Virulence Production in *Pseudomonas aeruginosa* is Influenced by Different *In vitro* Culture Conditions and by Treatment with Quorum Sensing Inhibitor Sodium Salicylate

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**Background:** In the developed world, approximately 1.5% of the total population will develop a chronic wound during their lifetime [1]. Infection contributes to the delayed healing, and although patients are often treated with antimicrobial agents they may not eradicate persistent biofilm infections [2]. Along with the increasing problem of antimicrobial resistance, there is an urgent need for alternative treatment strategies. Quorum sensing (QS) inhibition represents a potential treatment strategy for infected wounds. Interfering with the bacterial signalling system that regulates pathogenicity may disarm bacteria, by decreasing their toxicity, and increasing bacterial clearance by the host defence. The aims of the present study were: (i) to characterize the production of QS signals and virulence factors in clinical *P. aeruginosa* strains from wound infections, both under serum conditions and using standardized culture media; and (ii) to evaluate the effect of sodium salicylate on QS signal and virulence production.

**Methods:** The production of biofilm, alkaline protease, elastase, pyocyanin, iron siderophores and rhamnolipids were evaluated in 14 clinical wound isolates. The influence of culture conditions, namely solid agar versus liquid broth and the presence of serum, on virulence were also examined. The effect of sodium salicylate on QS inhibition and production of these virulence factors was investigated using a serum-containing wound-like media.

**Results:** The virulence production of the tested clinical isolates differed markedly, both between strains and culture conditions. The presence of serum in solid agar decreases pyocyanin and alkaline protease production, but increases iron siderophore production, after 24 h of culture. However, pyocyanin production seems to increase at later time-points under simulated wound conditions in comparison to general nutrient broth. Preliminary data showed that in the presence of serum, treatment with sub-inhibitory concentrations of sodium salicylate reduced biofilm formation on polystyrene as well as pyocyanin and iron siderophore production.

**Conclusion:** These results show that the virulence of clinical isolates is affected by serum, and stress the importance of using relevant culture conditions when evaluating potential treatments for clinical applications. The *in vitro* results of sodium salicylate treatment on bacterial virulence are promising and calls for further work, preferably using *in vivo* infection models, where both the effect on virulence inhibition and the immune system can be investigated.

Abstract:

Background: The failure of medical devices, such as implants and endoprosthesis, is often linked with the easiness to which bacteria can get in contact and adhere to its surface leading to biofilm formation. This, allied with the lack of efficacy of antibiotics, emphasizes the need to develop new locally acting anti-biofilm materials to prevent severe infections. To increase the susceptibility of biofilms to antibacterial treatments, we hypothesized that by combining an enzyme (E), able to hydrolyse exopolysaccharides present in the extracellular matrix of early stage biofilms, with other novel and potent bacteria-killing agents such as antimicrobial peptides (AMPs), one could enhance the anti-adhesion capacities of a material. Based on this, our goal was to develop surface modified polyhydroxyalkanoates (PHAs) combined with isocyanate-terminated star-shaped poly(ethylene oxide-stat-propylene oxide), (NCO-sP(EO-stat-PO), to covalently link bioactive and anti-adhesive agents, and ultimately produce scaffolds via electrospinning for medical applications. Methods and Results: On a first step, we screened the antibacterial activity of a novel synthetic AMP against Staphylococcus epidermidis which not only showed excellent bactericidal properties, but also the capacity to prevent more than 50% of biofilm formation when using a peptide concentration of 13.6 µg/ml. When assessing the enzyme effects against pre-formed biofilms we observed a decrease in the biofilm biomass by 71.3% with an enzyme concentration of only 3 µg/ml. Simultaneously, the cytotoxic effects of both AMP and E were evaluated against L929 murine fibroblasts over a period of 24h showing an IC50 of 117.4 µg/ml and 142.5 µg/ml, respectively. Afterwards, three different meshes were produced via electrospinning, namely, untreated PHAs-NCO-sP(EO-stat-PO), E loaded PHAs-NCO-sP(EO-stat-PO)-E and double loaded PHAs-NCO-sP(EO-stat-PO)-E+AMP. A viability higher than 70% was observed when assessing the cytotoxicity of the meshes against fibroblasts. The anti-adhesion performance of the meshes was tested in vitro by CFU counting after sonication for the detachment of the biofilm grown on the neat and loaded PHAs. The modified meshes showed a significant reduction (p<0.05) in the numbers of adhered bacteria in comparison with the untreated mesh. Fluorescent microscopy and SEM imaging confirmed an enhanced action on the double loaded mesh, PHAs-NCO-sP(EO-stat-PO)-E+AMP, by showing an increased reduction in the number and size of bacterial aggregates when compared with the E loaded mesh. Conclusions: These findings suggest that our strategy could be a step change in the current material technology; that the synergistic action of antimicrobial and antibiofilm compounds may be of benefit when targeting the development of anti-adhesive medical devices.
Dental caries, commonly stated as tooth decay, is one of the most prevalent chronic diseases of people worldwide. When dental caries occurs at the margin of an existing dental filling is called secondary caries (SC). SC may occur after initial caries has been removed and replaced by a filling. The replacement of the failed fillings accounts for approx. 60% of all fillings performed in the USA each year at an annual cost of over $5 billion. Tooth colored polymer-based direct placement materials, i.e., resin composites and dental adhesives have become the chosen material for restorative treatment of primary and secondary caries. Biofilm acids contribute to secondary caries, which is the main reason for dental restoration failures. The current resin based dental materials replace the affected tooth structure giving back the form and appearance of the tooth but do not promote biofilm modulation on the surrounding tooth. In fact, this peculiar group of dental materials has been associated with high susceptibility to RC. Therefore, preventing secondary caries involves control of microorganisms and/or the acid produced. An emerging alternative is the use of nanotechnology-based strategies for dental caries management. By releasing high levels of ions related to the small size and high surface area of the nanoparticles, nanoscale strategies such as nanoparticles of metals (i.e., silver-NAg) can impart antibacterial effect and thus helped to address negative effects of composites. Another important approach to face this problem involves the synthesis of a new dental composite containing quaternary ammonium dimethylaminohexadecyl methacrylate (DMAHDM). Here we review recent immediate and after 6 months results using silver nanoparticles or DMAHDM against broad-spectrum saliva based inoculum microcosm biofilm model and discuss how their application can improve and facilitate anticaries activity via dental material. A human saliva microcosm model was used to grow biofilms on composites and dental adhesives. Colony-forming unit (CFU) counts, live/dead assay, metabolic activity, and lactic acid production of biofilms were determined. Biofilm response expressed by metabolic activity and lactic acid production of the biofilm were nearly 85% of that on the control and RC (p<0.05). The combined monomers in the new composite decreased the CFU counts of biofilms (12x10^8) by 3 folds, compared to those on the control (p<0.05). Dental plaque biofilm colony-forming units (CFU) formed on the surface of dental adhesive containing different DMAHDM/ NAg have shown total microorganisms, total streptococci, and mutans streptococci expressive reductions. After 6 months, DMAHDM/ NAg reduced the biofilm CFU of the commercial control by two folds. The new formulations represents a promising approach of modulating the biofilm behavior of oral cariogenic biofilm, acid production, and secondary caries.
Background: Polymicrobial biofilms containing *Gardnerella vaginalis* as the primary bacterial constituent and *Atopobium vaginae* as a secondary pathogen are characteristic of bacterial vaginosis (BV) and may contribute to the resistance associated with standard BV treatments. TOL-463 is a novel boric acid (BA) based vaginal anti-infective enhanced with EDTA in clinical development for the treatment of BV and vulvovaginal candidiasis (VVC), with robust antibacterial/antifungal activity against single species biofilms demonstrated. The objective of this study was to develop a mixed species porcine *ex vivo* biofilm model closely mimicking human BV and evaluate the impact of TOL-463 against these established biofilms. Methods: A porcine model was chosen given the morphologic similarities between human and porcine vaginal mucosa. Vaginal tissue explants were sterilized and maintained in air liquid interphase prior to infection. Clinical isolates of *G. vaginalis* (JCP8151B) and *A. vaginae* (ATCC BAA-55) were used; the *G. vaginalis* strain was isolated from a BV patient and previously shown to induce a BV phenotype in mice. Inoculums and infection order were optimized to establish mixed species biofilms at relative compositions consistent with clinical BV. Explants were then divided into 3 treatment groups: TOL-463, BA (50 mg/ml) and vehicle (VEH), and treated every 24 h for 3 doses. *G. vaginalis* sialidase gene expression, a clinically relevant virulence factor, was also evaluated in the same model. Tissue samples were utilized for CFU assessment, RNA isolation, 16s rDNA quantification and measurement of sialidase expression. Results: Optimized mixed species biofilms were established with initial *G. vaginalis* colonization of vaginal epithelium for 12 hours and subsequent infection with *A. vaginae*, supporting growth of both pathogens *ex vivo* for up to 5 days. TOL-463 treatment resulted in a 6.62 log reduction in mixed biofilm load by the second dose (p<0.0001 vs. VEH) and was below the limits of CFU quantification by the third dose, compared with 4.62 logs CFU/mL of residual biofilm growth with BA (p<0.001 vs. TOL-463). TOL-463 also reduced expression of *G. vaginalis* sialidase virulence factor below detectable levels whereas BA induced expression relative to TOL-463 and VEH. Conclusions: TOL-463 effectively inhibits mixed biofilms of *G. vaginalis* and *A. vaginae* in the porcine *ex vivo* vaginitis model superior to BA, with complete eradication by the third dose. TOL-463 also displayed greater antivirulence capacity compared to BA, corresponding to its enhanced antibiofilm efficacy. These results conform with prior TOL-463 biofilm studies against BV and VVC pathogens and support the clinical efficacy of TOL-463 demonstrated in Phase II testing in women with vaginitis. Porcine vaginal model provided a biologically relevant substrate for studying polymicrobial biofilm infections *ex vivo*. 
Abstract: Glycoside Hydrolase DisH from *Desulfovibrio vulgaris* Degrades the *N*-Acetylgalactosamine Component of Diverse Biofilms

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The global costs of corrosion are more than $2.5 trillion every year (3.4% of the global gross domestic product), and a large part of corrosion (30%) is microbiologically influenced corrosion (MIC), which affects oil production, drinking water systems, and pipelines. MIC is commonly caused by sulfate-reducing bacteria (SRB) biofilms, and *Desulfovibrio vulgaris* is the model organism. Biofilms of sulfate-reducing bacteria (SRB) produce H\(_2\)S, which contributes to corrosion. The biofilm matrix of *D. vulgaris* consists primarily of proteins but we have identified it also contains the polysaccharides mannose, fucose, and *N*-acetylgalactosamine (GalNAc) (*Environ. Microbiol Reports*. 9:779-787, 2017). However, little is known about how to control its biofilm formation. Although bacterial cells in biofilms are cemented together, they often dissolve their own biofilm to allow the cells to disperse. Using *Desulfovibrio vulgaris* as a model SRB, we sought polysaccharide-degrading enzymes that disperse its biofilm. Using a whole-genome approach, we identified eight enzymes as putative extracellular glycoside hydrolases including DisH (DVU2239, dispersal hexosaminidase), an enzyme that we demonstrated here (DisH was previously unstudied), by utilizing various *p*-nitrooligosaccharide substrates, to be an *N*-acetyl-β-*D*-hexosaminidase. For *N*-acetyl-β-\(D\)-galactosamine (GalNAc), \(V_{\text{max}}\) was 3.6 µmol of *p*-nitrophenyl/min/(mg protein) and \(K_m\) was 0.8 mM; the specific activity for *N*-acetyl β-*D*-glucosamine was 7.8 µmol of *p*-nitrophenyl/min/(mg protein). Since GalNAc is one of the three exopolysaccharide matrix components of *D. vulgaris*, purified DisH was found to disperse 63 ± 2% biofilm as well as inhibit biofilm formation up to 47 ± 4%. The temperature and pH optima are 60°C and pH 6, respectively; DisH is also inhibited by copper and is secreted. In addition, since polymers of GalNAc and GlcNAc are found in the matrix of diverse bacteria, DisH dispersed biofilms of *Pseudomonas aeruginosa*, *Escherichia coli*, and *Bacillus subtilis*. Therefore, DisH has the potential to inhibit and disperse a wide-range of biofilms. DisH is the first glycoside hydrolase that has both GalNAc-ase/GlcNAc-ase degradation activities for diverse biofilms. Hence, we have found a key factor controlling *D. vulgaris* biofilm formation (*Environ Microbiol*. on-line, 2018).
Background: Auranofin is an FDA approved compound originally developed for treatment of severe rheumatoid arthritis. The drug has potent anti-inflammatory properties, and is now known to possess antimicrobial activity as well. The compound’s antibacterial properties appear to be due in part to an inhibition of thioredoxin reductase. Recent studies have suggested that auranofin is effective against mature bacterial biofilms, in contrast to standard antibiotics, which are largely ineffective against preformed communities. The combination of anti-biofilm activity and anti-inflammatory activity indicates that auranofin is a promising candidate for combating infections within wounds, as both the presence of resistant bacteria and aberrant immune responses are primary concerns during treatment.

Methods: The activity of auranofin was measured in vitro against Staphylococcus aureus biofilms using plate based assays. Biofilms were grown within polystyrene plates or on MBEC polystyrene pegs. Auranofin treatment was added at various time points during biofilm development. In vivo analysis of auranofin biofilm inhibition was performed using a S. aureus infected rat segmental defect model with an extended debridement time point to ensure persistent infection. Auranofin was added to infected defect sites via insertion of PUR scaffolds or direct application of powdered treatment. At 6 weeks post infection, animals were humanely euthanized and harvested samples were used to analyze bacterial burden and bone healing.

Results: In vitro data suggests that auranofin is effective at both preventing the initial formation of S. aureus biofilms and disrupting mature biofilm communities. The presence of auranofin was associated with a significant reduction of bacteria in multiple models. Analysis of MBEC biofilms treated with auranofin at concentrations of 12-50ug/mL showed a complete or near complete absence of recoverable CFUs following treatment at t=0hours and t=24hours. Crystal Violet based assays also showed a reduction in bacterial biomass when added to nascent biofilms. However, in vivo experiments showed that auranofin did not significantly decrease bacterial burden, either alone or in combination therapy. This was consistent between all conditions tested, including various inoculum sizes and delivery methods.

Conclusions: Auranofin is highly effective at inhibiting and dispersing S. aureus biofilms in vitro even at relatively low concentrations. However, auranofin does not appear to be an effective therapeutic against in vivo biofilms. Furthermore, significant side effects were seen in animals treated with auranofin or a combination of auranofin/vancomycin, suggesting that any potential effects that could be observed by increasing the dosage would likely be poorly tolerated. Therefore, auranofin does not appear to be a viable candidate for treatment of in vivo biofilms.
Abstract:
Chronic wound infections are an increasing problem within the United States. The treatment and care of these wounds costs millions of dollars annually and infections are associated with a high morbidity rate. Treatment frequently involves repeated debridement, multiple antibiotics, and in some cases limb amputation. Antibiotics often lack the ability to penetrate the biofilms and fight off the infection because the exopolysaccharide (EPS) secreted by bacteria within a biofilm can increase tolerance by up to one thousand percent. Thus, the ability to degrade this protective shield and allow antibiotics to penetrate the biofilm could potentially be a new therapy for chronic wounds. Glycoside hydrolases are enzymes that break down EPS and potentiate the efficacy of antibiotics and antimicrobials. Amylase and cellulase are two promising glycoside hydrolases that hydrolyze $\alpha$-1,4 and $\beta$-1,4 glycosidic linkages respectively, which are common within biofilm EPS. While we have demonstrated in vitro and in vivo efficacy of these enzymes to break down the biofilms in chronic wounds, their safety must be determined in order for this potential therapy to reach clinical trials. In this study we performed long term in vivo experiments, utilizing a murine chronic wound model, to characterize and measure the host response after administration of these enzymes. We assessed antibody production to amylase and cellulase, the inflammatory reaction after administration, and the effect of these glycoside hydrolases on wound healing. Thus far, no adverse effects on wound healing have been observed. As the safety of the administration of these glycoside hydrolases on the host continue, there has been no variation in the host response exhibited between the vehicle control (1xPBS) and treatment. We also extended our preclinical studies to test the efficacy of glycoside hydrolases on clinical strains of bacteria. In order to accomplish this, debridement samples were taken from the chronic wounds of patients and were treated with glycoside hydrolases. These samples were then analyzed for the bacterial community present. Our results clearly demonstrate that as the complexity of the wound population increased, the efficacy of glycoside hydrolase treatment decreased. This indicates that for complex, polymicrobial biofilm infections it will likely be necessary to target more than two of the conserved bonds in EPS, or more than just the EPS component of the biofilm matrix.
Bacterial Outer Membrane Vesicles Disrupt Competitor Bacterial Biofilms

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Abstract:

Previous studies indicate that bacteria within biofilms are 10- to 1000-fold more resistant to antibiotics, making it challenging but imperative to develop new therapeutics that can disperse biofilms and eradicate persistent microbes. Gram-negative bacteria shed outer membrane vesicles (OMVs) containing outer membrane and periplasmic components including small molecules, proteins, and lipids present on the surface or within the vesicle lumen. OMVs have been shown to play important roles in bacterial interactions with their environment, including interspecies competition. For example, OMVs isolated from *Pseudomonas aeruginosa* were shown to exhibit antimicrobial activity against competitor bacteria due to the presence of peptidoglycan hydrolases. Here, we examined the antimicrobial activity of OMVs derived from *Burkholderia thailandensis* (Bt), a soil saprophyte that is closely related to *P. aeruginosa* but non-pathogenic in animals and humans. We first examined the peptidoglycan hydrolase activity using peptidoglycan degradation assays. Interestingly, Bt OMVs significantly degraded purified peptidoglycan from *Staphylococcus aureus* but not from *Streptococcus mutans*. Nonetheless, Bt OMVs displayed significant antimicrobial activity against *S. mutans*. When *S. aureus* and *S. mutans* were treated with heat-inactivated OMVs, we still found potent antimicrobial activity against both live bacteria whereas the hydrolytic ability of OMVs against *S. aureus* peptidoglycan was abolished. These findings indicate the existence of both heat-stable and heat-labile (i.e. hydrolases) components in Bt OMVs that contribute to the killing of *S. aureus* and *S. mutans*. Additionally, we found that Bt OMVs significantly reduced *S. mutans* planktonic and biofilm cell viability in a time- and dose-dependent manner. Confocal microscopy imaging combined with COMSTAT 2.0 software analyses demonstrated significant reductions in total biofilm biomass, biofilm integrity, and bacterial cell viability in *S. mutans* biofilms after OMV treatment compared to control. Scanning electron microscopy also revealed altered biofilm and cellular morphology in OMV-treated *S. mutans* biofilms. Moreover, we observed a synergistic effect when combining OMV and gentamicin compared to either alone as quantified by minimal biofilm inhibition concentration assay. Based on these data, Bt-derived OMVs represent an untapped resource of novel therapeutics effective against biofilm-forming bacteria. Studies are ongoing to identify and characterize Bt OMV antimicrobial components.
Abstract:

**Background:** In recent years, antimicrobial peptides have gained great interest as options to counteract biofilms, thanks to their broad-spectrum of activity and low frequency of resistance. Peptides-coated medical devices can represent a new frontier to avoid biofilm formation, being stable and non-toxic. In this study, the anti-biofilm and anti-adhesive properties of a newly synthesized, 14-AA cyclic peptide, with sequence derived from a human GPCR protein, modified with polyethylene glycol and palmitic acid (grZ14s-nvCyc-3PEG-Pal) have been assessed in static and dynamic conditions against *Escherichia coli* isolates from catheter-associated urinary tract infections (CAUTIs).

**Methods:** Nine *E. coli* clinical isolates were analysed by crystal violet (CV) assay for biofilm formation. Minimum inhibitory concentration and CV assays on 96-well plates were performed for GrZ14s-nvCyc-3PEG-Pal solution (from 10 µM to 0.01 µM) to determine antimicrobial and anti-biofilm activities, respectively. Peptide adsorbed (10µM) onto 96-well plate and 1 cm-long segment of 100% silicone urinary catheter were used to investigate anti-adhesive properties by CV assay and CFUs counts, respectively. Bioflux system was employed to assess the ability of peptide (10 µM)-adsorbed silicone channel to interfere with the adhesion of Ec5-FSL isolate.

**Results:** No antimicrobial and anti-biofilm killing activities of the peptide's solutions were observed. On the contrary, among all the *E.coli* isolates, the adhesion of the more strongly adherent Ec5-FSL and Ec7-FSL strains was significantly reduced (P<0.01) on peptide-adsorbed wells. The more stable strong biofilm producer Ec5-FSL was selected to evaluate biofilm reduction when peptide grZ14s-nvCyc-3PEG-Pal was adsorbed onto silicone catheter and a 75,6±7,82% CFU/cm² reduction was observed. In Bioflux experiments with microfluidic channels perfused under 0.5 dyn/cm² shear flow, early Ec5-FSL bacterial clusters can be observed in the untreated channel within 2h, while a single cluster arose on the peptide-adsorbed channel after 6h. After 8 h, mature biofilm appeared in the control, while just few macrocolonies occurred in peptide-adsorbed channel. More interesting, by using 1 dyn/cm² shear flow for 14 h, a rich biofilm was observed in the control while no bacteria were detectable inside of peptide-adsorbed channel. **Conclusions:** The grZ14s-nvCyc-3PEG-Pal peptide showed remarkable anti-fouling properties on strong biofilm-producers *E. coli* isolates. Interestingly, this peptide, in static conditions, was able to significantly reduce the adhesion of a strongly adherent *E.coli* strain on silicone urinary catheter and, more important, to effectively delay or definitely counteract bacterial adhesion under very low shear flows (0.5 and 1 dyn/cm², respectively), that are conditions resembling those occurring in catheterized patients.
**Session:** Poster Session 3  
**Date & Time:** Wednesday, October 10, 2018, 4:00 pm - 6:00 pm  
**Poster Board #:** 010  
**Topic:** Antibiofilm Strategies  
**Abstract:** Pentadecanal Inspired Molecules as New Anti-biofilm Agents against *Staphylococcus epidermidis*  
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**Introduction:** *Staphylococcus epidermidis* is one the prevalent bacteria involved in orthopaedic device-related infections. A key factor contributing to the virulence of *S. epidermidis* is its ability to form biofilm. In this field, the interest in the development of new approaches for the prevention and treatment of bacterial adhesion and biofilm formation has increased. In a recent paper we reported the purification and the identification of pentadecanal able to impair *S. epidermidis* biofilm formation. One of the aims of this work was the design of some pentadecanal derivatives to enrich the arsenal of weapons to fight biofilm development.  
**Materials and Methods:** Pentadecanal derivatives used in this work were: pentadecanoic acid; methyl ester and dimethyl acetal. Biofilm formation of *S. epidermidis* was assessed by crystal violet and CLSM. Biofilm cell viability was also determined by the LIVE/DEAD® Kit. Synergy test of these molecules in combination with vancomycin was also evaluated by using checkerboard assay. Their biocompatibility on eukaryotic cells was investigated by toxicity assay.  
**Results:** The synthesized derivatives resulted to have anti-biofilm activity against *S. epidermidis* strains with different capability. This effect was further investigated by a CLSM analysis. The pentadecanal and its synthetic derivatives use in combination with antibiotics on mature biofilm were also explored. Results obtained showed that pentadecanoic acid modulated the antimicrobial activity of the vancomycin. In particular, the MBIC (minimal biofilm inhibition concentration) and MBEC (minimal biofilm eradication concentration) values were reduced by 2-fold in combination with pentadecanoic acid. The biocompatibility of pentadecanal and of its derivatives was investigated on fibroblasts and keratinocytes. All molecules resulted to be fully biocompatible under 50µg/mL.  
**Discussion and Conclusions:** All the synthesized molecules resulted to be able to prevent, to a different extent, the biofilm formation of *S. epidermidis*. Their effect on the *S. epidermidis* biofilm structure was evident, as the biofilm formed in their presence is strongly reduced and characterized by a porous structure containing many channels and voids. These results suggested to explore the use of aldehyde and its derivatives in combination with antibiotics to treat biofilm infections. The pentadecanoic acid resulted to be able to modulate the antimicrobial activity of vancomycin it was able to weaken the *S. epidermidis* biofilm structure making it less compact and homogeneous, and thus allowing the penetration of the vancomycin into the structure of the biofilm. This work endorses the pentadecanal and its derivatives as key molecules for the development of innovative approaches for the prevention and, in case of the pentadecanoic acid, for the treatment of *S. epidermidis* biofilm-associated infections.
**Background:** Approximately 95% of bacteria in drinking water systems are present in biofilms. Biofilm formation in drinking water treatment systems (DWTS) can lead to biofouling of pipework and filters (i.e. ultrafiltration membranes [UF]), reducing operation time. Therefore, biofilm management on DWTS infrastructure would be beneficial to consumers and operators of drinking water systems. The aim of this study was to determine whether in-situ dosing of electrochemically activated solutions (ECAS) or HOCl managed the formation of environmental biofilms within a model system.

**Methods:** Biofilms were grown for 48 hours in a Centre for Disease Control (CDC) reactor with polyethersulphone (PES) coupons (to replicate UF membrane material). Feed water was taken directly from a modified artificial water source on the University of the West of England, Bristol campus (UK). ECAS were generated through the electrolysis of weak saline solutions (1% w/v), producing solutions with an oxidation reduction potential (ORP) of >1130 mV. Biofilm systems were dosed in-situ with either HOCl or ECAS to achieve a final free chlorine concentration of 50 mg L$^{-1}$, or left untreated as a control. Biofilm samples were taken at 24 and 48 hours, whereby PES coupons were disaggregated through vortexing and sonication in 10 mL sterile quarter strength Ringer’s solution. Heterotrophic plate counts (HPC) of the feed water and disaggregated biofilms were plated onto R2A agar in duplicate and incubated at either 22°C or 30°C, for 5 or 3 days, respectively.

**Results:** The number of heterotrophic bacteria recovered from control PES coupons after 48 hours was 5.3 ± 0.06 (30°C plates) and 4.7 ± 0.28 (22°C plates) log$_{10}$ CFU coupon$^{-1}$. The biofilm density after 48 hours when dosed in-situ with ECAS was 2.3 ± 2.45 (30°C plates) and 2.3 ± 2.46 (22°C plates) log$_{10}$ CFU coupon$^{-1}$. No biofilm formation was observed when HOCl was dosed at 50 mg L$^{-1}$ over the 48 hour period. A significantly higher number of bacteria were recovered from the control biofilm (no dosing) compared to ECAS or HOCl treated ($P < 0.0001$). No significant difference was observed in biofilm formation after 24 hours between ECAS and HOCl dosed systems. After 48 hours, a significantly higher number of bacteria were recovered from the ECAS compared to the HOCl dosed system ($p = 0.0079$ [22°C] and $p = 0.0215$ [30°C]).

**Conclusions:** This study has demonstrated that both ECAS and HOCl had an inhibitory effect upon environmental biofilm formation over a 48 hour period. Although no significant differences between ECAS and HOCl at 24 hours was observed, after 48 hours, biofilm formed in the presence of ECAS dosing was significantly greater than HOCl dosing. Further studies are required to upscale and apply this to water treatment infrastructure such as pipework and UF membranes.
**Abstract:**

**Background:** The persistence of *Mycobacterium tuberculosis* (*M. tb*) against antibiotics underlines the requirement of multidrug chemotherapy in effective control of tuberculosis (TB). Biofilms formed by Mycobacterium harbor several non-tuberculous bacteria as well that may lead to secondary manifestations of TB. The survival of pathogenic microbial species within biofilms is influenced by their ability to develop subpopulation of persister cells that exhibit upto 500 times higher minimum inhibitory concentration (MIC), confer drug tolerance, endow propensity to evade host immune system causing chronic infections and are a cause for recalcitrance of 60% of all hospital associated infections. The inherent ability of the pathogen to evolve under selective drug pressure outpaces the rate of development of new drugs. The eminent solution is to expedite formulation of new drugs against classical and non-classical targets in *M. tb* proteome or repurpose the role of currently available drugs. The current study is directed to re-purpose FDA approved drugs against Biofilm forming pathogens. **Methods:** We have characterized an essential protein in *Mycobacterium tuberculosis* (*M. tb*), belonging to the class of cyclophilins, that play key role in biofilm formation. A high throughput analysis of suitable inhibitors against cyclophilin protein were tested from a library of approved FDA drugs and nanoparticle. The candidate FDA drugs and nanoparticle belonged to class of anti-diabetics or immuno-suppressants and allosteric competitor of Iron siderophores respectively. *In silico* and molecular docking studies, substantiated with surface plasma resonance (SPR) analysis, were done to assess if the candidate FDA drugs interact with cyclophilin protein. FDA drugs and nanoparticle were also tested in combination with anti-TB drugs to assess their efficacy in reducing MIC of anti-TB drugs. **Results:** *In silico* studies showed that FDA drugs interacted with cyclophilins. SPR studies confirmed high degree of physical interaction of cyclophilins with FDA approved drugs and nanoparticles. Molecular docking of *M. tb*cyclophilin protein with FDA drugs showed high degree of similarity in active site of the homologous proteins in other biofilm forming microorganisms, suggesting to their putative role as novel target against biofilms. Our results show that FDA approved drugs or nanoparticles suppressed biofilm formation in H37Rv, cells. We also demonstrated that treatment with FDA approved drugs or GaNP in combination with first line anti-TB drugs could reduce minimum inhibitory concentration of existing anti-TB drugs. **Conclusions:** FDA approved drugs and nanoparticle exhibits synergism with anti-TB drugs against *M. tb* species complex. Our results provide proof of principle that targeting cyclophilins could be masterstroke required for tackling biofilms in a wide spectrum of microorganisms.
**Abstract:**

**Background:** *Streptococcus pyogenes* or Group A Streptococcus (GAS) is a strict human pathogen causes mild to severe infections. *S. pyogenes* equipped with a vast array of virulence factors that help bacteria to thrive within the human host by evading the immune response system. In addition to virulence factors, the ability to form biofilm protects *S. pyogenes* from antibiotics and disinfectants. The highly competent environment within the biofilm helps *S. pyogenes* to acquire antibiotic resistance. Anti-infective agents targeting biofilms and virulence factors decrease the fitness of bacteria survive within the host. Inhibition of biofilm exposes *S. pyogenes* to the adverse host environment. Thus anti-biofilm agents can decrease invasiveness of *S. pyogenes* without affecting cell viability. Plants are a rich source of different chemical skeletons with bioactivity. Many developing countries in tropics have abundant plant resources. These resources can be channelized to reduce health burdens associated with infectious diseases. The Western Ghats is a mountain range situated along the western coast of India. It is one of the eight "hottest hotspots" of biological diversity in the world. Plant resources of Western Ghats are a rich source of bioactive compounds. The scientific exploration of plant compounds may unleash a vast number of plant compounds with pharmacological properties. In the present study, we demonstrate the anti-biofilm potential of fukugiside, a biflavonoid isolated from *Garcinia travancorica*. **Methodology:** MBIC determination, Light microscopy, Confocal microscopy, Protease quantification, MATH assay, RT-PCR studies, *In vivo* efficiency and toxicity studies using *Caenorhabditis elegans*. **Result:** Anti-biofilm activity of fukugiside identified and established using a crystal violet assay, light microscopy, and confocal laser scanning microscopy. At 80µg/ml concentration it prevented the formation of *S. pyogenes* biofilm formation. It also inhibits biofilm formation of clinical strains belongs to different M types. Electron microscopy revealed a reduction of EPS by fukugiside. The results for the microbial adhesion to hydrocarbon assay, cysteine protease quantification and altered regulation of the virulence genes (*speB, srv, dltA, and ropB*) suggested that fukugiside probably inhibits biofilm formation by decreasing cell surface hydrophobicity and destabilizing the biofilm matrix. Fukugiside reduced intra-blood survival of *S.pyogenes*. *In vivo* analysis in *Caenorhabditis elegans* established the non-toxic nature of fukugiside and its antivirulence potential against *S. pyogenes*. **Conclusion:** Fukugiside exhibits a potent anti-biofilm and antivirulence activity against different M serotypes of *S. pyogenes*. Fukugiside protected *C.elegans* from *S.pyogenes* infection without any toxicity.
Production of sessile biofilm is an important life style shift for planktonic bacteria in response to environment, nutrition and population behavior. Development of biofilms is often the cause of persistent bacterial infections, with biofilm enmeshed bacteria exhibiting high level of tolerance to host targeted antimicrobial mechanisms and conventional antibiotic therapeutics. The ability to alter the biofilm state of bacteria to successfully treat biofilm infection requires novel compounds that can alter and/or reverse the biofilm state of bacteria as well as successfully inactivate pathogens in the biofilms itself. In our pursuit for identifying novel agents that can alter the biofilm state of bacteria, we optimized a 96-well plate based fluorescence assay to quantify established biofilms. Compared to other pre-established assays this plate reader based approach provides high signal to noise ratio for biofilm detection and quantification. Using a broad host range of opportunistic and biothreat pathogens (Acinetobacter baumannii, Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus, Proteus mirabilis, Burkholderia mallei, Burkholderia pseudomallei, etc.) we also demonstrate that this assay technique is broadly applicable. To further establish the utility of this novel approach for identifying and screening biofilm inhibitors, we assayed several thousand compound library against A. baumannii ATCC19606 and B. pseudomallei Bp82 (ΔpurM, a BSL-2 derivative of B. pseudomallei 1026b) biofilm models. Using this screen we identified one or more lead compounds that are not bactericidal or bacteriostatic at the tested concentrations, but has strong potential to inhibit biofilm production. Employing this easy to adapt assay technique should greatly benefit the biofilm community in screening and evaluating antibiofilm agents.
**Background:** *Pseudomonas aeruginosa* infections can be challenging to treat due to the propensity of this organism to form biofilms, as well as its often having multiple underlying resistance mechanisms, including β-lactamase production. Ceftazidime is a broad-spectrum β-lactam used to treat *P. aeruginosa* infections. Avibactam is a β-lactamase inhibitor that when combined with ceftazidime can restore planktonic activity of ceftazidime in the presence of ceftazidime resistance, reducing the minimum inhibitory concentration of *P. aeruginosa* compared to ceftazidime alone. We hypothesized that ceftazidime-avibactam would be more active in preventing *P. aeruginosa* biofilm formation than ceftazidime alone. To test our hypothesis, we determined the ceftazidime and ceftazidime-avibactam minimum biofilm inhibitory concentration (MBIC) values of 66 clinical *P. aeruginosa* isolates. **Methods:** *P. aeruginosa* biofilms were grown for 3 hours on Nunc TSP 96 peg plates (Nuclon Delta Surface, Denmark). Pegs were rinsed in sterile PBS and placed into a 96 well plate containing two-fold dilutions of ceftazidime ranging from 512 to 0.5 µg/ml with or without 4 µg/ml of avibactam. Plates were incubated for 18 hours at 37°C and the MBIC determined by assessing the lowest concentration associated with absence of turbidity. MBIC results were reported as the range, the MBIC required to inhibit 50% of the isolates (MBIC$_{50}$), and the MBIC required to inhibit 90% of the isolates (MBIC$_{90}$). **Results:** Both ceftazidime and ceftazidime-avibactam had MBIC ranges of 1 to >512 µg/ml. The MBIC$_{50}$ of ceftazidime and ceftazidime-avibactam was 256 and 32 µg/ml, respectively. The MBIC$_{90}$ of ceftazidime and ceftazidime-avibactam was >512 and 512 µg/ml, respectively. The MBIC was at least one doubling dilution lower for ceftazidime-avibactam versus ceftazidime alone for 63 of the 66 isolates, and at least two doubling dilutions lower for 42 isolates. **Conclusion:** Ceftazidime-avibactam is more active in preventing *P. aeruginosa* biofilm formation *in vitro* than is ceftazidime alone.
Abstract:

Background: Biofilms are a critical component of catheter-associated urinary tract infections (CAUTI), costing the health system over $340MM annually while contributing to poor outcomes. Several approaches have recently emerged to mitigate these effects, however the incidence of CAUTI is rising. Sharklet and Cook Medical have developed and tested micropatterned catheters in a first-in-man clinical trial as a novel approach to reducing these infections. Here, we describe the research that led to the development and deployment of the Radiance® Clear Sharklet® Silicone Foley Catheter. Methods Initial prototypes were tested for microbial contamination, transfer, and biofilm formation using immersion inoculation followed bacterial recovery and dilution plating from biopsies of the sample to determine bacterial load. Bacterial migration along micropatterned rod segments was determined by a Petri dish migration assay. A single center, open labelled, randomized interventional study compared the Radiance® Clear Sharklet® Silicone Foley Catheter to the standard of care Foley catheter in 50 patients. Outcomes tracked in this study include: Incidence of CAUTI, Surface fouling and biofilm formation, and perceived patient pain. This study is registered in the clinical trial register (NCT02835456). Results: In vitro testing established that Sharklet micropatterned silicone rods significantly reduced initial adhesion of uropathogenic E. coli (UPEC) by 92% compared to smooth silicone rods. Additionally, Sharklet micropattern reduced the migration of both UPEC and P. aeruginosa along the rod by 99.9%. These results suggested that a Sharklet micropatterned catheter might be a viable approach to limit the bacterial colonization, biofilm, and migration that lead to CAUTI. A human clinical trial of 50 catheterized patients (25 Sharklet, 25 smooth) demonstrated that after an average of 8 days, Sharklet catheters accumulated significantly less biofilm than smooth catheters (Tip: P= 0.003, Middle part: P=0.013 and Base: P=0.013). Though no patients in the study developed CAUTI, patients with Sharklet catheters reported significantly less pain compared to the standard of care catheters (p=0.018). Conclusions: Urinary catheters have become an important part of treatment for many patients, however there are several complications that can lead to pain and poor healthcare outcomes. Coupled with the emergence of antimicrobial resistant strains, biofilm formation is a major risk for CAUTI. Innovative approaches are required to combat this issue and limit the risk during catheterization. This study has demonstrated that the Radiance® Clear Sharklet® Silicone Foley Catheter is an effective, safe, and novel approach to mitigating bacterial biofilms that are associated with CAUTI. It highlights the development pathway for new technologies to be implemented in an attempt to mitigate infections.
Abstract:

**Background:** Contamination of implanted medical devices by bacterial biofilms causes significant morbidity and mortality. These biofilm infections are resilient and adaptive, allowing them to resist both host defense and antimicrobial treatment. Currently, the standard of care to treat medical device infections is to surgically remove the device and replace it, which is costly and further increases morbidity and mortality. We have previously demonstrated that modest levels of heat may be a useful treatment in a static culture setting. Understanding biofilm development as the consequence of adsorption, growth and detachment with each effect governed by self-assembly, fluid mechanics and transport phenomena, we translate the use of heat as a potential anti-biofilm therapy from a static culture to a more physiologically relevant flow model. **Methods:** We developed an *in vitro* biofilm reactor system with precise control over flow rate and temperature to mimic the physiologic conditions surrounding a dialysis catheter. *Staphylococcus epidermidis*, the most commonly isolated species from medical device infections, was seeded and grown under low flow conditions (Reynolds number - Re = 1.6) at 37°C to establish a mature biofilm. Then the flow was increased to Re = 44 and the temperature of the infusate was held at 37°C or increased to 45°C, 50°C, or 60°C. Cell viability was determined by Live/Dead staining and confocal microscopy. Viability was also confirmed by complete dispersal of the biofilms within the flow cell followed by serial dilution, plating and colony enumeration. Biofilms morphology was characterized from confocal microscopy images using Fast Fourier Transform analysis. **Results:** The percentage of live cells decreased from 89% ± 2% at 37°C, to 87% ± 2% at 45°C, 64% ± 6% at 50°C and 27% ± 3% at 60°C treatments. There was a tenfold reduction in the number of colony forming units from 37°C to 45°C and a greater than three log reduction for both 50°C and 60°C treatments. We also observed a dramatic increase in the structural heterogeneity with elevated temperature treatment on macro-, meso-, and microscopic scales. **Conclusions:** Exposing biofilms to elevated temperatures changes both the morphology and cell viability of the biofilm. Understanding the response of these bacterial cells under thermal stress is a promising step toward the development of an *in situ* treatment/remediation method for biofilm growth in medical devices.
**Abstract:**

**Background:** *Pseudomonas aeruginosa* is an omnipresent and opportunistic pathogen causing infections due to their ability to adhere and form biofilm and its distinctive antibiotic resistance mechanisms. The extensive use of antibiotics against biofilm associated infections has lead to the emergence of multi drug resistant strains. Modify the interest in the development of alternative strategies needs to be developed. Plant Growth Promoting Bacteria (PGPRs) have been reported to modulate the synthesis of bioactive phytochemicals such as secondary metabolites, polysaccharides, and proteins. The aim of this study was to evaluate antibiofilm, antibacterial and anti QS activity of chickpea seedling protein.

**Method:** Crude protein extract from 7 day’s old root and seedlings were analyzed for their antibiofilm, antibacterial and anti QS activity against *P. aeruginosa* at sub-MICs. **Results:** Root crude protein extracts and seedling extract showed antibiofilm activity, 33.78 and 43.79 % of inhibition respectively compared to control. A significant antibacterial activity was recorded in the 7 days PGPRs treated root and seedling proteins (71.2 %) in *Bacillus amyloliquifaciens* and *B. subtilis* (85 %) in comparison to non-treated, positive and negative control respectively. The same approach would be helpful to meet the challenges of biofilm associated infections. PGPRs mediated phytochemicals probably displayed inhibitory activities at sub-lethal concentrations that would be further useful for investigation and to establish their potential in clinical application.
Gallium Composite Demonstrates Strong Potency against *Pseudomonas aeruginosa* and *Acinetobacter baumannii* Infections

Author: **M. I. Young**, A. Ozcan, P. Rajasekaran, M. Beazley, S. Santra; University of Central Florida, Orlando, FL.

Hospital acquired (nosocomial) infections are a serious health concern within the United States and the world at large. Every year there are over 2 million nosocomial infections and over 23,000 resulting deaths in the United States alone. Many of these infections are associated with microbial biofilms that exhibit higher resistance to antibiotics and therefore effective treatment becomes highly challenging. The objective of this study is to assess the efficacy of a gallium (Ga) composite as an antimicrobial agent against planktonic and biofilm forms of *Pseudomonas aeruginosa* PAO1 and *Acinetobacter baumannii* 19606. The Minimum Inhibitory Concentration (MIC) and Minimum Biofilm Eradication Concentration (MBEC) of *P. aeruginosa* and *A. baumannii* against planktonic and biofilm cells were determined using broth micro-dilution assay as described in the guidelines of the Clinical and Laboratory Standard Institute (CLSI) and ASTM E-2799 assay. The effect of the Ga composite on preventing biofilm formation was assessed using the traditional crystal violet assay. The MIC values of Ga composite varied with the most effective with a value under 4 µg/mL and comparable to traditional antibiotics. The Ga composite displayed a strong ability to prevent biofilm formation and disrupt established biofilms from both organisms. The cytotoxicity of the Ga composite were tested against multiple cell lines including Human Dermal Fibroblasts (HDF) and Murine macrophages (J774). Results showed that the Ga composite did not exhibit any toxicity against mammalian cell lines until very high concentrations, demonstrating a high therapeutic window. The uptake of Ga into bacteria from the Ga composite was conducted using ICP-MS. Overall, our preliminary studies suggest that our Gallium composite could be potentially used as an alternative to antibiotics for treatment of *Pseudomonas aeruginosa* and *Acinetobacter baumannii* infections. Further studies are planned to unravel new insights into the currently incomplete picture of gallium uptake and bactericidal mechanisms.
**Abstract:**

Background: Bacterial biofilms are able to tolerate high concentrations of antimicrobials and therefore new strategies for biofilm control must be implemented to overcome their global burden. Extracellular DNA (eDNA) is an integral component of many biofilm matrices including dental plaque. Many oral bacteria express deoxyribonuclease (DNase) enzymes to utilise eDNA whereas others require eDNA for attachment and colonization. Previously, we demonstrated that treatment of oral biofilms with a DNA-degrading enzyme, NucB from a marine strain of *Bacillus licheniformis*, strongly inhibited the accumulation of biofilms. Here, we identified and characterize SsnA, a DNase enzyme produced by the pioneer coloniser *Streptococcus gordonii* with a view to understanding the role of native extracellular DNases in dental plaque and the potential of exogenous and native DNase enzymes in oral biofilm control.

Methods: An *S. gordonii* ssnA null mutant was constructed and complemented by expression of *ssnA* from a plasmid. A quantitative fluorescence-based assay was employed to determine the activity of DNases under different pH conditions. Inhibition of biofilm formation by *S. mutans*, a caries pathogen, by SsnA and NucB was assessed by confocal laser scanning microscopy. A microfluidic system (BioFlux dual gas dual inlet) was employed to test SsnA and NucB efficacy in controlling of mixed species oral microcosms under most biologically relevant conditions. Results: An *S. gordonii* ssnA null mutant lacked extracellular DNase activity. Enzyme activity was restored in a genetically complemented strain, confirming that SsnA is the major extracellular DNase in *S. gordonii*. The presence of glucose, sucrose and maltose, but not galactose, during growth inhibited SsnA expression. Inhibition was alleviated in a *ccpA* knockout mutant that lacks carbon catabolite repression. Recombinant SsnA was most active at pH 9.5 and was inactive below pH 6. Biofilm formation by *S. mutans* was inhibited by SsnA and DNase I at pH 6 or 7, but only by DNase I at pH 5. SsnA exhibited a strong antibiofilm activity against oral microcosms grown under flow in natural human saliva. However, presence of sucrose inhibited the antibiofilm activity of SsnA. NucB on the other hand maintained its antibiofilm activity in the presence of sucrose. Conclusions: *S. gordonii* SsnA is an extracellular DNase that inhibits biofilm formation by *S. mutans* in the absence of sugars and at neutral to high pH. We propose that streptococcal DNases such as SsnA are important for maintaining microbial homeostasis by preventing colonization by pathobionts such as *S. mutans*, and that dietary sugars or the production of acid by *S. mutans* overcomes this beneficial effect. Addition of exogenous DNases that are active at lower pH values may provide an opportunity for controlling the integration of cariogenic species such as *S. mutans* into the biofilm.
Abstract: Structure-based Virtual Screening of Compounds with Potential of Inhibition of Adhesion Antigen I / II of Streptococcus mutans Dependent

Title: Structure-based Virtual Screening of Compounds with Potential of Inhibition of Adhesion Antigen I / II of Streptococcus mutans Dependent

Author Block: R. E. Rivera¹, W. Rivera², C. Rocha³, L. Padilla³, N. Cardona¹; ¹Antonio Nariño University, Armenia, COLOMBIA, ²Antioquia University, Medellin, COLOMBIA, ³Quindio University, Armenia, COLOMBIA.

Abstract: Antigen I/II of Streptococcus mutans has been implicated in bacterial adherence to constituents of the salivary pellicle, biofilm formation, collagen-dependent bacterial invasion of dentin and cariogenicity. Objective: To identify compounds with inhibitory potential of adhesion through the interaction with antigen I / II of Streptococcus mutans using in silico methods. Methodology: The crystallized structures of the A3VP1 (3IPK: PDB ID) and carboxy-terminus domains of the Ag I/II were selected to predict protein-ligand binding sites using metaPocket 2.0 and COACH meta-servers. Then, the compounds were searched from libraries containing small molecules, drug-like small molecules, and natural product molecules using molecular docking software running on the supercomputer at TACC (Texas Advanced Computing Center). The compounds were selected using two methods: the first one selecting manually the compounds with lower interaction energy and the second one using a script to select which interacted in the highest number of protein-ligand binding sites. Finally, the type of interactions presented between the compounds and the domains were analyzed by BIOVIA Discovery Studio software. Results: Structure-based virtual screening of approximately 883,551 compounds was performed resulting in 10 compounds for each 3IPK and 3QE5 domains; showing interaction energies between -9.6 and -12.8 kcal/mol respectively. Additionally, 10 compounds with high affinity were selected for multiple interaction sites of both domains, with an average energy of -8.3 kcal/mol. The most common interactions were hydrogen bonds in aminoacids such as GLY-ALA-LYS-ILE, followed by cation interactions with LYS and pi-pi with the TYR. Conclusion: 30 compounds were obtained with inhibitory potential of adhesion of S. mutans AgI/II dependent, resulting in 10 compounds with high affinity for specific sites at 3IPK domain as well as 3QE5 domain and 10 compounds that interact in both domains.
Abstract

**Title:** Magnetic Mitigation of *In Vitro* Oral Caries-associated Biofilms

**Author Block:** L. Heersema, H. D. Smyth; The University of Texas at Austin, Austin, TX.

**Background:** The increasing prevalence of antibiotic resistance is a primary concern in the health field and the development of novel therapies. The biofilm state of microorganisms is linked with increased antibiotic resistance due to many factors including reduced diffusion of chemotherapeutics through the extracellular polymeric substance barrier. In order to overcome this barrier and disrupt the microorganisms within biofilms, we have investigated the use of magnetic nanoparticles and fields against an in vitro multi-species oral caries-associated biofilm model. **Methods:** Multi-species biofilms, consisting of a 1:1:0.1 ratio of *Streptococcus gordonii* (DL1.1):*Streptococcus mutans* (UA159): *Candida albicans* (SC5314), were grown in 96 well microtiter plates for 24 h at 37°C and 5% CO₂ prior to dosing. Magnetic nanoparticles with various polymeric coatings were purchased from Chemicell and suspended in 1:1 RPMI:TSBYE 0.6% media at 100 µg/ml concentration. Static magnetic fields were generated using rare earth neodymium magnets of strengths from ±0.01 to 2.70 kG.

**Results:** Preliminary results demonstrate that potential of using magnetic nanoparticles and fields to disrupt orally relevant biofilms. Overall treatment with magnetic nanoparticles was most effective for 1.5-2.5 minute exposures. Nanoparticles with hydrodynamic diameters less than 110 nm were the most effective during this exposure time and comparable to treatment with an antiseptic mouthwash. The application of static magnetic field gradients indicates potential windows of enhanced efficacy against biofilms. **Conclusions:** The use of magnetic fields and nanoparticles presents an interesting solution to overcoming the barriers that frequently reduce the efficacy of many anti-biofilm treatments. Another important consideration when developing novel anti-biofilm therapies is the exposure time necessary to reduce biofilm viability. Our work with magnetic nanoparticles and fields demonstrates effectiveness within a relevant exposure time for oral cavity applications.
Abstract:

**Background** Salmonella is a major food borne pathogen commonly from poultry resulting in large number of outbreaks worldwide. Biofilm is ubiquitous in farms and food processing environment serving as crucial pathogenic and antimicrobial resistance mechanisms of bacteria. The global public health threat of multidrug resistant bacteria has led to the investments in the search for alternative natural (indigenous plants) antibacterial remedies. This study investigated the antimicrobial and biofilm inhibition effects of *Moringa olifera* leaf extract in *Salmonella* isolates from chicken. **Methods** Isolation, characterization and enumeration of bacteria were carried out on 334 chicken samples obtained from poultry farms in Lagos, Nigeria. The MIC of aqueous extracts of *Moringa* leaves was obtained followed by in-vitro sensitivity of graded concentrations to the isolates by the agar well diffusion method with ATCC 13311 salmonella as reference strain. The minimum inhibitory concentration (MIC) of these plants for each of the test organisms was evaluated. Biofilm assays of the isolates were performed in triplicate from overnight LB broth culture on Microtiter plate and incubated at 37°C aerobically for 96 hours. Also, activity of the leaf extracts against biofilm formation by *Salmonella* isolates was determined using the crystal violet assay. Biofilm quantification was obtained by O.D. values at 630 nm in microtiter plate reader. **Results** The prevalence of Salmonella spp. in poultry was 16.2% (n=54). The leaf extract inhibited the growth of 85.2% of the isolates with zones of inhibition ranging from 13.6 ±0.7 mm 17.5±0.7 mm. The highest antibacterial spectrum was produced by 100% extract while the MIC was 12.5% concentration. Biofilm formation was exhibited by 38 salmonella strains (O.D. = 0.05-0.12), with 16 strong biofilm producers, 22 strains weak producers and 16 non producers. Biofilm formation by the isolates varied with *Salmonella* strains and concentrations of extract. **Conclusions** The results of this study indicate that *Moringa olifera* can protect against chicken Salmonella by inhibiting their growth and biofilm formation. The nutraceutical values of *Moringa olifera* could be explore in food industry against bacteria food borne bacterial as natural edible additive to improve the quality and safety of poultry products.
Abstract:

Microorganisms can colonize almost all natural and artificial surfaces. If conditions are favorable, the attached cells build complex communities of surface, called biofilms. Initial adhesion is often reversible, so that the cells can break away from a surface if conditions change. The purpose of inhibiting microbial adhesion that prevents the initial steps of biofilm formation should be the most logical choice in infectious processes. The best results were found with the surface treatment or change surface properties with surfactants. Surfactants have potential to prevent bacterial adhesion. Biosurfactants have low toxicity, are biodegradable, exhibit chemical diversity and are more effective in environmental conditions. A major biological for producing biosurfactants sources is through bacterial strains. Furthermore, most studies biosurfactants, only a small part is dedicated to the anti-adhesive properties. Therefore, more studies on biosurfactants are required in order to design strategies to reduce bacterial adhesion that leads to the formation of unwanted biofilms. In the present work, rhamnolipids produced by bacterial strain Rn19a (partially identified as *Pseudomonas*) isolated from soil contaminated with hydrocarbons were evaluated. In the present work, rhamnolipids produced by the bacterial strain Rn19a isolated from soil contaminated with hydrocarbons were evaluated. The Rn19a strain has been partially identified as *Pseudomonas*. The rhamnolipids were used as a biosurfactant. Glass slides treated with 0.4% (w/v) rhamnolipids were used as a substrate. The slides were submerged vertically in sterile nutritive broth and individually inoculated with 6 strains isolated from urinary infections highly biofilm forming, and multiresistant called I3, I7, E26, I27, E30 and I31. Untreated slides were used as control. In order to evaluate the bacterial adhesion, a 36-hour kinetic was followed. Adhesion was evaluated every 6h and stained with 0.1% m / v violet crystal and 0.002% methylene blue (w/v) by optical density measurement. The results of crystal violet and methylene blue were consistent with each other. As a preliminary result, the finding that strains I3 and E26 increased their adhesion was found. Both strains have been identified by biochemical tests such as *Pseudomonas*. The above agrees with the fact that rhamnolipids contribute to the architecture of biofilms. This was expected since the rhamnolipids come from the same *Pseudomonas* family. On the other hand, strains I7, I27, I30 and I31, identified as *E. coli*, *Enterococcus*, *E. coli*, and *E. coli*, respectively, decreased adhesion to at least 1.0 optical density units. In conclusion, the above mentioned suggests that non-*Pseudomonas* strains are susceptible to rhamnolipids to interfere with or retard adhesion to surfaces.
Methicillin-resistant Staphylococcus aureus (MRSA) has been identified on the outer shell of firefighters’ turnout gear jackets, suggesting that contaminated gear may be an indirect transmission source. Using the washing procedures described in the NFPA 1851: Standard on Selection, Care, and Maintenance of Protective Ensembles for Structural Fire Fighting and Proximity Fire Fighting, the present study was conducted to develop a quantitative method to assess the antimicrobial efficacy of antimicrobials (Fire Soaps™ Fabric Sanitizer) in the decontamination of S. aureus from the outer shell (Gemini™ XT, Pioneer™ and Advance™) of turnout gear. After washing outer shell fabric with sanitizing solution according to specifications described in the ASTM E2274 Protocol for evaluating laundry sanitizers and disinfectants, the efficiency of Fabric Sanitizer was determined using standard microbial techniques and the quantitative real-time polymerase chain reaction (qPCR) to assess for viable and non-viable S. aureus that was washed from the fabric. To determine the amount of S. aureus that remained attached to the outer shell fabric, the bacteria was recovered by incubation of the fabric for five hours in Dey/Engle Broth (DEB) at 37°C. Using the ASTM E2274 Protocol, disinfection for 10 seconds reduced the viability of S. aureus on Gemini™ and Advance™ by 73% (0.6 log reduction) and 99% (2.2 log reduction), respectively. The reduction of viability was essentially unchanged after increasing the disinfection time to 10 minutes (68% reduction for Gemini™ [0.8 log reduction] and 97% for Advance™ [1.8 log reduction]). Scanning electron microscopy (SEM) was also used to observe the attachment of S. aureus to fabrics and characterize the stages of biofilm formation on the outer shell and that result in increased resistance to disinfection. Additional disease transmission risk from cross-contamination of turnout gear by other bacterial species was also revealed through the discovery of a sanitizer-resistant strain of Pseudomonas aeruginosa recovered from decommissioned turnout gear jackets. Moreover, the sanitizer-resistant strain of P. aeruginosa that was recovered from the decommissioned turnout gear jacket was shown to initiate biofilm formation on Gemini™ within 5 hours of incubation in DEB, demonstrating the potential for rapid biofilm formation in the field when worn by firefighters. A simple and inexpensive method that can be used by commercial firefighter laundering facilities to determine the effectiveness of outer shell sanitization in their washing machines is also proposed.
Antibiotic Resistance in Sewer Biofilms and Susceptibility to Disinfection as a Function of Pipe Material

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Sewers are not only conduits of wastewater; they are also complex bioreactors. Because wastewater treatment is designed to inactivate pathogenic microbes, fecal matter from patients with infectious diseases is allowed to enter the sewer systems. Here, these cells can get attached to pipes biofilm and exchange, acquire, and spread genetic material including antibiotic resistance genes (ARG). During wet weather, sewer overflows result in the release of these ARG that presents a public health risk. In this study, we used an annular biofilm reactor that simulates sewer conditions to assess the antibiotic resistance genomic composition of biofilms throughout time for two pipe materials: PVC and concrete.

Quantification of ARG in biofilm, water, and sediment samples was performed using qPCR. Results demonstrated that two ARG (sul1 and blaTEM) were detected in the biofilm samples on both pipes materials, while tet(G) was only detected on concrete. After 25 days of biofilm development, a disinfection experiment was performed by pumping a solution of 6.25% bleach into the reactor. ARG were quantified after the treatment using qPCR for total DNA and viability PCR that reduces the qPCR signal from cells with compromised membranes. Disinfection resulted in a reduction of ARG in the biofilm and can provide insight into the mechanism of disinfection in sewers (i.e., oxidation of cell membranes in biofilm vs. detachment). Overall these results indicate that sewer biofilms can serve as reservoirs of ARG that can persist after standardized disinfection protocols.
Abstract:

Transcriptomic, metabolomic, physiological, and computational modeling approaches were integrated to gain insight into the mechanisms of antibiotic tolerance in an in vitro biofilm system. *Pseudomonas aeruginosa* biofilms were grown in drip-flow reactors on a medium composed to mimic the exudate from a chronic wound. After three days, the biofilm was 114 μm thick and contained 9.45 log₁₀ cfu cm⁻². These biofilms exhibited tolerance to subsequent treatment with ciprofloxacin. The biofilm specific growth rate was estimated via elemental balances to be approximately 0.35 h⁻¹ or one-third of the planktonic maximum specific growth rate. Global analysis of gene expression indicated decreased anabolic activity in biofilms compared to planktonic cells. A focused transcriptomic analysis revealed the induction of multiple stress responses in biofilm cells including those associated with growth arrest, zinc limitation, hypoxia, and acyl-homoserine lactone quorum sensing. Metabolic pathways for phenazine biosynthesis and denitrification were activated in biofilms. A customized reaction-diffusion model was solved to characterize the distribution of oxygen inside the biofilm. It predicted that steep oxygen concentration gradients form when these biofilms are thicker than about 40 μm. Mutants deficient in Psl polysaccharide synthesis, stringent response, stationary phase response, and membrane stress response exhibited increased ciprofloxacin susceptibility in biofilms while many other mutants had no susceptibility phenotype. These results along with extensive literature supported a generalized conceptual model of biofilm antimicrobial tolerance with the following mechanistic steps: 1) establishment of concentration gradients in metabolic substrates and products through reaction-diffusion interactions, 2) active biological responses to these changes in the local chemical microenvironment through shifts in gene expression or alterations of enzyme activity, 3) entry of biofilm cells into a spectrum of states involving alternative metabolisms, stress responses, slow growth, cessation of growth, or dormancy, and 4) reduced susceptibility of microbial cells to antimicrobial challenges in some of these physiological states.
**Introduction:** An important contributing factor in *C. albicans* pathologies is their flexibility in adapting to different environmental conditions by virtue of growing preferentially as biofilms. In particular ECM biofilm formation by *C. albicans* carries important clinical repercussions in terms of treatment failure and burden on health-care budget. The purpose of our study is to isolate *C. albicans* from clinical specimens, and to find a correlation between their antifungal susceptibility and biofilm formation.

**Methods:** A total 300 *Candida albicans* isolated from patients with various clinical problems were included in the study. Confirmation of *Candida albicans* was done through CHROM Agar and API 20 C AUX. Antifungal susceptibility testing of Fluconazole and Voriconazole was undertaken by disc diffusion method recommended by CLSI M44A document. MICs of sessile cells was performed by the CLSI M27-A2 (yeasts) broth microdilution method. Biofilm formation was done in a 96-well flat bottom Antifungal susceptibility testing of Fluconazole and Voriconazole was undertaken by disc diffusion method recommended by CLSI M44A document microtiter plate with standarized cell suspensions in RPMI -1640 (Roswell Park Memorial Institute) that was incubated at 37ºC and was observed at different time intervals ranging from 02-48 hours.

**Results:** The mean age of the patients was 40 years. Male to female ratio was 1:1.2. Total *Candida albicans* isolated were 300. In our recent study fluconazole was susceptible to 116 (39%) and SDD susceptible dose dependent to 36 (12%) and resistant to 148 (49%) by disc diffusion method (Kirby-Bauer). The MIC sessile antifungal susceptibility of fluconazole was 87(29%) sensitive, 25(8%) SDD susceptible dose dependent and 188(63%) were resistant. Susceptibility of *Candida albicans* with respect to voriconazole was analyzed as sensitive in 230 (77%) of patients, it was intermediate in 15(5%) of patients and resistant in 55(18%) patients by disk diffusion. Sessile antifungal susceptibility to voriconazole was 89 (30%) sensitive, 42 (14%) were susceptible dose dependent (SSD) and 169 (56%) were resistant of the total isolates . Biofilm formation was observed in 285(95%) of the isolates and only 15 (5%) were observed as non-biofilm former.

**Conclusion:** Biofilm-based Candida infections are an emerging problem. High antifungal resistance was observed in biofilm-producing strains in this study. Using the variables affecting biofilm formation, tailored intervention strategies can be implemented to reduce biofilm-based Candidiasis.

**Key words:** Antifungal drugs, biofilm, *Candida, Candida albicans*,

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Date & Time: Wednesday, October 10, 2018, 4:00 pm - 6:00 pm
Poster Board #: 029
Topic: Biofilm Antimicrobial Tolerance
Abstract Title: Characterization of PA2915: A Putative Beta-Lactamase from Pseudomonas aeruginosa
Author Block: M. C. Goodyear, J. Van Loon, A. M. Berezuk, C. M. Khursigara; University of Guelph, Guelph, ON, CANADA.

Abstract:

**Background:** Pseudomonas aeruginosa is a Gram-negative bacterium that can cause chronic and multidrug resistant infections in immunocompromised individuals. *P. aeruginosa* possesses a number of intrinsic mechanisms of resistance that allow it to survive antibiotic treatments. These mechanisms include the ability to grow as biofilms and protein-based mechanisms such as antibiotic-degrading β-lactamase enzymes. A previous study in our laboratory compared the proteomes of biofilm and planktonic cultures of the *P. aeruginosa* laboratory strain PAO1 when grown for 24, 48, or 96h. The proteomics study identified a number of putative β-lactamases that showed biofilm-specific expression. We hypothesize that these uncharacterized β-lactamases contribute to the enhanced resistance of *P. aeruginosa* biofilms. Our studies aim to confirm the β-lactamase activity of these enzymes both *in vitro* and *in vivo*.

**Methods:** Current work is focused on PA2915, which showed increased abundance in biofilms at all three time points. Amino acid sequence analysis was used to show that PA2915 is a putative β-lactamase. A 6xHistidine tagged construct of PA2915 was made for over-expression in PAO1. Immobilized metal affinity chromatography and anion exchange chromatography were used to purify PA2915. Purified protein was then tested for β-lactamase activity *in vitro* using a nitrocefin assay. Nitrocefin is a chromogenic cephalosporin that mimics the structure of β-lactam antibiotics and can be cleaved by β-lactamases. When cleaved, the change in absorbance between substrate and product can be measured and used to confirm β-lactamase activity. To demonstrate the *in vivo* function of PA2915, a deletion mutant has recently been engineered in PAO1 using two-step allelic exchange.

**Results:** The amino acid sequence of PA2915 contains a Hx[DEH]xDH motif typical of metallo-β-lactamase enzymes that require a divalent metal cation cofactor. PA2915 was purified to homogeneity at high quantities (~25mg/mL). In preliminary nitrocefin assays, PA2915 showed zinc-dependent β-lactamase activity.

**Conclusions:** Sequence analysis and preliminary nitrocefin assays suggest that PA2915 is a metallo-β-lactamase. Metallo-β-lactamases are an increasing clinical concern as there are currently no inhibitors available for these enzymes. Susceptibility assays are currently underway to determine how the loss of PA2915 affects the resistance of PAO1 when challenged with β-lactam antibiotics in either the planktonic or biofilm mode of growth. The *in vitro* and *in vivo* workflows developed here will be used to characterize additional putative β-lactamases that showed biofilm-specific expression in PAO1. Together, these putative β-lactamases may represent a significant and understudied source of resistance in *P. aeruginosa* biofilms.
**Session:** Poster Session 3  
**Date & Time:** Wednesday, October 10, 2018, 4:00 pm - 6:00 pm  
**Poster Board #:** 030  
**Topic:** Biofilm Antimicrobial Tolerance  
**Abstract:** Microfluidic Study of the Effect of Disinfectants on *P. aeruginosa* Biofilms Grown on a Gradient Nutrient Level  
**Title:** Gradient Nutrient Level  
**Author:** E. Chiang, B. Sung Woo; National University of Singapore, Singapore, SINGAPORE.

**Abstract:**

**Background:** Bacterial biofilms have exhibited increased tolerance to antimicrobial agents. Studies using traditional biofilm cultivation devices require larger media volumes and lack spatial control, leading to risk of uneven exposure of disinfectants. This study proposes the use of a multi-channel polydimethylsiloxane(PDMS)-based microfluidic chip to create and maintain microenvironment for biofilm formation and stability. Therefore, the objectives of this study are (i) to evaluate the effect of chlorine on biofilm formation and dispersal in nutrient gradient-mixing channels and (ii) to examine their persistence to changes in chlorine concentration; chlorine levels are commonly increased to remove and suppress potential biofilm growth. **Methods:** The microfluidic chip consists of a PDMS layer mounted atop a cover slip, comprising of 5 parallel channels with diluted nutrient concentrations flowing from a gradient mixing network. Log phase *P. aeruginosa* cells were incubated for 2 hours prior to 1-day continuous flow of sterile LB at 10μL/min. Channels were exposed to 5mg/L NaOCl for 1 hour and subsequently 2mg/L. The biofilm structures were imaged using Zeiss LSM170 fluorescence microscopy during different conditions; live and dead biomass were calculated by iMaris (Bitplane Inc. Version 9.1.2); average biofilm thickness was measured by ImageJ. **Results:** The initial thinnest biofilm averaged 10μm uniformly, while the thickest biofilm averaged 37.5μm non-uniformly in mushroom-shaped clumps of *P. aeruginosa* biofilms, demonstrating gradient nutrient level induced biofilm thickness. Lower nutrient level prevented the growth of highly stable mushroom-shaped biofilms, possibly accounting for the dislodgement and subsequent 30μm thick aggregation only in the lowest nutrient channel, indicating its higher susceptibility to NaOCl. The thickest biofilm grew up to 77μm thick even when exposed to 5mg/L NaOCl but decreased to below 10μm during subsequent 2mg/L flow. The first exposure might be insufficient a shock to weaken the biofilm strength as the EPS may have neutralized the NaOCl and prevent physical stress, but continuous exposure may eventually compromise the EPS, allowing intrusion of NaOCl deeper into the biofilm. Subsequent LB flow overnight saw a rapid regrowth of biofilms up to 77μm. **Conclusion:** The microfluidic device as a promising platform for bacterial biofilm study was designed to elucidate high resolution observations of combined effects of nutrient levels and disinfectants on biofilm formation. The results have shown that increased nutrients enhanced biofilm’s stability and resistance to dispersal by NaOCl. A high NaOCl dose was ineffective in biofilm dispersal but longer exposure at a lower dose eventually removed more than 50% of the initial biofilm. A lower dose could be applied to weaken and disperse the EPS matrix before using a higher dose to kill the exposed cells.
**Session:** Poster Session 3  
**Date & Time:** Wednesday, October 10, 2018, 4:00 pm - 6:00 pm  
**Poster Board #:** 031  
**Topic:** Biofilm Antimicrobial Tolerance  
**Title:** Challenging *Staphylococcus aureus* Biofilms with different Dosing Patterns of Gentamicin in Combination with Rifampicin  
**Author Block:** W. Boot, R. G. Richards, T. F. Moriarty; AO Research Institute, Davos Platz, SWITZERLAND.

**Abstract:**

**Background:** Orthopaedic device-related biofilm infections (ODRI) caused by *Staphylococcus aureus* have a high treatment failure rate, despite prolonged systemic (intravenous and oral) and local application of antibiotics (e.g. antibiotic loaded bone cement, ALBC). In this study, we investigated the ability of two commonly applied antibiotics in the treatment of ODRI (gentamicin and rifampicin) to eradicate mature *S. aureus* biofilm after exposure to three distinct dosing patterns modelled after these different dosing options. **Methods:** Biofilms of a clinical *S. aureus* isolate were formed using the MBEC system for 5 days in TSB supplemented with 1% human plasma. Thereafter, biofilms were exposed to gentamicin alone or in combination with 3 µg/ml rifampicin as follows: 1) constant concentrations of gentamicin for 28 days (2000, 250, 15 µg/ml); 2) pulsed dosing of gentamicin (same concentrations), for two periods of two hours per day over 28 days; 3) burst release: initially high, but rapidly diminishing concentrations over time as may be expected from ALBC (2000 µg/ml on day 1, to 2 µg/ml at day 14). At each timepoint a total viable count (TVC) was performed from a total of 18 pegs per condition. Antibiotic resistance of viable bacteria was assessed by zone of inhibition. **Results:** The TVC of unexposed biofilms was ~6x10^8 CFU throughout the study. In the first test group, constant exposure of biofilms to gentamicin at 2000 µg/ml achieved a mean 7-log reduction in TVC at day 28. Twelve of 18 pegs were culture negative. Adjunctive rifampicin could not decrease TVC further, instead inducing rifampicin resistance by day 14. In the second group, pulsed exposure of 2000 µg/ml gentamicin achieved a mean TVC reduction of only 1 log by day 28 (0 culture-free pegs), but adjunctive rifampicin increased biofilm eradication to 13 out of 18 pegs (p<0.001 compared to control). Mean reduction was only 2 logs due to high numbers of rifampicin resistant bacteria in the culture positive pegs. In the third group, burst release of gentamicin reduced TVC by 1 log at day 7, however, the TVC recovered to control levels at day 14. Interestingly, adjunctive rifampicin reduced TVC 4 logs by day 14 (p<0.001 compared to control), with 6 out of 18 pegs culture negative. Again, culture positive pegs were colonised with rifampicin resistant bacteria. **Conclusion & Discussion:** Constant high concentrations of gentamicin were the most effective treatment of *S. aureus* biofilms; however, these concentrations are not achievable by systemic therapy. Adding rifampicin in this profile was ineffective and induced resistance. Interestingly, pulsed and burst release profiles were effective in achieving reductions in TVC or culture positive pegs when combined with rifampicin, even though total antibiotic exposure was less than the constant exposure profile. This data confirms the opportunities for optimising antibiotic pharmacodynamics against biofilms.
**Abstract:**

**Background:** Bacteria are continuously evolving new ways as survival strategies to avoid the effect of antibiotics and infections caused by bacteria are becoming more intense. Ability to form biofilm is one of such ways which potentiates the bacteria to establish infection and also to tolerate antibiotic treatment. Thus, bacterial biofilms cause chronic infections because they show increased tolerance to antibiotics and resist phagocytosis, as well as components of the innate- and the adaptive immune systems of a host. The bacteria in biofilms live in a self-produced matrix of extracellular polymeric substances (EPS) that form their immediate environment. EPS are mainly constituted of polysaccharides, proteins, nucleic acids and lipids; they provide the mechanical stability in biofilms and minimize effect of antibiotics. **Methods:** Biofilm formation by *Pseudomonas aeruginosa* isolates KPW.1-S1 and HRW.1-S3 was assayed in 24 well plates by Crystal Violet staining method. Scanning Electron Microscopy (SEM), Confocal laser scanning microscopy (CLSM) were performed for visualization and characterization of KPW.P1 biofilms. Extra-cellular Polymeric Substances (EPS) and generation of reactive oxygen species (ROS) in KPW.1-S1 and HRW.1-S3 biofilms were characterized biochemically. **Results:** *Pseudomonas aeruginosa* isolates KPW.1-S1 and HRW.1-S3, when grown in presence of sub-minimum inhibitory concentrations (sub-MICs) of tetracycline and gentamicin, intense biofilms were formed and the extent of biofilm formation, with respect to compactness and multi-layer structure and EPS formation was observed to be tetracycline and gentamicin concentration dependent. Increased levels of e-DNA and exo-proteins were found in biofilm structures grown in presence of tetracycline and gentamicin as compared to control biofilm structure. Interestingly, Sub-MIC concentration of gentamicin induced the growth of planktonic cells as well as biofilm formation by KPW.1-S1 and HRW.1-S3. **Conclusions:** Our findings show more compact biofilm formation by the isolated *Pseudomonas aeruginosa* strains in presence of antibiotics relative to control and increased biofilm formation was mediated through generation of ROS and increased production of eDNA and exo-proteins.
**Background:** *Enterococcus faecalis* is a commensal of the human gastrointestinal tract; it is also an opportunistic pathogen and one of the leading causes of hospital acquired infections. *E. faecalis* produces biofilms that are highly resistant to antibiotics and have been previously shown to exhibit altered 3D architecture in the presence of subinhibitory antibiotics, daptomycin in particular. A similar biofilm architecture was observed in an *epaOX* deletion mutant, which encodes a glycosyltransferase of the enterococcal polysaccharide antigen (*epa*) gene cluster and was shown to be important in biofilm-associated antibiotic resistance. These data suggested a model in which biofilm growth is associated with cell envelope stress and remodeling of biofilm architecture results from exacerbation of this stress by antibiotics or mutations affecting the cell wall. **Methods:** The model described above was examined using chemical treatments predicted to alter the cell envelope as well as examining other genes of the *epa* operon. Biofilm production of OG1RF and mutant derivatives was examined in the presence of subinhibitory daptomycin, gentamicin, and sodium cholate (a component of bile) and biofilm architecture was observed after growth on Aclar membranes using fluorescence microscopy. **Results:** Exposure to both sodium cholate and daptomycin, substances that alter the cell envelope, resulted in altered biofilm architecture in OG1RF. A mutant in *epaQ*, which encodes a hypothetical membrane protein, showed reduced biofilm production in the presence of daptomycin relative to OG1RF. Moreover, Δ*epaQ* exhibited a more extreme altered biofilm architecture than previously observed with Δ*epaOX*. Biofilm production of Δ*epaOX* was reduced in the presence of both sodium cholate and daptomycin. Additionally, altered biofilm architecture was observed upon depletion of *epaOX* expression in a pre-established biofilm. To determine how this stress is sensed, we examined a pathway previously proposed to be involved in cell wall stress response, which includes IreK and the two-component regulatory system CroRS. Preliminary data shows that disruption of this pathway alters the response of OG1RF to sodium cholate and daptomycin, suggesting this pathway is important for modulating changes in biofilm architecture in response to cell wall stress. **Conclusions:** Our cumulative results indicate that biofilm growth of *E. faecalis* may entail cell envelope stress not encountered in planktonic growth, and the *epa* polysaccharide modulates this stress to allow robust biofilm growth and architecture. *E. faecalis* produces an altered biofilm architecture in response to heightened cell wall stress caused by either chemical exposure or genetic disruption of the normal cell wall structure.
**Background:** To study filamentous fungal biofilms that are representative of those in the built environment, we established a method for engineering biofilms in a controlled reactor under low-shearing force on a glass coverslip. The purpose of this project is to assess cell viability and quantify the biofilm features of *Aspergillus niger* biofilms and those exposed to antimicrobial agents. *A. niger* is being studied because it is ubiquitous and a model organism. **Methods:** Cell viability quantification in *A. niger* biofilms has not been reported, thus we are comparing two different methods to determine which is optimal. One method utilizes the LIVE/DEAD Yeast Viability Kit containing FUN1 cell stain that exhibits orange-red fluorescent intravacuolar structures in metabolically active cells, while dead cells fluorescence green-yellow. The second method involves using the LIVE/DEAD BacLight Bacterial Viability kit containing SYTO9, a green fluorescent stain with a capacity to penetrate the active cell walls, and Propidium Iodide (PI), a red fluorescent stain that penetrates the damaged cell membrane. To determine the efficacy of antimicrobial agents on fungal biofilms, we used sodium hypochlorite solutions and stained with SYTO9 and PI. Confocal Microscopy and the computer program COMSTAT 2.1 are being used to visualize fluorescently labeled cells and quantitating biofilm structures. **Results:** Biofilms were stained using both methods and were compared for reliability. The center portion of the biofilm contained more live cells when compared to the edge portion. Also, the edge portion contained conidigenous cells and conidiophores. The viability of the edge portion was not homogenous, a majority of the biofilm cell was viable, but a minority of red fluorescent nonviable cells was also noted. This indicated the stage of maturation. In antimicrobial test analysis, we found that it was impossible to distinguish live cells stained with SYTO9 from dead cells due non-fluorescence of dead cells stained with PI. This is because sodium hypochlorite damaged the DNA. If the nucleic acid gets damaged by sodium hypochlorite, it will not be able to get linked with the PI fluorochrome. **Conclusion:** The combination of nucleic acid stains SYTO9 & PI is more reliable for imaging and live-dead cell differentiation. In the case of sodium hypochlorite efficacy test, SYTO9 stained with a capacity to penetrate cell walls with relative ease and stained the cell green regardless of their viability. In comparison, PI cannot stain the cell with nucleic acid damage.
**Background.** Biofilm forming, antibiotic-resistant strains of bacteria represent an increasingly serious threat to global public health. Bacterial biofilm sensitivity to antibacterial agents may decrease 10-1000 times compared to planktonic forms, leading to multi-drug resistant bacterial spread and ineffective treatment. Therefore new treatment methods are needed against chronic infections caused by bacterial biofilms. With the development of nanotechnology, it is possible to obtain nano-sized particles of different substances, including smaller versions of antibiotics, that differ from its full-length particles by extremely enhanced chemical activity and increased membrane fluidity. The aim of this study was to establish experimental conditions for *Staphylococcus aureus* biofilm formation and for ultrastructural analysis of such cultured biofilms for future use in studies investigating bactericidal effects of nanoparticles.

**Methods.** Experimental biofilm was obtained from isolated pure *Staphylococcus aureus* cultures from children’s stool samples and delivered to the Bacteriological Laboratory of the Republican Center of Hygiene and Epidemiology for further processing. The suspension of microorganisms (2x10^8 colony forming units as determined by McFarland turbidity standards) was added onto an aluminum specimen mount soaked in meat-peptone broth for biofilm generation.

**Results.** At day 1, the aluminum-mounted biofilm was fixed with Gentian violet and electron micrographs obtained by scanning electron microscopy (JEOL-40 microscope). Biofilms from the same culture of *St. aureus* were studied at additional timepoints as well (i.e., days 1, 2, 3, 5 and 10). At early time points (days 1 and 2), the bacterial cells were scattered. Formation of biofilm-specific intercellular primary strands was observed at days 1 and 2 at 20,000x magnification. Subsequently, at days 3 and 5, dense surface colonization, reminiscent of a multilayer frame made of spherical bacterial cells, was revealed. This previously described multilayer frame formation has been shown to hinder recognition of antibiotic targets and could be used in future studies to monitor the effects of nanoparticles on biofilm integrity.

**Conclusions.** The work described here demonstrated a methodology for ultrastructural analysis of aluminum-mounted biofilm samples and follow-up of biofilm formation stages for these infectious agents. This is critical to evaluate nanoparticles as shortened versions of antibiotics in in vitro therapeutic studies or other antibacterial agents on experimentally obtained and native bacterial biofilm. Our in vitro methodology can especially assist in screening nanoparticle-sized antibiotics against biofilm antibiotic resistance and help identify these “shorter antibiotic” compounds showing more fluidic passage through biofilm channels and potentially more efficacious.
Abstract:
Microbial biofilms form complex spatial structures such as wrinkles and fruiting bodies. How do these structures influence the evolutionary fitness of the microbial population and the relative importance of chance in its evolution? Measuring spatially-resolved evolutionary lineages can give us insight into the evolutionary history of these biofilms and an understanding of how it was influenced by these structures. Existing methods of spatial lineage tracing are limited by genetic engineering techniques or to small population sizes (order 1000 individuals), making it challenging to learn about emergent population-level behavior. We have developed a label-free method to infer evolutionary lineage trees in biofilms composed of trillions of cells using population-level time lapse microscopy. We measure local velocity fields from the collective motion of cells and calculate stream lines that are then converted to lineages. This method allows us to show that in wrinkled *Bacillus subtilis* biofilms, lineages get lost from the population faster at a wrinkle than far away from a wrinkle. This result suggests that wrinkles are local population bottlenecks, where the relative effect of natural selection is reduced in comparison to that of chance.
Microfluidic-based Transcriptomics Reveals Rheosensitive Bacterial Gene Expression

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Title: Microfluidic-based Transcriptomics Reveals Rheosensitive Bacterial Gene Expression

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Abstract: Bacteria must contend with flow to colonize new environments, such as hosts and associated medical devices. However, it was previously unknown if bacteria could actively respond to flow independent of surface sensing. Here we used microfluidic-based transcriptomics to temporally resolve the rheosensitive response of the human pathogen Pseudomonas aeruginosa. Flow rapidly induced expression of genes throughout the genome and we show that the flow-induced transcriptome is distinct from the surface-induced transcriptome. Many flow-induced genes are also induced during human infection, including the previously uncharacterized operon froABCD (flow responsive operon). Single-cell analysis confirmed that fro induction is surface-insensitive and revealed that fro induction does not require surface sensors PilY1 or type IV pili. Instead, fro induction by flow is mediated by a previously uncharacterized anti-sigma factor FroI and sigma factor FroR. P. aeruginosa is capable of dynamically tuning fro expression to shear rates of 40-400 sec⁻¹, which closely matches shear rates found in the human host. Thus, P. aeruginosa mounts an active response to flow independent of surface sensing that tunes gene expression to environmental shear rate.
Abstract: Establishment of a Biofilm in a Soil Column is Correlated to Reduced Permeability

Author: L. F. Caslake, M. J. Roth

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Background: The growth and maintenance of a biofilm may be an answer to some civil and environmental engineering problems. Groundwater seepage is associated with failures of dams, levees, and contaminant containment systems. Traditional engineering methods to reduce groundwater seepage in the field can be expensive and may create significant environmental concerns. The use of microbiological processes to modify the properties of in-situ soils has shown significant potential in the laboratory. A biofilm that blocks portions of the void space in soil will reduce the soil permeability and limit seepage. Methods: Our experimental setup consisted of six vertical sand columns confined in acrylic tubes with sampling ports along the length of the column. Nutrients were added to four sand columns stimulating the growth of the extant population of microbes; two columns served as controls and received only de-ionized water. To monitor permeability, the apparatus requires not only a location where the soil is contained and mechanisms that permit flow through the sample, but also allows the measurement of the volume of fluid passing through the sample and the measurement of pressure differences across the soil sample. Nutrients were initially provided daily to the columns; however, clogging occurred above the sample at the location where the nutrients entered the soil and significantly reduced fluid flow. Once the clogging was removed, the nutrients and de-ionized water were provided to the columns on alternate days. Columns were maintained under this schedule for over 11 weeks and permeability was measured daily. Samples of the column fluid were taken through the sampling ports with an 18 Ga needle and syringe, placed on a slide, stained for DNA (with SYTO-9) and EPS (with Con-A conjugated Alexa Fluor 633) and visualized using confocal microscopy. Results: Sustainable permeability reductions of up to one order of magnitude were measured in each experimental column. Although the biofilm was likely disrupted by sampling methods, confocal microscopy confirmed the presence of bacteria and EPS throughout the experimental columns. Conclusions: Biofilm growth in soils may be a low-cost alternative to traditional engineering field methods of permeability reduction.
**Abstract:** Biophysics of *Candida albicans* Adhesion to Nanofibers-coated Surfaces

*Candida albicans* is a human commensal and opportunistic fungal pathogen. *C. albicans* biofilms on implanted devices are less susceptible to antimicrobials and can cause systemic infection with substantial mortality rates. Prior works by us and others have shown that micro/nano-scale topographical features influence microbial retention on surfaces. However, a quantitative model describing the effects of the geometry and size of features on microbial retention is lacking. Furthermore, the effect of surface topography on near-surface behavior of *C. albicans* is largely unexplored. Through synergistic experimental and theoretical investigations, we examined *C. albicans* adhesion, at single-cell and population-level, to micro/nano-fiber coated surfaces as a function of the geometry (i.e. diameter) and configuration (i.e. inter-fiber spacing) of the surface features. *C. albicans* interaction with polystyrene (PS) surfaces coated with PS nanofibers of uniform diameter (500 nm-2 µm) and spacing (1-3 µm) was studied using high spatiotemporal resolution optical microscopy to gain insight into the mechanisms involved in response to surface topography in absence of motility. Population-level cell behavior on nanofibers-coated surfaces was characterized using 24-hour dynamic retention assays to quantify cell attachment. A biophysical model was also developed to describe the changes in the total free energy (adhesion energy and stretching energy) of the adherent *C. albicans* as a function of the diameter and spacing of the fibers, surface energies of the cell and substrate, and cell stretching modulus. Our single-cell experimental studies show that a non-Brownian biologically-driven motion enables *C. albicans* to adhere in distinctly preferred locations. Through biophysical modeling of cell-surface interactions, we demonstrate that these preferred adhesion locations minimize the total energy of the adherent cells. Comparison of the retention assay and modeling results shows that the cell attachment density trend closely correlates with the theoretically predicted adherent single-cell total energy. The nanofiber coating (1.2 µm diameter, 2 µm spacing) that maximized the total energy of the adherent cell resulted in the lowest microbial retention. Using our biophysical model, relative importance of the surface topography, and cell and surface physicochemical properties were explored and a set of non-dimensionalized curves was developed to cohesively relate all the variables. We show that *C. albicans* responds to surface topography by adjusting its geometry and relative position to minimize its total free energy. We further demonstrate that the single adherent cell total free energy quantification enables prediction of the population-level cell retention, which can be utilized towards *ab initio* design of surfaces that resist biofilm growth for medical applications and beyond.
**Session:** Poster Session 3  
**Date & Time:** Wednesday, October 10, 2018, 4:00 pm - 6:00 pm  
**Poster Board #:** 040  
**Topic:** Biofilm Metabolism  
**Abstract Title:** Deciphering a Global Role of RNA Chaperone Hfq in the *Pseudomonas aeruginosa* Transcriptome: Comparative CLIP-seq of Planktonic and Biofilm Forms  
**Authors:**  
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Small non-coding RNA (sRNA) associated with post-transcriptional regulations in bacteria has attracted attention for its role in the control of bacterial metabolism and adaptation to stressful environments. In *Pseudomonas aeruginosa*, some general sRNAs play an important role in biofilm development and adaptation. For example, RsmA protein titrating sRNAs RsmY/Z indirectly and positively regulate biofilm formations. Generally, RNA chaperone Hfq helps sRNA associate with target RNAs and modulate translation efficiency depending on the physiological state. In a previous report, the comparative RNA-seq of wild type *P. aeruginosa* PAO1 and Δhfq strain revealed that Hfq regulates numerous biological processes. However, since comprehensive Hfq-dependent RNA targets are still elusive, we sought to understand the global role of Hfq in planktonic and biofilm forms in the *P. aeruginosa* PAO1 transcriptome. To reveal Hfq-dependent RNA targets and background RNA expression, in vivo UV crosslinking immunoprecipitation followed by high-throughput sequencing (CLIP-seq) and total RNA-seq were performed in *P. aeruginosa* PAO1 late-exponential cultures and colony biofilms. In the CLIP-seq, UV crosslinking and coimmunoprecipitation were applied to efficiently recover the Hfq-RNA complex. Furthermore, this approach can be used to identify binding sites at high resolution by an RNase treatment process. Next-generation sequencing followed by bioinformatics analysis revealed various genes and biological processes under the control of Hfq in the two physiological states. The difference in known *Pseudomonas* sRNA affinities was estimated by disentangling Hfq peaks from background expression, and the results suggested that sRNA PhrS and PrrF1 have significantly high affinities in planktonic and biofilm forms, respectively. Affinity variations depending on physiological states may be due to target RNA expression, a fact reminiscent of the target-centric perspective in higher eukaryotes. Furthermore, CopraRNA, a tool for sRNA target prediction, was used for five known *Pseudomonas*sRNAs: ErsA, NrsZ, PhrS, PrrF1, and RgsA. The comparison between CopraRNA predictions and Hfq peaks detected by CLIP-seq extracted novel sRNA targets with previously validated ones. Intriguingly, Hfq associates with some tRNAs specifically in the biofilm, despite there being no significant difference in the expression of these tRNAs between the two phenotypic conditions. The global screen thus suggested novel post-transcriptional regulation in *P. aeruginosa* metabolism and supported a critical role of Hfq during the adaptation to biofilm environments. The current work has focused on validating how sRNA-target interactions affect phenotypic properties.
Background: Because current wastewater treatment processes often fail at removing anthropogenic chemicals, chemicals such as antibiotics are frequently discharged into the environment. To improve removal efficiencies, we need to better understand the metabolic capabilities of wastewater biofilms. For example, the role soluble and matrix-bound extracellular enzymes play in the biotransformation of chemicals during biological wastewater treatment is poorly understood. In this study, we aim to explore the roles of different fractions of enzymes in the biotransformation of selected antibiotics. We anticipate that a fast, extracellular transformation of antibiotics reduces the formation of antibiotic resistance in both wastewater treatment plants and environmental systems.

Methods: We used high-resolution mass spectrometry to measure biotransformation rates and transformation pathways for six amide-containing antibiotics. To assess the contribution of different enzyme fractions to the biotransformation of these antibiotics, we developed a method to separate soluble and matrix-bound enzymes from the entirety of enzymes from wastewater microbial communities derived from full-scale wastewater treatment plants. Furthermore, we used high-throughput fluorescence-based techniques to test for a link between peptidase activity and antibiotic transformation rate.

Results: We found that the removal rate constants in biological wastewater treatment strongly differed among the tested antibiotics. Interestingly, the observed trends were similar across wastewater treatment plants that were operated under different conditions. Furthermore, we found that while all tested antibiotics were to some extent transformed in intact sludge, only a small subset of the antibiotics was transformed by extracellular enzymes attached to the extracellular matrix of the wastewater biofilm. None of the tested antibiotics were readily transformed by the soluble enzymes. This finding correlated well with the measured peptidase activities, which decreased in the following order: intact sludge, matrix-bound enzymes, soluble enzymes.

Conclusions: Assuming that the rate of antibiotic resistance formation negatively correlates with the removal rate of the antibiotic during wastewater treatment, the observed difference in transformation rates highlights the importance of considering biotransformation rates when selecting antibiotics for use in human or veterinary medicine. The approach presented here is furthermore key for other metabolomics experiments assessing the transformation potential of extracellular enzymes in biological wastewater treatment plants. Knowledge on the susceptibility of certain functional groups to biotransformation will improve the design of chemicals for a sustainable future and will improve our understanding of this important biofilm system.
Abstract: Characterization of the Role of \textit{pstB1} in \textit{Enterococcus faecalis} membrane Stress and Biofilm Formation

\textit{Enterococcus faecalis} is a gram-positive commensal resident of the gastrointestinal tract that has emerged as a leading healthcare-associated pathogen. \textit{E. faecalis} clinical isolates are often resistant to antibacterial agents and readily form biofilms, making enterococcal infections difficult to treat. Understanding the genetic and regulatory mechanisms that enable \textit{E. faecalis} to form and maintain biofilms will be critical for finding novel methods to treat \textit{E. faecalis} infections. Through the use of recombination-based \textit{in vivo} expression technology (RIVET) screens, our lab has identified specific \textit{E. faecalis} promoters that are up-regulated in \textit{in vitro} biofilm assays and rabbit biofilm infection models (endocarditis and subcutaneous abscess). A putative promoter upstream of the \textit{pstB1} ORF was identified in all three screens. This suggests that expression of PstB1, which is annotated as an ATP binding protein in \textit{E. faecalis}, may be important in enterococcal biofilm formation. \textit{pstB1} is located within the \textit{pst-phoU} locus, a putative operon that encodes a predicted, well-conserved inorganic phosphate (Pi) ABC transport system. The \textit{pst-phoU} locus is known to play a role in Pi uptake in a variety of bacterial species. Furthermore, phosphate homeostasis is understood to be an important contributor to bacterial virulence, including biofilm formation. However, the roles of Pi and the \textit{pst-phoU} locus in \textit{E. faecalis} biology have not been determined. Therefore, we have generated an in-frame \textit{ΔpstB1} deletion mutant strain and have initiated phenotypic characterization studies to evaluate the role of \textit{pstB1} in \textit{E. faecalis} membrane stress and biofilm formation conditions. When compared to the wild-type strain (OG1RF), the \textit{ΔpstB1} strain exhibited increased susceptibility to bile salts and SDS when plated on solid medium. Unexpectedly, this phenotype was not present when the strains were exposed to bile salts or SDS during growth in broth. The wild-type and \textit{ΔpstB1} strains also had identical survival phenotypes in the rabbit subcutaneous abscess infection model, which indicates that \textit{pstB1} is not essential for \textit{in vivo} foreign-body abscess infection. In order to determine the role that Pi plays in \textit{E. faecalis} biofilm formation, we generated a reduced-phosphate BHI broth. OG1RF and \textit{ΔpstB1} grown in the reduced-phosphate BHI did not readily form biofilms compared to bacteria grown in normal BHI. Together these findings demonstrate that Pi plays an important role in the ability of \textit{E. faecalis} to form biofilms and that deletion of \textit{pstB1} contributes to membrane disruption by detergents on solid surfaces.
Abstract: The health and environmental hazards associated with water body contamination by industrial effluents cannot be over emphasized. This study investigated the bacteriological and physicochemical qualities of effluents from Ebonyi Fertilizer and Chemical Plant and the impact on Azuiyiokwu Creek, located in Abakaliki, Ebonyi State, Nigeria between May, 2010 and March, 2011. Standard microbiological techniques were used for the isolation and identification of microbial isolates. The physicochemical parameters detected from all the samples investigated include pH, Ca, Cu, Pb, Ni, As, Al, Hg, Co, Zn, Fe, Cr, Na, P, Mg, and Mn. The study shows that the concentrations of the metals were constantly higher in fertilizer effluents, than Azuiyiokwu Creek and the control. There was a significant difference (P≤0.05) in concentration of metals between fertilizer effluents, Azuiyiokwu and the Control Creeks. The results also show that metallic contaminations of Azuiyiokwu Creek could be as a result of the infiltration of effluents from Ebonyi Fertilizer and Chemical Plant into Azuiyiokwu Creek. The study revealed higher bacterial load in the Ebonyi Fertilizer and Chemical plant effluents than Azuiyiokwu and Control Creeks. There was a significant difference (P≤ 0.05) in the microbial counts between the Control and Azuiyiokwu Creek. Bacteria genera encountered in this study include: *Staphylococcus*, *Proteus*, *Klebsiella*, *Pseudomonas*, *Bacillus*, *Escherichia*, *Salmonella*, *Vibrio* and *Aeromonas*. This shows that the effluents received by Azuiyiokwu creek have low bacteriological and physicochemical qualities and the range of microorganisms isolated in this study raise more serious concern about the public health implications. This reflects the possible pathetic condition of most water bodies in Nigeria. Therefore, adequate measures should be put in place to help ameliorate the deplorable state of our water bodies. **Keywords:** abakaliki, azuiyiokwu, bacteria, effluents, fertilizer, physicochemical, water, nigeria
Abstract: Biofilms formed by nontypeable Haemophilus influenzae (NTHI) bacteria play an important role in a number of respiratory tract diseases, including chronic otitis media (OM) in children. We aimed to better understand the structure and formation of these biofilms by developing a computational model of NTHI biofilms based on statistical physics. The model was validated by closely comparing computational results to experimental images of in vivo NTHI biofilms recovered from the middle ears of the chinchilla model of experimental OM. NTHI biofilms are known to contain extracellular DNA (eDNA); in vivo the source of the eDNA includes both the host immune response and the bacteria themselves, and the eDNA network grows denser over the course of the infection. In our simulations, the bacterial growth was modeled with a given eDNA network in place, generated to mimic properties of eDNA seen experimentally, in order to understand the role of the eDNA network on the final biofilm morphology. The model is based on the following rules hypothesized to describe the behavior of NTHI: (1) biofilm-resident bacteria are resistant to the host’s immune response and do not die on the timescale of the simulation; (2) bacteria attached to the eDNA network can move to adjacent locations with eDNA via a “twitching” mechanism using type IV pili; and (3) bacteria disperse (become planktonic) via a quorum sensing mechanism, and these planktonic bacteria may be either killed by the host or reattach at other sites. Similar to what has been observed experimentally, the morphology was stable on the timescale of weeks. At intermediate times the in silico morphologies displayed fractal or self-similar organization of bacterial cells at the surfaces of the bacterial clusters. This was also observed in the analysis of confocal images of biofilms formed by NTHI in vivo. We also validated the model further by comparing model predictions regarding biofilm morphology at early stages (e.g., 4 days) of biofilm development. The fractal interfaces can potentially help NTHI survive in the nutrient poor host environment by increasing its exposure to nutrients, however this increased surface area may also increase exposure of biofilm-resident bacteria to antibiotics or immune effectors as well. We expect that simple models that reproduce known bacterial biofilm morphologies can deepen our understanding of bacterial behavior in biofilms, which may lead to improved, targeted treatments for NTHI-related diseases in the future. Funding source: This work was supported in part by the NIGMS grant R01GM103612 to JD.
Poster Session 3

Wednesday, October 10, 2018, 4:00 pm - 6:00 pm

045

Biofilm: From Nature to Models

Title: Anidulafungin Increases the Activity of Tigecycline against Staphylococcus aureus in Polymicrobial Candida albicans-Staphylococcus aureus Device-Associated Peritonitis

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The emerging use of medical devices encounters an increased occurrence of biofilms-related infections. Both the bacterium Staphylococcus aureus and the yeast Candida albicans are key players in the cause of hospital-acquired infections due to their extreme ability to inhabit diverse host niches especially in immunocompromised individuals. It is crucial to study and understand the behavior of these pathogens when coexisting together and to discover a viable option for treatment of not only single species but also mixed species biofilms. In the present study, we demonstrate for the first time the activity of the antifungal drug anidulafungin and the antibiotic tigecycline against dual species C. albicans - S. aureus biofilms developed in a novel model of intra-abdominal foreign body infection. We provide insight into the pathogenesis of this dual-species biofilm-associated infection. We show that this novel model of mixed biofilm infection is characterized by bacterial dissemination into the vital organs within 24 hours which remains persistent over 21 days. In addition, flow cytometry data reveals significantly greater neutrophil influx upon polymicrobial intraperitoneal device-associated infection in comparison to single species infection. In search of an effective treatment strategy we demonstrate that tigecycline acts synergistically when combined with anidulafungin against in vivo biofilms. We show that anidulafungin impaired synthesis of poly-β(1-6)-N-acetylglucosamine (PNAG), a major constituent of S. aureus biofilm matrix. Therefore, we hypothesize that the effect of anidulafungin on fungal and bacterial polysaccharides production may contribute to the synergism between these two drugs.
Abstract:

*Pseudomonas aeruginosa* frequently colonizes the respiratory tract of cystic fibrosis patients where excessive amounts of mucus build-up obstruct the airways. *P. aeruginosa* is known to form aggregates within this mucus plug, causing chronic infections. Previously, *P. aeruginosa* cultured in 0.8% w/v agar was observed to grow as suspended aggregates (Staudinger BJ *et al.*, 2014), which leads us to hypothesize that growth in high viscosity environments induce these aggregations. In this study, we further expanded this observation to better understand the characteristics of these aggregates. Based on our observations, we found that the aggregate sizes are dependent on the concentration of nitrate which serves as an alternative electron acceptor. Aggregates were small in the absence of nitrate and their sizes grew larger as nitrate concentrations were increased. This phenotype is overridden in part by the overexpression of pyocyanin, which may be suggestive of pyocyanin’s role in aiding *P. aeruginosa* anaerobic respiration. Furthermore, the aggregates do not require biofilm extracellular polysaccharides PEL and PSL to form. High c-di-GMP strains, which are well-characterized as hyper-biofilm forming strains due to the overexpression of PEL and PSL, have identical aggregate phenotypes, raising a provocative question of whether suspended aggregates are to be categorically classified as a type of biofilms.
**Abstract:**

**Background:** Catheter-associated urinary tract infection (CAUTI) is one of the most common nosocomial infections, resulting in over 560,000 infections, 8,000 deaths, and upwards of $1.7 billion in added medical costs each year in the US. Despite several decades of research, a urinary catheter designed to inhibit biofilm formation continues to elude clinical adoption. One reason for this poor track record relates to the in-vitro models employed for urinary catheter research, which have mostly relied upon nutrient-rich defined media, and laboratory bacterial strains. These in-vitro models poorly mimic in-vivo conditions under which CAUTI develops, and lead to failed therapeutic candidates in the clinical domain. To address this problem, we have devised a more clinically relevant in-vitro model for assessing biofilm inhibition on non-vital surfaces.

**Methods:** A total of 46 subjects met the clinical criteria for urinary tract infection (UTI) and were enrolled from an urban emergency department. 100 mL of UTI urine was collected and transported to the laboratory. 1 cm² flat silicone surface that was either uncoated or coated with one of two enzymes previously shown to inhibit biofilm formation were individually incubated in 5 ml of fresh uti urine for 4 days at 37°C with rocking. Subsequently, each silicone surface was then removed, stained with a fluorescent nuclear stain and imaged with an epifluorescence microscope. Biofilm images were evaluated with Image J software. Samples were stored and subsequently DNA was extracted for additional biofilm assessment using universal 16S primers in conjunction with ddPCR. Urine culture results were extracted from patient medical records for use in data analysis. Of enrolled subjects, 37 had culture results indicating uropathogens and were included in data analysis.

**Results:** Silicone surfaces coated with amylase, (active amylase has previously shown antibiofilm activity), demonstrated a significant increase in biofilm coverage whether all uropathogens were evaluated, or just those urine samples that grew out E. coli. Silicone surfaces coated with acylase, an AI-1 inhibitor, showed a similar trend which, however, did not reach statistical significance.

**Conclusion:** We have shown that biofilm formation on silicone surfaces by clinical uropathogens in a clinically relevant medium (UTI urine) can be assessed via image analysis. Further we have shown that engineered enzymatic surface coatings previously shown to inhibit biofilm formation by representative strains of biofilm-forming bacteria, did not inhibit biofilms on silicone surfaces in our model. Work to assess biofilms with greater sensitivity using ddPCR is currently ongoing.
Pathogenic biofilms are detrimentally relevant in over 80% of bacterial infections in the human body. Biofilms are composed of both bacterial cells and a bacterially-secreted extracellular matrix composed of multiple biopolymers. This “extracellular polymeric substance” (EPS) creates a barrier to protect the bacteria from many external threats including antibodies, and cellular arms of the immune system. More importantly, bacteria deep within the biofilm become metabolically quiescent due to nutrient limitation and thus trigger the stringent response. The upregulation of oxidative stress response genes as part of the stringent response makes bacterial biofilm infections resistant to even the strongest antibiotics. Because of this, it is estimated that biofilm infections cost the United States health care system on order of 5 billion dollars per year as they can only be treated through debridement. Developing new drugs against biofilms requires high-throughput methods for growing and analyzing biofilms. Standard methods involve growing bacterial strains in 96-well plates. This method has limitations i.e. bacteria cannot be cultured for an extended period of time. Currently only one system is available on the market which enables the growth of bacterial biofilms for long time periods in the context of a high throughput 96 well plate. Due to the lack of competition that system is expensive to purchase and designed for a single use. Proposed Solution: We have developed a technology, based on individual metal pins, that can be arrayed to fit into 96-well plated and which can be controlled (moved either individually or in full array mode) with magnets because of their ferromagnetic properties. We have shown that bacterial biofilms can be grow robustly and indefinitely on these metal pins, through the serial and facile transfer of the magnetic plate/pin apparatus to fresh media on a daily (or other regular) basis. The biofilms grown on the pins can be quantified by standard biofilm staining methods, confocal and electron microscopy. This technology gives the user the ability pull out and analyze any single pin, or combinations of pins from the 96 well plate without effecting any of the other pins. With current technology it would be necessary to brake and destroy the entire 96 plastic pin assembly just for the analysis of a single pin. In addition, the small metal pins are relatively inexpensive and furthermore they can be washed, sterilized and reused which greatly reduces the total cost of use. The usage of small electromagnets will allow future automation and greater possibility for high throughput screening. The technology was used to evaluate anti-biofilm properties of newly designed compounds (see D.C. Hall Jr. poster) and *E. coli* C biofilm (see J.E Krol poster).
Deep-bed biofiltration technology has been broadly applied in water industry for decades. Biofiltration can effectively remove soluble and particulate organics and nutrients using biofilms and their supporting media (e.g., granular activated carbon) through particle deposition, adsorption and biodegradation. During operation, biofilm growth and particle deposition will increase headloss across the filter, which can negatively impact contaminants removal. Periodic backwash of the biofilter is typically performed in response to headloss buildup. At many full-scale facilities, backwash has been a significant energy and maintenance burden. Thus, there is a need to develop strategies that optimize filter design and operation with respect to backwash requirements. However, the industry still currently lacks a systematic tool for helping biofilter design and optimization. In this study, we developed a biofiltration process kinetic model to quantify contaminant removal while simultaneously predicting headloss development. This model not only considered particle deposition and adsorption on filtration media, but also the biofilm growth during biodegradation of contaminants. The model includes inputs like flowrate, temperature, influent contaminants including organics (e.g., TOC, DOC or COD), nutrients (e.g., N, P), and suspended solids (TSS or turbidity), and can predict the headloss as well as contaminants profile along biofiltration depth and time. Application of the model to full-scale biofilter data was performed. Analyses indicated that the contributions of particle deposition on headloss accumulation were negligible in this system whose biofilter influent had low particulate content. Instead, biofilm growth was the key contributor to headloss accumulation in this system. Moreover, it was found that contaminants breakthrough could be attributed to the reduced hydraulic retention time caused by bed porosity decrease as a result of biofilm growth. The outcome of this study will shed light on prediction and optimization of headloss accumulation as well as contaminant control in deep-bed biofiltration for water and advanced wastewater treatment.
Abstract: Surgical site infections in orthopaedics are a great problem for both physicians and patients where infection rates as high as 30% lead to high morbidity and mortality. This study focuses on a clinically-relevant visual and quantitative model to understand the development of biofilms, and serve as a platform for testing eradication techniques. Utilizing Confocal Laser Scanning Microscopy and Scanning Electron Microscopy, we assessed the biofilm formation of Pseudomonas aeruginosa, Staphylococcus aureus, and Staphylococcus epidermidis on Polyether Ether Ketone (PEEK) in a spacio-temporal manner. Methods: Bacterial Culture and Biofilm Inoculation: P. aeruginosa, S. aureus, and S. epidermidis were propagated in Tryptic Soy Broth overnight at 37°C. Sterile PEEK disks were inoculated with a stock concentration of 1x10^8, 2x10^7 and 1x10^7 CFU/mL for S. epidermidis, P. aeruginosa and S. aureus, respectively, with rinses and media replacement at timed intervals. Biofilm Labeling and Confocal Imaging: Biofilms were stained with TOTO-1 Iodide to visualize Extracellular DNA, SYPRO Ruby Biofilm Stain for proteins, and Concanavalin A 635 conjugate for exopolysaccharides. Samples were imaged with an Olympus FV-1000 MPE Multiphoton Microscope. Scanning Electron Microscopy (SEM) Imaging: After confocal imaging, samples were imaged on a Hitachi 2700 SEM at both 1,000X and 5,000X magnification. Data Analysis: Images were processed via ImageJ (NIH) for the visual presence of each macromolecule, and quantification of the overall volume at each time point. Results: The biofilm formation of P. aeruginosa, S. aureus and S. epidermidis on PEEK were observed via the combination of CLSM and SEM. At 4 hours, P. aeruginosa permanently adheres to the substrate, demonstrating stage 2 biofilm. Subsequent time points, show the formation of microcolonies and organized vertical growth and exopolysaccharide secretion. of extracellular DNA, protein, and exopolysaccharide can be observed. SEM analysis of the same structures confirmed the presence of microfibers and matrix components. S.aureus biofilms form at a significantly slower rate than P. aeruginosa, but followed a comparative pattern of biofilm formation. Conclusions: The combination of CLSM and SEM allowed for informed assessment of a very dynamic biofilm development cycle. This approach allowed for the visualization and quantification of the biofilm composition in three dimensions and detailed topographical information for each stage of biofilm and a very dynamic cycle of biofilm formation on orthopaedic relevant materials, resulting in a platform for which to test irrigation and debridement techniques. References: 1. Darouiche RO. Treatment of infections associated with surgical implants. New England Journal of Medicine. 2004;350:1422-9.
**Abstract:** Biofilms associated with chronic disease states often exist as consortia of bacteria and other microorganisms reliant on mutualistic relationships to ensure growth and survival. In the oral cavity, biofilms are associated with many disease states. While there are many *in vitro* models used to study specific aspects of caries-associated biofilms, our study focused on developing a reproducible multispecies model within a 96 well microtiter plate to allow for high-throughput screening of anti-biofilm therapies. **Methods:** The microbial consortia developed in this model consisted of *Streptococcus gordonii* (DL1.1), *Streptococcus mutans* (UA159), and *Candida albicans* (SC5314) grown in 96 well microtiter plates for 24 h at 37°C and 5% CO₂. Nine common microbiological media were tested for mono- and multispecies biofilm growth and reproducibility. Additionally, inoculum concentrations and growth kinetics were evaluated for the multi-species biofilms. In addition to biofilm viability and biomass measurements, microenvironment pH and structural properties of the biofilms were assessed. **Results:** Our results indicate that the 1:1 RPMI:TSBYE 0.6% media supported growth of the mono-species and three concentrations of multispecies biofilm best of the nine media tested. This was assessed using both viability measurements and biomass over three independent studies. Inoculum studies confirmed the mutualistic effects of *C. albicans* with both *S. gordonii* and *S. mutans*. Overall the three concentrations of multispecies biofilms tested had increased viability and biomass production compared to the mono-species biofilms. Microenvironment pH measurements showed that *S. mutans* mono-species biofilms on average had the lowest pH over a 48-hour period. Structural assessments using rheology found that multi-species biofilm response to deformation was intermediate of the streptococci mono-species biofilms and *Candida albicans* mono-species biofilms. **Conclusions:** We have successfully developed a reproducible and physiologically-relevant in vitro caries associate biofilm model for high-throughput screening applications. The model uses a defined consortia and laboratory microbiological media to allow for consistent comparison of anti-biofilm therapies.
A Study of Antibiotic Susceptibility & Biofilm Production of Bacteria Isolated from Diabetic Foot Ulcer Patients

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Diabetic foot ulcers (DFUs) are the most common complications of diabetes. Ulcers of diabetic extremities are often followed by amputation due to treatment difficulties. Diabetic foot infections are commonly associated with a poly-microbial etiology (biofilm production). Antibiotic susceptibility profile of biofilm producers and non-biofilm producer isolates were performed against several antimicrobial agents (choice of which depending on Gram-reaction) using the Kirby-Bauer disk diffusion method on Muller Hinton agar. Three different methods were used to screen for biofilm formation by all isolates (quantitatively and qualitatively). The two quantitative methods were the microtiter plate biofilm formation assay (MTP) and Tissue culture plate method (TCP). In addition, qualitative detection of biofilm production was performed using Congo red agar (CRA) method. A total of 86 Gram-positive and Gram-negative bacterial isolates were recovered from 60 DFU patients (60 clinical isolates of fresh pus sterile cotton swabs). These non-healing diabetic foot ulcers were processed and yielded positive cultures. An average 1.43 organisms per specimen was obtained. 60% of the 60 clinical specimens yielded a single microorganism while the rest showed poly-microbial growth. In the microtiter plate method, it was shown that, 81.39 % of the 86 isolated Gram-positive and Gram-negative bacteria were biofilm producers. The tissue culture plate method showed that 83.7% of the isolates were biofilm producers. The qualitative Congo red agar (CRA) method of biofilm production by the isolates, indicated that 54 of 86 isolated bacteria were biofilm producers. Multi Drug Resistance (MDR) was in same frequent among Gram-negative and Gram-positive isolates. Multi-drug resistant organisms (MDROs) prevalence rate was 75.58% of overall. In addition, 89.2% of the MDROs were biofilm producers. Gram-negative bacteria were less prevalent than Gram-positive bacteria. Staphylococcus aureus was the most frequent Gram-positive microorganism, while Proteus mirabilis was the most frequent Gram-negative microorganism. There was no statistically significant association between biofilm formation and multi-drug resistance among isolates obtained from diabetic foot infections.
**Abstract:** The multidrug resistant opportunistic pathogen *Stenotrophomonas maltophilia* is a potent biofilm forming and gram-negative bacterium. It can contribute significantly to disease progression in cystic fibrosis patients, but it is also found in wounds and on catheter surfaces. For an improved understanding of processes and genes involved in the biofilm formation within the genus *Stenotrophomonas*, we are currently analyzing the genomes of over 350 clinical and 40 environmental isolates. In parallel we are investigating the biofilm profile of these isolates. Up to date we have established the genome sequences of over 100 isolates (Steinmann, Front Microbiol. 2018; 9:806) and the genome sequencing of additional 250 isolates is ongoing. The genome data together with the biofilm analysis and other phenotypic and metabolic data will generate the largest data set of *S. maltophilia* and its biofilm formation on a genus and pangenome-wide level.

**Methods:** Biofilm assays were done in microtiter plates and flow cell analyses. Genomes were sequenced using NGS technologies and phylogenetic trees constructed as previously published (Steinmann, Front Microbiol. 2018; 9:806). Transcriptomes were generated using RNAseq and *S. maltophilia* mRNA extraction protocols (Abda, Front. Microbiol. 2015; 6:1373). **Results:** The microtiter and flow cell analyses of 390 clinical and environmental *S. maltophilia* isolates revealed a strong variation in biofilm forming ability among the isolates. 13.5 % of all isolates formed very strong biofilms, while 10.4 % formed strong, 63.8 % formed moderate and 12.3 % formed rather weak biofilms. Clinical and environmental isolates did not differ in their biofilm formation abilities. Most interestingly, analyses of 3D-structures of biofilms grown in flow cells identified high levels of heterogeneity within the biofilm matrix and appearance independent of the strains and their phylogenetic position within the genus. Furthermore, no correlations between biofilm formation abilities, the 3D-structure and resistance to the antibiotic colistin were observed. Additional transcriptome data for selected isolates are underway to estimate the biofilm formation on a global level. **Conclusions:** *S. maltophilia* isolates both clinical and environmental display a diverse biofilm phenotype. Thus, we speculate that under *in vivo* conditions *S. maltophilia* also reveals varying biofilm architectures on a strain-specific level. The strain specific heterogeneity may just be another strategy to escape antibiotic treatment. The high variation in the 3D biofilm structures of different clinical isolates may correlate with *S. maltophilia* strain specific expression patterns.
**Abstract:** Skin burn wounds are highly susceptible sites for opportunistic colonization and biofilm formation by bacteria and fungi. About 10-20% of burn wound infections are caused by *Candida* spp. Although sepsis following failed treatment of such infection is not uncommon and may result in up to 70% mortality of the infected individuals, the factors contributing to burn wound candidiasis are currently unknown. Recent studies show that *Candida albicans* can rapidly neutralize the extracellular pH *in vitro* and within the macrophage phagosome. This process is controlled by the transcription factor Stp2 and results in a morphogenetic switch from yeast to the more virulent hyphal form. As an acidic pH is critical for wound healing, this study aims to investigate the virulence determinants of burn wound candidiasis, including environmental pH modulation by *C. albicans* at the wounded site. **Methods:** *C. albicans*-optimized luciferase was integrated in the *C. albicans* wild type (wt) SC5314 and in the pH modulation deficient *stp2Δ* strain. In order to identify the factors involved in burn wound candidiasis, we established protocols for two skin burn wound infection models: an *ex vivo* human native skin model and an *in vivo* rat burn wound model. Histopathological PAS staining and TUNEL staining using immunohistochemistry were used for analysis of skin damage and fungal burden, while progression and distribution of infection was monitored using D-luciferin. pH at the burned site was monitored to test if *C. albicans* infection could interfere with wound healing. **Results:** Our results show that the luciferase expressing *C. albicans* wt and *stp2Δ* strains have no phenotypic differences to the strains expressing empty vector. Application of $1 \times 10^5$ fungal cells/ml at the wounded site resulted in persistent infection, since the luminescence signals measured up to six days post infection were about ten times higher than the mock-treated skin. In contrast to the invasive burn wound infection with marked biofilm formation caused by the wt strain, infection with the *stp2Δ* strain was less aggressive. The pH at the burn site was lower in uninfected wounds and upon infection with the *stp2Δ* strain compared to wt infected burns, suggesting that *C. albicans* modulates the wound pH in a Stp2-dependent manner. **Conclusion:** *C. albicans* causes Stp2-dependent progressive burn wound infection and can interfere with the wound healing process by increasing the environmental pH.
The aims of this study were to evaluate the susceptibility of Klebsiella pneumoniae clinical isolates to antibiotics and biocides (benzalkonium chloride and deconex) under biofilm and planktonic conditions and also to monitor antibiofilm activities and biofilm eradication time of these two biocides. A total of 85 K. pneumoniae were isolated from patients in 4 referral hospitals in Kerman, Iran during six months. We found that, 15% (n=12) of the isolates showing strong, 40% (n=35) moderate, 30% (n=26) weak and 15% (n=12) no biofilm activities. Both the biocides had profound inhibitory activities on planktonic cells (average MIC 0.062±0.4 mg/ml for deconex and average MIC 0.031±0.1 mg/ml for benzalkonium chloride), however, exerted least antibiofilm effect at sub-MIC concentrations (0.015 mg/ml).

Nevertheless, biofilm formation reduced considerably in acidic pH and at low temperature (15 °C). Those strains that formed high amounts of biofilm also harbored cepA gene (p≤0.05), although some weak and no-biofilm formers also carried copA gene. Furthermore, we studied Minimum Inhibitory Concentration (MIC) of 12 antibiotics for the cells showing high amounts of biofilm under biofilm and planktonic cells growths, we found that there was considerable increase in MIC to piperacillin/tazobactam, tetracycline, chloramphenicol and cefotaxime for the cells taken from 24 h biofilm as compared to planktonic condition but all these isolates were sensitive to colistin and tigecycline. Most of the biofilms was eradicated from microtiter wells within 30 min exposure to these biocides. From above data, we suggest, benzalkonium chloride and deconex may serve as good hospital disinfectant for removal of planktonic but not biofilms related contaminations at in use concentrations.
Poster Session 3

Wednesday, October 10, 2018, 4:00 pm - 6:00 pm

Poster Board #: 056

Topic: Biofilms and Infection

Title: Antibody Fragments Directed against a Bacterial DNA-binding Protein Resolve Experimental Otitis Media

Author Block:

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Background: The DNABII family of DNA-binding proteins serve as critical structural elements to the extracellular DNA scaffold within biofilms formed by all 18 human bacterial pathogens tested to date. Removal of DNABII proteins, including integration host factor (IHF), from biofilms with antibody directed against the DNA-binding ‘tip’ region of this protein induces catastrophic collapse of the biofilm and release of resident bacteria. Biofilms confound the treatment of chronic diseases, including otitis media; therefore, this antibody-mediated biofilm disruption strategy has tremendous clinical utility. A potential adverse outcome of antibody therapy is development of neutralizing anti-antibody; therefore we examined the utility of antibody fragments, specifically the antigen-binding domains of anti-IHF IgG (i.e. ‘Fab’ fragments) to disrupt bacterial biofilms. Methods: Nontypeable Haemophilus influenzae (NTHI) biofilms were established in vitro and incubated with 5 μg Fab fragments directed against the DNA-binding tip domain of NTHI IHF, or as negative controls, either Fab fragments against carboxy-terminal (tail)-region of IHF or naive serum. Also, in an experimental model of otitis media, NTHI biofilms were first established in the middle ears of chinchillas, then Fab fragments were delivered directly to this site. Two doses were delivered at 24 hr intervals and animals sacrificed 1 or 7 days later. Results: In vitro, incubation of NTHI biofilms with 5 μg tip-directed Fab fragments resulted in a 78% reduction in biomass. In vivo, one day after receipt of the second dose of Fab fragments, a significant 3-log reduction in biofilm-resident NTHI and significantly less biofilm biomass was observed in the middle ears of animals administered tip-directed Fab fragments, compared to Fab fragments against the tail domain or naive serum. This outcome was enhanced over an additional seven days despite no additional treatment, thus residual NTHI within the middle ears of animals that received tip-directed Fab fragments did not re-form a biofilm. Moreover, whereas the middle ear mucosa from animals that received tail-directed Fab fragments exhibited considerable inflammation and middle ear fluids were enriched with pro-inflammatory cytokines, the mucosa from animals administered tip-directed Fab fragments appeared similar to that of a naive animal and the cytokine profile was anti-inflammatory. Conclusions: These data demonstrated the efficacy of IHF tip-directed Fab fragments to both resolve NTHI biofilms in vitro and in vivo. Moreover, the sole need of just the antigen binding domain of IgG and not the Fc portion recapitulates our model that binding of the DNABII proteins is the exclusive mechanism of biofilm disruption. As such, these Fab fragments could serve as a powerful biofilm-targeted therapeutic for resolution of recalcitrant diseases. Support: NIH R01 DC011818
Culture-based and Molecular Techniques in Biofilm Consortia

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Urinary catheter and ureteral stents insertion remains two of the most common urological procedures, simultaneously being considered a predisposing factor for urinary tract infection. We analyzed almost 350 urinary catheter- and stent-related samples, treated with sonication, using both routine culture and broad-range 16S rRNA PCR followed by Denaturing Gradient Gel Electrophoresis and Sanger sequencing (PCR-DGGE-S). The results of all methods were compared. In 338 positive samples, out of which 86.1% were polybacterial, 1295 representatives of 153 unique OTUs were detected. Gram-positive microbes were found in 46.5% and 59.1% of catheter- and stent-related samples, respectively. In comparison to standard culture, PCR-DGGE-S showed overall higher specificity (95% vs. 85%, p<0.01) and lower sensitivity (50% vs. 69%, p<0.01), making it appropriate as a complementary method for urinary tract-related sample analysis. This study extends our knowledge of biofilm consortia composition by analyzing large urinary catheter and stent sample sets using both molecular and culture techniques. This work was supported by grant of Ministry of Health of the Czech Republic (16-31593A).
**Abstract:**

**Background:** Chronic wounds affect over 6.5 million patients with an estimated $25 billion in healthcare costs, annually. Biofilms in chronic wounds significantly hinders wound healing. Recently, there have been several novel approaches to developing electroceutical wound dressings with electric currents to remediate biofilms. These dressings are at various stages of commercialization; however, a significant knowledge gap remains in how electrical current may impact bacterial biofilms in soft tissues, where there is diffusion limitation. In this study, we developed a simple agar based model to mimic biofilm growth on soft tissue using bioluminescent and antibiotic resistant strains of *Pseudomonas aeruginosa*. **Methods:** Two silver (Ag) electrodes were embedded under tryptic soy agar (TSA) polystyrene petri dishes and spread with PA to grow lawn biofilms for 24h. A 6V battery with 1kΩ ballast resistor was used to treat biofilms for 1 or 24h. Colony forming units (CFU) and scanning electron microscopy (SEM) was performed on lawn biofilms at 0 h before applying current, when current was stopped at 24h, and after incubation without current until 48h. EDS elemental analysis was performed to determine the distribution of elements within the agar to provide clues as to the nature of the inhibitory compounds. Since hypochlorous acid (HOCl) is produced when current is passed through a saline solution, we looked for the presence of 3-chlorotyrosine, which is formed when hypochlorous acid reacts with proteins by western blot. The amount of killing of PA biofilms was also determined in agar made with 40% human serum (HS) or bovine synovial fluid (BSF) to represent physiologically relevant chemistry. **Results:** Loss of bioluminescence with a significant 4-log reduction in CFU was achieved over the anode. Current treatment was also effective in reducing biofilms of antibiotic resistant strains of PA. SEM showed damaged cells and disrupted biofilm architecture. The antimicrobial activity continued to spread from the anode even 24 h after turning off the current. EDS suggested that silver was not responsible for biofilm killing. Chlorotyrosine was detected using western blot analysis suggesting the role of HOCl in the reduction of PA biofilms. **Conclusions:** The *in vitro* model could serve as a platform for fundamental studies to explore the effects of electrochemical treatment of biofilms by electroceutical dressings in a diffusion dominated environments such as found in wounds.
Abstract: Rapid Interstrain Recombination in Pseudomonas aeruginosa generates Adaptations to Biofilm Growth during Chronic Infections

Background: High biofilm forming variants have been associated with pathogen resistance to host immune defenses and antibiotics as well as worse outcomes in chronic infections like cystic fibrosis and wounds. A model microorganism for studying biofilms and their relevance to clinical outcomes is Pseudomonas aeruginosa. Loss of function screens and experimental evolution studies of Pseudomonas species have elucidated a number of well-characterized SNPs or short indels in genes encoding phosphodiesterase and/or diguanylate cyclase enzymes that increase intracellular cyclic-di-GMP production and confer high biofilm forming phenotypes. However, the contribution of recombination to cyclic-di-GMP-regulating pathways and biofilm regulation is largely unknown. Here, we present evidence for interstrain recombination as an underappreciated evolutionary mechanism for rapid adaptation to the biofilm lifestyle in chronic infections.

Methods: Six strains of P. aeruginosa, ranging from environmental to clinical isolates, were co-inoculated into a porcine full-thickness burn wound model. High biofilm producing stable variants were isolated 3, 14, and 28 days post infection and whole genome sequencing was conducted on representative isolates. Genetic targets of selection that conferred the high biofilm producing phenotypic change in the isolated variants were determined by short (Illumina) and long read sequencing (Oxford Nanopore).

Results: Of the six strains inoculated into the burn wound, only strains PA14 and PAO1 evolved high biofilm forming variants over the course of the experiment. Short read genome sequencing revealed a remarkable level of mutational parallelism in the well-characterized wsp pathway in PA14. However, no mutations were detected by short read genome sequencing in the high biofilm PAO1 variants. Long read sequencing and de novo assemblies revealed that recombination occurred in PAO1 with exogenous genetic material introduced from the four other P. aeruginosa strains that eventually went extinct over the course of the experiment. These recombination events were detected at the earliest sampling time, day 3 after infection, indicating rapid adaptation in the wound. The sites of insertion were most commonly in untranslated regions containing repeat sequences directly upstream of phosphodiesterase genes. These mutations are predicted to inactivate the phosphodiesterase, leading to increased cyclic-di-GMP levels and consequently high biofilm production. Therefore, these recombination events served primarily to disrupt existing genes rather than to provide new genetic material.

Conclusions: We propose that interstrain recombination is an important and often overlooked mechanism of rapid adaptive evolution, and that these mutations can cause drastic shifts in phenotypes associated with poor clinical outcomes.
**Abstract:**

**Background:** *Pseudomonas aeruginosa* is an opportunistic pathogen and a causative agent of persistent infections due to its capability to form biofilms. The self-produced extracellular matrix enables embedded bacteria to efficiently withstand antimicrobial treatment as well as host immune responses. The ecological success is based on the remarkable capability of *P. aeruginosa* to adapt and to survive in a broad range of diverse and challenging habitats. The large genome and the high proportion of transcriptional regulators enable a versatile lifestyle and flexible changes in bacterial behavior. **Methods:** In this study, we used confocal microscopy to determine the biofilm phenotypes of >400 clinical isolates. According to their biofilm structure, the isolates were grouped into three major clusters and extensively characterized in respect to various bacterial phenotypes. These included motility and virulence in the *Galleria mellonella* infection model. Furthermore, we analyzed the transcriptional profiles of the clinical isolates of the three distinct biofilm clusters under planktonic and biofilm conditions and performed global comparison studies. **Results:** The distinct biofilm clusters showed a remarkable correlation with an array of virulence-related phenotypes (e.g. *in vivo* virulence in *G. mellonella*, *in vitro* cytotoxicity, elastase secretion, proteolytic activity, pyocyanin production) as well as motility and c-di-GMP levels. Interestingly, transcriptome data recorded under biofilm- but not under planktonic conditions revealed group-specific transcriptional signatures. For each group, unique differentially expressed genes were identified, whereas all three biofilm clusters shared only a small biofilm core transcriptome. **Conclusions:** Our work reveals that discrete *P. aeruginosa* populations adapt specific biofilm phenotypes by following similar evolutionary paths. Most interestingly, no group-specific differences could be observed if the bacteria were cultured under planktonic conditions, indicating that acquired mutations shape the biofilm structure but do not impact growth under rich medium conditions. Distinct biofilm clusters show barely any overlap in the gene expression. Our findings indicate that the biofilm status is not per se selecting for a particular transcriptional profile.
**Abstract:**

**Background:** Chronic wounds affect approximately 2% of the worldwide population and incur healthcare costs in the billions. Owing to an aging population and a substantial rise in predisposing factors such as obesity, diabetes and cardiovascular disease, chronic wounds have been described as a silent epidemic, the full financial impact of which is immeasurable. Key to their persistence is the formation of microbial biofilms, which are accounted for in nearly 80% of all non-healing wounds. A paradigm shift in wound-care management has resulted in the emergence of smart dressings, which effectively monitor the wound condition without physical intervention. The smart dressing presented herein aims to detect a range of volatile infection protagonists, with a striking colour change that can be visualised with the naked eye, providing 24/7, non-invasive monitoring of biofilm development and antimicrobial treatment efficacy.

**Methods:** A porcine skin wound biofilm model was optimised and validated. A range of coloured indicator films housing dyes responsive to biofilm-derived volatile analytes in the wound headspace were developed and tested against porcine skin inoculated with two common wound pathogens, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Digital images of the indicator film were captured regularly over a 16-hour period and the resulting images were aligned and split into red, green and blue (RGB) colour channels to yield semi-quantitative data. Scanning electron microscopy and confocal laser scanning microscopy was used to verify the presence of biofilm on porcine wound explants. **Results:** A CO2-sensing film comprising xylene blue dye underwent a marked colour change from blue to yellow within 12 hours of inoculation with PAO1, whilst indicators monitoring uninoculated control skin remained blue (no colour change). Studies on indicator films monitoring *S. aureus* are ongoing. In addition, correlation of biofilm bioburden and indicator sensitivity data indicate a high level of sensitivity to the presence of *P. aeruginosa* in the wound model. GC-MS will be exploited to identify additional volatiles for incorporation into the smart dressing design. **Conclusion:** This *ex vivo* biofilm wound model is suitable for growing and studying pathogenic biofilms. This method is simple, reproducible and the materials used are affordable and easily sourced. The marked colour change exhibited by each indicator film is easily visualised by eye and can be digitally analysed to provide semi-quantitative data. This early warning, point-of-care technology is a promising candidate in combatting biofilm development in wounds.
**Abstract:**

**Background:** *Pseudomonas aeruginosa* and *Enterococcus faecium* are identified as the most common species isolated from complex polymicrobial wound environments of traumatically injured soldiers, 2011-2016 (TIDOS Study). In addition, at the point of injury, Coagulase-negative *Staphylococcus* spp. have also been identified. Each of these bacterium are independently known to utilize biofilm formation as a strategy to survive stressful conditions preventing the adequate delivery of antibiotics and promoting resistance. However, the interspecies ability to inhibit or exacerbate biofilm formation, within complex, polymicrobial communities, is unknown. The main objective of this project is to investigate the interspecies effects on biofilm production according to predetermined formula: Gram- + Gram- + commensal.

**Methods:** All the isolates used are clinical isolates provided by Multidrug Resistant Organism Surveillance Network. Seven strains of *P. aeruginosa* and 20 strains of *E. faecium* and *S. epidermidis* were tested in a compatibility assay. Strains that showed no antagonistic (i.e. growth inhibition) relationships were chosen to investigate the effects of two and three species polymicrobial biofilms compared to monomicrobial biofilms. Overnight cultures of each strain were grown and inoculum adjusted to ~1.3 x 10^6 colony forming units (CFU)/well. Each individual strain, and all combinations of two species and three species were grown for 24 and 48h in incubator (37°C, 5% CO2). At these time points, biomass was measured (OD600), numbers of surviving bacteria enumerated, and plates were stained with crystal violet to determine biofilm production (OD590). All experiments were performed in triplicate and results expressed as mean ± standard deviation. **Results:** *S. epidermidis*, inoculated singly, produces less biofilm than when grown in the presence of other bacteria. *S. epidermidis* CFUs significantly increase in *P. aeruginosa* co-infection, while their CFUs drop to less than half in *E. faecium* co-infection. When grown individually, *P. aeruginosa* has similar biofilm and CFU when grown with other species. *E. faecium* shows significant increase in all measured parameters when grown in the presence of *P. aeruginosa*. However, when grown with *S. epidermidis* absorbance values remain similar but CFUs are significantly less. **Conclusions:** Data provides evidence that there are varying effects on biofilm production, biomass, adhesion units, and CFU counts when comparing interspecies infections to monoculture and co-culture conditions. When considering two and three species co-cultures, biofilm properties and CFU counts (increase or decrease) based upon the specific species combination of bacteria present in the inoculum. Initial data must be further explored with regards to therapeutic development, clinical therapeutic application, and initial battlefield combat casualty care guidelines.
**Abstract:**

*Clostridium difficile* is a leading cause in antibiotic-induced diarrhea, particularly in hospitalized patients. Following antibiotic treatment, the native flora (gut microbiome) of the patient is eradicated, leaving *C. difficile* spores free to colonize the gut and cause persistent infection. The human gut microbiome provides some resistance to *C. difficile*; however, the specific intestinal bacteria that do this and their mechanisms of resistance are still unclear. Here, we are modeling microbial dynamics between beneficial and pathogenic cells involving *C. difficile* in artificial microniches. Using a microfluidic-based technique, thousands of nanoliter-sized polydimethylsiloxane (PDMS)-based microspheres are generated to encapsulate bacterial cells including *Escherichia coli* Nissle 1917, *Staphylococcus aureus* and *C. difficile*. PDMS provides mechanical and chemical transport properties, which enable the long-term study of microbiome dynamics such as *C. difficile* persistence in the mock community. After antibiotic treatment of the nanocultures, the nanocultures are imaged for a 24-hour period, capturing the microbial growth dynamics in the presence or absence of antibiotics effective against *C. difficile*. This platform has potential to investigate microbial pathogenesis by monitoring *C. difficile* sporulation and growth based on microenvironmental changes as occurring in the human microbiome.
Abstract:

Adhesion is a process that allows bacteria to attach to other cells and surfaces. It is an important step in the colonization and biofilm formation of a new host or environment and can contribute to bacterial pathogenesis. The aims of this in vitro study were to investigate a) the impact of bacterial binding on the viable counts in the surrounding suspension, b) the bacterial binding to wound dressing materials at different incubation times and c) the role of the surrounding environment on bacterial binding. The following hydrophobic dressing materials were evaluated for bacterial binding: a silicone film (SF, contact angle (CA) 124°), a polyurethane film (PUF, CA 105°) and a dialkyl carbamoyl chloride (DACC, CA 107°) coated dressing fabric. To evaluate the impact of the test medium, the dressing materials were placed on top of two different suspensions containing $10^5$ colony forming units (CFU) of *Staphylococcus aureus* and *Pseudomonas aeruginosa* either in Phosphate Buffered Saline (PBS) or Simulated Wound Fluid (SWF, 50% fetal calf serum and 50% Maximum Recovery Diluent) and incubated for 1 and 24 hours. Suspensions without any material served as controls. Bacteria adhering to the materials and remaining in suspension were quantified by serial dilution and plating. The results show that the quantity of viable bacteria in the surrounding suspension was barely affected by any of the tested materials, relative to the growth control. The only exception was for *P. aeruginosa* suspended in PBS, where a reduction of about 0.5 log units was observed in the suspension after 1 hour incubation. The reduction of viable counts in the suspension seemed to be affected by the growth dynamics of the bacteria in the suspension, rather than adhesion to the hydrophobic surface of the tested materials. Overall, the bacterial binding to the dressing materials did not significantly reduce the viable counts in the surrounding environment, nor were the materials capable of inhibiting growth over time. Furthermore, the results show that the test medium composition, the incubation time and type of bacteria seem to have a larger impact on the binding and proliferation than the dressing material itself. Proteins, as contained in the SWF, will also bind to the materials and may thereby alter the surface properties leading to a change in hydrophobicity and affinity towards bacterial binding. In addition, larger bacterial adhesion was observed with the longer incubation time, irrespective of the dressing material. This can be explained in part by more bacteria adhering to the surface over time but also by the proliferation of bacteria on the surface. The type of bacteria has very little impact on the binding at 1 hour. However, after 24 hours, *P. aeruginosa* was found to bind significantly more to all materials compared to *S. aureus*, which most probably is a result of the former’s higher capability of growing in the different test media.
**Background:** Biofilms are recognized across many industries as a costly, damaging, and potentially hazardous occurrence whose presence is often difficult to detect and treat. Although the important differences between biofilm-associated bacteria and planktonic bacteria are increasingly acknowledged by both industry and regulatory bodies, there are many applications that rely on monitoring of planktonic bacteria as a measure of the health of the system. This poses a challenge for innovative biofilm prevention or biofilm remediation technologies: how can the efficacy of products targeting biofilm be demonstrated most effectively, both in the lab and in the field? **Methods:** We use both (modified) standard lab-scale tests and tests adapted for possible use in the field, including the single-tube method, chlorine demand tests, as well as tests that probe material properties like heat transfer efficiency or flow. **Results:** We will discuss the ability of various tests to capture the level of biofilm present on a given surface (reported as CFU/surface area), with particular interest paid to how relevant that particular test may be to industrial applications.
**Abstract:**

**Background:** *Batrachochytrium dendrobatidis* (*Bd*) causes chytridiomycosis, a lethal amphibian skin infection. The fungus transitions from the flagellated motile zoospore to a sessile reproductive sporangium with rhizoids and may adhere to surfaces as a film. To study how the fungus survives in the absence of the host and based on our observations in growth media in the laboratory, we hypothesized that the chytrid forms a biofilm. Biofilms formed by microorganisms take multiple forms and serve a variety of purposes. **Methods:** In *vitro* biofilms were characterized using the crystal violet assays, light and electron microscopy, and gas chromatography/mass spectrometry. Differential expression of genes: zinc-responsive activator, polysaccharide synthase, alcohol dehydrogenase, chitin synthase were carried out using Real-time PCR. The resistance of biofilms, zoospores, and sporangia to a variety of treatments: high temperature, pH, sodium chloride, amphotericin B, and active ingredients of common spices: curcumin, 6-gingerol, and allicin was tested by measuring the cell viability using XTT ((2, 3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide) reduction. **Results and Discussion:** Kinetic analyses of biofilms showed the density-dependent biofilm formation and biomass increased over the time. Mature *Bd* biofilms displayed complex, a heterogeneous structure mainly consisting of different developmental stages of sporangia. Microcolonies embedded in a thin layer of extracellular matrix were clear in scanning electron and fluorescence microscopy. The glycosyl composition analysis of exopolymeric material revealed the composition of the matrix consisting of five relatively abundant sugars: ribose, xylose, mannose, galactose, and glucose. Overall, the expression levels of specific genes were higher in zoospores except chitin synthases were upregulated in biofilm and sporangia. This was expected as chitin synthases involved in the cell wall production when wall-less zoospores transition to sporangia and then to biofilms. Overall, biofilm-associated cells and sporangia showed higher resistant to high temperature and pH, and antifungal agents tested. Biofilm-associated cells were significantly highly resistant to temperature (28°C), pH (pH=9), and allicin (3.375 µg/ml). All cell types showed similar susceptibility to sodium chloride. Combination of all three phytochemicals at their MICs and MIC/2 equally affected all three cells types than individual chemicals. **Conclusion:** Our results strongly supported our hypothesis that *Bd* can form a biofilm. We propose that *Bd* biofilm formation may secure the survivability of the fungus in the absence of host by attaching to solid surfaces in ponds and streams while facilitating its group dispersal.
Ethanol Induces Biofilm Behaviors in *Pseudomonas aeruginosa*

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*Pseudomonas aeruginosa* causes infections in compromised individuals, burn wounds, and the lungs of individuals with cystic fibrosis (CF). In clinical settings, such as the CF lung, *P. aeruginosa* encounters other species of bacteria and fungi that produce bioactive metabolites including ethanol (EtOH). We show that when exposed to 1% EtOH, *Pseudomonas* induces a rapid and sustained induction of biofilm pathways even in planktonic cells. In medium with EtOH, cells have higher c-di-GMP (cdG) levels (2.5- and 1.9-fold (p<0.0001) at one and 16 hours, respectively) a second messenger known to negative regulate motility and promote biofilm formation. EtOH reduces WT *Pseudomonas* motility in a swim agar assay by 30% (p<0.0001), and a 2-fold increase in flagellar reversal rates is also observed (p<0.01). A genetic screen identified genes involved in the EtOH-induced repression of flagellar motility. We found that two diguanylate cyclases, SadC and GcbA, involved in the initial stages of biofilm formation and surface response, both participated in motility repression by EtOH and a ΔsadCΔgcbA mutant shows a greatly reduced stimulation of cdG in response to EtOH. We also found that the motility repression was dependent on two cdG effector proteins, FlgZ and PilZ.

The reduction of swimming motility in response to EtOH not only requires the stator complex, MotAB, but occurs via inhibiting proper flagellar function that rapidly transitions the cells to being immobile instead of motile. We have previously published that EtOH stimulates biofilm matrix production via WspR and we found that SadC, but not GcbA also participates in this response. In addition, we have identified upstream components, and domains within those components, that are necessary for the stimulation of cdG levels and the reduction in motility. Together, these data point to a complex network in which EtOH primes planktonic cells for the switch from a motile to a sessile lifestyle thereby promoting co-colonization of *P. aeruginosa* with EtOH-producing microbes. These data may lead to new insights into how mixed species communities in the CF lung lead to worse patient outcomes. The data may also point to the relevance of EtOH in biofilm initiation and maturation in disease.
**Abstract:**

*Myxococcus xanthus* possesses a form of surface motility powered by the retraction of the type IV pilus (T4P). Additionally, exopolysaccharide (EPS), the major constituent of bacterial biofilms, is required for this T4P-mediated motility in *M. xanthus* as the putative trigger of T4P retraction. The results here demonstrate that the T4P assembly ATPase PilB functions as an intermediary in the EPS regulatory pathway composed of the T4P upstream of the Dif signaling proteins in *M. xanthus*. A suppressor screen isolated a pilB mutation that restored EPS production to a T4P mutant. An additional PilB mutant variant, which is deficient in ATP hydrolysis and T4P assembly, supports EPS production without the T4P, indicating PilB can regulate EPS production independently of its function in T4P assembly. Further analysis confirms that PilB functions downstream of the T4P filament but upstream of the Dif proteins. *In vitro* studies suggest that the nucleotide-free form of PilB assumes the active signaling conformation in EPS regulation. Since *M. xanthus* PilB possesses conserved motifs with high affinity for c-di-GMP binding, the findings here suggest that c-di-GMP can regulate both motility and biofilm formation through a single effector in this surface-motile bacterium.
Revealing Bacterial Surface Structures with Interferometric Scattering Microscopy

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The surfaces of bacteria are covered with extracellular appendages helping them explore and physically interact with their surroundings. These thin structures are actively involved in a variety of behaviors such as swimming, cell-to-cell interaction, surface exploration and attachment. Visualization of these nanometric filaments is challenging and relies mainly on labeling. However, labeling protocols are invasive and often require mutations within filament subunits and visualization is subject to bleaching. Therefore, to observe dynamics of unperturbed extracellular structures, there is a need to develop a label free and more sensitive microscopy technique. Here we demonstrate label-free dynamic visualization of these filaments in live cells by interferometric scattering microscopy (iSCAT). iSCAT is more sensitive than any other “brightfield-like” method as it uses interference to highlight the photons scattered by small objects, for example pili. We adapted this method to the visualization of living cells by performing various modification decreasing its phototoxicity.

With iSCAT, we could visualize multiple structures involved in *Pseudomonas aeruginosa* biofilm formation. For example, we obtained high temporal resolution visualization of monotrichous helical flagella, from which we could observe its dimension as well as a translation of the patterns over time due to rotation. Furthermore, we could reveal type IV pili, which are micrometer-long retractile filaments only about 5 nanometers thick. TFP enable *P. aeruginosa* to explore surfaces through twitching motility during early biofilm formation. We could use iSCAT to visualize the dynamics of these filaments at high spatial and temporal resolutions. Using these visualizations, we could probe how TFP coordinate extension and retraction to optimize displacements onto the surface. Altogether, we demonstrate that iSCAT is a powerful microscopy technique to study extracellular structures of bacteria. We hope to expand the use of iSCAT to the visualization of the many other surface structures of bacteria.
Abstract:

Background: Bacterial surface proteins termed adhesins play essential roles in promoting host colonisation and biofilm-associate chronic infections. These adhesins include the well characterized >1 µm fimbrial adhesins along with their more abundant smaller ~10 nm non-fimbrial adhesin counterparts. The largest group of non-fimbrial adhesins include the autotransporters which overall are the most abundant outer membrane proteins in Gram-negative bacteria. Despite their prevalence and clear roles in host colonisation, there remains only one example of a molecular mechanism used by these highly prevalent adhesins to facilitate adhesion and biofilm formation. Self-association of the Antigen43a adhesin between neighboring uropathogenic E. coli (UPEC) cells was found to promote bacterial aggregation and biofilm phenotypes. Methods: We have employed a multidisciplinary approach that combines X-ray crystallography, analytical ultracentrifugation, ligand screening, binding assays, mutagenesis, mass spectrometry along with microbiology techniques, to further uncover new mechanisms of action for diverse autotransporter adhesins. Results: Our investigation has now revealed a second and completely new molecular function for an autotransporter adhesin. Our first X-ray crystal structure of UpaB from UPEC was found to incorporate unique features that allows it to interact with both human fibronectin and glycosaminoglycans, to promote direct colonisation of UPEC to the urinary tract epithelium. Furthermore, our latest research has finally revealed the molecular mechanism for yet another autotransporter adhesin TibA from enterotoxigenic E. coli (ETEC). Unusually, TibA had been found to be glycosylated by its own glycosyltransferase TibC. Further, TibA is a multifunctional autotransporter, that can promote both bacterial aggregation/biofilm formation in addition to binding/invasion of the intestinal epithelium. Our findings, which include crystal structures of TibA in both its glycosylated and unglycosylated forms, now show how TibA can mediate these two distinct mechanisms of action via regulation by glycosylation. Discussion: Overall, we have now elucidated in molecular detail two distinct mechanisms used by autotransporter adhesins to promote colonisation and biofilm formation on host surfaces. Furthermore, we have uncovered a new regulation system used to switch the function of autotransporter adhesins at the level of post-translational modification. These mechanisms and forms of regulation are likely to be found throughout other bacteria and provide missing details on bacterial adhesion and biofilm formation that could now be used to develop new types of therapeutics.
Background/Purpose: Dental caries is a polymicrobial infectious disease that affects the tooth. The disturbance of host-microbe homeostasis is the central factor initiating caries, there is a shift in microbial balance of the biofilm resulting in increased proportions of acidogenic and aciduric bacteria. It is a costly disease and a major problem to health service providers. The study isolates and identifies some microorganisms found in carious lesions and compares same with caries-free subjects in Enugu, Nigeria.

Materials and Methods: The study was carried out in the Microbiology laboratory of University of Nigeria Teaching Hospital Ituku/Ozalla Enugu. A total of 336 samples comprising of 211 patients with caries and 125 caries-free subjects were randomly collected from patients attending some dental clinics in Enugu metropolis. Cheesy portion of carious lesion was excavated with sterile excavator and then swabbed and processed. Standard cultures and biochemical techniques were used for isolation and identification. Standardized questionnaires were used to record the demographic data and other risk factors. Statistical analysis was done using Graph prism Version 6.

Results: All samples yielded microorganisms. A total of 635 and 254 microorganisms were isolated from carious lesions and caries-free subjects respectively. Of the 211, 207 (98.1%) were polymicrobial while 4 samples were monomicrobial. Lactobacillus spp (141, 66.8%) was the most predominant pathogen followed by Streptococcus mutans (104, 49.2%) Veillonella spp. 46 (21.8%), Candida albicans 38 (18.0%), Peptostreptococcus anaerobius 38 (18.0%), Actinomyces naeslundii 34 (16.1%). Streptococcus sanguis ranked highest (41, 32.8%) in caries-free subjects. The prevalence of caries was higher in females, though the difference was not statistically significant. Age was found to be a significant factor. A high prevalence occurred within the first 31 years with a cluster of cases between 16-31 years of age with prevalence tapering with advancing age.

Conclusion: Finding highlights the polymicrobial nature of carious lesion. There is need to consider the diversity of these microorganism in prevention and treatment of patients. Public awareness, introduction of oral health education in schools and use of good fluoridated tooth paste are advocated. Keywords: Biofilm, Dental caries, Lactobacillus spp, Streptococcus mutans.
The Role of Biofilm and Resident Bacterial Populations of Human Jawbone in Dental Implant Outcomes

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Abstract: Statistical ordination revealed ecological separation of different clinical groups. Native, pristine sites of congenital tooth absence provided benchmarked homeostasis as a health control. Surgical debridement beyond sclerosis progresses the clinical healing closer to the health controls. Surgically debrided groups ordinate closer to ecological health control with increased community diversity, suggesting surgical debridement beyond sclerosis may be responsible for microbial ecological shift and provides a mechanism for restoration of predisturbance condition - ecological resilience. It creates a “cleaner” healthy balanced microbial ecosystem, improves bone quality and osseointegration outcome, contributes to implant success. Non-debrided ordained groups have significantly different genera in their compositional structure to the health microbiota. These groups ordinate closer to disease with lower community diversity, ecosystem stability and osteolytic/osteosclerotic bone quality correlating with biofilm impaired healing. Conclusions: Dental implant failures are biofilm infections of the biomedical surface accompanied by chronic osteomyelitis. Sterile bone theory is invalid and spontaneous cure does not happen. Pathology in the dental implant bone bed is consistent with the expectations of biofilm science where a commensal (resident) bacterial biofilm population is subject to colonisation following disturbance and population shift.
Humidity is known to degrade Navy ship electronic equipment, especially in hot moist environments. If left untreated, it can cause significant and permanent damage. Even rigorous inspection and frequent clean-up would not prevent further equipment contamination and degradation because of the constant presence of favorable growth conditions for many microorganisms. Generally, relative humidity levels of less than 60% will inhibit corrosion in electronic equipment, but because NAVY electronics often operate in hot and humid environments, prevention via dehumidification is not always possible. Currently, there is no defined research that fully describes key mechanisms which cause electronics and its coating degradation. The corrosive action of most bacteria is mainly developed through (i) mycelium adherence to the metal plates, (ii) facilitation the formation of pitting areas, (iii) production of organic acids such as citric, iso-citric, cis-acontic, alpha-ketoglutaric, which are corrosive to electronic equipment and its components. Our approach studies corrosive action in electronic equipment: circuit-board, wires and connections that are exposed in the humid environment that gets worse during condensation. In our new approach the technical task is built on work with the bacterial communities in public areas, bacterial genetics, bioinformatics, biostatistics and Scanning Electron Microscopy (SEM) of corroded circuit boards. Based on these methods, we collect and examine environmental samples from biofilms of the corroded and non-corroded sites, where bacterial contamination of electronic equipment, such as machine racks and shore boats, is an ongoing concern. Sample collection and sample analysis is focused on addressing the key questions identified above through the following tasks: (1) laboratory sample processing and evaluation under scanning electron microscopy (2) initial sequencing and data evaluation; (d) bioinformatics and data analysis. Preliminary results from scanning electron microscopy (SEM) have revealed that metal particulates and alloys in corroded samples consists mostly of Tin (< 40%), Silicon (< 4%), Sulfur (< 1%), Aluminum (< 2%), Magnesium (< 2%), Copper (< 1%), Bromine (< 2%), Barium (<1%) and Iron (< 2%) elements. We have also performed X 12000 magnification of the same sites and that proved existence of undisrupted biofilm organelles and crystal structures (Fig 1). Non-corrosion sites have revealed high presence of copper (< 47%); other metals remain at the comparable level as on the samples with corrosion. We have performed X 1000 magnification on the non-corroded at the sites and have documented formation of copper crystals.
Enhanced Antimicrobial Production during Respiratory Virus Co-infection Promotes Bacterial Competition in Polymicrobial Biofilms

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Persistent polymicrobial infections are common in individuals with chronic respiratory disease, including chronic rhinosinusitis (CRS), chronic obstructive pulmonary disease and Cystic Fibrosis (CF). *Staphylococcus aureus* and *Pseudomonas aeruginosa* are the most frequently isolated bacterial pathogens from CF children and adults, respectively. *P. aeruginosa* acquisition correlates with seasonal respiratory virus infections, and CF patients experience severe exacerbations during viral coinfection. We observed *P. aeruginosa* biofilm growth on CF airway epithelial cells (AECs) is enhanced during respiratory virus coinfection and hypothesized virus infection also alters microbial community dynamics in the CF airways. Using co-culture systems, we observed that cultured individually with CF AECs, *P. aeruginosa* and *S. aureus* each exhibit enhanced biofilm growth on RSV-infected cells. However, when *S. aureus* and *P. aeruginosa* are co-cultured, RSV co-infection leads to a dramatic reduction in *S. aureus*. We observed *P. aeruginosa* exhibits enhanced production of the antimicrobial pyocyanin (PYO) during RSV co-infection. Exogenous PYO addition at concentrations observed during *P. aeruginosa*-virus co-infection decreases *S. aureus* biofilms on CF AECs. When co-cultured with a *P. aeruginosa* PYO mutant, *S. aureus* populations did not decrease during virus co-infection. We are currently investigating if this outcome is mediated by specific *P. aeruginosa* and host interferon-mediated antimicrobial mechanisms activated during respiratory virus infection. We observe RSV infection increases expression of the host interferon stimulated gene IDO1, resulting in elevated levels of the secondary metabolite kynurenine. IDO1 controls tryptophan metabolism during infection via the kynurenine pathway and has antimicrobial effects towards a range of pathogens, and *P. aeruginosa* utilizes kynurenine to produce the quorum-sensing signal PQS, which regulates PYO production. Over-expressing IDO1 in CF AECs increases apical kynurenine, and co-culture on IDO1 over-expressing cells leads to enhanced PYO production and a decline in *S. aureus* populations, as observed during virus co-infection. Dual host-pathogen RNA sequencing to evaluate transcriptomic changes in CF AECs, *P. aeruginosa* and *S. aureus* during virus co-infection confirmed that in CF AECs, expression of IDO1 and SLC6A14, encoding the kynurenine transporter hATB⁶⁺, is increased during co-infection with all three pathogens. *P. aeruginosa* genes *pqxABCD* and *phz*M also show increased expression, supporting our hypothesis that these pathways mediate antimicrobial activity during polymicrobial infections. This work will further our understanding of how virus coinfection alters interactions between bacterial pathogens and shapes host-associated polymicrobial communities.
**Background:** In Cystic Fibrosis (CF), the lung microbiome becomes dysbiotic because of a mutation in the gene for the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR), a chloride channel, leading to bronchiectasis, inflammation and oxidative stress. A hallmark of CF disease pathology is chronic *Pseudomonas aeruginosa* lung infections characterized by bacterial growth in biofilms. Biofilms provide protection from innate immune defense, antibiotics, and facilitate production of high titers of virulence factors. Virulence factors can be directly toxic to the host or can act indirectly to influence innate immunity. For example, virulence factors such as Cif protein can exacerbate CFTR trafficking issues and modulate host-derived inflammatory mediators (oxylipins), leading to chronic inflammation. The objective of the present study was to determine the effect of *P. aeruginosa* biofilms on activation of innate immunity in CF host bronchial epithelium (CFBE).

**Methods:** CFBE41o- cells, homozygous for the ΔF508 mutation, and CFBE41o-pCEP-WT cells, complemented with WT-CFTR, were provided by Dr. Dieter Gruenert at the University of California San Francisco. *P. aeruginosa* PA14 and a Cif deletion mutant (ΔCif) were provided by Dr. George O’Toole at Dartmouth University. In all studies, CFBE were grown as monolayers using standard cell culture techniques. Separately, *P. aeruginosa* biofilms were grown in artificial sputum media (SCFM2) and cell-free supernatant was collected. Then, CFBE monolayers were treated with biofilm supernatants. Following co-culture, culture medium and cell lysates were collected and analyzed. Non-targeted and targeted metabolomics were performed on biofilm supernatants and culture medium, respectively, to reveal the suite of virulence factors produced and their subsequent effect on pro-inflammatory oxylipin profiles. qRT-PCR and Western blotting were used to determine effect of co-culture on oxylipin metabolic gene and enzyme expression. Finally, Redox Western blots were performed to look at the effect of biofilm supernatant treatment on compartmental redox balance. **Results:** CFBE41o-CFBE challenged with *P. aeruginosa* PA14 biofilm supernatants display lower oxylipin metabolic gene expression, protein expression, and a lower titer of pro-inflammatory oxylipins compared to CFBE41o-pCEP/WT CFBE. Additionally, increases in mitochondrial, cytosolic, and membrane oxidation were observed following treatment. When CFBE41o-CFBE was challenged with ΔCif-PA14 biofilm supernatants, levels of innate immune activation were increased and oxidative stress was decreased compared to WT-PA14 biofilm supernatant treatment. **Conclusions:** Cif protein and other enzymatic virulence factors represent a key link between chronic infections and the damaging inflammatory and oxidative environment observed in cystic fibrosis.
**Session:** Poster Session 3  
**Date & Time:** Wednesday, October 10, 2018, 4:00 pm - 6:00 pm  
**Poster Board #:** 076  
**Topic:** Regulation of Biofilm Development  
**Abstract Title:** The Impact of Chlorine on the Microbiome of Drinking Water Biofilms  
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**Abstract:** Disinfection residuals (commonly chlorine) are used to manage the planktonic microbiology of drinking water distribution systems (DWDS) in order to safeguard water quality and public health. However, the majority of the DWDS microbial load exists in biofilms that form on the inner pipe-wall. Biofilms are ubiquitous and can degrade water quality during its transportation through the DWDS. In particular, biofilms are emerging as important drivers in the accumulation and release of material which causes discoloration of drinking water, a leading example of water quality degradation worldwide. A common perception is that disinfectant residuals convey some level of protection/regulation against biofilm development but the impact of chlorine concentration upon biofilms and the discoloration risk they present is unknown. This study reports results from a full-scale DWDS experiment facility in which biofilms were developed under different Chlorine regimes; Dechlorinated (low/no chlorine), No-dosing (medium chlorine) and Chlorine-boost (high chlorine). At the end of a 28-day growth phase, the bacterial and fungal communities of biofilms from each chlorine regime were quantified (via qPCR) and their taxonomic composition compared (via Illumina Mi-Seq). After the growth phase, biofilms were exposed to elevated shear stress forces to determine their discoloration response in the bulk-water, quantified via turbidity, iron and manganese concentrations. Increasing the chlorine concentration reduced biofilm bacterial concentration but did not affect fungal quantities. All biofilms were dominated by bacteria genes and the chlorine regime shaped the community structure and composition of both bacteria and fungi. Certain taxa were common to biofilms from each chlorine regime but their abundance was influenced by the chlorine residual such that at the end of the growth phase the communities from each regime were distinct, potentially presenting a different risk to water quality. Surprisingly, the greatest discoloration response was observed from the Chlorine-boost regime, which supported the lowest concentration of microorganisms in the biofilm. Overall, these results offer critical insights into the interactions between chlorine and the DWDS biofilm microbiome and highlight that chlorine boosting should be considered carefully. The data generated has potential implications for management of DWDS biofilms, particularly disinfection control strategies.
Background: Bacillus subtilis has been considered a model organism to study formation of complex multicellular structures called biofilm. Impaired respiration is one of the environmental signals triggering biofilm formation in B. subtilis. Vitreoscilla hemoglobin (VHb) is known to supply oxygen to respiratory chain and hence improves aerobic growth of variety of bacteria including Bacillus spp. Although VHb improves respiration, very little efforts have been made in elucidating its effect on biofilm formation. Here, we report the effect of incorporation of VHb on biofilm formation in B. subtilis DK1042 in different conditions. Methods: B. subtilis DK1042 was genetically modified to develop two Integrants NRM1113 and NRM1114 containing vgb-gfp operon under 2 and 5 copies of P43 promoters, respectively, at amyE locus by double cross over events. Promoter strength was characterized by measuring the GFP fluorescence of both the integrants. Effect of VHb on biofilm formation by integrants and wildtype (WT) was assessed on both solid and pellicle biofilm in lysogeny broth (LB) and LB supplemented with 1% glyceol and 0.1 mM manganese (LBGM). Pellicle biofilms were assayed by crystal violet staining method. Solid biofilms were monitored for the development of complex architecture. Sporulation efficiency was determined by plate count method. Effect of VHb was also monitored by exposing the cells to 6 % salt stress in solid LB medium. Results: Time dependent analysis of cell growth and fluorescence of B. subtilis integrants revealed that integrant NRM1114 having higher copies of P43 promoter upstream of vgb-gfp operon showed two fold increase in fluorescence/OD600 as compared to NRM1113 in both minimal medium and Luria Bertani medium. Moreover, production of brown pigment upon sporulation was reduced in integrants as compared to WT in minimal medium. Biofilm formation was significantly reduced on LB and LBGM in integrants as compared to WT. Integration of vgb also decreased biofilm-associated sporulation in both the integrants as compared to WT on LB and LBGM agar as well as LB agar supplemented with 6% NaCl. Conclusion: Biofilm formation is an adaptation by microorganisms to withstand variety of environmental insults. Reduced biofilm formation and sporulation by both the integrants harboring vgb suggests better capacity to survive adverse environment. VHb is known to improve antioxidant status of the host cells. Thus, improved aerobic metabolism and detoxification of ROS by constitutive expression of VHb together contribute to maintaining the vegetative state longer than the WT. Incorporation of VHb in other Bacillus spp. may have similar beneficial effects during their sessile lifestyle in rhizosphere that may enhance their performance as Plant Growth Promoting Rhizobacteria (PGPR). It would be interesting to understand the molecular processes involved in VHb modulated biofilm formation.
Potential of Marine Actinomycetes for the Reduction of Biofilm Formation

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Background: Biofilms are complex communities of microorganisms embedded in extrapolymeric substances (EPS) matrix. Due to their inherent resistance to antimicrobial agents and their ability to form on a variety of surfaces, biofilm formation poses a serious problem to the industry, marine transportation, public health, and medicine. Although majority of clinically useful drugs have been obtained from terrestrial natural sources, recently marine actinomycetes are being tapped as the new emerging and underdeveloped source of novel compounds with promising pharmaceutical potentials. Marine bacteria produce antibacterial compounds that may inhibit human pathogens and detrimental biofilm formation. Up to date, few studies have been reported on the isolation of actinomycetes from marine environments most especially in mangrove ecosystem. Material/methods: In this study, nine isolates of actinomycetes were isolated from soil and sediment samples in a mangrove swamp. Morphological and biochemical characterizations have been performed resulting to five different actinomycete isolates coded as BA01, BB02, CB02, CC03, and CG07, respectively. Each isolate was tested for antibacterial activity against the known biofilm forming bacteria; *Escherichia coli* (ATCC 8739), *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 25923), *Bacillus subtilis* (ATCC 6633), *Salmonella typhimurium* (clinical isolate), and *Klebsiella pneumoniae* (clinical isolate). Results: Isolates BA01 and BB02 exhibited activity against *B. subtilis* and *P. aeruginosa*. The rest of the isolates (CB02, CC03, and CG07) did not manifest any effects on the test bacteria. Each of these 5 isolates was mass produced in starch casein broth, using ethyl acetate as the extracting solvent and then further tested for antibacterial activity using the disc diffusion assay. Ethyl acetate extract showed partially active to active activity against *B. subtilis* and *P. aeruginosa*. Biofilm inhibition assay were done using the 96-well microtitre plate. All of the isolates were able to inhibit the biofilm formation of the test organisms but greater inhibitions were observed in *E. coli*, and *B. subtilis* most especially *P. aeruginosa*. Conclusions: These five marine Actinomycetes can be utilized further for the discovery of pharmaceutically important compounds.
**Session:** Poster Session 3  
**Date & Time:** Wednesday, October 10, 2018, 4:00 pm - 6:00 pm  
**Poster Board #:** 079  
**Topic:** Regulation of Biofilm Development  
**Abstract:**

**Title:** In Response to Phosphate Starvation, *Escherichia coli* O157:H7 Modifies Outer Membrane Components that are Involved in Biofilm Formation  
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Under nutrient-deprived conditions bacteria can grow as biofilms. These structures provide protection against physical stresses and allow slowed growth and metabolism, which extends survival. In open environment such as water, *E. coli* O157:H7 respond to the phosphate (Pi) starvation by inducing the Pho regulon controlled by PhoBR. The Pst (phosphate specific transport) system serves as a sensor of the extracellular Pi concentration. In the *pst* mutant, the regulator PhoB is constitutively activated and the expression of genes from the Pho regulon is modulated. O157:H7 *E. coli* strain EDL933 grown in low Pi condition and its *pst* mutant grown in excess of Pi displayed an increased ability to auto-agglutinate and to form biofilm. Analysis of double *pst phoB* mutant indicated that the increased biofilm and auto-agglutination phenotype were dependent on *phoB*. The goal was to identify specific *E. coli* factors that are involved in the increase of biofilm formation in response to phosphate starvation. 5118 transposon mutants derived from the *pst* mutant of EDL933 were screened to isolate 30 mutants defective in auto-agglutination and biofilm formation. Transposon insertion sites were identified by high-throughput sequencing. The transcriptome profiles of the *pst* mutant and its parental strain were also compared to each other. O157 LPS modifications were analysed by SDS-PAGE and MALDI-TOF. For several auto-agglutination and biofilm defective mutants, the transposon was inserted in genes *waaF, waaC, waaD, waaE, waaG* and *waaQ* that are involved in the synthesis of the lipopolysaccharide (LPS) core. Global transcriptomic studies of *pst* mutant revealed that genes involved in the biosynthesis and export of LPS were down regulated. Some of these genes contain Pho box binding sites. LPS analysis by SDS PAGE had shown that the O chain was absent and lower bands corresponding to rough types LPS were observed. Absence of O antigen units was confirmed by immunoblotting and MALDI analysis. The main LPS molecular species corresponded to R3 core. Thus the LPS core of *pst* mutant is truncated and lacks the O157 antigen. Our results suggest that truncated LPS core plays a role in the biofilm and auto-agglutination phenotype of *pst* mutant. Although *waaH* gene, responsible of glucuronic acid (GlcUA) core modification of *E. coli* K12 strain, was highly upregulated in *pst* mutant, addition of GlcUA on LPS was not detected. Nonetheless, *waaH* mutant in low Pi and *pst waaH* double mutant displayed a decreased ability to form biofilm suggesting a role for this glycosyltransferase in biofilm formation in response to Pi starvation. In response to low Pi environment, *E. coli* O157 outer membrane undergoes several modifications controlled by the Pho regulon leading to increase the potential of biofilm formation. The biofilm ability of *E. coli* O157 may play a role in its transmission, persistence and virulence.
Abstract:

Background: *Porphyromonas gingivalis* is a Gram-negative anaerobe that persists within the subgingival biofilm adjacent to the epithelium in the human oral cavity. This bacterium is asaccharolytic, but highly proteolytic; it utilizes protein substrates as a main source for energy production and growth. A type IX secretion system (T9SS) has been shown to be essential for the secretion of a variety of proteases and protein modifying enzymes. Included in this repertoire are the arginine and lysine gingipains as well as a unique peptidylarginine deiminase (PPAD). PPAD is an enzyme that converts positively charged arginine residues within proteins to neutral citrulline residues, which can subsequently affect the conformation and function of proteins. Although the removal of free L-arginine from the environment has been shown to alter *P. gingivalis* physiology and inhibit biofilm formation, the impact of removing peptidylarginine via citrullination is not known. Here we report that the increased presence of peptidylarginine in a PPAD deletion mutant (Δ8820) enhances biofilm formation. **Methods:** Biofilm formation by the wild type (strain 381) and Δ8820 was measured by staining with safranin and with the LIVE/DEAD BacLight Bacterial Viability Kit. PPAD and gingipain enzymatic activities were measured using colorimetric assays. Fimbriae and adhesin protein levels were assessed under different growth conditions using western blot analysis. Mass spectrometry was performed on cell lysates to identify citrullinated proteins. 381 and Δ8820 cells from colony biofilms grown on plates were observed by transmission electron microscopy (TEM). **Results:** During early stages of biofilm development, more Δ8820 cells attached to surfaces compared to the parent strain 381. Mature Δ8820 biofilms were comprised of a higher density of cells, larger microcolonies, and less void space. Gingipain-derived adhesin proteins were predicted by mass spectrometry to be citrullinated and citrullination of these proteins by strain 381 was confirmed in vitro. Furthermore, Δ8820 biofilms contained more gingipain-derived adhesin proteins than 381 biofilms. Surprisingly, Δ8820 colony biofilms produced extracellular fibers that looked to form a matrix when observed by TEM. **Conclusions:** The enhanced biofilm phenotype of Δ8820 is due, in part, to the lack of citrullination or retention of arginine residues within T9SS-secreted proteins known to play a role in attachment and biofilm formation, including gingipain-derived adhesin proteins. Overall, our data indicates that citrullination of T9SS cargo proteins modulates attachment and biofilm development in *P. gingivalis* biofilm.
**Session:** Poster Session 3  
**Date & Time:** Wednesday, October 10, 2018, 4:00 pm - 6:00 pm  
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**Topic:** Regulation of Biofilm Development  
**Abstract**  
Serine Hydroxymethyltransferase ShrA (PA2444) Controls Rugose Small-Colony Variant Formation in *Pseudomonas aeruginosa*  
**Title:** Formation in *Pseudomonas aeruginosa*  
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*Pseudomonas aeruginosa* causes many biofilm infections, and the rugose small-colony variants (RSCVs) of this bacterium are important for infection. We found here that inactivation of PA2444, which we determined to be a serine hydroxymethyltransferase (SHMT), leads to the RSCV phenotype of *P. aeruginosa* PA14. In addition, loss of PA2444 increases biofilm formation by two orders of magnitude, increases exopolysaccharide by 45-fold, and abolishes swarming. The RSCV phenotype is related to higher cyclic diguanylate concentrations due to increased activity of the Wsp chemosensory system, including diguanylate cyclase WspR. By characterizing the PA2444 enzyme *in vitro*, we determined the physiological function of PA2444 protein by relating it to S-adenosylmethionine (SAM) concentrations and methylation of a membrane bound methyl-accepting chemotaxis protein WspA. A transcriptome analysis also revealed PA2444 is related to the redox state of the cells, and the altered redox state was demonstrated by an increase in the intracellular NADH/NAD⁺ ratio. Hence, we provide a mechanism for how an enzyme of central metabolism controls the community behavior of the bacterium, and suggest PA2444 protein should be named ShrA for serine hydroxymethyltransferase related to rugose colony formation (*Frontiers Microbiol.* 9:315, 2018).
Abstract: *Pseudomonas aeruginosa* is an opportunistic pathogen that can form robust biofilms in environments as varied as domestic showerheads and human airways. Biofilm development involves discrete steps: initial surface attachment and commitment, followed by microcolony growth, biofilm maturation and dispersion. While the early regulatory steps of biofilm development have been explored, relatively little is known about the mechanisms required for *P. aeruginosa* to sustain a mature biofilm, a state associated with antimicrobial tolerance and environmental persistence. We have identified three genes that play putative roles in biofilm maturation - all three of these genes encode proteins that impact the level of the bacterial second messenger, c-di-GMP. Using 96-well and flow-cell based biofilm assays, we found that inactivation of these genes - PA14_7500 and *morA*, encoding proteins with motifs associated with both diguanylate cyclase (DGC) and phosphodiesterase activity (PDE), and PA14_10820, encoding a HD-GYP domain typically associated with phosphodiesterase activities - compromises biofilm architecture, resulting in the strains being unable to sustain a mature biofilm. Additionally, loss of these genes uncovered a non-canonical relationship between c-di-GMP signaling and biofilm formation whereby gene inactivation increased concentrations of c-di-GMP prior to the disintegration of the mature biofilm. These findings provide new insights into the genetic mechanisms through which mature biofilms are regulated and suggest a previously unknown role for these c-di-GMP signaling proteins in this process.
Title: Hfq-Assisted RsmA Regulation is Central to *Pseudomonas aeruginosa* Biofilm and Motility

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Abstract: Expression of biofilm and motility genes is controlled by multiple regulatory elements, allowing bacteria to appropriately adapt a sessile or motile lifestyle. In *Pseudomonas aeruginosa*, the post-transcriptional regulator RsmA has been implicated in the control of various biofilm- and motility-associated genes, but much of the evidence for these links is limited to transcriptomic and phenotypic studies. RsmA binds to target mRNAs to modulate translation by affecting ribosomal access and/or mRNA stability. Here we trace the global regulatory role of RsmA to the inhibition of Vfr - a key transcription factor required for efficient production of two other transcriptional regulators, namely FleQ and AlgR. FleQ and AlgR, in turn, directly control flagella and pili genes, respectively. FleQ also controls biofilm-associated genes that encode that PEL polysaccharide biosynthesis machinery. Furthermore, we show that RsmA cannot bind *vfr* mRNA alone, but requires the RNA chaperone protein Hfq. This is the first example where a RsmA protein family member is demonstrated to require another protein for RNA binding.
To permanently attach to surfaces and form robust biofilms, *Caulobacter crescentus* produces a strong adhesive, the holdfast. The timing of holdfast synthesis is developmentally regulated by cell cycle cues. When *C. crescentus* is grown in a complex medium, holdfast synthesis can also be stimulated by surface sensing, in which swarmer cells rapidly synthesize holdfast in direct response to surface contact. In contrast to growth in complex medium, here we show that surface contact does not trigger holdfast synthesis when cells are grown in a defined medium. In this study, we investigate the role of the flagellum and its rotation in adhesion and holdfast synthesis in *C. crescentus* under different nutrient conditions. We compare mutants lacking the flagellum (Fla−) or the stator required for flagellum rotation (Mot−) to wild-type cells. Though both mutants exhibit non-motile phenotypes, their adhesion phenotypes reveal several differences that are conditional upon media composition. Both Fla− and Mot− mutants display similar attachment deficiencies in complex medium, while Fla− mutants form more robust biofilms over time when grown in defined medium, despite impaired initial adhesion. We find that the overall enhanced adhesion phenotype in Fla− mutants in defined medium is due to an increase in the number of cells that synthesize holdfast, and to a premature production of holdfast during the cell cycle. Our results show that the status of flagellum synthesis regulates holdfast production via transcriptional control of the holdfast inhibitor HfiA. *hfiA* transcription is reduced in Fla− mutants, and this reduction is modulated by the diguanylate cyclase developmental regulator PleD. Our data support a model in which flagellum assembly feeds back to control holdfast synthesis via HfiA expression in a c-di-GMP dependent manner under defined nutrient conditions.
Protein Lysine Acetylation Plays a Regulatory Role in Bacillus subtilis Biofilm Formation

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Protein lysine acetylation is a post-translational modification (PTM) that alters the charge, conformation, and stability of proteins. Published lists of acetylated proteins, or acetylomes, in bacteria have demonstrated that protein lysine acetylation occurs on proteins with diverse function, including central metabolism, DNA transcription and binding, chemotaxis, and cell size. Bacillus subtilis is a non-pathogenic soil bacterium, and model organism for sporulation, motility, biofilm formation, and multicellular development. The genetic regulation of biofilm formation has been extensively studied, however, regulation by PTM has not yet been explored. To further investigate protein acetylation, we generated an acetylome under biofilm-inducing conditions and identified acetylated proteins, many of which are involved in biofilm development. Various single and double mutants of genes known to encode enzymes involved in global protein lysine acetylation in B. subtilis demonstrated that lack of acetylation impacts biofilm development. Deletion of acuA and pta, the genes that encode an acetyltransferase and a phosphotransacetylase, respectively, showed a decrease in biofilm matrix production based on visible phenotype and quantitative β-galactosidase assay of matrix gene expression. Lastly, we targeted three known acetylated biofilm proteins; SinR, the biofilm master transcriptional regulator, YmcA, a biofilm regulatory protein, and GtaB, a UTP-glucose-1-phosphate uridylyltransferase that synthesizes a nucleotide sugar precursor for exopolysaccharide synthesis. To determine the importance of acetylated lysine residues for the function of these proteins in biofilm development, we performed site-directed mutagenesis of the acetylated lysine codons and assayed the cells containing those point mutants for biofilm formation. Results demonstrated severe biofilm defects upon mutation of lysine residues SinR Lys7 and Lys13, YmcA Lys64, and GtaB Lys89 and Lys191 suggesting the importance of these residues for protein function in biofilm formation. Here, protein lysine acetylation is presented as a potential regulatory mechanism for bacterial protein function during biofilm development.
Abstract:

**Background:** Polyamines are polycationic molecules that are important in a variety of cellular processes, including formation of bacterial biofilms. One enzyme that is present in a diverse set of bacteria, called SpeG, catalyzes the acetylation of these polyamines. We previously showed that SpeG is an allosteric protein that binds polyamines in both allosteric and active sites. While the *speG* gene has been shown to be important for pathogenicity for some bacteria, to our knowledge the role of the SpeG protein and its products are still unclear. It currently appears that different pathogens may use *speG* and SpeG in different ways. **Methods:** To gain a greater understanding of how the recombinant SpeG proteins from different pathogenic bacteria catalyze their reactions, we performed a kinetic characterization of the recombinant proteins and compared their kinetic parameters. **Results:** We found that turnover, catalytic efficiency, and sigmoidicity of SpeG from different pathogens varied even though they are homologs and catalyze the same reaction. Additionally, we found that pH affected these kinetic parameters. **Conclusions:** Our kinetic analysis of SpeGs from different pathogens show that the enzyme behaves differently across organisms and should be further studied to understand how these variances may contribute to polyamine levels and biofilm formation within each organism.
Background: Gradually increasing antibiotic resistance is a phenomenon observed worldwide in the past few decades that threatens to reverse the advancements of anti-infective therapies, a keystone of modern medicine in general. In addition, antibiotic treatment regularly fails to cure patients suffering from infections caused by adaptively resistant microbial communities, referred to as biofilms. Even though at least two thirds of all clinical infections are associated with biofilms, there are no biofilm-specific therapies on the market or in clinical trials. *Pseudomonas aeruginosa* is a remarkably antibiotic resistant, nosocomial pathogen and biofilm-former that causes morbidity and mortality especially in cystic fibrosis, nosocomial pneumonia and immunocompromised patients. This project aims to identify the genes associated with drug resistance in *P. aeruginosa* biofilms and to provide novel biofilm-specific targets for the design of potent drugs.

Methods: Genome-wide screens using transposon-sequencing (Tn-Seq) pools of *P. aeruginosa* strain PA14 and the cystic fibrosis epidemic isolate LESB58 were performed to identify genes involved in adaptive antibiotic resistance associated with biofilms. Tn-Seq mutants growing under planktonic but not under biofilm conditions will have lost the ability to form biofilms; thus the inactivated genes in these mutants are potential candidates required for biofilm formation. Treatment of biofilms with sub-inhibitory concentrations of antimicrobial compounds allowed only the survival of mutants retaining adaptive resistance, whereas more susceptible mutants survived within untreated biofilms. I will discuss regulatory genes involved in both processes as well as genes corresponding to the known resistome of antimicrobials. Results: Random insertion of promiscuous, mariner-based transposons into the genomes of *P. aeruginosa* strains PAO1, PA14 and LESB58 resulted in the generation of Tn-Seq pools that each contained more than 200,000 mutants. To identify and quantify transposon mutants present in the pools, we developed a method combining the amplification of transposon-genome junctions with high-throughput sequencing. Preliminary analysis of the three generated Tn-Seq pools suggested that ~90% of all genes were successfully mutagenized in each respective genome. Furthermore, biofilm growth conditions were established for an *in vitro* Tn-Seq screen of *P. aeruginosa* PA14 in synthetic cystic fibrosis medium on hydroxyapatite discs, a substrate mimicking bone tissue and teeth, and sequencing suggested nearly 600 genes were essential for biofilm formation including dozens of regulatory proteins. Conclusion: The *P. aeruginosa* Tn-Seq pools are enabling us to screen the whole genome for genes associated with biofilm growth and adaptive antibiotic resistance.
c-di-GMP Receptor PlzC Affects Diverse Cellular Processes in *Vibrio cholerae*

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Abstract:
A bacterial global second messenger cyclic dimeric guanosine monophosphate (c-di-GMP), play important roles in the physiology of many bacterial pathogens. c-di-GMP is produced by diguanylate cyclase (DGC) proteins containing the GGDEF domain and degraded by phosphodiesterase (PDE) proteins bearing the EAL or HD-GYP domains. Receptor proteins or RNAs sense c-di-GMP, and then interact with a downstream protein to affect a particular cellular function. PilZ domain proteins are a class of c-di-GMP receptors, which have RxxxR, and D/NxSxxG consensus motifs required for c-di-GMP binding. *Vibrio cholerae*, the causative agent of the disease cholera, has five PilZ domain proteins (PlzA-E); we do not yet know how PilZ domain containing c-di-GMP receptors controls specific downstream biological processes. In this study, we analyzed the role of PlzC in c-di-GMP-regulated cellular processes and found that ΔplzC mutant exhibit altered motility, biofilm formation, and whole genome expression profile compared with wild type. Additionally, we found that intracellular c-di-GMP levels of the ΔplzC mutant decreased compared to the wild type suggesting that PlzC can function not only as a c-di-GMP receptor but also as a modulator of cellular c-di-GMP levels. To investigate how PlzC regulates diverse cellular processes, we evaluated interactions between PlzC and a set of proteins involved in motility/chemotaxis and c-di-GMP synthesis/degradation using bacterial two-hybrid system. We found that PlzC interacts with specific c-di-GMP metabolizing enzymes. These results suggest that PlzC controls diverse cellular processes by modulating intracellular c-di-GMP levels and by participating in protein-protein interactions with DGCs, PDEs.
**Background:** *Bacillus thuringiensis* are ubiquitous Gram-positive bacteria that are agriculturally and medically important as they produce insecticidal Cry proteins, thus have bio-control applications. Previous studies reported that the ubiquitous carbon source glucose could induce restricted motility and fractal pattern formation in the growing colonies of the isolated pH, salt and arsenate tolerant *Bacillus thuringiensis*KPW.P1. As bacteria are evolved with the ability to exhibit multicellular behaviour and biofilm formation under harsh or limiting conditions as survival strategies, our present study was focused on exploring the effect of glucose in biofilm formation by *Bacillus thuringiensis* KPW.P1. **Methods:** Biofilm formation by *Bacillus thuringiensis* KPW.P1 was assayed in 24 well plates by Crystal Violet staining method. Scanning Electron Microscopy (SEM), Confocal laser scanning microscopy (CLSM) and cell surface hydrophobicity assay (MATH assay) were performed for visualization and characterization of KPW.P1 biofilms. Extra-cellular Polymeric Substances (EPS) in KPW1.P1 biofilms were characterized biochemically. **Results:** With respect to control, six times more biofilm load was marked for *Bacillus thuringiensis* KPW.P1 in presence of 2 % of glucose. Interestingly, it was observed that the effect was glucose specific as other sugars could not induce any significant increase in KPW.P1 biofilm load, although all sugars tested, could increase the planktonic growth of KPW.P1 in a dose-dependent manner. Scanning Electron- and Confocal Laser Scanning microscopic studies revealed increased densely packed microcolonies of KPW.P1 inside of exopolymeric substances (EPS) in presence of higher concentrations of glucose. It was also observed that increased glucose contributed increased EPS production by KPW.P1 and increased hydrophobicity and adherence properties in KPW.P1cells. **Conclusions:** The present study, clearly states that presence of glucose in growth media induces biofilm formation by *Bacillus thuringiensis* KPW.P1 and such effect is glucose specific as the presence of other sugars in growth media could not result in the induction of biofilm formation by KPW.P1. The observed glucose induced increased biofilm formation is most likely mediated through the adherence property of the bacteria and more EPS production resulting in more biofilm formation. These outcomes can give a major premise to a more exact investigation of the biofilm formation of this Cry producing bacterium *Bacillus thuringiensis* on plant surfaces in response to glucose.
Biofilms represent the predominant mode of growth of most, if not all, bacterial species. The ubiquity of the biofilm trait, however, raises the question of whether there are genes that contribute to biofilm formation in a similar manner across species boundaries. If such genes exist, we surmised that these genes should be increased in transcript abundance under biofilm growth conditions, and conserved in sequence. To identify biofilm-specific genes, we first determined the transcriptomic profile of \textit{Pseudomonas aeruginosa} planktonic and biofilm cells by RNA-seq. The analysis revealed several genes that were highly expressed in \textit{P. aeruginosa} biofilms that were conserved in other bacterial species including \textit{Escherichia coli}.

Three pairs of \textit{Pseudomonas} and \textit{E. coli} homologs, PA0918 and \textit{yodB}, PA2184 and \textit{yciE}, and PA3915 and \textit{moaB}, were selected for further study. To determine if these genes are conserved in function we evaluated the role these genes in attachment, biofilm formation, and swarming in two species, \textit{P. aeruginosa} (PAO1) and \textit{E. coli} (BW25113). Inactivation of PA0918 or \textit{yodB} had no effect on attachment and swarming, but resulted in decreased biofilm biomass accumulation. Inactivation of PA2184 or \textit{yciE} coincided with reduced attachment and mutant biofilms demonstrating an altered biofilm architecture relative to wild type. Moreover, \Delta PA3915 and \Delta \textit{moaB} mutants were characterized by reduced attachment, biofilm biomass accumulation, and increased swarming relative to wild type. Moreover, biofilms formed by \textit{P. aeruginosa} mutants \Delta PA2184 and \Delta PA3915 were impaired in their tolerance to tobramycin. Given the similarity in function, we also determined whether the \textit{E. coli} homologs are capable of complementing the \textit{P. aeruginosa} mutant phenotypes associated with biofilm formation and tolerance. Expression of either the \textit{P. aeruginosa} or \textit{E. coli} version of the genes restored the respective mutant phenotypes including tolerance to wild-type levels. Overall, our findings suggest that homologs of biofilm-specific genes that contribute to biofilm phenotypes by \textit{P. aeruginosa} carry out similar functions in other biofilm-forming bacteria. Moreover, our findings suggest the likely existence of genes that contribute to the biofilm trait that are conserved in sequence and function.
Abstract: The ability to form biofilms is an important virulence trait of oral bacteria. These oral biofilms are diverse, multi-species structures that are regulated by bacterial communication processes. Oral biofilms are enriched in outer membrane vesicles (OMVs), which carry biomolecules including toxins and other virulence factors, peptidoglycan, and nucleic acids. The ability of OMVs to transport these molecules to host cells and the role of this process in bacterial virulence has been well-studied, but the process of OMV delivery to bacterial cells within a biofilm and the impact of this process on biofilm formation has not yet been studied. To investigate this process, we looked at the ability of *Aggregatibacter actinomycetemcomitans* OMVs to alter biofilm production by *Streptococcus mutans*, the etiologic agent of dental caries. *A. actinomycetemcomitans* is a Gram negative organism associated with localized aggressive periodontitis. Strains that produce more leukotoxin (LtxA), a secreted protein that specifically kills human immune cells, are more closely associated with disease than those that do not produce this toxin. Because competitive interactions between these two organisms have been reported, we hypothesized that *A. actinomycetemcomitans* OMVs would inhibit the ability of *S. mutans* to form biofilms.

Methods: We collected OMVs from two strains of *A. actinomycetemcomitans*: JP2, a highly pathogenic strain that produces LtxA, and JP2-1704, an isogenic *ltxA* mutant. Purified *A. actinomycetemcomitans* OMVs were then added to *S. mutans* Clarke (ATCC 25175) cultures at various time points, before biofilm production was initiated and after the biofilm had been established, and biofilm production was quantified using crystal violet staining. We also investigated interactions between these OMVs and planktonic *S. mutans* cells using flow cytometry.

Results: We determined that *A. actinomycetemcomitans* JP2 OMVs associated with *S. mutans* cells in culture, but this association had no effect on planktonic growth. However, when *S. mutans* was grown in the presence of JP2 OMVs, biofilm production was inhibited in a dose-dependent manner. These JP2 OMVs also disrupted established *S. mutans* biofilms. Interestingly, JP2-1704 OMVs, which do not contain LtxA, increased rather than decreased biofilm production by *S. mutans*, suggesting that this toxin may play a role in the competitive interactions between these organisms. Purified LtxA, however, did not affect *S. mutans* biofilm production, likely due to transport limitations through the biofilm.

Conclusions: This work presents a new understanding of the ability of *A. actinomycetemcomitans* to regulate the virulence of *S. mutans* by altering its ability to form biofilms. The importance of LtxA in this process suggests a new role for this toxin that is dependent on its packaging in these bacterial vesicles.
**Abstract:**

**Background:** Many chronic inflammatory diseases, including periodontal infections, are biofilm-based pathologies mediated by commensal microbiota persisting within complex host-associated microbial communities. The central hypothesis for this project is that the availability of L-arginine is a key signal that directs colonization and expression of virulence determinants by the periodontal pathobiont *P. gingivalis*. Our studies have shown that L-arginine removal or addition are fundamental signals that impact colonization and expression of key surface structures (fimbriae) known to be required for biofilm development and pathogenicity. Yet, how *P. gingivalis* senses L-arginine and the regulatory mechanisms that transmit and control the response have not been identified. Our current goal is to determine how *P. gingivalis* senses L-arginine in its environment and how it responds in regards to its metabolism and biofilm formation.

**Methods:** For detecting arginine-interacting protein(s), we employed arginine affinity chromatography followed by SDS-PAGE electrophoresis and mass spectrometry. *In silico* analysis was used to identify arginine transport systems. By generating mutants and in trans complementation, the function of relevant genes were studied with regard to biofilm formation. Moreover, the impact of the availability of free arginine on generated mutants and wild-type were studied via biofilm assessment and growth rates. In addition, the metabolic status of the mutants and wild-type were analyzed via untargeted global metabolomic analysis. **Results:** Our results show that under certain growth conditions free arginine can inhibit biofilm establishment and development. We show that the major outer membrane protein RagA, which is predicted to be a macromolecular transporter binds arginine. We also show that a putative ATPase/kinase (PGN_1641) predicted to be involved in the transport of arginine, and the response regulator RprY (PGN_1186), which has been shown to regulate PGN_1641; both negatively regulate biofilm formation. The degree of inhibition by the availability of free arginine was found to be dependent on the physiological state of cells before and after establishment of biofilm. Lastly, metabolomic analysis discovered that the production of polyamines is significantly altered when arginine is available.

**Conclusions:** Our data indicate that RagA, RprY, and a predicted ATPase/kinase (PGN_1641) mediate or control arginine sensing. In the context of pathogenesis, arginine levels can directly impact the expression of virulence determinants and survival.
Session: **Poster Session 3**  
Date & Time: **Wednesday, October 10, 2018, 4:00 pm - 6:00 pm**  
Poster Board #: **093**  
Topic: **Antibiofilm Strategies**  
Abstract Title: **Bacterial cyclic diguanylate signaling networks sense temperature**  

**Author Block:**  
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Many bacteria use the intracellular second messenger cyclic diguanylate (c-di-GMP) to control virulence, motility, and extracellular polymer production. Although a growing body of work has elucidated the biochemistry of c-di-GMP synthesis, degradation and effector function, little is known about how external stimuli are perceived by c-di-GMP regulatory networks. Here we report the discovery of c-di-GMP signaling proteins that function to perceive temperature. The archetype of these proteins is the thermosensory diguanylate cyclase (TdcA), identified in a human isolate of *Pseudomonas aeruginosa*. TdcA thermostats c-di-GMP biosynthesis with catalytic rates that change up to 10-fold per 10 °C. These enzyme kinetics enable rapid physiological changes over narrow temperature ranges, and challenge long-standing theory for universal enzymatic rate-temperature dependencies. Domain-swapping analyses indicate that heat-sensing is mediated by a cofactorless Per-Arnt-SIM (PAS) type III domain, which is a previously undescribed function for this widespread protein domain family. Using intravital imaging, we demonstrate that tdcA allows *P. aeruginosa* to suppress early innate immunity in the murine lung. This immune evasion depends on the c-di-GMP-regulated extracellular polysaccharides PEL and PSL. TdcA orthologues are widespread in bacteria, and putative heat-sensing PAS domains are linked with hundreds of predicted diguanylate cyclases and c-di-GMP-specific phosphodiesterases in the PFAM database. We propose, therefore, that thermotransduction is a prevalent function of bacterial c-di-GMP signaling networks.
Inter-Species Interactions in the Oral Cavity: Friends or Foes?

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Background: The oral microbiome contains highly diverse microorganisms with certain subsets predominating at different habitats. It has been shown to play a crucial role in maintaining oral and systemic health. Aside from causing diseases in the oral cavity, certain oral commensals can be implicated in systemic diseases, including cardiovascular diseases, aspiration pneumonia, stroke and diabetes. Although these oral commensals can be found in healthy and disease-affected subjects, the portion of certain bacteria, e.g. lactic acid bacteria, appears to substantially increase in oral disease patients, suggesting an imbalance among oral ecosystems. Moreover, it has been recognized that caries, periodontitis and other dental infections are caused by consortia of organisms in a biofilm rather than a single pathogen. Therefore, it is essential to understand the ecology of the oral commensals not only in oral diseases but also in the healthy oral cavity. Bacterial commensal Corynebacterium spp. has been shown to play a protective role by, at least in part, utilizing human triacylglycerols and releasing antimicrobial free fatty acids, resulting in altering the environment and inhibiting the growth of the pathogenic strains, such as Streptococcus pneumoniae. To our knowledge, the inter-species interactions of oral commensals, in particularly, Corynebacterium spp.

Abstract: and Streptococcus spp., and the effect of such interactions on dental diseases have never been investigated. In this study, we aim to understand the molecular mechanism(s) of different taxa interactions and the implications on oral diseases. Corynebacterium durum (Cd) and Streptococcus sanguinis (Ss), the two common commensals found in the oral cavity and dental plaque, have been used. Methods and Results: So far, we have discovered that, when treating Ss with culture supernatant collected from Cd, Ss chain length was substantially elongated. We additionally tested other oral streptococci but none of them showed any morphological alterations, suggesting species-specific interaction between Cd and Ss. By using real-time PCR, an alteration of genes predicted to be involved in glycerol and lipid metabolism, and cell division has been observed in Cd-supernatant treated Ss compared to the untreated Ss. An increase in cell aggregation of Cd-supernatant treated Ss was also observed. Interestingly, an ability to phagocytose of monocyte/macrophage-like RAW 264.7 cells was decreased when challenging with co-cultured Ss with Cd in comparison to Ss. Conclusions: Our findings so far have suggested that there is inter-species interactions between both dental commensals Cd and Ss, at least in part, for the potential benefits of oral commensal colonization which can prevent colonization of pathogenic strains.
Abstract:

Although the majority of biofilm studies has examined mono-species cultures, the biofilm formation is intra- and inter-species phenomena that require dynamic interactions between bacteria in mixed biofilm communities. However, little is known to understand molecular interactions between biofilm bacteria. *Escherichia coli* strain Nissle 1917 (EcN) is a probiotic bacterium that has antagonistic effects on adherence, growth, and biofilm formation of other *E. coli* strains. In this study, we investigated the ability of EcN to outcompete with the biofilm formation of pathogenic bacteria such as pathogenic *E. coli* (EHEC), *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *S. epidermidis*. When dual-species biofilms were formed, EcN inhibited the EHEC biofilm population by 14-fold compared to EHEC single-species biofilms. This figure was 1,100-fold for *S. aureus* and 8,300-fold for *S. epidermidis*; however, EcN did not inhibit *P. aeruginosa* biofilms. In contrast, commensal *E. coli* did not exhibit any inhibitory effect toward other bacterial biofilms. We identified that EcN secretes DegP, a bifunctional (protease and chaperone) periplasmic protein, outside the cells and controls other biofilms. Although three *E. coli* strains tested in this study expressed *degP*, only the EcN strain secreted DegP outside the cells, and the purified DegP directly repressed EHEC biofilm formation. Hence, probiotic *E. coli* outcompetes pathogenic biofilms via extracellular DegP activity during dual-species biofilm formation. In addition, through transposon mutagenesis screening of EcN, we identified that four genes related to arginine and serine biosynthesis of EcN are involved in repressing biofilms of EHEC as well as *S. aureus* by modulating growth competition between EcN and those pathogens. These results provide insights in applying and developing probiotics as an effective treatment for the biofilm-related infectious diseases.
Abstract: The opportunistic pathogen P. aeruginosa, employs a hierarchical quorum-sensing network to regulate virulence factor production which cooperatively benefit the population at a cost to the individual. Quorum-sensing suppression was therefore proposed as an attractive anti-virulence target. Furthermore, it was argued that the evolution of a cooperative mutant in a quorum-sensing-suppressed population would be hampered through its exploitation by neighboring non-mutant cells. It remains unclear whether mechanisms which overcome this exploitation exist. We investigated the regain of quorum-sensing cooperation by combining rational design of candidate strains and directed evolution of a mutant of the lasR master quorum-sensing regulator. We find that the most effective mutations pleiotropically combined regain of cooperation and private benefit. We find one such mutation which led to significant conflict between regain of cooperation and antibiotic resistance. Reference: Oshri et al, ISME, 2018, "Selection for increased quorum-sensing cooperation in Pseudomonas aeruginosa through the shut-down of a drug resistance pump"
Communication between Two Bacillus subtilis Biofilm Colonies in Proximity

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Different soil bacteria are known to produce small chemical signals that can regulate itself and also the organisms around it. These chemical signals are also known to regulate biofilm formation. Bacteria in the environment are predominantly found as biofilms. Bacillus subtilis, a gram positive model bacterium for studying biofilm. They differentiate into different subpopulations with response to environmental cues but are genetically identical cells. This indicates the existence of various signaling molecules which can regulate its metabolism when in a biofilm. We aim to identify and study such signals of chemical nature in and between Bacillus subtilis biofilms. To investigate this we isolated a new strain of Bacillus subtilis confirmed by 16s rDNA sequencing and whole genome sequencing. Two Bacillus subtilis biofilm communities were grown nearby to check for any chemical communication molecules. Using chemical indicators and ion chromatography we were able to confirm ammonia as a communicator molecule between two biofilm communities that are placed nearby. Ammonia is already known to be correlated to the metabolic state of a Bacillus biofilm but not known to be a communicator molecule between two communities of biofilms. Ammonia gas was also seen to induce fruiting body formation in the newly isolated Bacillus subtilis strain. We also observed interstrain communication with ammonia which is reported here. Here we suggest the Bacillus subtilis colonies produce ammonia which creates a temporary alkaline gradient which is perceived as a signal by a neighboring colony. The colony growth in the direction of amplified signals is inhibited. Hence colonies orient themselves towards the area which minimize the competition for nutrients.

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**Background:** When bacteria dwell in biofilms on the surface of an implanted medical device or surgical site, they produce an extracellular matrix (EM) that encases the cells and acts as a layer of protection against antibiotic infiltration. Recent evidence suggests that certain biofilm-associated bacteria utilize β-sheet-rich amyloid proteins as a scaffold to reinforce the EM. Amyloids are typically associated with mammalian neurodegenerative disease, but they play a functional role in the EM to help biofilms resist dispersion by chemical or mechanical agents. Consequently, these functional amyloids represent a novel target to interrupt medical biofilm formation. We hypothesize that the pathway to amyloid formation is characterized by specific physicochemical motifs; therefore, peptides engineered to bind these motifs should inhibit fibril formation and destabilize the biofilm.

**Methods:** We applied a combination of computational predictions and biophysical assays to identify regions of high aggregation propensity in functional bacterial amyloids. We also used molecular dynamics (MD) simulations to characterize conformational changes in several amyloid proteins. These data were then applied to design peptides that inhibit amyloid formation by targeting unique secondary structures. **Results:** In amyloid proteins from *P. aeruginosa*, mutation of amyloidogenic motifs to a designed, non-amyloidogenic motif suppresses fibrillation. Further, MD simulations suggest that a variety of amyloids adopt a non-standard secondary structure, termed “α-sheet”, in the early stages of aggregation. Accordingly, peptides designed to incorporate complementary α-sheet structure suppress amyloid formation in vitro. These peptides also inhibit biofilm formation in *S. aureus*, *P. aeruginosa*, and *E. coli* by targeting functional amyloids in the EM. **Conclusions:** The results reported here expand our understanding of the mechanisms of bacterial amyloid formation, particularly the role of α-sheet structure in this process. Designed anti-α-sheet peptides represent a novel, non-killing strategy to combat medical biofilms.
Title: Changes in Cell Wall Compositions of Staphylococcus aureus and Enterococcus faecalis during Biofilm Formation

Abstract: Changes in the cell wall compositions of Staphylococcus aureus and Enterococcus faecalis were characterized using combined solid-state NMR and liquid chromatography-mass spectrometry (LC-MS). Solid-state NMR was used to characterize whole cells and cell walls of S. aureus during planktonic, immature biofilms (formed after 24 h after stationary phase), and mature biofilms (after 60 h after stationary phase) labeled either by (i) [15N]glycine and L-[1-13C]threonine, or in separate experiments, by (ii) L-[2-13C,15N]leucine. We then measured 13C-15N direct bonds by C{N} rotational-echo double resonance (REDOR). The increase in peptidoglycan stems that have bridges connected to a surface protein was determined directly by a cell-wall double REDOR difference. For both planktonic cells and the mature biofilm, 20% of pentaglycyl bridges are not cross-linked and are potential sortase-mediated surface-protein attachment sites. None of these sites has a surface protein attached in the planktonic cells, but one-fourth have a surface protein attached in the mature biofilm. Thus, a primary event in establishing a S. aureus biofilm is extensive decoration of the cell surface with surface proteins that are linked covalently to the cell wall and promote cell-cell adhesion. We also examined changes in peptidoglycan (PG) composition of Enterococcus faecalis associated with the biofilm formation. For accurate analysis, we developed “Stable Isotope Labeling by Amino Acids in Bacterial Culture” (SILAB) optimized for bacterial cultures with incomplete amino acid labeling. The analysis was carried out by labeling E. faecalis in biofilm with heavy-Lys (L-[13C6, 2D9, 15N2]Lys) and planktonic bacteria with natural abundance L-Lys, then mixing the equal amount of bacteria from each condition and carrying out cell-wall isolation and mutanolysin digestion for LC-MS. SILAB analysis of 47 pairs of PG fragment ions from isolated cell walls of planktonic and biofilm samples show increased PG cross-linking, increased N-deacetylation of GlcNAc, decreased O-acetylation of MurNAc, and increased stem modifications by d,d- and l,d-carboxypeptidases in E. faecalis biofilm.
Session: Poster Session 3
Date & Time: Wednesday, October 10, 2018, 4:00 pm - 6:00 pm
Poster Board #: 101
Topic: Synthesis and Assembly and Function of the Biofilm Matrix
Abstract: A Biofilm Matrix-Associated Protease Inhibitor Protects *Pseudomonas aeruginosa* from proteolytic Attack
Title: B. Tseng¹, C. Reichhardt², G. E. Merrihew², S. A. Araujo-Hernandez¹, J. J. Harrison³, M. J. MacCoss², M. R. Parsek²;
Author: ¹University of Nevada Las Vegas, Las Vegas, NV, ²University of Washington, Seattle, WA, ³University of Calgary, Calgary, AB, CANADA.
Abstract: *Pseudomonas aeruginosa* produces an extracellular biofilm matrix that consists of nucleic acids, exopolysaccharides, lipid vesicles, and proteins. In general, the protein component of the biofilm matrix is poorly defined and understudied relative to the other major matrix constituents. While matrix proteins have been suggested to provide many functions to the biofilm, only proteins that play a structural role have been characterized to date. Here we identify proteins enriched in the matrix of *P. aeruginosa* biofilms. We then focused on a candidate matrix protein, the serine protease inhibitor ecotin (PA2755). This protein is able to inhibit neutrophil elastase, a bactericidal enzyme produced by the host immune system during *P. aeruginosa* biofilm infections. We show that ecotin binds to the key biofilm matrix exopolysaccharide Psl and that it can inhibit neutrophil elastase while matrix-associated. Finally, we show that ecotin protects both planktonic and biofilm *P. aeruginosa* cells from neutrophil elastase-mediated killing. This may represent a novel mechanism of protection for biofilms to increase their tolerance against the innate immune response.
Abstract: Extracellular Z-DNA and not B-DNA is the Important Extracellular DNA Configuration that maintains the Structural Integrity of Mature Biofilms

Author: S. D. Goodman, J. Buzzo, A. Devaraj, J. A. Melvin, L. O. Bakaletz; Nationwide Children's Hospital, Columbus, OH.

Background: Biofilms consist of bacteria embedded in a self-produced extracellular polymeric substance (EPS) that is comprised of often species-specific polysaccharides and proteins, as well as extracellular DNA (eDNA). We have shown that eDNA and the ubiquitous DNABII family of DNA-binding proteins are universal components of biofilm EPS, and together provide essential structural support for biofilm development and maintenance. While DNA nucleases can prevent efficient biofilm formation, mature biofilms are typically resistant to nucleases. This phenomenon has been attributed to a diminished structural role for eDNA in mature biofilms. However, abundant eDNA is present in mature biofilms, and removal of DNABII proteins disrupts mature biofilms. Thus, eDNA provides essential structural support to mature biofilms, and must instead have adopted a nuclease-resistant form. DNA structure is in equilibrium among interconvertible configurations. B-form is the canonical and dominant configuration that consists of a nuclease sensitive right-handed double helix, whereas the Z-form consists of a left-handed helix with distinct geometry that is nuclease resistant. While not abundant intracellularly due to the high intrinsic energy of this state, Z-form DNA can exist under defined conditions such as in the presence of certain biogenic amines, e.g. spermidine. Since spermidine is available at high concentrations extracellularly, we hypothesized that eDNA adopts the Z-form as biofilms mature, which provides resistance to nuclease-mediated disruption. Methods: DNABII protein stabilization of Z-DNA was assessed by circular dichroism spectroscopy. To assay the role of biofilm matrix components in Z-DNA formation and its correlation to biofilm stabilization, we grew bacteria in chambered coverglass and detected matrix components and eDNA structures in the resultant biofilms by immunofluorescence microscopy. To quantify biofilm formation, we stained biofilms grown in chambered coverglass with Live/Dead™ stain, imaged biofilms by confocal laser scanning microscopy, and analyzed images with COMSTAT software. Results: DNABII proteins facilitated conversion of DNA to the Z-form through DNA-binding. Further, as bacterial biofilms matured, extracellular spermidine concentrations increased concomitantly with the appearance of Z-form eDNA, albeit at consistently lower levels than B-form eDNA. Remarkably however, nuclease digestion of mature biofilms that fails to disrupt biofilm structure completely eliminated B-DNA and predictably preserved eDNA in the Z-form. Finally, conditions that stabilize eDNA in the Z-form facilitated biofilm formation whereas conditions that favor B-form DNA impeded bacterial biofilm maturation and growth. Conclusions: We contend that eDNA in the Z-form is the critical configuration for structural stability of a mature bacterial biofilm EPS.
Session: Poster Session 3
Date & Time: Wednesday, October 10, 2018, 4:00 pm - 6:00 pm
Poster Board #: 103
Topic: Synthesis and Assembly and Function of the Biofilm Matrix
Abstract Title: Extracellular Polymeric Substances from Biofilm Forming Marine *Streptomyces* sp and their Radionuclide Bioremediation Application
Author Block: K. Kamala, P. Sivaperumal, R. Tilagaraj; SRM Institute of Science and Technology, Kattankulathur, INDIA.
Abstract: Extracellular Polymeric Substances (EPS) producing marine actinobacterium has been isolated from a marine natural biofilm matrix and identified as *Streptomyces* sp. CuOff24 through 16S rDNA sequencing. Approximately 450 mg L$^{-1}$ EPS were produced and major content of carbohydrate followed by protein, nucleic acid and unidentified compounds (68.6%, 12.3%, 10.4% and 8.7% respectively) were quantified. The spectroscopy study also confirmed the presence of carbohydrate functional groups on the EPS surface and GCMS showed that the EPS comprised of mannose (45.2%), glucose (28.8%) and arabinose (26%). The 1g EPS could remove 86.7±0.61% of Cs$^+$ ion from test solution having 10mM CsCl$_2$ with KCl. The biosorption of Cs$^+$ ion in marine actinobacterial EPS with different environmental factors (pH, temperature, time interval) were also tested. SEM coupled with EDS also confirmed the Cs$^+$ ion adsorption by EPS obtained from *Streptomyces* sp. CuOff24. Maximum of the cesium ion was found to be matrix with EPS and it could suggest that, the radionuclide ions were interact with surface active groups. The high Carbohydrate compounds in marine actinobacterial EPS could be used for the large scale remediation of radionuclides contaminated environment.
Background: Psychrotrophic pseudomonad species are the dominant bacterial component on aerobically stored chilled meat. Biofilm formation by pseudomonads during chilled storage and transportation results in slime formation which is a major spoilage characteristic. Limited in-situ characterization of the structure and matrix composition of biofilms, which may aid in understanding their dominance on chilled meat, has been undertaken. We studied the structural changes of biofilms formed on meat with time and analyzed the matrix composition of *Pseudomonas fragi* (n=2) and *Pseudomonas lundensis* (n=2) using confocal laser scanning microscopic (CLSM) imaging, cell counts and confocal Raman spectroscopy. Methods: Sterilized beef samples were inoculated separately with each of the species at cell numbers similar to those on retail meat (10^4 CFU/cm²) and incubated aerobically at 10°C and 25°C for 7 days. On each day, biofilms were stained with Live/Dead fluorescent stains and imaged with CLSM. At the same time, numbers of bacteria in biofilms formed under identical conditions were determined by total plate counts from disrupted and sonicated biofilms. To study the matrix composition, biofilms of same meat isolates were grown at the same temperatures on sterile nitrocellulose membranes placed on sterile beef cuts to minimize the contamination from the meat. Raman spectra were obtained and principal component analysis was conducted to identify differences between matrix components based on temperature and species level. Results: Bacterial numbers in the meat grown biofilms increased rapidly in all the strains and after reaching a population maximum of around 10^11 CFU/cm², began to decline. The changes in cell count data correlated well with CLSM images which showed that all the strains produced thick biofilms at both temperatures. After populations reached maximum levels, biofilm structures began to degrade and live/dead ratio declined with time. Preliminary Raman spectroscopic data showed differences in matrix composition between biofilms grown at 10°C and 25°C in both species. Analysis confirmed that the spectral changes are associated with protein, polysaccharide and DNA/RNA contents obtained in different biofilm samples. Conclusion: Despite the abundant access to nutrients from raw meat, biofilms formed by the proteolytic *Pseudomonas* species dispersed with time. The signal for dispersal could be the depletion of meat muscle with glucose and/or production on nitrogenous compounds as a result of proteolysis during the latter stages of biofilm growth. The differences between Raman spectra of biofilms grown at 10°C and 25°C suggest that selected psychrotrophic spoilage pseudomonads respond to cold stress by modifying the matrix composition. Key words: Biofilm dispersal, biofilm matrix, Chilled meat, *Pseudomonas fragi*, *Pseudomonas lundensis*
Aspergillus fumigatus (AF) is a ubiquitous saprophytic fungus and the second most common causative agent of fungal infections in hospitals. Its ability to resist currently available antifungal drugs and evade the immune system is facilitated by its ability to form a biofilm. A key component of the AF biofilm is the exopolysaccharide adhesin galactosaminogalactan (GAG), a heteropolysaccharide composed of α-1,4-linked galactose, N-acetylgalactosamine, and galactosamine. In A. fumigatus, GAG biosynthesis and export across the cell membrane is facilitated by the protein products of a five-gene cluster. One of these genes, ega3, encodes a glycoside hydrolase (GH) belonging to the GH114 superfamily. To understand the role of Ega3 in GAG export and its enzymatic mechanism, structural and functional studies were performed. Recombinant Ega3 was expressed and purified using Pichia pastoris and crystallization screens conducted. Ega3 crystals diffracted to 1.76 Å, and the structure was solved with a distant homolog using the ARCIMBOLO-SHREDDER software. The atomic structure reveals a characteristic (β/α)8 fold with an electronegative cleft that binds the sugar polymer. In addition, co-crystallization with galactosamine revealed a flexible loop that folds over to coordinate the sugar monomer. In vitro activity assays performed with purified Ega3 support that it is an endo-acting hydrolase that can disrupt A. fumigatus biofilms. Mutation of the conserved residues in the putative binding cleft supports a central aspartic acid (D189) and glutamic acid (E247) as the catalytic residues. Our combined results suggest that Ega3 is involved in GAG processing during export and can be repurposed as a novel therapeutic for biofilm related infections.
**Title:** The Effect of Morphology-Changing Compounds on Biofilm Density and Antibiotic Susceptibility of *Escherichia coli*

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**Abstract:**

**Background:** In the United States alone, more than two million people become infected by antibiotic-resistant bacteria each year, leading to at least 23,000 deaths as a result. Biofilms, a matrix of bacterial cells, act to further protect bacterial infection from any kind of antimicrobial treatment. These protective structures serve as a physical means of antibiotic resistant. MreB, an essential protein for proper morphological development of all gram-negative bacteria, plays a key role in forming biofilms. In healthy *E. coli*, the protein causes cells to hold a rod-like shape rather than the circular shape they hold without it. Another important protein is ftsZ, although it plays a different role than MreB. FtsZ is a necessary protein for the cell division process, establishing where exactly the cell will be divided during binary fission. Together, these two proteins serve to properly form biofilms. **Methods:** The present study seeks to compare the effects of the inhibition of the two proteins on K-12 *E. coli*. This inhibition will come through the application of two inhibitory chemicals; A22 Hydrochloride inhibiting MreB and PC190723 inhibiting ftsZ. The efficacy of the chemicals will be measured through biofilm density, quantified using a crystal violet staining assay on 24-well plates. The stains will be put into solutions which will then read be read a spectrophotometer. **Results:** After experimentation, it was found that a mid-level dosage of A22 significantly decreased biofilm growth while there was no dose response to PC190732. This signifies that the inhibition of MreB was more effective than that of ftsZ. **Conclusions:** To further study biofilms and their antibiotic-resistant abilities, testing should be done on the dual inhibition of MreB and ftsZ. This conjunct inhibition would allow for any synergistic or additive effect to become present. Additionally, testing should be done to determine if the protein inhibition will increase antibiotic susceptibility to Amoxicillin. This future experiment would allow insight into the effectiveness of antimicrobial treatment after the biofilm density has been decreased, truly showing the practical application.
**Background:** According to the World Health Organization, nearly fifteen percent of all hospitalized patients worldwide acquire nosocomial infections. A particular area of concern for bacterial build up in hospitals is sink drains. The moist, microbiologically active environment of drains promotes the formation of biofilms that are difficult to target with standard chemical disinfectants. Bacteriophage, however, show potential to be used as a disinfecting agent in hospital drains. Not only do bacteriophage increase in titer as they infect, spreading to hard-to-reach surfaces, numerous phage have been shown to degrade the extracellular polymeric substances of biofilms and gain access to underlying bacteria. Water channels in the extracellular matrix of various biofilm, such as *E. coli* biofilms, allow entry of phage T4. This research explores the potential of a bacteriophage-based disinfectant to eradicate biofilms in an environment modeling a sink drain by comparing the efficacy, thoroughness, and durability of the phage disinfectant to a chemical disinfectant. **Methods:** *E. coli* biofilms were grown in M9 minimal media placed in sink P-traps. The P-traps were divided into three treatment groups: one group was treated with bacteriophage T4, a second group with a chemical disinfectant, and a control group with deionized water. To compare the efficacy of treatments, the biofilms were quantified at five time points - 1 hour, 12 hours, 24 hours, 72 hours, and 1 week. This process involved washing to remove planktonic cells, applying crystal violet to stain biofilms, adding 30% acetic acid to solubilize biofilms, and using a spectrophotometer wavelength 570 to measure optical density. **Results:** Crystal violet staining revealed that one hour after application of treatment, both the chemical disinfectant and bacteriophage were able to reduce biofilm growth compared to the negative control, with the chemical disinfectant being slightly more effective. With heavier biofilm growth at longer time intervals after treatment - such as forty-eight hours to 1 week -- the bacteriophage began exhibiting the same effectiveness at removing biofilm as the chemical disinfectant. Upon visual examination, sink P-traps treated with bacteriophage showed a tendency for more uniform destruction of biofilm across the drain compared to P-traps treated with chemical disinfectant. **Conclusion:** Overall, this work highlights the potential of bacteriophage as an alternative to conventional chemical disinfectants for long term biofilm control in settings such as hospital drains. Future work will be done to quantify the distribution of biofilm elimination across the P-trap.
Abstract: Background: Biofilms are aggregations of bacteria living together surrounded by a matrix and connected to a surface. They are highly antibiotic resistant, and this comes at a great cost to the public as many infections are potentially biofilm-related; this is seen particularly in nosocomial infections, with 34.7% of health-care-associated infections in 2014 being surgical-site or urinary tract related, two areas where biofilm formation is common. This high antibiotic resistance is coupled with an increased chance of the bacteria developing resistance to whatever treatment used due to factors such as a higher rate of mutagenesis and plasmid transfer compared to planktonic bacteria. One field of treatment that has been explored is the use of antimicrobial peptides, including a peptide derived from bee venom, melittin. Melittin has been demonstrated to have antimicrobial effects on both bacteria in a planktonic state and bacteria in a biofilm state. On biofilms, however, melittin has been demonstrated to have a minimum inhibitory concentration that is too high for it to be applicable in a clinical setting. The use of an agent to break down the biofilm matrix has been previously demonstrated to improve the efficacy of antimicrobial peptides when used in combination with them. DNase I is one such agent, as eDNA is a structural component of many biofilm matrices, including that of E. coli. Methods: Whether DNase I could be used to increase the efficacy of melittin and make it a clinically possible treatment was investigated, as well as the best way to use them in combination. Melittin and DNase I were applied to E. coli biofilms in 24-well plates, both individually and in combination with each other. The combination treatments were melittin and DNase I applied simultaneously and DNase I applied 7 hours before the melittin was. The amount of biofilm in each well was analyzed using a crystal violet assay and 24 and 48 hours, with the optical density of the crystal violet dissolved in ethanol being determined using a spectrophotometer. Results: Through comparison of the treatment wells to wells that had only the buffers of melittin and DNase I applied to them, preliminary results show that DNase I was the most effective treatment. Ongoing research is investigating the propensity of E. coli biofilms to become resistant to DNase I treatment. Conclusions: Data collected indicates that DNase I and melittin used in combination is not an effective biofilm treatment, but that DNase I may be a good option for clinical use despite concerns about the possibility of it increasing biofilm dispersal and recolonization.
Abstract: The formation of a model cheese-rind biofilm involves a reproducible succession of bacteria and fungi. As the growing biofilm breaks down cheese proteins and releases ammonium, the pH of the cheese increases drastically, from pH 5 to pH 8. This dynamic abiotic environment can modulate the nature of interactions between biofilm residents in two possible ways: either by removing or imposing a reliance on another species, or by altering the mechanism by which two species interact. As a multi-species biofilm undergoes such abiotic and biotic dynamics, it can be expected that the structure of the biofilm will reflect and further impact these dynamics. Using a 7-member in vitro cheese rind community as a model, growth assays of all pairwise combinations reveal species pairs that interact in a pH-dependent versus pH-independent manner. To understand how these changing interactions are reflected in the spatial organization of species pairs and whether these patterns are maintained in a complete community, fluorescence in situ hybridization methods have been optimized to visualize in vitro cheese rind biofilms consisting of two- to seven-microbe biofilms. These results may have implications for the resultant pattern of succession observed in the complete 7-member community.