dependent Quorum-Sensing System (ODS) of *Pseudomonas aeruginosa*. We have shown that ODS regulates important phenotypes of *P. aeruginosa*, such as biofilm formation, virulence and antibiotic resistance, provided that oleic acid, the precursor of the oxylipin autoinducers, is present in the bacterial environment. Quorum sensing is a known mechanism of bacterial cell-to-cell communication, which involves the production and detection of diverse small molecule autoinducers. Our data show that ODS uses oleic acid scavenged from host tissues as a substrate to synthesize the oxylipins autoinducers, which in turn control the expression of a gene subset in a cell density-dependent manner. We identified the LysR-type transcriptional regulator encoded by gene PA2076 in the model strain of *P. aeruginosa*, PAO1, as the primary receptor of the oxylipin signal. ODS is unique not only because it uses prokaryote oxylipins as autoinducers, but also because these signaling molecules are synthesized extracellularly by *P. aeruginosa*, as opposed to all other autoinducers described thus far that are synthesized intracellularly, and because the ODS is regulated independently of the hierarchical quorum sensing network of this bacterium.
Abstract: The extracellular polymeric substances (EPS) matrix of biofilms is a complex mixture of compounds, with varying composition depending on environmental conditions and bacterial species present. The common practice in EPS modelling is to treat it as a pool of organic carbon, neglecting its complex chemistry. This work presents a novel methodology for modelling EPS production, by proposing a general EPS chemical formula and using a microbial thermodynamics framework. Methods: The EPS matrix is assumed to contain only proteins (general formula $C_{16}H_{24}O_8N_5$) and polysaccharides (general formula $C_{6}H_{10}O_5$), neglecting humic acids, eDNA or lipids. The EPS chemical formula (i.e. $CH_{1.8}O_{0.5}N_{0.2} + c HCO_3^- + d H_2O + e H^+ + f EPS$) is proposed by considering the ratios of protein to sugars experimentally reported in activated sludge treatment systems. The thermodynamic approach represents metabolism as the sum of two simplified reactions: the anabolism, in which biomass is synthesized, and the catabolism, which provides the energy for anabolism. EPS formation is modelled as a product in the anabolic reaction, eq. (1).

$$a C_{16}H_{24}O_8N_5 + b N- \text{source} \rightarrow CH_{1.8}O_{0.5}N_{0.2} + c HCO_3^- + d H_2O + e H^+ + f EPS$$

The stoichiometry is obtained by solving the element and charge balance, for a given stoichiometric coefficient of EPS. The thermodynamic properties of EPS (enthalpy, entropy and free energy of formation) are estimated using empirical relations. The stoichiometric coefficient of the electron donor in the overall growth reaction represents the reciprocal of the biomass yield on substrate. Biofilm development is modelled using a 2D individual based model. For the simulations, heterotrophic bacteria using glucose as electron donor and ammonia as nitrogen source were used. Results: The influence of the protein to polysaccharides ratio of EPS on bacterial growth yield was examined. The computed free Gibbs energy is lower for polysaccharides than for proteins. As a result, higher maximum yields correspond to EPS with lower protein content. The stoichiometric coefficient of EPS in the anabolic reaction is varied between 0 to 1, to study biofilm growth with EPS production. Despite the energetic burden, bacteria able to synthesize high EPS amounts produce higher biofilms, in the same simulation conditions. The lower density of EPS is responsible for better diffusion of the nutrients in the biofilm and the better performance of high EPS producers. Conclusions: Modelling the EPS formation using a thermodynamic framework is a novel approach that allows the quantification of the energy cost of its production on the development of microorganisms. The model can be improved by considering the effect of EPS chemical composition on the matrix’s physical properties (e.g. density, viscosity). References: 1. Gonzalez-Cabaleiro, R., et al. (2015). ISME J 9(12), 2630-2641. 2. Battley, E. H. (1999). Thermochimica Acta 326(1-2), 7-15.
**Abstract:**

**Background:** Standard image analysis programs are required to analyze and compare the effects of various treatments on biofilms grown in vitro. Many in vitro biofilm model systems combine confocal laser scanning microscopy (CLSM) with image analysis tools to study biofilms. However, currently, there is no standard approach to analyze biofilm images grown in vitro. This study introduces and evaluates an in-house developed image analysis software that we call BAIT (Biofilm Architecture Inference Tool). BAIT quantifies the architecture of oral multi-species biofilms enabling quantitative comparisons of biofilms grown under different treatment conditions. **Methods:** In vitro biofilms representative of oral biofilms were developed over the course of 22 hours using a 24-well Bioflux™ system. Pooled human saliva served as both the growth medium and initial inoculum. During development, nascent biofilms were either untreated or treated with a formulation 8 and 18 hours into development. Treatment formulations included water (negative control), sodium gluconate (placebo) or stannous fluoride (anti-biofilm agent) at active stannous concentrations of 1,000, 3,439, and 10,000 PPM. Mature biofilms were stained with LIVE/DEAD and imaged with CLSM. Digital 3-dimensional image stacks were then quantified with the BAIT software and evaluated for seven outcomes: biovolume, surface area, number of objects, fluffiness, connectivity, convex hull porosity, and viability. **Results:** Treatment with 3,439 and 10,000 PPM stannous formulations visibly and significantly decreased bioburden. Analysis with BAIT demonstrated that biovolume, surface area, number of objects, and biofilm connectivity decreased while fluffiness increased (p<0.01). The lowest concentration of stannous, 1,000 PPM, did not visibly decrease biofilm burden, but BAIT indicated an altered biofilm architecture: the number of objects and fluffiness increased while connectivity decreased (p<0.05), suggesting fragmentation of biofilm. **Conclusions:** In conclusion, BAIT was able to measure clearly visible as well as more subtle changes in in vitro biofilm architecture. The software enhances the analysis of 3-dimensional biofilm images and can quickly evaluate the efficacy of candidate antimicrobial and anti-biofilm agents.
Background: An *ex vivo* porcine dermal model of mature biofilm has been implemented, in several variations, for the evaluation of the efficacy of wound care therapies. The major advantages of this model system are the use of a natural porcine skin matrix and the relatively high throughput format of the assays in determining the effect of wound care technologies on biofilm prevention and/or the eradication of existing biofilm. Here we describe the development and use of this model system to study the efficacy of a nitric oxide (NO)-releasing polyvinylpyrrolidone (PVP) with respect to anti-biofilm efficacy. Microbial biofilms play an important role in the prolonged inflammation state of wounds, and NO has been recognized for its broad-spectrum efficacy, where even sub-bactericidal concentrations lead to biofilm dispersal. The goal of this study was to devise a series of assays that would serve as a prediction of success in the *in vivo* studies, taking into consideration the relevant wound size and the formulation of the active material. Method: Aliquots of the porcine dermal tissue were prepared either by punching out the tissue using a 7/16" biopsy punch or by preparing square shaped pieces approximately 2x2” in size. In the assay using the small tissue, the artificial wound was created using a Dremel tool, while wounds in the larger tissue were done using a scalpel. The artificially wounded tissue samples were then excessively washed and sterilized using chlorine gas. The biofilm was established by inoculating sterilized tissue with approximately 10^5 CFUs of the bacteria or yeast and incubated for up to 1-3 days on soft agar in the presence of appropriate antimicrobial agents. The biofilms were then challenged in multiple formats with solutions containing the active material or wound care appropriate formulations thereof. Results: We have developed and implemented several variants of an *ex vivo* porcine dermal model of mature biofilm, including *C. albicans* and mixed species biofilms comprised of *P. aeruginosa* and *S. aureus*. The size of the artificial wound was varied from approximately 2-15 mm in diameter to provide a scenario that more closely resembles the wounds found in the clinic. While aqueous solutions of PVP/NO ranging from 0.5% to 5% showed very potent anti-biofilm efficacy with ≥ 6 log reduction of the biofilm load, the efficacy of PVP/NO semi-solid formulations was significantly lower, especially when the larger wound model was used. Ongoing efforts are geared towards optimizing the NO release from the formulations, the key factor needed for the efficacy of the material. Conclusion: The *ex vivo* porcine dermal model of mature biofilm is a particularly useful tool for guiding the optimization of formulations for wound care applications prior to *in vivo* studies. Additionally, the selection of appropriate experimental set up (i.e., “wound” size) is critical when considering the clinical use of the technology.
Background: Subgingival plaque plays a primary role in initiation and progression of gingivitis and periodontitis. It is a complex and diverse biofilm formed predominantly by normal oral flora in addition to small amounts of potentially pathogenic microbes. The objective of this study was to optimize growth conditions for developing a biofilm model that mimic the human subgingival plaque. Methods: Subgingival plaque was used as a seed culture and was collected using paper points and placed into pre-reduced transport medium. Biofilms were grown from the subgingival plaque sample on saliva pre-coated pegs of a MBEC assay 96 well plate (formerly called Calgary device) in 5 different culture media; modified SHI (mSHI), mSHI with glucose (GmSHI), Brain Heart Infusion supplemented with vitamin K, hemin and mucin (sBHI), mucin-only medium supplemented with 10% serum (MS10) or 20% serum (MS20). Biofilms were allowed to grow for a period of 14 days in anaerobic conditions, with a replacement of medium every 3.5 days. The microbiomes were then harvested and DNA was extracted. 16S rRNA gene sequencing (Illumina, V1-V3, 2x300 bp chemistry) was performed on DNA from biofilms as well as from original subgingival plaque sample. Post sequencing data cleaning, taxonomic assignment and diversity analysis were carried out using MOTHUR, BlastN and QIIME, respectively. Results: An average of 234 species were identified in the original subgingival sample, whereas the number of species identified in the in vitro microbiomes were in the range of 64-94. All 5 media showed an excess growth of Firmicutes (Parvimonas, Peptostreptococcus, Solobacterium and Mogibacterium). sBHI harbored Veillonella, Prevotella and Fusobacterium in proportions close to that observed in subgingival sample; mSHI captured the TM7s. Shannon and Simpson alpha diversity indices were lowest for MS10 and MS20 media, and highest for mSHI and sBHI. Principal coordinate analysis (PCoA) based on unweighted unifrac and weighted unifrac distance matrix biofilms grown in mSHI and sBHI were closest to the original sample on the PC1 while those grown in MS10, MS20 and mSHI were closest on the PC2 and PC3. Conclusions: Both alpha and beta diversity analyses indicate that biofilm developed using mSHI and sBHI media were closest to the original plaque sample. However the overgrowth of some species and absence of growth of some others was observed. Further optimization and development of this model can be useful for understanding the complex nature of oral biofilms and can have implications for the screening of microbiome modulators.
Abstract: Catheter-associated urinary tract infections (CAUTI) are the most common cause of hospital-associated infections in the US and can result in significant morbidity, including bacteremia, and in some cases death. Increasing drug resistance among uropathogens and their ability to form recalcitrant, biofilm-associated diseases have made appropriate management of CAUTI difficult. Problematically, the presence of a urinary catheter increases the risk of infection, especially to atypical uropathogens. One such pathogen that has received little attention is *Staphylococcus aureus*. Recent studies show that urinary catheterization is the strongest predisposing factor for the development of *S. aureus* UTI and the majority of isolates are methicillin-resistant *S. aureus* (MRSA). The frequency with which *S. aureus* CAUTI results in bacteremia and toxic shock and the difficulty in treating these infections is particularly concerning. Thus, this study aimed to gain a better understanding of the mechanisms by which urinary catheters facilitate MRSA CAUTI. 

Methods: We adapted a mouse model of CAUTI to assess the bacterial load on urinary implants and in the bladders, dissemination to other organs, and the immune responses following urinary catheter implantation and MRSA infection. Additionally, we collected patient urinary catheters to confirm the results from our mouse model accurately recapitulated human disease. 

Results: Our adapted MRSA CAUTI model indicated: i) MRSA required a catheter to establish persistent UTI; ii) the host protein fibrinogen (Fg), which plays a major role in clotting and wound healing, accumulated in the bladder and subsequently coated the catheter; iii) MRSA co-localized with Fg deposited on catheters and the bladder epithelium; and iv) MRSA rapidly disseminated to bacteremia and subsequently colonized the spleen and heart. Furthermore, the clumping factor B (ClfB) adhesin and the urease enzyme significantly contributed to MRSA CAUTI. While, ClfB contributes to CAUTI by interacting with Fg recruited following catheter-induced damage, the role urease plays is less clear. Urease breaks down urea in urine, which induces crystal formation, and results in catheter encrustation. Urease mutants grow comparably to wildtype in human urine in vitro but in the CAUTI mouse model results in a 2 log decrease in bacterial load at 1 dpi. Importantly, analysis of patient catheters indicates that *S. aureus* is present on catheters and long term colonization results in urease-induced crystal formation, which the pathogen attaches to form biofilm. 

Conclusions: These studies indicate the catheterized bladder facilitates MRSA infection by providing additional binding ligands and MRSA employs several mechanisms to establish CAUTI. These studies will provide insights into the development of more effective treatment options to prevent or treat MRSA CAUTI.
**Background:** Typhoid fever is a major health concern that affects 21 million individuals and causes 200,000 deaths each year. Acute illness is primarily caused by *Salmonella enterica* subspecies *enterica* serovar Typhi (S. Typhi). The only known reservoir for S. Typhi is chronically-infected human carriers, who harbor the organism in the gallbladder and are usually asymptomatic but shed and transmit the organism via the fecal-oral route. A hallmark of chronic S. Typhi infections is the ability to produce biofilms on cholesterol gallstones that are encased in *Salmonella*-produced extracellular polymeric substances (EPS). Biofilms are thought to permit bacterial evasion of host immunity, but the involvement of specific EPS components in immune tolerance is poorly characterized. In particular, knowledge on subversion of the innate immune response during early stages of infection is lacking. **Methods:** We characterized S. Typhi and S. Typhimurium for susceptibility to serum complement and to the antimicrobial peptides (AMPs) polymyxin B and melittin. These immune factors attack bacterial surfaces by pore-forming mechanisms. Following exposure to each immune factor in the planktonic or biofilm state, bacterial viability was quantified by CFU analysis. We further investigated the contribution of individual EPS components by deleting genes essential for expression of curli fimbriae, cellulose, O-antigen capsule, colanic acid, or Vi antigen. **Results:** The biofilm lifestyle clearly enhances tolerance to serum complement and to AMPs. EPS mutations do not affect tolerance to serum complement. Loss of curli fimbriae, O-antigen capsule, and colanic acid reduces tolerance to polymyxin B. However, only loss of curli fimbriae reduces tolerance to melittin, suggesting curli fimbriae should be a prioritized target for anti-biofilm agents. S. Typhi Vi antigen mutants have hyper-biofilm forming phenotypes. Despite having excess biofilm, these mutants are more susceptible than wildtype to AMPs, indicating a protective function of Vi antigen and that biofilm recalcitrance cannot be attributed to biomass alone. **Conclusions:** By identifying mutants exhibiting less tolerance than wildtype *Salmonella*, we have identified EPS components crucial for the biofilm immunotolerant phenotype. These data improve our understanding of biofilm-mediated recalcitrance to innate immunity and provide new directions for therapeutics that specifically target critical EPS components.
Bacterial biofilms play a key role in the persistence of infections of the upper and lower respiratory tract. Primary ciliary dyskinesia (PCD) is a genetic disorder characterized by abnormal ciliary function and low airway nitric oxide (NO) levels compared to healthy individuals. Although mechanistically distinct from cystic fibrosis, PCD is also associated with persistent bacterial colonisation of the airways, causing localized inflammation and airway damage. Nontypeable *Haemophilus influenzae* (NTHi) is the most common pathogen isolated from PCD patients and we hypothesized that patients with PCD are predisposed to biofilm infections with NTHi. We developed a primary human airway epithelial cell co-culture model to evaluate this hypothesis and determine the specific contributions of abnormal ciliary motility and NO production in PCD epithelial cells in response to NTHi colonization. Epithelial cells from PCD and non-PCD patients were differentiated to ciliation by culture at an air-liquid interface and co-cultured with a biofilm-forming NTHi clinical strain for 72 hours. NTHi adherence was significantly greater on ciliated PCD epithelial cells compared to ciliated non-PCD cells (p<0.05) and the volume of aggregated NTHi in biofilms on PCD epithelium was significantly increased (p<0.001). Apart from defective ciliary motility, PCD epithelial cells were not significantly different from non-PCD cells in the degree of ciliation, epithelial barrier integrity, cytokine or antimicrobial peptide production. Notably, while nitric oxide synthase expression and NO production were also similar, treatment of differentiated PCD epithelial cells with exogenous NO combined with an antibiotic significantly increased the sensitivity of NTHi to antibiotic killing (p<0.05). These studies indicate impaired ciliary function is a primary defect in PCD airway epithelial cells underlying susceptibility to NTHi biofilm development and that PCD is another disease highlighting the importance of diminished mucociliary clearance in biofilm development and chronic bacterial infection in the human respiratory tract. Our data further suggest a potential role for NO in treating these infections as well as the prospective value of the co-culture model to investigate polymicrobial infections and anti-infective/biofilm therapies.
**Abstract:**

Catheter-associated urinary tract infections (CAUTI) is an alarming hospital based disease with the increase of multidrug resistance (MDR) strains of Proteus mirabilis. Cases of long term hospitalized patients with multiple episodes of antibiotic treatments along with urinary tract obstruction and/or undergoing catheterization have been reported to be associated with CAUTI. The cases are complicated due to the opportunistic approach of the pathogen having robust swimming and swarming capability. The latter giving rise to biofilms and probably inducible through autoinducers make the scenario quite complex. High prevalence of long-term hospital based CAUTI for patients along with moderate percentage of morbidity, cropping from ignorance about drug usage and failure to cure due to MDR, necessitates an immediate intervention strategy effective enough to combat the deadly disease. Several reports and reviews focus on revealing the important genes and proteins, essential to tackle CAUTI caused by P. mirabilis. Despite longitudinal countrywide studies and methodical strategies to circumvent the issues, effective means of unearthing the most indispensable proteins to target for therapeutic uses have been meager. Here, we report a strategic approach for identifying the most indispensable proteins from the genome of P. mirabilis strain HI4320, besides comparing the interactomes comprising the autoinducer-2 (AI-2) biosynthetic pathway along with other proteins involved in biofilm formation and responsible for virulence. Essentially, we have adopted a theoretical network model based approach to construct a set of small protein interaction networks (SPINs) along with the whole genome (GPIN) to computationally identify the crucial proteins involved in the phenomenon of quorum sensing (QS) and biofilm formation and thus, could be therapeutically targeted to fight out the MDR threats to antibiotics of P. mirabilis. Our approach utilizes the functional modularity coupled with k-core analysis and centrality scores of eigenvector as a measure to address the pressing issues.
**Background:** Biofilms contribute to the chronicity and recurrence of many diseases and eradication of these bacterial communities is confounded by a multicomponent extracellular polymeric substance which shields the resident bacteria from antimicrobials and host immune effectors. The bacterial DNABII protein, integration host factor (IHF), is a critical element of the extracellular DNA matrix that supports the biofilm structure. Whereas the adaptive immune response to IHF is against non-protective epitopes within the carboxy-terminal (or ‘tail’) region, antibodies against the DNA-binding ‘tip’ regions induce catastrophic collapse of biofilms formed by all human pathogens tested. To therefore redirect the adaptive immune response away from the non-protective tail region and toward protective tip domains within IHF, we designed a chimeric peptide immunogen comprised of 20-mer portions within the tip regions of IHF α and β subunits. 

**Methods:** Nontypeable *Haemophilus influenzae* (NTHI) biofilms were established *in vitro* then incubated with anti-tip chimer peptide or anti-whole native IHF protein. Antibodies against a ‘tail chimer peptide’ that represented non-protective domains within IHF were also tested. Further, we used an experimental model of otitis media wherein NTHI biofilms established within the middle ears of chinchillas were treated with IgG-enriched anti-tip chimer peptide, anti-IHF or anti-tail chimer peptide that was delivered directly to this site. 

**Results:** *In vitro*, NTHI biofilm biomass was significantly reduced within 5 min of exposure to anti-tip chimer peptide or anti-IHF compared to naive serum, whereas biofilms incubated with anti-tail chimer peptide sera were not disrupted. Moreover, at the earliest (5 min) and latest (60 & 120 min) time points tested, biofilms incubated with anti-tip chimer peptide were significantly reduced compared to anti-IHF. *In vivo*, middle ears administered anti-tip chimer peptide or anti-IHF had a significant 4-log fewer NTHI adherent to the mucosa and within biofilms compared to receipt of naive serum or anti-tail chimer peptide. Mucosal biofilms were significantly reduced in animals that received anti-tip chimer peptide or anti-IHF. Importantly, anti-tip chimer peptide antibodies were more effective than anti-native whole IHF to induce mucosal biofilm clearance and eradication of NTHI from the middle ear, which validated the premise for the design of this chimeric immunogen.

**Conclusions:** Whereas the natural immune response to IHF is directed towards non-protective domains of this protein, re-direction of the adaptive immune response by immunization with a peptide that represents defined immunoprotective regions was highly effective and avoided augmentation of a pre-existing non-protective response that would have likely resulted following immunization with native IHF. Support: NIH R01 DC011818
HVAC (heating, ventilation, and air conditioning) condensate can be a beneficial source of water that is currently going down the drain. As warm air is cooled by an air conditioning system, humidity in the air forms condensation that is essentially distilled water. Typically, this high-quality water is drained into wastewater systems, but it can alternatively be collected to support water conservation and sustainable water use. In hot, humid weather, an air conditioner may produce three to ten gallons of water per day per 1000 square feet of air-conditioned space. The goal of this study was to assess if HVAC condensate water was safe for various reuse purposes by testing bulk water samples and surface biofilms for opportunistic bacterial pathogens. The bulk condensate water from four air handling units in two buildings in North Carolina was monitored biweekly from May through October, 2017. Additionally, 36 biofilm samples were collected a single time from condensate surfaces (100 cm² swabbed), collection pans (25 cm² swabbed), and drain pipes (variable sized areas swabbed) of each handler using sterile swabs. Microbial analyses included heterotrophic plate counts (HPC), culture and qPCR for nontuberculous mycobacteria (NTM), and qPCR for *Legionella pneumophila* serogroup 1 (Lp SG1). The average HPC counts for the bulk condensate water were 6.05 x 10³ CFU/ml. The average HPC counts for biofilm drain pipe samples were 1.83 x 10⁶ CFU/ml, 4.15 x 10³ CFU/cm² for collection pans, and 6.24 x 10³ CFU/cm² for condensation surfaces. No biofilm samples and 2.5% (1/40) of condensate samples were positive for *Mycobacterium avium* (MA) by qPCR. Six percent (2/36) of biofilm samples and 7.5 % (3/40) of condensate samples were positive for *M. intracellulare/chimaera* (MI/C). Lp SG1 was detected by qPCR in one biofilm sample and one condensate sample, both quantities below the assay’s limit of quantification. Nearly 50 putative mycobacteria were isolated from condensate water samples and two biofilm samples. In this study, the microbiological quality of untreated HVAC condensate was evaluated to determine its safety for reuse purposes. Heterotrophic bacteria are not necessarily pathogenic but concentrations were higher than the <500 CFU/mL recommended for potable water. While species-specific pathogen detection by qPCR (MA, MI/C, Lp SG1) was rare and concentrations were low, clinically relevant species were also isolated by the general *Mycobacterium* culture method. Disinfection could provide sufficient bacterial log reduction to utilize HVAC condensate water safely for many non-potable uses including irrigation, toilet flushing, and cooling tower makeup water. This research will inform States on concentrations of opportunistic bacterial pathogens and non-pathogens in untreated HVAC condensate and aid in recommending fit-for-purpose treatment options for this type of reclaimed water.
Abstract:

*Pseudomonas aeruginosa (Pa)* is a ubiquitous gram-negative bacterium that thrives in diverse environments. The ability of *Pa* to exist in these different environments can be linked to its ability to adhere to surfaces and produce a protective matrix. The *Pa* biofilm matrix is composed of proteins, exopolysaccharides (EPS), and self-produced extracellular DNA (eDNA). In addition to occupying environmental niches, *Pa* can cause chronic upper respiratory infections in immunocompromised individuals, such as cystic fibrosis (CF) patients. We performed immunohistochemistry staining of sputum and explanted lungs of CF patients and confirmed that the EPS Pel is produced by *Pa* in CF airways and that it remains localized to *Pa* aggregates. Previous work in our lab revealed that the *Pa* EPS Pel is composed of N-acetyl-glucosamine and N-acetyl-galactosamine and carries a positive charge at pH<6.9. Producing Pel and no other EPS is sufficient to support *Pa* biofilm structures and Pel was also found to be a major contributor to the antibiotic resistance of structures containing multiple EPS. However, the interactions between Pel and other matrix components or host products remains largely unexplored. Since the upper airway of CF individuals is known to be mildly acidified and contain high concentrations of eDNA from lysed host cells, we hypothesized that Pel could interact with host eDNA in the airway. We observed that positively charged Pel can form ionic aggregates with eDNA in a cell-free system. Additionally, eDNA bound by Pel is protected from nuclease degradation. In biofilms grown under continuous flow, exogenously added eDNA exclusively colocalizes with Pel and does not bind to structures lacking Pel. Furthermore, Pel-containing biofilms displayed an increased tolerance to tobramycin when grown with excess eDNA. Our work demonstrates that Pel produced in the CF airway can interact with host eDNA and that this may influence *Pa* susceptibility to antibiotic treatment.
The opportunistic pathogen *Pseudomonas aeruginosa* forms chronic biofilm-based infections in the lungs of cystic fibrosis (CF) patients. *P. aeruginosa* biofilms in the CF lung environment are often characterized by the overproduction of the exopolysaccharide alginate, due to acquired mutations in *mucA*. MucA inhibits the sigma factor AlgU, and it is thought that the clinical mutations in *mucA* lead to truncated proteins that are fully degraded, which results in misregulation of AlgU and overproduction of alginate. Paradoxically, our work shows that a portion of *mucA* is required for bacterial viability in a variety of *P. aeruginosa* strains. Here we examine why *mucA* is essential. Our results show that *mucA* was no longer essential in a strain lacking *algU* and that *mucA* alleles that abolished interaction with AlgU were not sufficient for viability. These results suggest that the loss of the MucA-AlgU interaction results in bacterial cell death because MucA is no longer available to regulate AlgU, leading to aberrant expression of AlgU-regulated genes. To determine if alginate is responsible for *mucA* essentiality, we deleted *algD*, a key alginate biosynthetic gene. We also created a strain in which three transcription factors that regulate alginate production (*algB*, *algR*, and *amrZ*) were deleted. In both strains, *mucA* was still essential, suggesting that alginate overproduction is not, at least solely, responsible for the *mucA* viability defect. To help determine the cause of the cell viability defect, we imaged cells depleted of MucA. We observed that MucA-depleted cells were shorter, suggesting that the cells were under some form of stress. Because MucA is involved in the response to envelope stress and removal of the envelope stress response via the deletion of *algU* suppresses *mucA* essentiality, we wondered if removal of other stress response pathways would alleviate the requirement for *mucA*. We, therefore, deleted two transcription factors involved in stress response, *rpoS* and *oxyR*. We found that *mucA* was no longer required in these backgrounds. While more work is still needed to understand why *mucA* is required for bacterial viability in *P. aeruginosa*, our results suggest that in the absence of the ability to respond to certain stresses, *mucA* is no longer required for bacterial viability.
Background: Urinary tract infections (UTIs) are mostly caused by the uropathogenic *E. coli* (UPEC). UPEC infect the urinary tract by forming Intracellular biofilm like communities (IBC) in urothelial bladder cells. Between 15-25% of hospitalized patients receive urinary catheters during their hospital stay. The most important risk factor for developing a catheter-associated UTI (CAUTI) is prolonged use of the urinary catheter. An important virulent factor in uropathogenesis and CAUTI is biofilm, the initial step of this is adhesion that requires pili. 

Methods: Bacterial assemblages like the adhesion, biofilm, swarming motility, and auto-aggregation were measured in this study. The UPEC biofilm formation on plastic surface or catheter was analyzed either by crystal violet assay, fluorescent microscopy, and Scanning Electron Microscopy. Auto-aggregation was tested by auto aggregation assay and Boarder crossing assay was done to test swimming and swarming motility.

Results: Two compounds, 1-amino-4-hydroxyanthraquinone (MB 07) and 2-Chloro-4-(methylsulfonyl) benzoic acid (MB10) are commercially available shows better inhibition at 10 and 20 µg ml⁻¹ against the adhesion respectively and effective inhibition of biofilm formation without reducing the planktonic growth of UPEC. The compounds MB 07 and MB 10 reduced the autoaggregation of the UPEC significantly at concentration of 10 µg ml⁻¹. It was observed that swimming was inhibited at 10 µg ml⁻¹ and swarming motility was inhibited by 20 µg ml⁻¹ concentration of both the compounds, as assessed by the border crossing method.

Conclusion: Chemicals are screened against adhesion to stop the biofilm formation. In adhesion type 1 pili plays an important role. These chemicals may be targeting the pili to stop the adhesion. So CAUTI can be controlled by coating the chemical in catheter which will act as Anti-adhesive compound.
Background: *Bordetella pertussis* is a gram-negative human pathogen and the primary etiological agent of the disease whooping cough or pertussis. Despite widespread and very high levels of vaccination coverage, pertussis has re-emerged with significant morbidity and mortality in infants, adolescents and adults. We have hypothesized that *B. pertussis* biofilms allow bacteria to escape from immune defenses and vaccine-mediated immunity thereby enhancing their persistence, transmission, and continued circulation. Little is known about how *Bordetella* biofilm growth has adapted with respect to time, region, and changing immunization regimens. To address this, a side-by-side comparison of the biofilm-forming abilities, structures and pathogenic phenotypes of currently circulating strains from the USA, Mexico and Argentina, three countries utilizing different vaccines and vaccination schedules, was conducted. Methods: *B. pertussis* clinical isolates recovered in USA, Mexico and Argentina, from 2001 to 2014, the laboratory reference strains Tohama I and Bp536, and an avirulent mutant strain (Bp369) were used in this study. Biofilm forming capacity was evaluated by the microtiter dish assay, and by confocal microscopy. In addition, autoaggregation index and biofilm-promoting and -inhibitory factors (Filamentous hemagglutinin and Adenylate cyclase) were determined. Adhesion to respiratory epithelial cells A549 and colonization of the mouse respiratory tract were also analysed. Results: Irrespective of the country of origin and compared to the reference laboratory strains, all the recently isolated strains formed hyperbiofilms. However, biofilm structural analyses revealed country-specific differences, with strains from the USA forming more structured biofilms. Bacterial hyperaggregation and reciprocal expression of biofilm-promoting and -inhibitory factors were also observed in current isolates. Strikingly, hyperbiofilm capacity of current isolates was associated with augmented epithelial cell adherence and higher levels of bacterial colonization in the mouse nose and trachea. Conclusions: Taken together, our data provide a link between increased biofilm formation in *B. pertussis* clinical isolates with altered pathogenic phenotypes and a colonization advantage in an animal model, even among those isolated in countries with different vaccination regimens.
Abstract:

**Background:** Every year, there are up to six million bone fractures in the United States; many of these fractures require surgical fixation, which contribute to the nearly two million fracture fixation devices deployed annually. In addition to fracture fixation devices, there are millions of prosthetic joint replacements inserted yearly. The burden of infection for these surgeries is one of the biggest problems in orthopedics. Infection rates can climb to nearly 2% for closed fractures, 30% for open fractures, and cause nearly 26,000 prosthetic joint infections yearly. These infections can extend patient hospital stays by 9.7 days per patient, cost $20,000 extra in hospital costs per person, and decrease overall quality of life. The current methods used to diagnose these infections are traditional bacterial culturing, PCR, and gram staining. However, these methods have issues with accuracy, efficiency, and cost. This study aims to assess the capabilities of a rapid visualization assay using fluorescently conjugated antibodies and Confocal Laser Scanning Microscopy (CLSM) to detect bacterial presence on surgical explants, tissue, and synovial fluid. **Methods:** With an IRB approved protocol, samples from 6 faculty surgeons at Rhode Island Hospital were collected and stored in 10% neutral buffered formalin. Synovial fluid samples were fixed onto slides with methanol and stained with a cocktail of serums, while explants and tissue were stained in 15 mL falcon tubes. Fluorescently conjugated anti-LPS (Dylight 594 and AlexaFluor 568) and anti-LTA (FITC 488) antibodies were added to the samples to label gram negative bacteria and gram positive bacteria. A DAPI stain was used to mark the presence of eukaryotic cells. Positive controls of Methicillin Sensitive Staphylococcus aureus and Acinetobacter baumannii and negative controls lacking bacteria are used to determine antibody quality and viability. Images were quantified through Confocal Laser Scanning Microscopy, analyzed with ImageJ (NIH), and compared to the results of the hospital data. **Results:** In the course of our experiment, we have obtained 43 synovial fluid samples and 32 hardware and tissue samples. Our assay results agree with hospital data and gram staining trials in 100% (43 out of 43) of cases for synovial fluid samples. For the hardware and tissue samples, hospital data found that 31.25% (10 of 32) of samples were infected, but our assay showed that 65.63% (21 of 32) had significant bacterial presence. **Conclusion:** This assay has demonstrated the ability to visualize bacteria on surgical explants, tissue, and synovial fluid with high contrast and specificity in 30 minutes. The assay also allows for the determination of bacterial morphology regardless of sample heterogeneity. In solid samples, the technology is able to pinpoint bacterial presence directly on the samples, allowing for directed debridement and localize treatment.
Testing Clin. Antibiotic Prophylaxis against Biofilms in a Sheep Model of Open Fracture

**Abstract:**

**Background:** Managing infections of orthopedic implant sites remains a considerable challenge despite the abundant availability of therapeutic compounds in this antibiotic era. A poignant example is Type IIIB open fractures reduced with internal fixation, displaying infection rates upwards of 50%. These high rates of infection have scarcely changed since the Type IIIB fracture was defined in 1984; persisting because significant environmental biofilm contamination at the time of injury overwhelms clinical approaches. A sheep model for open fracture biofilm contamination was developed by Williams et al. and has previously been used extensively to test experimental anti-biofilm approaches. We used this sheep model of biofilm contamination to test a clinical prophylactic course of antibiotics typical for open fracture cases.

**Methods:** The sheep model used *S. aureus* biofilms grown on polyether ether ketone (PEEK) membranes using a modified CDC biofilm reactor; the two PEEK-biofilms were each placed on the medial aspect of the right tibia and secured atop with two corresponding metal fixation plates. One of the biofilm-plate combinations was removed post-necropsy for microbiological analysis; the other remained on the bone and was processed for histological analysis. Each PEEK membrane contained $1.58 \pm 0.42 \times 10^{10}$ CFU at the time of inoculation. There were two study groups (n=2 sheep/group). The first group did not receive any post-procedural antibiotics whereas the second group received a 48 h prophylactic dose of IM antibiotics: gentamycin 10mg/kg every 24 h and cefazolin 25 mg/kg every 8 h. Sheep were sacrificed at 21 days. **Results:** Both groups had an approximate 4 log10 reduction in CFUs in the PEEK-biofilms, with little difference between the group which received prophylactic antibiotics and the positive control group. The PEEK-biofilms from the antibiotic group were slightly more robust at $4.0 \pm 1.2 \times 10^{6}$ CFU/membrane compared with $0.6 \pm 0.2 \times 10^{6}$ from the group which received no antibiotics (Figure 1). Histology is currently being performed. **Conclusions:** Clinical prophylactic antibiotics had no measurable effect on the biofilm bacteria used in this model of open fracture infection. The model simulated well the difficulty to treat infections, and may indicate why open fracture infections remain upwards of 50%. Results validated the utility of the model and may benefit future work to develop antibiofilm technologies.
Biofilm Formation, Component and Inhibition in Bartonella Henselae Using Real Time Monitoring and Microscopy

**Abstract:**

*Bartonella henselae* (*Bh*) is a Gram-negative rod that is typically transmitted to humans by a scratch from the common house cat. Infection of humans with *Bh* can result in a range of clinical disease including lymphadenopathy observed in cat-scratch disease and more serious disease from persistent bacteremia. It is a common cause of blood-culture negative endocarditis as the bacterium is capable of growing as aggregates and forming biofilms on infected native and prosthetic heart valves. Its aggregative growth requires a high molecular weight trimeric autotransporter adhesin (TAA) called Bartonella Adhesin A (BadA), a member of the TAA's found in all *Bartonella* species and other Gram-negative bacteria. Using *Bh* Houston-1, *Bh* Houston-1 ∆badA (a complete badA deletion mutant) and *Bh* Houston-1 ∆badA/pNS2PTrcbadA (a partial complement of badA consisting of the N-terminal head region, a truncated neck region and the C-terminal membrane anchor), we analyze bacterial adhesion, biofilm formation, and biofilm composition. Immunofluorescence microscopy using anti-BadA primary antibody and fluorophore conjugate secondary antibody demonstrated surface expression of the truncated version of the badA expressed by the partial complement. Real time monitoring using ACEA Biosciences’ xCelligence RTCA system and microscopy experiments both show that *Bh* Houston-1 quickly adheres and forms biofilm more efficiently than the *Bh* Houston-1 ∆badA. The partial complement *Bh* Houston-1 ∆badA/pNS2PTrcbadA displayed an intermediate ability to form biofilms indicating an incomplete restoration of the parental phenotype. In addition, a novel 3D nano-fibrous scaffold biofilm model was stained with the cationic dye Alcian Blue and employed to preserve the EPS structure and show biofilm formed by *Bh* Houston-1. The deletion of the badA gene significantly decreased adhesion, aggregation and biofilm formation in-vitro that was partially restored with a partial complement of the badA gene. Addition of proteinase K and DNAse1 both reduced adhesion and biofilm formation for all three strains suggesting the presence of both protein and extracellular DNA as components of the *Bh* biofilm EPS. We conclude that badA is required for optimal adhesion, autoagglutination and biofilm formation by *Bartonella henselae*.
Dispersion is a mechanism by which bacterial cells leave the biofilm in response to various environmental endogenous and exogenous cues including carbon sources and nitric oxide, resulting in a phenotypic switch of dispersing cells returning to the planktonic mode of growth. Compared to biofilms dispersed cells have been demonstrated to have reduced c-di-GMP levels, increased motility, reduced matrix, and altered virulence and susceptibility. While is now apparent that dispersion occurs as a result of complex spatial differentiation and molecular events in biofilm cells, little is known about the mechanism contributing to dispersion. The aim of the current study was to characterize dispersal mechanisms by elucidating genes necessary to enable dispersion. We therefore determined the transcriptome of planktonic, biofilm, and dispersed cells by using RNA-Seq, with dispersion being induced by nitric oxide or glutamate. Genes identified to be induced upon induction of dispersion or found to be dispersion-specific comprised those involved in adaptation and protection, transcriptional regulators, and matrix degradation. The latter comprised genes encoding secreted degradative enzymes including nucleases (EndA, EddA) and hydrolases (PslG, PelA, PA3429 and PA0480). Given that dispersion coincides with the release of bacteria from biofilms enmeshed in a polymeric matrix composed of polysaccharides and eDNA, we asked whether genes encoding matrix degrading enzymes are required for dispersion. While inactivation of eddA only impaired dispersion in response to glutamate, biofilms by endA::IS were impaired in the dispersion response regardless of the dispersion cues used. Moreover, induction of endA gene expression coincided with dispersion and an overall reduction in eDNA present in biofilms. We furthermore asked if degradation of polysaccharides present in the biofilm matrix were necessary for the dispersion response. While inactivation of PA0480 only reduced dispersion in response to nitric oxide, inactivation of PA3429 impaired the dispersion response to both cues. Moreover, while inactivation of pel and psl coincided with biofilms being impaired in the dispersion response, only the induction of the Pel specific hydrolase pelA but not pslG, the Psl specific hydrolase, resulted in dispersion. Our findings indicate that nucleases and hydrolases are essential to the liberation of bacterial cells from the biofilm. Moreover, our findings suggest bacteria to be actively released through specific regulation of degradative factors resulting in the overall reduction specific components of the biofilm matrix.
**Abstract:**

**Background:** Outer membrane vesicles (OMVs) are ubiquitously produced by Gram-negative bacteria and have extensive roles in virulence and pathogenicity. For these reasons, elucidating what factors control OMV biogenesis is critical for developing therapeutic strategies against pathogenic bacteria. Our lab and others have shown that the *Pseudomonas* quinolone signal (PQS) induces the formation of OMVs in *Pseudomonas aeruginosa* as well as in other species. PQS is part of a hierarchy of quorum sensing pathways, and its production is dynamic and dependent on many genetic and environmental cues. This work identifies a novel effector of PQS induced OMV formation: cyclic dimeric guanosine monophosphate (c-di-GMP). This universal molecule acts as a lifestyle switch for bacteria inducing the shift from planktonic to biofilm growth. Previous studies have shown that c-di-GMP downregulates the virulence factor regulator (Vfr). Separately, Vfr has been shown to upregulate LasR, which is a positive regulator of PqsR. As a result, we hypothesized that PQS induced OMV production is negatively regulated by c-di-GMP. **Methods:** PQS induced OMV production was measured under low and high c-di-GMP conditions. These conditions were achieved using c-di-GMP mutants in planktonic conditions and a natural biofilm model. In our genetic approach, ΔwspF (which overproduces c-di-GMP due to its inability to regulate the WspR diguanylate cyclase) and PAO1/pMJT-PA2133 (which overproduces the PA2133 phosphodiesterase and depletes intracellular c-di-GMP levels) were analyzed after 12 hours of planktonic growth. In our natural model, tube reactor biofilms that have different c-di-GMP concentrations at each stage of biofilm formation were analyzed for PQS, OMVs, and c-di-GMP. PQS was extracted with ethyl acetate and quantified by TLC. OMVs were isolated by differential centrifugation and measured by nano-particle tracking analysis (NTA) and lipid analysis. C-di-GMP was extracted with ethanol and quantified by HPLC. **Results:** In our planktonic studies we found that under high intracellular c-di-GMP conditions (ΔwspF) PQS production significantly decreased. Consequently, we also observed that OMV production was reduced in ΔwspF. On the contrary, when planktonic cultures were grown under low c-di-GMP conditions (PAO1/ pMJT-PA2133) PQS production significantly increased. This increase was accompanied by a significant increase in OMV formation. We were very excited to show that the trends observed in planktonic conditions upheld in a biofilm model. We observed that as biofilms developed through attachment, maturation and dispersion, PQS production was inversely correlated to intracellular c-di-GMP concentration. **Conclusion:** This work provides new insights into PQS-controlled OMV biogenesis as it connects this mode of OMV production to a major modulator of pathogenicity in *P. aeruginosa*, c-di-GMP.
Abstract:
The pathogen *Pseudomonas aeruginosa* (PA) exhibits complex social behaviors. Although the physiological basis of processes like biofilm formation has been studied for several years, it is still unclear how gene content differences shape this process across different environments and selective pressures. We used a 138-strain library of sequenced PA isolates from environmental and human sources and phenotypically characterized these isolates biofilm formation and antibiotic resistance. Using a combination of LASSO (with Bayesian parameter estimation) and phylogenetic signal assignment we were able to estimate genetic predictors for all the traits based on the size of their effect and their phylogenetic correlation. This mapping of genotypes to phenotypes enabled us to explore the evolution of biofilm formation. We found that several transcription factors which shape these processes and cluster together in the PA regulatory network. Based on these findings we used mutual information to build a co-occurrence network of genes. This network indicates that traits evolve in a modular fashion. We also found instances where modules with predicted effects biofilm formation have an inverse effect on antibiotic resistance. Finally, we mapped the genes of PA to lower phylogenetic levels to date the age of genes involved in biofilm formation and characterize the historical times where most genes appear.
**Background:** Autotransporter (AT) proteins make up the largest group of non-frimbrial adhesins in Gram-negative bacteria. The autotransporter Antigen 43 (Ag43) is a highly abundant AT, which through self-association between neighbouring *Escherichia coli* cell surfaces was known to promote both cell aggregation and biofilm formation. Ag43 is found in all *E. coli* pathotypes, including uropathogenic *E. coli* (UPEC), the main cause of urinary tract infections and enterohaemorrhagic *E. coli* (EHEC), causative agent of severe foodborne diseases. The chronic forms of these infections involve bacterial biofilms, which promote bacteria persistency in hostile environments and resistance against the effects of conventional antibiotics. **Methods:** We have investigated and compared two distant Ag43 homologues from UPEC and EHEC in order to define similarities and differences in their molecular mechanisms for forming bacterial aggregates and biofilms. We have accomplished this by utilising a unique combination of X-ray crystallography, biophysical techniques, mutagenesis and cell-based assays. **Results:** My two new X-ray crystal structures of Ag43 from UPEC and EHEC have shown a surprising degree of structural conservation of their three stranded beta-helix structures and L-shaped bending. Despite this similarity, both adhesins remarkably showed significant differences in their mechanism of self-association, when their interfaces were mapped using site-directed mutagenesis. We further found that these differences in Ag43 self-association caused variations in their dimerization affinities. These differences in structure and mechanism of action translated into differing bacterial aggregation phenotypes. **Conclusions:** The different modes of dimerisation observed in Ag43 may reflect the adaptations to the different environmental niches that the they colonize. These findings suggest that there is a possibility of further diversity in the self-association of autotransporter. These differences and common themes of self-association could be used to create both narrow and broad spectrum anti-microbials.
Listeria monocytogenes is a biofilm-forming microorganism capable of survival under a wide range of conditions. Even at high bile salt concentrations with antimicrobial activity, it did not prevent its biofilm growth in the gallbladder. The biofilm formation and the ability to attach to abiotic surfaces or biotic surfaces are facilitated by extracellular polymeric substances, the biofilm matrix. The matrix contributes to the resilience of the microorganism to stress conditions, including bile. Therefore, we aimed to assess the effect of bile on biofilm formation and each matrix component. The biofilm formation of 25 strains was assessed with and without the presence of bile. The abundance of the matrix components was evaluated with and without the application of bile using spectrophotometry. All experiments were performed at least in three independent replicates and evaluated statistically. The biofilm treated with bile was visualized by confocal laser scanning microscopy. The ability of strains to form a biofilm increased in the presence of bile (p<0.001). The preliminary tests revealed that the total amount of proteins was similar in both treatments. Surprisingly, the overall eDNA content was higher after the application of bile in contrast to saccharides that decreased significantly. The information about the proportion of biofilm matrix components could expand the understanding of matrix functions in the presence of bile.
**Title:** The Conformation of Fibronectin Determines the Success of Bacterial Attachment

**Abstract:**

**Background:** *Staphylococcus epidermidis* is responsible for many implant-associated infections, and its pathology is linked to its ability to form biofilms on the implant. Attachment to the implant occurs through specific interactions with adsorbed host proteins. One important receptor for *S. epidermidis* is the giant extracellular matrix binding protein (Embp), which binds to fibronectin (Fn). Embp, like many other bacterial adhesins, bind to host proteins that are available in solution in the blood as well as adsorbed to the surface of biomedical implants. So how can interaction with these proteins promote attachment to the surface? We hypothesized that Embp only mediates attachment to immobilized fibronectin, and that the distinction between soluble and immobilized fibronectin lies in the availability of binding domains in Fn. These domains may become exposed only when the protein adsorbs to an implant surface and undergoes conformational changes that lead to fibrillation. **Methods:** To investigate these hypotheses, we first used fluorescently labelled Fn and confocal microscopy to show that *S. epidermidis* interacted with adsorbed but not soluble Fn. Soluble Fn is in a globular conformation, while adsorbed Fn can either remain globular, or change conformation to form fibrils in a similar way as it does in the extracellular matrix of host tissue. To study the bacterial interaction with Fn in these two conformations, we produced surfaces coated with (poly)methyl acrylate (PMA) and (poly)ethyl acrylate (PEA), which adsorb Fn in the two different conformations. **Results:** Atomic force microscopy confirmed that Fn adsorbed to PMA remained globular, while Fn adsorbed to PEA fibrillated. We then quantified Embp-mediated bacterial attachment to the two surfaces, using *Staphylococcus carnosus* expressing a recombinant fragment of Embp. Fibrillar Fn promoted bacterial attachment while globular Fn did not. These differences were also reflected in the adhesion forces. **Conclusions:** This result supports our hypothesis that adsorption-induced conformational changes dictate if a host protein promotes or prevents bacterial attachment to an implant surface. Our results underline that the materials properties of implants affect biofilm formation indirectly by making host proteins available in the right or wrong conformation. This knowledge adds a new layer to the considerations made in materials design for novel implant materials that prevent biofilm infections.
Background: During the infection process, bacterial pathogens are exposed to a variety of host factors that may influence their susceptibility to antimicrobial agents. While many compounds that modulate antibiotic activity are known, the influence of the local environment at the host-pathogen interface on bacterial responses to antibiotics is still poorly understood. Gaining insights into these host factors that influence the efficacy of antibiotics may help improve our understanding of why there is such a poor correlation between antibiotic activity in vitro and in vivo. This is particularly relevant for biofilm-associated infections for which antibiotic therapy chosen based on susceptibility assays frequently does not lead to clinical improvement, as is the case in for example respiratory tract infections by Pseudomonas aeruginosa in people with cystic fibrosis.

Study goals: This study aims to evaluate the influence of lung epithelial cell secretions on antibiotic activity against P. aeruginosa biofilm formation. We investigated (i) whether lung epithelial cell secretions modulate the activity of antibiotics, (ii) which host factors are responsible, and (iii) what the underlying mode of action is.

Methods: We assessed antibiotic activity against P. aeruginosa in the presence of conditioned medium of an in vivo-like 3-D lung epithelial cell model (3-D CM). Antibiotic activity in the presence of 3-D CM or control medium was tested using a biofilm inhibition assay and time-kill curve. Tobramycin uptake was measured using BODIPY-labelled tobramycin in combination with flow cytometry analysis. The intracellular pH was determined using a 2,7-bis(2-carboxyethyl)-5(6)-carboxyfluorescein-acetoxymethyl (BCECF-AM) assay. A series of chemical inhibitors of metabolic pathways were used to assess which compounds produced by the host were responsible for increased tobramycin activity.

Results: The 3-D CM potentiated the biofilm inhibitory and bactericidal activity of aminoglycosides, including tobramycin. The effect was observed for the reference strain PAO1 and most of the tested clinical and environment isolates of P. aeruginosa. Mechanistic studies indicated that 3-D CM enhanced intracellular pH of P. aeruginosa (hereby increasing the ΔpH component of the proton motive force, PMF), resulting in an enhanced tobramycin uptake. Finally, our data suggest that metabolites of the host, generated through pyruvate metabolism, stimulate bacterial metabolism - hereby increasing the PMF and aminoglycoside uptake in P. aeruginosa biofilm cells.

Conclusions: We demonstrate that lung epithelial cells contribute to the innate defence against biofilm-grown P. aeruginosa by acting in concert with antibiotics.
Abstract:

Staphylococcus aureus, in both methicillin sensitive and methicillin resistant form, is the common pathogen associated with chronic wound infections. Despite the fact that S. aureus is the most prevalent microorganism in multiple cutaneous diseases associated with biofilm, the mechanisms by which this pathogen is causing persistent skin infections remain largely unknown. We examined the role of recently-discovered anti-microbial protein, Perforin-2 (P-2) a membrane-attack-complex-perforin domain containing protein important for cutaneous innate immunity, in S. aureus wound biofilms. We have previously shown that wound infection with S. aureus in P-2 deficient mice results in systemic bacterial dissemination and death. Necrosis and inflammation were absent in P-2 KO despite systemic dissemination and massive replication of S. aureus in vivo. We further analyzed P-2 expression in human skin wound model and biofilm infected chronic ulcers at a single cell resolution. We describe a novel approach for the measurement of host repose to biofilm infection by analyses of P-2 mRNA within individual skin cells using an amplified fluorescence in situ hybridization (FISH) technique. The unique aspect of this approach (FISH-Flow) is simultaneous detection of P-2 mRNA in combination with immune-phenotyping for cell surface proteins using fluorochrome-conjugated antibodies. We detected P-2 transcript in both hematopoietic (CD45+) and non-hematopoietic (CD45-) cutaneous cell populations, confirming the P-2 expression in both professional and non-professional phagocytes. Next, we investigated P-2 role in restoration of barrier function utilizing human ex vivo wound model. We found induction of P-2 during early inflammatory phase of wound healing confirming its role in cutaneous immunity. In contrast to healthy skin and non-infected wounds, FISH-Flow analyses from human infected chronic diabetic ulcers revealed P-2 suppression in CD45+ cells and basal keratinocytes, suggesting their inability to eliminate S. aureus biofilms. The P-2 mediated response to S. aureus biofilms was measured in human ex vivo wound infection model. Human wound infection with S. aureus biofilm resulted in suppression of P-2 expression in a cell specific manner, revealing a novel mechanism by which S. aureus escapes cutaneous immunity to cause persistent biofilm wound infections. Our data suggest that P-2 may have dual property during wound healing, acting as an innate immunity effector preventing biofilm formation and also wound healing stimulator, while its suppression by S. aureus contributes to chronicity of wound infections. Thus, P-2 may be a new target for prevention of staphylococcal skin infections and associated complications.
Poster Session 4

Date & Time: Thursday, October 11, 2018, 4:00 pm - 6:00 pm
Poster Board #: 075
Topic: Host Microbe Biofilms
Abstract Title: Extracellular HMGB1 Controls Biofilms in Mammalian Hosts
Author Block: S. D. Goodman, A. Devaraj, F. Robleda-Avila, L. A. Novotny, J. Buzzo, L. Mashburn-Warren, S. Partida-Sanchez, L. O. Bakaletz; Nationwide Children’s Hospital, Columbus, OH.

Background: We show that the eukaryotic HMGB1 protein which is known to function as a pro-inflammatory effector, is also the primary guardian of the innate immune system to control bacterial biofilms by virtue of its potent anti-biofilm activity. HMGB1 is a ubiquitously expressed and highly conserved eukaryotic nuclear protein that functions as a structural component in a gamut of nucleoprotein interactions in the intracellular milieu by virtue of its ability to bind to and bend DNA. HMGB1 can be secreted and/or released into the extracellular milieu by activated immune cells, epithelial cells and fibroblasts and is associated with neutrophil extracellular traps to combat microbial infection. We have previously demonstrated that the bacterial DNABII proteins ubiquitously expressed by prokaryotes bind to and bend DNA in the minor groove and are integral to the structure of biofilms formed by multiple human pathogens. HMGB1 shares no sequence or structural homology to DNABII proteins, yet has been shown to functionally complement DNABII proteins in vitro. Moreover, whereas both the DNABII proteins and HMGB1 bind DNA in the minor groove, HMGB1 binds on the convex surface of DNA while the DNABII proteins bind on the concave side to implement a bend. Methods: To evaluate its effect on bacterial biofilms in vitro, we added HMGB1 (or its anti-inflammatory variant; 200 nM) to pre-formed biofilms at 24h. At 40h, the biofilms were stained with LIVE/DEAD® and analyzed using confocal laser scanning microscopy. To assess the ability of HMGB1 to eradicate (0.2 nmole HMGB1 added to an established biofilm) or prevent (HMGB1 added simultaneously with the bacteria) biofilm development in vivo, we employed a chinchilla model of otitis media and a mouse model of lung infection. Results: We demonstrated that recombinant HMGB1 disrupts preformed biofilms in vitro (formed by multiple bacterial species including the ESKAPE pathogens) and in vivo (non-typeable Haemophilus influenzae biofilms in a chinchilla model of otitis media, and Burkholderia cenocepacia biofilms in murine airways). Despite the latter successes, HMGB1 induced a substantial inflammatory response akin to its native function, hence we engineered a mutant HMGB1 variant (mHMGB1) to inactivate its proinflammatory properties. The engineered variant mHMGB1 retained the anti-biofilm function against multiple human pathogens but exhibited an attenuated inflammatory response in both animal models. Conclusions: Thus, we hypothesize that while HMGB1 has a native extracellular role in both biofilm control and inflammatory response, the latter function can be sufficiently attenuated, which demonstrates the potential to deliver mHMGB1 and thereby provide a significant therapeutic benefit against disease caused by multiple human bacterial pathogens without induction of hyper-inflammatory sequelae.
Abstract: A Suppressor Mutation of Eep-Mediated Lysozyme Resistance Leads to Permanent Alterations of the Enterococcus faecalis Cell Surface

Author: C. N. Rouchon, A. Weinstein, K. L. Frank; Uniformed Services University of the Health Sciences, Bethesda, MD.

Enterococcus faecalis is an opportunistic pathogen that is resistant to lysozyme, an important antimicrobial of the host innate immune system. Previous studies demonstrated that the E. faecalis Eep membrane metalloprotease, a biofilm infection-associated virulence factor, confers lysozyme resistance through a signal transduction cascade that involves activation of the alternative sigma factor SigV via cleavage of the anti-sigma factor RsiV. Our lab has isolated suppressor mutants of Δeep (Δeep lysR) that regain the ability to thrive in the presence of lysozyme. The goal of this study is to elucidate the molecular mechanisms that confer the Δeep lysR phenotype. Using a time-kill assay, in which E. faecalis planktonic cultures were grown in broth containing 2.5 mg/ml lysozyme, we found that the number of viable Δeep cells decreased by >2 log10 CFU/ml relative to the inoculum at 6 hours post-exposure. However, after 24 hours of exposure to lysozyme, the number of Δeep cells recovered to a level similar to that of WT, for which growth was unaffected. Colonies of Δeep from 24-hour cultures remained resistant to killing by lysozyme after passage in non-selective, rich medium. These experiments suggested that the observed re-growth of Δeep in the 24-hour lysozyme cultures was due to selection of genetically stable, lysozyme-resistant Δeep suppressor mutants (Δeep lysR). Whole genome sequencing analysis of Δeep lysR isolates revealed that a gene required for techoic acid biosynthesis was mutated in these strains. Using a microtiter well biofilm assay, we found that the biomass of 24-hour Δeep lysR biofilms was reduced relative to that of the lysozyme-sensitive Δeep parent strain. Additionally, through confocal microscopic imaging, we observed that the cell envelope of Δeep lysR biofilms stained with an Alexa Fluor 594-wheat germ agglutinin conjugate fluoresced more intensely than that of similarly-labeled WT and Δeep cells. Further examination of the Δeep lysR cells by transmission electron microscopy demonstrated that the cell surface of these isolates lacked the electron dense structures observed on the surface of WT and Δeep cells. Furthermore, analysis of purified cell wall carbohydrates indicated that Δeep lysR exhibits altered polysaccharide content relative to WT and Δeep cells. The combined findings of this study demonstrate that in the absence of functional Eep protein, Δeep lysR suppressor mutants utilize an alternate mechanism of lysozyme resistance that is associated with stable alterations to the cell surface. Our on-going studies are focused on examining the fitness of Δeep lysR cells following exposure to cell wall-targeting molecules and characterizing the lectin-binding abilities of these isolates.
Symbiotic strains of *Vibrio fischeri* must coordinate the expression of multiple traits, including production of a biofilm matrix, in order to enter into a mutualism with the squid, *Euprymna scolopes*. We utilized an experimental evolution approach to examine whether non-native and symbiosis impaired strains could adaptively evolve under squid host selection and subsequently identified the paths of adaptability in evolved lineages (Pankey, Foxall et al.; eLife 2017;6:e24414 doi: 10.7554/eLife.24414). This led to the discovery of convergent gain-in-function mutations in the gene encoding the BinK biofilm repressor underlay adaptation. The breadth of phenotypes associated with adaptive *binK* alleles implied a potential pliability of sensory transduction partnerships could underlie improved symbiotic fitness. We used comparative genomics to identify potential signal transduction partnerships, which revealed a paralogous, ancestral sensor kinase to BinK we call RemK. BinK and RemK differed in sensory perception architecture and had divergent REC output domains that could promote phosphotransfer with different downstream signaling partners but had a conserved core domain sequence that could promote cross-talk. *In vivo* protein interaction assays indicated that BinK and RemK were capable of forming a heterodimer and genetic analysis indicated that BinK repressed biofilm and squid colonization by heterodimerization with RemK. BinK and RemK co-repressed cellulose and symbiotic polysaccharide (Syp) production and loss of either paralog increased expression of *sypA*. However, loss of *remK* additionally enhanced the expression of all *syp* promoters activated by the key regulator SypG. We predict that the adaptive mutations impaired BinK homodimer activity and also impaired heterodimer repressor activity thereby extending the BinK-mutant regulon to include impaired RemK repression of biofilm and that these mutations were favored over mutations that eliminated BinK through the greater impact on biofilm matrix production that included both Syp and cellulose.
**Abstract:**

**Background:** Campylobacter jejuni has been reported as the leading cause of bacterial foodborne infections worldwide, with significant increase over the past years. Despite the fastidious growth requirements, C. jejuni is able to survive in the environment without permanent loss of viability and virulence. The mechanisms responsible for its survival remain unknown, survival strategies might be associated to adhesion to inert surfaces and biofilm formation. The objectives of this study was to assess the effect of dioxygen (O₂) on adhesion and biofilm formation of C. jejuni. In order to understand the underlying mechanisms of dioxygen on adhesion and biofilm formation, the role of the virulence factor Campylobacter adhesion to fibronectin CadF and the essential transcriptional factor Campylobacter oxidative stress regulator CosR identified to pilot the sub-system of reactive oxygen species (ROS) detoxification in C. jejuni were inspected.

**Methods:** BioFilm Ring Test was used to assess the adhesion to inert surfaces and Confocal Laser Scanning Microscopy (CLSM) was applied to analyse biofilm formation and spatial structuration. Effect of oxygen-enriched conditions (OEC) on membrane proteome was approached using proteomics analyses. CadF-knock out mutant and CosR-overexpressing transformant were used to investigate their role in adhesion to inert surface and biofilm maturation, respectively. **Results:** A high intra and inter-species variability among strains of Campylobacter was observed for adhesion capability. Among the adherent strains, biofilms could be formed within 17 h of incubation. Biofilm architecture could differ from finger-like structure with voids and channels to compact multilayer-like structure. Dioxygen and oxidative stress conditions favoured adhesion to inert surface and biofilm formation. Pre-conditioned cells to oxygen-enriched conditions (OEC) were less favourable to biofilm development than cells incubated in OEC during biofilm formation. CadF was over-expressed under OEC and demonstrated to be involved in the adhesion process to inert surface. The orphan two-component regulator CosR was found to contribute to the entrance to the maturation phase of biofilm in an O₂-independent manner. **Conclusions:** C. jejuni is able to survive by forming biofilms. Cellular mechanisms involved in adhesion and biofilm development are enhanced by O₂. Entrance in maturation phase seems to be crucial for biofilm development of C. jejuni. While the microaerophilic Campylobacter is sensitive to oxidative stress, oxygen-enriched conditions enhanced adhesion to inert surface and biofilm formation. Biofilm might play an important role in O₂ distribution and availability to maintain Campylobacter viable in the environment. This adaptation capability might contribute to disseminate pathogenic species of Campylobacter in the environment and food plants.
Abstract: Clostridium perfringens is a Gram-positive spore-forming anaerobic pathogen that causes gas gangrene and food poisoning. Biofilm formation and self-produced extracellular polymeric substances (EPS) confer increased tolerance, and thus biofilms are widely recognized as causes of infectious diseases and environmental contaminants. We found that C. perfringens forms biofilms with different structures in response to temperatures. At 37°C, cells adhere densely to the surface, forming thin biofilms. In contrast, at 25°C cells produce a threadlike extracellular EPS that facilitates the formation of a thick, elastic, pellicle-like biofilm. Temperature is an environmental cue that alters between the outside and inside of the host, which suggests that the morphological changes of biofilms are involved in the pathogenesis of C. perfringens. Here, we identified the gene responsible for production of the threadlike extracellular EPS and pellicle-like biofilm formation. We constructed fluorescent protein-fusion reporters to detect the ESP gene expression in C. perfringens. Fluorescent reporter analysis indicates that there are two types of cells within the overall population. In one type, the EPS gene promoter is activated. In the other type, the EPS gene promoter is repressed, attaching to the surface. The sub-population size of EPS-expressing cells gradually increase as the temperature decreases. Interestingly, we observed that cells expressing EPS overlie the EPS-repressed cells, which are located near the surface by confocal laser scanning microscopy. The deletion of the gene involved in type IV pili, which are involved in the attachment in C. perfringens, activates the EPS gene expression in the whole population through the positive feedback regulation via a two-component system. Furthermore, we also found that a quorum sensing system is also involved in fine-tuning of the EPS gene expression. Heterogeneity within the population determines cell fate; cells may either attach to the surface or produce the threadlike EPS. This heterogeneity is controlled by temperatures and quorum sensing, which greatly influences biofilm morphology. C. perfringens may modulate EPS expression to induce morphological changes in the biofilm structure as a strategy for adapting to the inter-host and external environments.
Abstract: Biofilms are surface-associated groups of microorganisms that adhere to surfaces and interact with each other using an extracellular polymeric substance matrix. Microorganisms have developed complex mechanisms to sense and react to their constantly changing environment under these conditions. One key regulatory cue for them is temperature. Studies have shown that different factors, such as temperature, can cause behavioral and morphological changes in the microbial communities. *Pseudomonas aeruginosa* is a common nosocomial gram-negative bacterium, that can cause various serious diseases in infected humans. The severity of the infections is compounded by *P. aeruginosa*'s ability to form robust biofilms in all the various niches it occupies. Biofilm-associated infections are particularly recalcitrant to clearance by both antimicrobial therapy and immune function. We hypothesize that the fluctuations in temperature in the different niches that *P. aeruginosa* occupies drive the formation of biofilms specifically adapted to survival within that niche. Using MALDI IMS, we have demonstrated that biofilms grown under these different temperature conditions exhibits dramatically different protein expression profiles, which supports the contention that these biofilms are uniquely adapted to different niches. The objective of this project is to elucidate the genes involved in the temperature regulation of biofilm formation of *P. aeruginosa*. For this purpose, a biofilm screen was run on a commercially-available transposon mutant library containing over 5,000 unique mutants of *P. aeruginosa* at four different temperatures (room temperature, 30°C, 37°C, and 40°C) to identify genes required for temperature-dependent biofilm formation. This temperature range was chosen to simulate conditions relevant to both medical and industrial settings. These strains were replicated out into 96-well plates and incubated for two days at the specified temperatures. Finally, total biomass and biofilm growth were monitored using specific absorbance readings combined with staining analysis. The biofilm assay becomes more variable as temperature increases, but we were still able to identify potential primary screen hits that exhibit reproducible biofilm phenotypes. We identified mutants with temperature-dependent as well as few with temperature-independent phenotypes. The potential hits were then categorized into groups based on their function and carried forward towards the secondary screen. Effectively this project will reveal the genetic mechanisms utilized by *P. aeruginosa* to establish biofilm growth at temperatures relevant to medical, industrial, and natural environments and will provide a wealth of information regarding the adaptive potential of *Pseudomonas aeruginosa* towards the colonization of various niches including the human.
Oxygen-Regulated Subpopulations Direct the Formation of Architecturally Complex Biofilms Capable of Withstanding Exogenous Insults

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Rather than existing as a phenotypically uniform population, bacteria within biofilms differentiate into spatially segregated and metabolically distinct subpopulations. As a result of this metabolic specialization, there is a division of labor within biofilms that is essential for the formation of structurally complex communities capable of withstanding exogenous stressors. The presence of chemical gradients within biofilms is a major driver of metabolic differentiation. Previous studies detected a steep oxygen gradient within biofilms that results from consumption of oxygen by respiring bacteria near the surface of the biofilm. In turn, this oxygen gradient regulates bacterial redox-state and influences expression of key adhesive molecules and matrix components. We hypothesized that the presence of such gradient results in distinct subpopulations that express different respiratory components and are organized along the oxygen gradient. Consistent with this hypothesis, we demonstrate that cytochrome bd, a high affinity quinol oxidase required for aerobic respiration under hypoxic conditions, is the most abundantly expressed respiratory complex in uropathogenic Escherichia coli biofilms. Strikingly, loss of cytochrome bd causes marked disruption biofilm architecture and leads to global dysregulation of protein expression within the biofilm. In addition, the loss of cytochrome bd alters the composition of the biofilm extracellular matrix, such that the mutant biofilms permit increased penetration by aqueous solutions and, as a result, are more susceptible to antibiotic therapy. These data indicate that oxygen gradients spatially regulate expression of respiratory components within biofilms and that the cytochrome bd expressing subpopulation is central to formation of structurally intact biofilms capable of withstanding antibiotics and other exogenous stresses.
Session: Poster Session 4  
Date & Time: Thursday, October 11, 2018, 4:00 pm - 6:00 pm  
Poster Board #: 082  
Topic: Regulation of Biofilm Development  
Abstract: Isolation of Biofilm Producing Microorganisms from Packaged Water Sold in the Open Market Within the Three Senatorial District of Lagos Metropolis  
Title: Within the Three Senatorial District of Lagos Metropolis  
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Background: Bacteria growing in a biofilm are linked with protracted and recurring human infections; and have been shown to be highly resistant to antimicrobial agents. Although biofilm has become a popular research topic in many areas in recent years; it is still opaque in the continent of Africa as very little or no data is available in this area in most African countries. Objective: This study was conducted to detect biofilm forming Microorganisms in packaged water sold in retail store in Lagos, Nigeria. Method: Samples (130 bottled and 170 sachet) water was purchased using the simple random technique and isolation of microorganism was carried out using the filtration method recommended by WHO; while biofilm formation was detected using the tube method. Results: A pH range of 3.5 was recorded for the 300 samples at 25°C. 82% of the 130 bottled water brands conformed to WHO maximum contamination level while 29% of the 170 sachet water did not. A total of 112 bacteria were isolated of which approximately 70% were capable of producing biofilms of varying degree. Among the strong biofilms producing isolates are E. coli, Klebsiella pneumoniae, P. aeruginosa, Salmonella typhi and Enterococcus faecalis while S. aureus S. epidermidis were moderate biofilm formers whereas species of Bacillus and Pseudomonas were non/weak biofilm producers. Conclusion: The results of the present study exposes the lack of attention paid to biofilms formation within storage tanks and pipes of water distribution system; by this small and medium scale packaged water companies; sprouting up every now and then within the metropolis. We therefore call for strict and routine monitoring of the fast growing packaged water industry within the metropolis with a view that ensures adequate periodic back washing activity and anti-biofilms equipment are used within the industry, in order to safeguard public health.
Abstract: Prophages are regions in bacterial genomes that have originated from lysogenic viral infections. Recent research showed that in a pool of 2110 bacterial genomes, almost half of the genomes contain such regions. Among *Pseudomonas aeruginosa* strains, the prevalence of prophages is even higher; at least one prophage region was found in every strain analysed. To examine the contribution of prophage genes, a comprehensive screening was done on five *P. aeruginosa* strains (laboratory and clinical strains) using several bioinformatic tools, which revealed a total of ~750 prophage-derived genes. Two of these genes, Prevent host death protein (*phdP*) and Plasmid stabilization system protein (*pssP*) were found in two of the examined strains (PAO1 and C3719), and are estimated to constitute a Toxin-Antitoxin (TA) system. TA systems are genetic elements that consist of two components - a toxin and antitoxin that counteract the activity of the toxic protein. There are a variety of functions that have been assigned to TA systems, ranging from persistence to DNA stabilization to protection against mobile genetic elements. PssP is assumed to be a member of the ParE toxin family, which is toxic towards DNA gyrase, but is neutralized by the antitoxin. We provide experimental evidence demonstrating that PssP acts as a toxin most likely through interaction with GyrA. Another pair of genes, Accessory Cholera Enterotoxin (Ace) and Zona Occludens Toxin (Zot), were found in three of the examined strains (PAO1, C3719 and 2191), and are localized in one operon along with coat protein A of bacteriophage Pf1. Ace and Zot, together with Cholera Toxin, are known to comprise the *Vibrio cholerae*’s "virulence cassette", which originated from bacteriophage CTXΦ. A previous study suggested that Zot might not have virulent functionality in *P. aeruginosa* and has an additional role which was speculated to be related to phage assembly. We characterized the role of Ace and Zot on a range of *P. aeruginosa* phenotypes including biofilm formation and motility.
Abstract:

Mycobacterium tuberculosis (Mtbd), the causative agent of tuberculosis, is among the first clonally purified bacterial species described to form multicellular communities, appearing like parallel bundles of cords. Our work over the last decade have led us to define these communities as surface-associated, matrix-encapsulated, and antibiotic tolerant three-dimensional architecture, providing consistency with the broader framework of microbial biofilms. However, factors regulating the architecture development of Mtb biofilms and their relationship, if any, to the emergence of antibiotic tolerance remained unclear. Using a high throughput TnSeq-based approach, we screened a mutant library of Mtb to rank fitness of individual mutants in biofilms, relative to planktonic culture. Mutants thus identified were further analyzed for their sensitivity to rifampicin (RIF) in planktonic cultures. Majority of mutants with deficiency in biofilm formation were also hypersensitive to RIF, suggesting that growth of Mtb in biofilms enriches drug tolerant clones at the expense of their drug-sensitive counterparts. A proof-of-concept at molecular levels was offered by mutants of genes involved in phosphorous homeostasis. While mutations in the phosphorous sensing pathway cause hypersensitivity to RIF through a constitutive activation of RegX3-SenX3 two-component system, these mutations also abrogate biofilm-specific induction of a gene cluster involved in remodeling of the cell envelope during architecture development. In summary, our findings provide a direct linkage between architecture development and emergence of drug tolerant cells in Mtb biofilms.
Around the half of the world’s population is infected by *Helicobacter pylori* which is a major cause of duodenal and gastric ulcers as well as gastric cancer. *H. pylori* has an impressive ability to persist chronically in the human stomach and to have increased tolerance towards conventional antibiotic treatments. Similar characteristics are associated with biofilm formation in other bacteria. However, *H. pylori* biofilm process is poorly understood. To gain insight into this mode of growth, we carried out comparative transcriptomic analysis between *H. pylori* biofilm and planktonic cells, using the mouse colonizing strain SS1. Optimal biofilm formation was obtained with low serum and three-day growth. RNA-seq analysis found that 8.18% of genes were differentially expressed between biofilm and planktonic transcriptomes. Biofilm-downregulated genes included those involved in metabolism and translation, suggesting cells in that setting have low metabolic activity. Biofilm-upregulated genes included those whose products were predicted to be at the cell envelope, involved in regulating different stress response, and surprisingly, genes related to formation of the flagellar apparatus. Using scanning electron microscopy, flagella appeared to be in high abundance and an integral component of the biofilm matrix, supported by the observation that an aflagellated mutant displayed a less robust biofilm with no apparent filaments. We observed flagella in the biofilm matrix of additional *H. pylori* strains, supporting that this is a common occurrence. Flagella are known to play a role in initial biofilm formation, but are often considered to be down-regulated after this stage. In this study, we demonstrated that *H. pylori* have co-opted these structures for non-motility roles, including biofilm and thus support recent studies in *Escherichia coli* that suggested an architectural role of flagella in biofilms.
Title: Controlling Chronic Pseudomonas aeruginosa Infections by Strategically Interfering with the Sensory Function of SagS

Abstract: The hybrid sensor SagS plays a central role in the formation of Pseudomonas aeruginosa biofilms, by enabling the switch from the planktonic to the biofilm mode of growth, and by facilitating the transition of biofilm cells to a highly tolerant state. In agreement with the transition to the sessile lifestyle having been associated with global shifts in virulence, SagS has been furthermore shown to contribute to the virulence of P. aeruginosa. However, the contribution of SagS to P. aeruginosa pathogenicity is not well understood. The goal of this study was to determine whether SagS contributes to virulence in a manner dependent on biofilm formation, biofilm drug tolerance, or both. To address this question, we made use of the finding that SagS contributes to biofilm formation and drug tolerance via two distinct sets of amino acid residues located within the HmsP sensory domain of SagS. Specifically, residue D105 was found to only contribute to drug tolerance, while residue L154 affected the ability of P. aeruginosa to transition to the surface-associated lifestyle. ΔsagS mutant strains harboring the empty CTX integration vector or expressing intact sagS or sagS variants (harboring alanine substitutions in residues D105 and L154 affecting drug tolerance and biofilm formation, respectively), under the control of the native promoter, were used. Virulence phenotypes were assessed using romaine lettuce, murine lung and agarose bead-based chronic bronchopulmonary mouse infection models. The transcript abundance of select genes was determined using qRT-PCR. Virulence was first assessed using a murine model of chronic pneumonia. Three days post infection, the bacterial burden in mice infected with ΔsagS was significantly reduced relative to the complemented strain. Attenuation of sagS was further supported by ΔsagS only being 17% as competitive as the parental strain PAO1 using competitive index infection assays. Moreover, alanine substitution of residue D105 had no effect on the bacterial burden while expression of sagS-L154A coincided with attenuation. Given that SagS also contributes to the drug tolerance of biofilms in a manner dependent on residue D105, we repeated the virulence study in combination with antibiotic treatment. Tobramycin treatment greatly affected the bacterial burden in mice infected with ΔsagS and ΔsagS expressing sagS-D105 than strains expressing sagS or sagS-L154A. Interestingly, no difference in the disease progression or bacterial burden was noted post 24h of infection of romaine lettuce or the murine lung. Moreover, with the exception of biofilm marker genes, only minor differences in the expression of virulence genes were detected, regardless of the mode of growth tested. Our findings suggest that interference with the biofilm or tolerance regulatory circuits of the sensory domain of SagS affects P. aeruginosa pathogenicity in chronic, but not acute, infections.
Abstract: Exploring Stochastic and Temporal Expression of Virulence Genes during Staphylococcus aureus Biofilm Development

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Background: The intricate process of biofilm formation in S. aureus involves distinct stages during which a complex mixture of matrix molecules including polysaccharides, proteins, and extracellular DNA (eDNA) is produced and modified throughout the developmental cycle. Early in biofilm development the accumulated cells have been shown to detach from the surface of the microfluidic chamber in an event termed “exodus”. This event is mediated by the production of a secreted staphylococcal nuclease, which degrades the eDNA within the matrix, causing the release of cells, and allowing for the formation of metabolically heterogenous microcolonies in subsequent stages of development. Recent studies have shown that the nuclease gene (nuc) is regulated by the SaePQRS multi-component regulatory system, which is also responsible for regulating the transcription of several additional virulence factors. Methods: To characterize virulence gene expression patterns within the dynamic biofilm context, fluorescent gene reporter plasmids were introduced into strains of interest. These strains were then grown in a Bioflux 1000 microfluidic system which supplies the bacterial populations with a constant flow of fresh media while simultaneously performing time-lapse microscopy to image these developing biofilms in five-minute intervals. To assess the impact of various regulatory systems influence on gene expression within this context, mutant strains in which these systems have been rendered non-functional were transduced with the same plasmids and compared to their wildtype counterparts. Results: Our results indicate the nuc gene is expressed in only a subpopulation of biofilm-associated cells via an unknown mechanism involving the SaePQRS system. Interestingly, these studies also demonstrate that SaePQRS coordinates temporal and stochastic expression of other virulence genes during biofilm development. Additional experiments have focused on exploring the effects of SaePQRS as well as the Agr quorum sensing system on expression of virulence factors during biofilm development and have shown that the two regulatory systems have distinct effects on spatial and temporal expression. Conclusions: The results of this study demonstrate that stochastic gene expression in S. aureus is not limited to the nuc gene. Rather, multiple virulence genes were shown to be expressed in this specialized subpopulation of cells. Importantly, these studies also demonstrated that virulence genes regulated by the SaePQRS regulatory system are stochastically expressed, while Agr-regulated genes are generally expressed more homogenously within growing micro-colonies. Overall our results indicate that the Sae system may function as a bistable switch similar to regulators controlling competence gene expression in Bacillus subtilis and persister cell formation in Escherichia coli.
Session: Poster Session 4
Date & Time: Thursday, October 11, 2018, 4:00 pm - 6:00 pm
Poster Board #: 088
Topic: Regulation of Biofilm Development
Abstract Title: Unraveling O104:H4 Escherichia coli Chemical Language during Biofilm Formation
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Abstract:
Enteroaggregative Escherichia coli O104:H4 Stx+ has caused a major diarrheagenic hemolytic-uremic syndrome (HUS) outbreak in many countries in Europe in 2011. Specifically, the C227-11 O104:H4 strain produces Shiga toxin (Stx2a subtype), also found in enterohemorrhagic E. coli O157:H7 serotype, a deadly combination for humans. The two-component system QseBC is closely related to expression of virulence genes in Enterobacteriaceae. The chemical signaling here occurs via Autoinducer-3/Epinephrine/Norepinephrine employing the QseC sensor kinase, as previously described by our group (Curtis et al., 2014). The VisP periplasmic protein is a pleiotropic function player during chemical signal and virulence of different pathogenic E. coli strains (Moreira et al., 2013). A novel compound described by our group, the LED209, blocks the QseBC signaling pathway by directly affecting virulence in Gram-negative pathogens (Rasko., 2008). Therefore, this study aimed to investigate the chemical signaling virulence in EAEC O104:H4 C227-11 (Stx2a+) and EAEC O104:H4 BA3826 (Stx-) in vivo. We have orally challenged C57BL/6 mice with distinct O104:H4 Stx+ and Stx-. This treatment of O104:H4 employing the LED209 compound have shown distinct colonization during a total period of 14 days pi and the expression levels of qseC and visP genes during in vivo challenge. The qseC overexpression was observed in throughout in vivo experiment, both in the presence and absence of Shiga toxin. It was 3.8-fold higher by C227-11 at day 5 and 9.5-fold for the BA3826 strain. Noteworthy, the qseC expression reduction at day 14 in the absence of Shiga toxin, thus the BA3826 strain, with approximately 1-fold decrease. The visP expression was highly upregulated in the presence of LED209, upon its potential to block the sensor kinase QseC, C227-11 strain between day 3-14 pi has peaked at 30-fold increase. Moreover, the BA3826 strain has shown higher levels of visP expression, specifically at day 5 pi, but much lower in comparison to the Shiga toxin O104:H4 strain. To date, the VisP important role in enteric bacterial stress response seems accentuated upon LED209 in vivotreatment. The QseC sensor kinase may trigger alternative mechanisms of intestinal colonization, bacterial survival and overexpression of important chemical signaling players. Herein, we highlighted the importance of QseC sensor kinase in the O104:H4 (Shiga +) outbreak E. coli strain during colonization in vivo and in the presence of a promising anti-virulence drug to intestinal pathogens.
Abstract:

**Background:** Biofilm formation occurs in natural and man-made environment, on wide variety of surfaces including medical devices, water system piping, aquatic environments, etc. Its formation in food processing environments and surfaces can lead to pre or post production contamination of foods which is of public health significance. It is of great concern because of the contamination of foods which may lead to food poisoning and resistance to antimicrobials, disinfectants and other cleaning agents. *Escherichia coli* (E. coli) O157:H7 is an organism which is capable of forming biofilm and causes food borne disease outbreaks. It is one of the major milk borne pathogens and is capable of causing diseases. Ginger is well known for its medicinal and preservative values but its use for inhibition and dispersion of *E. coli* O157:H7 biofilm has not been sufficiently explored. This study was therefore designed to evaluate the biofilm inhibition and dispersion activities of *Zingiber officinale* crude extract and fractions on biofilms of *E. coli* O157:H7 isolated from dispensed powdered milk in Ibadan, South-West Nigeria. **Methods:** Ginger root samples were obtained, identified (UIH-22558), extracted, fractionated using four solvents (hexane, chloroform, ethyl acetate and ethanol) and concentrated. Three *E. coli* O157:H7 isolates were obtained from dispensed powdered milk and a reference strain (*E. coli* ATCC 35218) was obtained from microbiology laboratory. Biofilm quantification was done using the crystal violet binding assay. The effect of the plant extracts on biofilm formation and biofilm dispersal were tested at 37°C for 48 h and 24h respectively. The experiment was done in triplicates. **Results:** All the isolates developed biofilm (0.08±0.07). Biofilm development was suppressed with ethanolic (0.01±0.00) and crude extracts (0.05±0.01), while highest biofilm dispersion was exhibited with the ethanolic (0.04±0.05) and crude extract fractions (0.05±0.06). The chloroform fractions had little or no effect on biofilm formation and dispersal at 50mg/ml conc. **Conclusion:** Ginger (*Zingiber officinale*) showed potential use for dispersion of already formed biofilm by *E. coli* O157:H7 strains, hence, can be used in food processing plants, surfaces and industries to combat biofilm forming organisms, disperse their biofilms and enhance food safety.
Identifying Genes Important for Bacterial Biofilm Formation in *Pseudomonas aeruginosa*

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Surface-bound bacteria in matrix-coated aggregates, called biofilms, are up to 1,000 times more resistant to antibiotics than planktonic cells (Rasmussen 2006). Rather than attempting to create new antibiotic treatments, disrupting biofilms would allow for a more effective way to use already existing ones. *Pseudomonas aeruginosa* is a model organism in biofilm research due to its proficiency forming biofilms, its mechanisms for antibiotic resistance, and its medical impact in chronic infections. The formation and survival of a biofilm is paramount on having a strong initial cell-surface attachment by way of exo-polysaccharides (Palmer 2017). We hypothesize that interrupting bacterial attachment to surfaces will reduce disease burden. RNA sequence results of cells attached within the first hour showed that 437 genes were regulated upon surface contact in the initial attachment phase of biofilm formation. Transposon mutants of these genes were screened for attachment to polyvinyl chloride. Of the 437 mutants of surface-regulated genes, 36% exhibit enhanced attachment and 15% have an attachment defect compared to the parental strain, PAO1. Through prioritization by magnitude of attachment alteration, 8 mutants were selected for clean deletions of highest priority genes. Future work will test the biofilm formation of clean deletion mutants. Identification of essential genes for biofilm formation will facilitate the development of treatments specifically preventing biofilms.
Biofilm formed by Streptococcus mutans is recognized to play important roles in the development of dental caries. Streptococcus salivarius BD3900, isolated from the oral cavity of a healthy volunteer, displayed the potential to be an oral probiotics. The bacterial strain BD3900 displays strong hydrophobicity (95.8%) as well as high surface charges (≥88.6%), which endure its high capability to adhere in the oral cavity. Furthermore, the bacterial strain BD3900 exhibited high auto-aggregation (15%-20%) and co-aggregation (13.1%-64.3%) with S. mutans. When co-cultured with S. mutans in vitro, the biofilm formation by S. mutans could be reduced by 67.1%. To explore the substances and/or mechanism involved in the inhibition of S. mutans biofilm formation by S. salivarius BD3900, the cell free supernatant of the cultivated M17 broth supplemented with 0.5% (w/v) sucrose by BD3900 was precipitated by ammonium sulphate at 60% saturation. The active components responsible for the inhibition of S. mutans biofilm in the precipitated substance were with molecular mass exceeding 10kD. When heated at 70°C for 5min, the precipitated substances lost almost all of the inhibitory activity on the formation of S. mutans biofilm, whilst the existence of Fe³⁺, Zn²⁺, Ca²⁺ could enhance the antibiofilm activity. When the precipitated substances were co-incubated with S. mutans at the presence of sucrose, the biofilm formed by the latter was significantly decreased, and also the amount of insoluble exopolysaccharides synthesized by S. mutans. Therefore, it is postulated that some enzymes secreted by S. salivarius BD3900 might interfere the synthesis of insoluble exopolysaccharides by S. mutans and thus inhibited the biofilm formation by the latter. Keywords: Streptococcus salivarius BD3900; Streptococcus mutans; biofilm; dental plaque.
**Title:** A Global Analysis of Gene Expression within *Staphylococcus aureus* Biofilms Across Multiple Clonal Lineages

**Abstract:**

*Staphylococcus aureus* is a highly virulent, opportunistic pathogen and a leading cause of both nosocomial and community acquired infections. Although a variety of methods exist for clonally typing widely different isolates of this organism, the CDC derived USA lineages is perhaps most commonly used in North America. When comparing these clonal lineages, it is clear that infection characteristics vary greatly as demonstrated by distinct disease progression and altered expression profiles of virulence determinants. A key characteristic of *S. aureus* infections, and one that also varies phenotypically between clones, is that of biofilm formation, which aids in bacterial persistence through increased adherence and immune evasion. Using newly developed real-time tracking technology known as xCELLigence, we measured biofilm initiation, progression, and dispersal across the 5 major *S. aureus* CDC-types (USA100-USA500). In so doing we observed patterned adherence phases that markedly differed between the various strains. To gain insight into this, we used RNA-seq based transcriptomic profiling of these isolates, compared to planktonically growing counterparts (and to each other), at a variety of different time points. As a consequence, we uncovered distinct and specific global expression profiles for each clonal lineage that reveals complex and unique regulation across the different *S. aureus* lineages. This data is currently being used to compile a global and comprehensive map of regulatory circuits during *S. aureus* biofilm growth that has the potential to inform not only on the basic mechanism of this aggregate lifestyle but may also provide new insight into future therapeutic targets.
Bacteria spend most of their lives attached to surfaces as multicellular communities called biofilms. In a biofilm, bacteria can share nutrients, synchronize behaviors, and stay protected from the environment and xenobiotic stresses. *Caulobacter crescentus* is a freshwater bacterium that produces an adhesive holdfast to permanently attach to surfaces and form biofilms. *C. crescentus* asymmetrically divides and produces two different cell types, a motile swarmer cell and a sessile stalked cell, at each division. The swarmer cell harbors a polar flagellum and several pili at the same pole. It differentiates into a replication-competent stalked cell by shedding its flagellum, retracting its pili, and synthesizing a holdfast and a stalk at the same pole of the cell. Thus, holdfast synthesis is developmentally regulated by cell cycle cues. Furthermore, holdfast synthesis can also be stimulated by surface sensing, in which swarmer cells rapidly synthesize holdfasts in direct response to contact with a surface.

The goal of this study is to better understand the regulation of the transition between motile and sessile lifestyles and holdfast synthesis in *C. crescentus*. The intracellular signaling molecule c-di-GMP is involved in the switch between motile and sessile lifestyles in *C. crescentus*, signifying proper flagellum functions and holdfast synthesis. c-di-GMP plays a role in both the developmentally regulated and the surface contact stimulated pathways that lead to holdfast production. In this study, we are investigating the connection between c-di-GMP signaling, flagellum and holdfast production. We determined that, while more than 60% of a WT cells population harbor a holdfast in a mixed culture, this number drops to less than 10% in a ΔpleΔflgEΔmotB triple mutant that produces lower amounts of c-di-GMP (pleD deletion) and lacks its flagellum (flgE deletion) and flagellar motor (motB deletion). This number is similar to the number of holdfasts produced by a mutant unable to produce c-di-GMP (rcdG0). To gain a better understanding of the interplay between c-di-GMP, the holdfast, and flagellum, we constructed a mutant transposon library in the ΔpleΔflgEΔmotB triple mutant and screened for strains where the low holdfast production phenotype was suppressed. We screened more than 24,000 mutants and isolated eight strains with increased holdfast production and biofilm formation compared to the parent strain. Once we have characterized the mutations, we will determine how the identified genes are involved in the regulation of flagellum and holdfast production by c-di-GMP. This study will provide a better understanding of the pathway that starts with c-di-GMP production and leads to the switch between motile and sessile lifestyles. We expect to identify novel players that play a role in this complex regulatory cascade.
The bacterial pathogen *Staphylococcus aureus* is a leading cause of antibiotic-resistant nosocomial infections and is often found growing as a biofilm in catheters and chronic wounds. Cells in the biofilm are held together by an extracellular matrix that consists of recycled cytoplasmic proteins that moonlight as components of the matrix and extracellular DNA (eDNA), which forms an electrostatic net. Taking an unbiased genetic approach, we previously identified the gene (*gdpP*) for cyclic-di-AMP phosphodiesterase as being required for eDNA release. Using a riboswitch biosensor, we now find that cyclic-di-AMP levels drop prior to biofilm formation and that this drop is prevented in a *gdpP* mutant. Conversely, artificially depleting cyclic-di-AMP levels by overexpressing *gdpP* is sufficient to promote eDNA release under growth conditions that normally do not support biofilm formation. Together, these results support a model in which a drop in c-di-AMP is both necessary and sufficient for eDNA release during biofilm formation *S. aureus*. We have also identified genes upstream and downstream of cyclic-di-AMP in the pathway governing eDNA release.
Cargo Transport Shapes the Spatial Organization of a Microbial Community

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The human microbiome is an assemblage of diverse bacteria that interact with one another to form communities. Bacteria in a given community are arranged in a three-dimensional matrix with many degrees of freedom. Snapshots of the community display well-defined structures, but the steps required for the construction of these structures are not understood. Here, we show that this construction is carried out, in part, by gliding bacteria that carry non-motile bacteria on their surface as public cargo. Gliding is defined as the motion of cells over a solid or semi-solid surface without the necessity of growth or the aid of pili or flagella. Genomic analysis suggests that gliding bacteria are present in human microbial communities. We focus on Capnocytophaga gingivalis which is present in abundance in the human oral microbiome. By adhering to a mobile cell-surface adhesin, SprB, cells of non-motile bacterial species attach to the surface of C. gingivalis and are propelled as cargo. The cargo cell moves along the length of a C. gingivalis cell, looping from one pole to the other. Multi-color fluorescent spectral imaging of cells of different live but non-motile bacterial species reveals their long-range transport in a swarming polymicrobial community. Some non-motile bacterial species use this public transport more efficiently than others. Tracking of fluorescently-labeled single cells and of gas bubbles carried by fluid flow shows that the swarms are layered, with cells in the upper layers moving more rapidly than those in the lower layers. Thus, cells also glide on top of one another, arranging themselves in three-dimensional space.
Title: Multidrug Resistant Microbial Consortia Isolated from Intl. Space Station (ISS) Potable Water: Multi-Dpecies Interactions, Biofilm Formation, Metabolic Characteristics, and Hemolytic Subpopulations

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Abstract:
Understanding the impact of spaceflight on microbial ecosystem dynamics and biofilm formation is critical for spacecraft design, operations, and astronaut health. The ISS is an isolated closed environment that has been inhabited by more than 200 international crew members and their accompanying microbes since 2000. This habitat relies on sustainable life support systems (including those that provide potable water) to support crew health and can be compromised by microbial contamination and overgrowth. The latter can lead to materials degradation, biofouling, operational systems failure, and infections, all of which have been reported on the ISS. The purpose of this study is to understand 1) how microbial communities and their population dynamics have developed and been sustained in the ISS potable water system under low nutrient conditions, and 2) how these complex microbial ecosystems may adapt and evolve in the spaceflight environment. The ultimate goal of this project is to provide a better understanding of the effects of spaceflight on microbial ecosystems in ISS regenerative potable water, their effective control, and implications for spaceflight systems integrity and crew health risks, and to advance our understanding of the microbiome of built environments on Earth. We received 16 ISS potable water microbial isolates from the NASA Johnson Space Center and profiled the growth kinetics, drug sensitivities, and halotolerance of each of the individual strains. Over 20 antimicrobial compounds were tested at a range of concentrations and assayed for microbial colony forming units (CFUs). Based on our assays, the tested strains are classified as MDR (multidrug resistant) or XDR (extensive drug resistant). No single antibiotic tested in this study completely killed the ISS water microbiota, and a combination of antibiotic cocktails are necessary for complete elimination. Interestingly, our results revealed an association between antibiotic tolerance and changes in distinct phenotypes. We also investigated key metabolic characteristics, biofilm formation, changes in colony morphology of polymicrobial communities, and multispecies interactions showing synergistic or competitive behaviors. Unexpectedly, we discovered subpopulations in selected Burkholderia species that differed in colony morphology, biofilm formation, sensitivity to antibiotics, and hemolytic potential. This is the first thorough evaluation of microbial characteristics of microbial isolates from the ISS potable water system. This study is funded by a postdoctoral fellowship award from the Alfred P. Sloan Foundation.
**Introduction:** Caries and periodontitis are recognized as polymicrobial diseases caused by an altered abundance of species present in the oral microbiome. This shift is driven by synergistic and antagonistic interactions among the oral species. Streptococci are known as early colonizers of the dental surface. Pyruvate oxidase (SpxB) positive species form H$_2$O$_2$ and thus inhibit the growth of competing bacteria like *Streptococcus mutans* (Sm), a major contributor of initial enamel caries. Objectives: The deletion of *spxB* and external addition of H$_2$O$_2$ uncovered the presence of a so far unknown phenotype of *Streptococcus sanguinis* SK36 (SK36) and *Streptococcus gordonii* DL1 (DL1). Both, SK36 and DL1 were able to detoxify H$_2$O$_2$ from the surrounding and thus protect nearby bacteria from its inhibitory effect. Therefore, we aim to investigate cause and ecological meaning of such contradictory phenotype. 

**Methods:** We investigated *ccpA, spxB* and *ccpA/spxB* double mutants as well as further deletion mutants of SK36 and DL1. H$_2$O$_2$ release was quantified by a chromogenic assay. Growth inhibition assays as well as H$_2$O$_2$-inhibition and H$_2$O$_2$-protection assays (disc diffusion) were performed by co-culturing SK36 or DL1 derivatives with Sm or other streptococcal / staphylococcal isolates. Further, the H$_2$O$_2$-detoxifying capacity of crude extract was determined by the survival rate of an epithelial cell line (OKF4) challenged by H$_2$O$_2$. Directed and transposon based random mutants were constructed and tested to identify candidate genes involved in regulation, release or synthesis of the protective agent. 

**Results:** Due to the lack of H$_2$O$_2$ production *spxB* mutants of SK36 and DL1 fail to inhibit other bacteria. Remarkably, the external addition of H$_2$O$_2$ revealed a protective effect against H$_2$O$_2$ mediated growth inhibition. Investigation of different growth conditions uncovered this protective substance to be oxygen, glucose and biofilm dependent regulated. Analyses of crude extract revealed the protective substance to be water soluble and heat resistant. The protective effect against H$_2$O$_2$ mediated killing was verified for several bacterial species as well as for eukaryotic cells. Directed and random mutagenesis identified 29 genes involved in regulation / release or synthesis of the protective agent (*ccpA, dps, nox* etc). 

**Conclusion:** Our findings uncovered the presence of a so far unknown phenotype of SK36 and DL1. The release of components protecting against H$_2$O$_2$-mediated killing provide a new aspect in bacterial mutualism in oral mixed-species-biofilms. Furthermore, considering the importance of H$_2$O$_2$ as an antimicrobial as well as regulatory molecule of the innate immune response, the identified effect might also play a central role in the host-microbe interaction or even have a direct modulatory influence in the immune defense in the oral cavity.
Session: Poster Session 4
Date & Time: Thursday, October 11, 2018, 4:00 pm - 6:00 pm
Poster Board #: 098
Topic: Social and Asocial Interactions in Biofilms
Abstract Title: Characterization of Multi-species Biofilms of Significant Bacterial Pathogens
Author Block: V. Fuchsova, O. Chlumsky, K. Zdeňkova, K. Demnerova; University of Chemistry and Technology Prague, Prague 6, CZECH REPUBLIC.

Abstract: During their existence bacteria evolve in order to adapt to the surrounding environment. Permanent microbial modification causes an emergence of formation called biofilm. Its composition and structure provide advantages manifested by changes in the phenotype and physiology of microbial population, high stability and resistance. In our environment the prevalent forms of life are multi-species biofilms, which are more complex, virulent, stable and resistant in comparison to mono-species biofilms. These multi-species communities can represent very beneficial part of human microflora. Bacterial biofilms can contribute for example to processes of bioremediation in the industry. However, some pathogenic microorganisms, which cause human diseases (such as Escherichia coli, Staphylococcus aureus and Listeria monocytogenes), also have the ability to form biofilm. The stability of biofilm, the inability of the detection by classical cultivation techniques and the resistance to the antimicrobials have become a major problem in many branches. The main objective of this study was to characterize multi-species biofilms of the E. coli, S. aureus and L. monocytogenes. Biofilms of these bacteria were detected in food and medical industry. In this work the classical microbiologic methods, the molecular-biologic method and the confocal laser scanning microscopy (CLSM), were used to study multi-species biofilms of above mentioned bacterial pathogens. We evaluated the ability of bacterial strains to form a mono- and dual-species biofilms and afterwards we measured biomass of 24 hours biofilms. Biofilms were cultivated under the static condition of 37 °C in tryptone soya broth (TSB). The range of absorbance values was detected by using the crystal violet staining. Most of tested samples had the ability to form a biofilm. In comparison the biomass amount of mono- and dual-species biofilms four species combinations increased the biofilm biomass. The maximum level of biomass was observed in food isolate S. aureus strain 1241 in combination with food isolate L. monocytogenes strain 149. For the monitoring of spatial distribution of multi-species biofilms by CLSM the transformations of plasmids containing genes sequences coding iLOV and mCherry fluorescence proteins were also successfully executed. According to confocal microscopy results, it seems that used bacteria form dual-species biofilms with microcolonies arranged in layers. This study has been supported by GACR project 17-15936S and from specific university research (MSMT No 21-SVV/2018).
**Abstract:**

**BACKGROUND:** The human oral cavity hosts a diverse microbiome where synergistic and antagonistic interactions between bacterial species governs the balance between health and disease. Health-associated commensal streptococci can antagonize the growth of pathogens through the production of H$_2$O$_2$ and other factors that promote their persistence. In the case of dental caries, they suppress the growth of acidogenic and acid tolerant organisms that create localized acidic microenvironments while enmeshed in sucrose-derived exopolysaccharide (EPS) matrices. We report here the development of tools that will aid in the study of how interbacterial interactions between competing *Streptococcus* species affects the spatial organization and behaviors of species within microenvironments when these microbes are co-cultured in biofilm communities.

**METHODS:** To visualize individual species within biofilms, a series of fluorescent reporters were developed using constitutive promoters (Pveg, P23) and common fluorescent genes (*gfp, dsRed*) on the *Streptococcus* shuttle vector pDL278. Following transformation of these plasmids into health-associated *Streptococcus gordonii* DL1, *Streptococcus sanguinis* SK150 and *Streptococcus A12*, and into the caries-associated pathogen *Streptococcus mutans* UA159, competitive fitness of the health-associated streptococci against *S. mutans* was monitored and compared in 24 h planktonic and biofilms cultures with or without sucrose. 24-h biofilms were imaged by confocal laser scanning microscopy and 3D-images of the resulting biofilms reconstructed.

**RESULTS:** Health-associated streptococci were present in comparable numbers to *S. mutans* in planktonic growth conditions, but became less competitive when grown in biofilms. The growth inhibition was further exacerbated by the presence of sucrose. Microscopy showed that the architecture and organization of bacterial species was markedly different with glucose versus sucrose, with *S. mutans* forming microcolonies that excluded the health-associated strains in the presence of sucrose with enhanced microcolonies only in the presence of other streptococcal species rather than when *S. mutans* strains were cultured together. Addition of Alexa Fluor 647-labeled dextran conjugate showed that EPS matrix formed in the presence of sucrose associated preferably with *S. mutans* but not with commensal strains.

**CONCLUSIONS:** Our data confirms and builds upon earlier work showing that *S. mutans*-produced EPS-matrix modulates the architecture and spatial heterogeneities of oral biofilm communities through the formation of *S. mutans*-rich microcolonies. Establishment of these tools will allow for closer examination of how health-associated streptococci that could be used in probiotic applications interact with disease-associated oral microbiome members, such as *S. mutans*. 
**Abstract:**

**Background:** Co-infection studies show increased virulence, and multispecies infections are correlated with worse patient outcomes. A common hypothesis is that pathogen synergy results from competition and communication between multiple species at an infection site. Outer Membrane Vesicles (OMVs) are mediators of competition and communication among many species and are increasingly associated with multiple modes of virulence. OMV biogenesis in *P. aeruginosa* is induced by secretion and intercalation into the outer membrane of a self-produced small molecule: the Pseudomonas Quinolone Signal (PQS). The biophysical underpinnings of this model are easily generalizable, raising the possibility that the mechanism is widespread. We developed a tightly controlled experimental system to test the interaction of bacterially-produced factors with target cells. Results from these studies inform on the generality of small molecule-induced OMV biogenesis as well as highlight the role of cross-species induction of OMV biogenesis in pathogen synergy.

**Methods:** A panel of recipient bacteria were exposed to pre-solubilized PQS at low concentration for short duration and resultant OMV production was analyzed by both nanoparticle tracking and lipid analyses. *P. aeruginosa* was exposed to supernatants from donor species and OMV production was likewise analyzed. Pairs of species were cultured together and OMV production was compared to monocultures of either species. Co-culture OMVs were harvested and tested for THP1 cytotoxicity by trypan blue staining and compared to that of monoculture OMVs.

**Results:** We show that multiple species respond to PQS by increasing OMV formation, that PQS accumulates in all induced vesicles, and that other bacteria secrete OMV-promoting factors. Analysis of induced vesicles indicates that recipient-mediated mechanisms exist to control vesicle size and that relatedness to the producer organism can dictate susceptibility to OMV-inducing compounds. This work provides evidence that small molecule induced OMV biogenesis is a widely conserved process and that cross-talk between systems may influence OMV production in neighboring bacteria. We extended this work to show that several pairs of bacterial species produce more OMVs per cell when co-cultured than when alone, and that co-culture OMVs are more cytotoxic against THP1 monocytes than monoculture OMVs.

**Conclusions:** Our work demonstrates reciprocal cross-species induction of OMV biogenesis mediated by secreted factors. The physiological consequences of this phenomenon were explored, and we showed that such interactions between co-cultured species resulted in more OMV production and greater cytotoxicity toward host cells. Thus, “peer pressure” to produce greater numbers of more potent OMVs in bacterial communities likely contributes to the pathogen synergy observed in many clinical infections.
Session: Poster Session 4
Date & Time: Thursday, October 11, 2018, 4:00 pm - 6:00 pm
Poster Board #: 101
Topic: Synthesis and Assembly and Function of the Biofilm Matrix
Abstract Title: Regulation of Bacterial Amyloid Biogenesis by Multitasking Molecular Chaperone DnaK
Author Block: S. Sugimoto1, K. Arita-Morioka2, A. Terao1, K. Yamanaka3, T. Ogura3, Y. Tanaka2, Y. Kinjo1, Y. Mizunoe1;
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Background: Curli are functional amyloid fibers that assemble on the extracellular surface of enteric bacteria such as Escherichia coli during biofilm development and colonization. Unlike pathogenic amyloids that result from protein misfolding, curli are generated via a secretory nucleation-precipitation mechanism, also called the type VIII secretion system. In E. coli, seven proteins encoded by two operons, csgBAC and csgDEFG, regulate curli expression, export, and assembly. The major curli subunit CsgA has three domains: an N-terminal signal peptide, the recognition sequence for the curli-specific translocation channel CsgG, and five imperfect amyloidogenic repeats. Following translocation across the cytoplasmic membrane through the Sec translocon, the signal peptide is cleaved, yielding a ~13-kDa mature CsgA subunit that is exported across the outer membrane by CsgG. Exported soluble CsgA is nucleated by CsgB, the minor curli subunit, to induce amyloid assembly. The csgDEFG operon encodes CsgD, a master transcriptional regulator of curli biogenesis that acts as a positive regulator of the csgBAC operon, the periplasmic accessory protein CsgE, the extracellular accessory protein CsgF, and CsgG. Recently, we found that molecular chaperone DnaK is involved in curli biogenesis and that Myricetin (IC50 = 46.2 μM), a DnaK-inhibiting polyphenol, and Epigallocatechin gallate (IC50 = 5.9 μM), a derivative of Myricetin, prevent curli-dependent biofilm formation by E. coli K-12 (Arita-Morioka et al. Antimicrob. Agents Chemother. 2015, Sci. Rep. 2018). However, molecular mechanisms how DnaK regulates curli biogenesis was unclear. Methods and Results: Transcription and immunoblotting analyses using E. coli BW25113 and its isogenic ΔdnaK strains showed that DnaK positively regulates the expression of CsgA and CsgB via quantity and quality control of CsgD and RpoS, a stationary phase-specific alternative sigma factor promoting expression of the csgDEFG operon. Cytological analysis with fluorescent protein reporters revealed that DnaK also keeps CsgA and CsgB in a translocation-competent state by binding to their signal peptides prone to aggregation, facilitating their translocation in the cell. Molecular interaction analyses by surface plasmon resonance and peptide-scanning indicated that DnaK strongly binds to the N-terminal 8-amino acid peptides of CsgA and CsgB. In vitro translation/folding analysis demonstrated that DnaK suppresses the aggregation of CsgA, but other chaperones GroEL and SecB, both of which facilitate the protein export in E. coli, do not. Conclusion: Our findings indicate that DnaK controls the homeostasis of curli biogenesis at multiple stages to organize the biofilm matrix (Sugimoto et al. Commun. Biol. 2018). Collectively, these results may lead to the development of drugs to treat chronic biofilm-associated infections.
Alcohols are natural major end products of some microbial fermentations. The acetone/butanol/ethanol (ABE) or the isopropanol/butanol/ethanol (IBE) fermentation using solventogenic Clostridium strains have a long industrial history but an efficient fermentation system is still required. Recent publications highlight that immobilized cells are the preferred option for obligate anaerobe such as the solventogenic strains belonging to this genus. This operating mode can indeed increase cell density and productivity by maintaining high cell concentrations in the bioreactor (Dolejš et al., 2013; Jiang et al., 2009). However, relatively few data are available regarding the physiological state of the Clostridium cells present in such biofilms. Our study aimed at validating a flow cytometric (FC) approach to analyze cellular viability in biofilms of the isopropanol producing strain Clostridium beijerinckii DSM6423. Two couples of fluorescent probes, Propidium Iodide [PI] associated with carboxyfluorescein diacetate [cFDA] or Bis-(1,3-dibutylbarbituric acid)trimethine oxonol [Dibac], were validated to screen suspended cells of Clostridium beijerinckii in batch or continuous fermentations. As FC analysis require suspended cells, a pretreatment of the biofilm was required. Physical pretreatments were tried but a comparative analysis of cell/event counting, respectively by plating and cytometry, revealed a strong difference. This may be due to the presence of EPS in the cell suspension which can interfere with the FC analysis. Moreover, a significant quantity of extracellular DNA (eDNA) was detected in the biofilm matrix, disturbing the propidium iodide cell staining. Another approach, using sequential enzymatic treatments, were allowed us to limit the background noise linked to the presence of EPS but more particularly eDNA. Interestingly, FC analysis revealed that only a fraction of the cells was viable in Clostridium beijerinckii DSM6423 biofilm, suggesting that only a part of the biofilm is permanently active. This new protocol provides a rapid and efficient method to measure cell viability and monitor immobilized cell fermentation. Dolejš I., M. Rebroš, M. Rosenberg. 2013 Immobilisation of Clostridium spp. for production of solvents and organic acids. Chem. Pap., 68: 1-14. L. Jiang, J. Wang, S. Liang, X. Wang, P. Cen, Z. Xu. 2009 Butyric acid fermentation in a fibrous bed bioreactor with immobilized Clostridium tyrobutyricum from cane molasses. Bioresour. Technol., 100: 3403-3409.
Abstract:

**Background:** *Klebsiella pneumoniae* (*Kp*) from the *Enterobacteriaceae* family, is a frequent cause of hospital-acquired infections (HAI). It has emerged as an “urgent threat” to public health due to antibiotic resistance. Biofilm formation by *Kp* is a major concern in clinical settings as *Klebsiella* forms biofilms on urinary catheters, ventilators etc. *Kp* is a major etiological agent found in HAI, especially in CAUTIs (70%). To prevent and eradicate biofilms, the dynamics of biofilm formation on different materials needs to be understood. The aim of the present study was to evaluate the biofilm-formation capacity of clinical isolates of *Klebsiella* associated with UTI. Biofilm components were quantified to evaluate the contribution of each component in formation of biofilm matrix. The EPS (extracellular polymeric substances) plays an important role in the resistance and strength of the biofilm. **Methods:** Pathogenic isolates of *Klebsiella* spp. (*n*=30) from patients suffering from UTI were collected from Gujarat, India and identified using 16s *rRNA* gene sequencing. Biofilm was studied by crystal violet assay on 96 well-plate and isolates were categorized into 3 categories: weak, moderate and strong biofilm formers using statistical analysis. Biofilm formation capacity of isolates was studied on latex and silicone catheters. Biofilms formed on catheters were further characterized by quantification of each component of biofilms including EPS, extracellular DNA, living and dead cells. Scanning electron microscopy and fluorescent microscopy were also performed to study the difference in the composition of biofilm matrix on different materials and to compare the matrix of weak and strong biofilm. Extraction and quantification of EPS produced by each isolate were done. **Results:** 30 isolates were identified as *Klebsiella* spp. by 16s *rRNA* gene sequencing. Among these, 41%, 44% and 15% isolates were found to be strong, moderate and weak biofilm formers respectively. Biofilm formation was found to be more on latex-based catheters than silicone-based catheters. 50% decrease was seen in biofilms formed on silicone. More number of live cells were found to be present in weak biofilms, while in case of stronger biofilms, a large number of dead cells were present. In case of EPS quantification, a wide range of EPS production was observed among the isolates (0 µg EPS/mg protein to 210.79 µg EPS/mg protein) and the isolate with highest EPS (M-27) has strong biofilm and lowest EPS (M-20) has very weak biofilm. **Conclusion:** A high level of variation in EPS production and biofilm formation among the *Klebsiella* isolates were observed. Interestingly, dead cells embedded in the biofilm matrix seem to play a major role in the formation of strong biofilms by *Klebsiella*. Further work to study the role of cell death in strong biofilm formation is warranted.
Abstract:

**Background:** In their natural environments, biofilms regularly encounter the presence of viral pathogens, termed bacteriophages which use bacteria as their host for self-replication. Our aim was to understand the interaction of phages and biofilms, as well as their coexistence. **Methods:** In order to study phage-biofilm interactions we developed a method to visualize phage spread inside living *E. coli* biofilms. By insertion of sfGFP into the T7 phage genome, the conversion of susceptible to infected cells can be visualized spatiotemporally at the single-cell level. Using a combination of bacterial genetics, molecular biology, and fluorescent reporters, we were able to understand key elements of phage-biofilm interactions. **Results:** We discovered that biofilm susceptibility to phage infection is dependent on the stage of biofilm development and the production of biofilm matrix. *E. coli* biofilms that were grown for 48 h or less were rapidly eradicated due to phage infection. By contrast, biofilms grown for 60 h and more experienced no biomass reduction in the presence of phages. The removal of curli fibers, a major component of the *E. coli* matrix, generated biofilms that were susceptible to phage infection, regardless of the age of the biofilms. Visualization of curli fibers during biofilm growth further demonstrated a dynamic change in matrix composition. The development of phage tolerance in biofilms coincided with the production of curli fibers. We further discovered that curli fibers protect bacterial communities via two mechanisms: (1) Curli prevent phages from diffusion inside biofilms, and (2) curli fibers protect individual cells from phage infection. **Conclusion:** Our results demonstrate that a single component of the biofilm matrix can provide individual as well as collective protection against viral infection.
**Abstract:**

**Background:** Staphylococcus aureus are among the most frequent causes of infections on indwelling medical devices, which characteristically involve biofilms. Phenol soluble modulins (PSMs) have been identified as one of the key regulators for the S. aureus biofilm lifecycle. As monomers, PSMs promote biofilm dissociation, but they can self-assemble to form extracellular amyloid fibrils that fortify the biofilm matrix to better resist disassembly by enzymes and mechanical stress. Despite the recent advances made in understanding the roles of PSMs and their aggregation in biofilm control, many crucial aspects are still not clear. Here, we discuss recent progress on the molecular mechanisms of PSMs’ self-assembly to amyloid fibers in S. aureus biofilm, using molecular dynamics (MD) simulations. **Method:** PSMα1 and PSMα3 were studied respectively using classical all-atom MD simulation in explicit solvent. We studied dimer configurations to understand the early stages of oligomer formation, then single, double, and triple sheets protofibril structures consisting of 10, 20 and 30 dimers to analyze the structural changes emerging from the fibril growth. The stability of different dimers’ configurations was investigated using well-tempered Metadynamics. Protofibrils were extensively equilibrated and then simulated to capture slow structural rearrangements. MD simulations were performed using the NAMD, employing the CHARMM general force field; well-tempered metadynamics were performed using PLUMED plugin. **Results:** PSMα1 and PSMα3 dimerization is driven by the intermolecular interactions between hydrophobic groups, but the monomers retain a large degree of flexibility in the terminal amino acids. For the transition to single sheet protofibril structure, hydrogen bonds (and potentially salt bridges) among dimers play an important role. While general characteristics of the protofibril agree with crystallographic data, it was found to form a helical structure in water with 0.15M NaCl. This arrangement is barely detectable in the double sheet protofibril and it disappears in the triple sheet structure. As the number of sheets in the protofibril increase, individual peptides become less organized in the central part of the fibril. These two factors suggest that the number of sheets composing the fibril is generally limited to 3-4, which is equivalent to a width of 12-16 nm, in agreement with the average diameter of PSM fiber observed in experiments. **Conclusion:** We have investigated critical aspect of the molecular mechanism of amyloid fiber formation via self-assembly of common bacterial peptides, PSMα1 and PSMα3 from S. aureus biofilm. Dependencies on the structure and size of the peptides were discovered suggesting potential avenues to target PSMs for the development of anti-staphylococcal biofilm agents.
**Abstract:**

*Salmonella enterica* serotype Typhimurium is a Gram negative, motile bacterium that causes infection via the fecal oral route. These bacteria, as well as other *Enterobacteriaceae*, produce amyloid proteins called curli as a major proteinaceous component of their biofilm. Amyloids, like curli, can be produced by bacteria as well as humans. Accumulation of amyloids in humans can be found in complex human diseases such as Alzheimer’s Disease. It is thought that the amyloids seen in humans are the result of misfolded aggregates, however bacterial amyloids have been found to be functional. Amyloid curli complexes with extracellular DNA within the biofilm. When purified from the matrix, these complexes are recognized by a variety of receptors. Previous studies have shown that the amyloid portion of these curli complexes is recognized by the Toll Like Receptor 2 (TLR2)/TLR1/CD14 complex as well as the NLRP3 inflammasome, leading to the production of proinflammatory cytokines such as interleukin-1 beta (IL-1β). Additionally, the eDNA within the complex has been shown to induce type I Interferon (IFN) production and autoimmunity through activation of TLR9.

Recently, we determined variable amounts of DNA within the curli prepared in the lab. We have been testing two protocols and were able to manipulate the amount of DNA in the curli complexes. We found treatment with DNase and RNase in cultures is able to disrupt aggregation of the curli/eDNA complexes making for a weaker biofilm. Using fluorescent microscopy we are able to see the aggregates in our high DNA preps are larger and more robust than those of the low DNA preps. We hypothesize that this difference in aggregate size may affect the downstream recognition and signaling of the complexes. To test this, we will treat bone marrow derived macrophages that are wild-type, TLR2-/-, TLR9-mutant, or TLR2-/-/9mutant with low and high curli to see if there are differences in response between the different complexes. We expect that the low DNA curli will not activate TLR9 mutant macrophages as well as the high DNA curli due to lesser DNA incorporation. Additionally, we will look at internalization by these macrophages to see if the aggregate size difference affects TLR2 signaling and entry into the cell. Downstream effects on type I IFN response and in vivo autoantibody production in mice will be investigated to understand how DNA incorporation to produce mature biofilm structure affects the functionality of the complexes.
Pseudomonas aeruginosa biofilms have been linked to a number of chronic infections. The biofilm matrix is composed of exopolysaccharides (EPS), eDNA and proteins, where research historically concentrated in studying EPS. Non-mucoid P. aeruginosa produce two EPS, Pel and Psl, and recent studies determined their chemical structure. Conversely, the role of proteins in P. aeruginosa remains largely understudied. Considering that EPS is the main component of the matrix, proteins that interact with sugars are potentially important for aggregate formation. Currently, CdrA is the only structural protein shown to bind Psl and contribute to the aggregate stability. Additionally, P. aeruginosa is known to produce two small soluble sugar-binding proteins, designated as LecA and LecB. Interestingly, mutation of LecA and LecB has been linked to abnormal biofilm formation suggesting that they play a biofilm-specific role. However, the underlying mechanism for this biofilm phenotype is unclear. Previous sugar-binding studies of LecA and LecB revealed that they have binding affinities to monosaccharides present in Pel and Psl, respectively. The aim of our study was to determine if LecB could bind to Psl and if these interactions would contribute to biofilm development. First, we assessed the binding of LecB to saccharides via ELISA-based assays and co-immunoprecipitation, and we demonstrated that LecB binds to Psl. These results led us to assess if LecB could interact with Psl in situ using biofilms grown under flow. When fully grown aggregates were stained with LecB-FITC, we observed by microscopy that LecB and Psl were localized in the same areas, suggesting that LecB interacts with Psl in situ. Next, we investigated the redundancy of LecB and the adhesin CdrA. When both LecB and CdrA were mutated, Psl was not retained in the matrix and a thin layer of cells was formed. When the double mutant was partially complemented with LecB, aggregate architecture appeared similar to wild-type PAO1. Furthermore, when the double mutant was partially complemented with CdrA, aggregate formation was impaired with the appearance of a thick layer of cells that contained Psl. These results support the hypothesis that LecB interacts with Psl and this interaction is crucial for Psl placement in the matrix and consequently biofilm development.
**Session:** Poster Session 4  
**Date & Time:** Thursday, October 11, 2018, 4:00 pm - 6:00 pm  
**Poster Board #:** 108  
**Topic:** Antibiofilm Strategies  
**Abstract Title:** Eradicating Bacterial Biofilms by Wireless Electrostimulation  
**Author Block:** H. Wang, D. Ren; Syracuse University, Syracuse, NY.

**Background:** Bacteria such as *Pseudomonas aeruginosa* and *Staphylococcus aureus* can form biofilms on implanted medical implants and cause serious infections that are incurable by conventional antibiotics. Low-level electric currents have been shown to have potent antimicrobial activities and synergy with antibiotics in bacterial killing. However, electrochemical treatment *in vivo* requires skin-piercing to introduce the wires for delivering electric current from an outside power source. This can cause discomfort and complications including secondary infections, which hinder the clinical applications of this technology. To address this challenge, we developed a wireless system for more effective control of biofilms. **Methods:** Both *in vitro* and *ex vivo* studies were conducted to evaluate the killing activities against *P. aeruginosa* and *S. aureus* biofilm cells using electric currents generated by wireless electromagnetic induction. We also designed and constructed a prototype device, and validated its anti-biofilm performance. The CRL5803 lung epithelial cells were treated under the same condition to evaluate the cytotoxicity to human cells. **Results:** After treatment with 6 µA/cm² of wirelessly delivered DC *in vitro* for 6 h, the viability of biofilm cells was reduced by 87% and 91% for *P. aeruginosa* and *S. aureus*, respectively. Clear synergy between low-level DC and antibiotics in biofilm killing was also observed in concurrent treatment. For example, the viability of *P. aeruginosa* biofilm cells was reduced by 2.5 logs after treatment with 6 µA/cm² wirelessly delivered DC and 4.5 µg/mL tobramycin. In comparison, treatment with DC or tobramycin alone only showed 0.9 and 0.6 log of killing, respectively. The viability of *S. aureus* biofilm was reduced by 2.2 logs after concurrent treatment with 6 µA/cm² DC and 10 µg/mL chlorhexidine, while treatment with DC or chlorhexidine alone only showed 1.1 log and 0.6 log of killing, respectively. When treated with 50 µA/cm² of wirelessly delivered DC in an *ex vivo* model with pork skin, the viability of biofilm cells on the surface of prototype device was reduced by approximately 4 logs and 2.6 logs for *P. aeruginosa* and *S. aureus*, respectively. Existence of pork skin tissue (1 - 5 mm thick) did not show significant effects on wireless DC delivery; and these conditions were found safe to tested human cells. **Conclusions:** With the capability to kill bacteria without using a directly connected power source, this platform technology has potential applications in developing new systems and devices to effectively control biofilm infections such as those associated with pacemakers, cochlear implants, gastric stimulators and other implanted medical devices.
Abstract: Clinical Efficacy of a Biofilm Disrupting Surgical Lavage in Reducing Bacterial Plate Counts in Total Knee Arthroplasty Revision Surgery in Known Cases of Prosthetic Joint Infection

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Background: Total joint arthroplasty (TJA) is one of the most common surgical procedures performed in the US and worldwide. In 2010, an estimated 332,000 total hip arthroplasty (THA) procedures and over 600,000 total knee arthroplasty (TKA) were performed in the United States. Periprosthetic Joint Infection (PJI) affects 1-2% of total joint arthroplasty patients and remains one of the most serious complications of TJA. While rare, the condition incurs substantial morbidity and costs, and a significant portion of sufferers will bear consequences for the remainder of their lives. By 2020, the predicted cost for infected revision procedures may reach as high as $1.6 billion. Bactisure Wound Lavage solution has been developed to improve removal of planktonic and biofilm bacteria from the articular joint space. Bactisure is a mixture of surfactants, chelating agents and salts to disrupt and dissolve contaminants, providing a cleanser to a wound (articular space) and potentially reducing the risk of future infection.

Methods: A 40-patient clinical trial was designed for patients undergoing the first stage of a 2-stage revision for PJI. To date, 36 patients have completed the surgical portion of the trial. Infection was confirmed following TKA with the PJI occurring within 30 days of the primary procedure or 1 year with primary implant retention. Bactisure lavage was performed at the end of the procedure, prior to closure, and then followed by saline lavage. 3 mL fluid cultures were obtained from deep in the surgical wound both before the Bactisure lavage and after the saline lavage. White blood cell (WBC) counts were obtained from the fluid as a surrogate marker for bioburden. Plate counting was performed to determine the bacterial colonization of the surgical site and DNA analysis was used to identify the bacteria. A 90-day follow-up period for infection is ongoing to determine the effectiveness in preventing recurrence of PJI.

Results: There was a substantial (>99%) reduction in the WBC counts, (2.3 log reduction, 4.2 +/- 0.5 log to 1.9 +/- 0.7 log, p-value <0.01). There was a dramatic decrease in the number of colony forming units in the surgical site. For those patients with a positive culture in the pre-lavage test, 74% had no countable bacteria in the post-lavage test (p-value < 0.01, 19/36 culture positive in the pre-lavage population, 14 of those 19 were culture negative after lavage). For patients with positive cultures, the average plate count decreased by 99.6% (2.4 log), with the log CFU of bacteria being reduced from 2.9 +/- 1.6 log to 0.5 +/- 1.2 log (p-value < 0.01). For all patients, including those with 0 count pre-lavage, the average plate count decreased by 96% (1.6 log), with the log CFU of bacteria being reduced from 1.9 +/- 1.9 log to 0.3 +/- 1.0 log (p-value < 0.01).

Conclusions: The use of Bactisure prior to closure significantly reduces the bioburden and bacterial count within the surgical site.
Pseudomonas aeruginosa is an opportunistic pathogen that affects a large proportion of cystic fibrosis (CF) patients. CF patients have dehydrated mucus within the airways that leads to the inability of the mucociliary escalator to expel inhaled microbes. Once inhaled, P. aeruginosa can persist in the lungs of the CF patients for the remainder of their lives. During this chronic infection, a phenomenon called mucoid conversion can occur in which P. aeruginosa can mutate and inactivate their mucA gene. As a consequence, transcription of the alg operon is highly expressed, leading to the copious secretion of the alginate exopolysaccharide, which is associated with decreased lung function and increased CF patient morbidity and mortality. Alginate biosynthesis by P. aeruginosa is post-translationally regulated by bis(3′-5′)-cyclic dimeric guanosine monophosphate (c-di-GMP), which binds to the receptor protein Alg44 to activate alginate production. The identification of small molecules that disrupt the binding of c-di-GMP to Alg44 could inhibit the ability of P. aeruginosa to produce alginate. In this work, a class of thiol-benzo-triazolo-quinazolinone compounds that inhibited Alg44 binding to c-di-GMP in vitro was identified after screening chemical libraries consisting of ~50 000 chemical compounds. Thiol-benzo-triazolo-quinazolinones were shown to specifically inhibit Alg44-c-di-GMP interactions by forming a disulfide bond with the cysteine residue in the PilZ domain of Alg44. The more potent thiol-benzo-triazolo-quinazolinone had the ability to reduce P. aeruginosa alginate secretion by up to 30%. These compounds serve as leads in the development of novel inhibitors of alginate production by P. aeruginosa after mucoid conversion.