Montana State University

Center for Biofilm Engineering

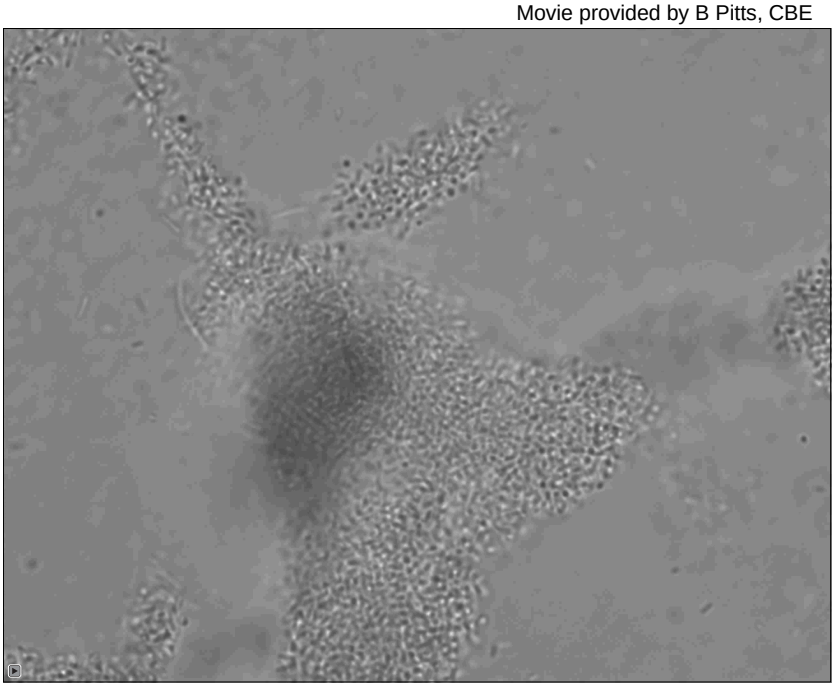
Bozeman

montana biofilm SCIENCE & TECHNOLOGY meeting

July 12–14, 2011

proceedings

Movie provided by B Pitts, CBE



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Speaker Abstracts

SESSION 1: Biofilm Dispersion

Escape from the matrix!

Presenter: Richard Losick, Professor, Biology
Affiliation: Harvard University, Cambridge, MA

Bacillus subtilis forms thick and structurally complex biofilms at air/liquid interfaces. These pellicles naturally disassemble by eight days of incubation. My collaborators and I have identified small molecule factors in conditioned medium from aged cultures that cause biofilm disassembly. One such factor is a mixture of D-amino acids that are incorporated into the peptidoglycan, where they trigger the release of the protein component (amyloid fibers) of the extracellular matrix. The second factor is a specific polyamine that works by targeting the exopolysaccharide. Mutants blocked in both D-amino acid production and polyamine production form long-lived pellicles, and the application of a mixture of both factors to a mature pellicle triggers biofilm breakdown after several hours. Thus, biofilm disassembly is mediated by small molecule factors that separately target the two principal components of the extracellular matrix. The mechanism of action of both factors and their efficacy in preventing biofilm formation by unrelated gram-positive (*Staphylococcus*) and gram-negative (*Pseudomonas*) bacteria will be discussed.

Marine alkaloid derivatives that inhibit and disperse bacterial biofilms

Presenter: Christian Melander
Affiliations: Co-Founder and Chief Research Officer, Agile Sciences, and
Associate Professor, Chemistry, North Carolina State University, Raleigh, NC

Marine alkaloid derivatives based upon the 2-aminoimidazole scaffold have demonstrated the ability to inhibit and disperse both gram-positive and gram-negative bacterial biofilms, fungal biofilms, and mixed species biofilms. The development of these compounds, as well as applications in various sectors (agriculture, dental, pharmaceutical, etc.) will be presented. Data will also be presented that demonstrate that these compounds work synergistically with conventional antibiotics and microbicides to eliminate bacteria in their biofilm state as well as their planktonic brethren.

Imaging biofilm dispersion...or not!

Presenter: Betsey Pitts, Research Associate and Microscope Facilities Manager
Affiliation: Center for Biofilm Engineering, Montana State University, Bozeman, MT

Chemical or enzymatic dispersion of biofilms is of interest across a broad range of biofilm contexts and is currently a heavily published research topic. We used time-lapse confocal microscopy to directly visualize the effects of several agents reported to disperse *Staphylococcus aureus* biofilms. Biofilms of the green fluorescent protein-expressing *S. aureus* strain AH1979 were grown in a capillary flow cell reactor for 48 hours at 37^{o}C, at which time the 1 mL/min flow of medium was switched to a treatment solution and time-lapse imaging began. Applied treatments included: 20 mg/L proteinase K, 8 KU DNase I, 25 mg/L lysostaphin, 500 mg/L lysozyme, 1,000 mg/L trypsin, 50 mg/L sodium hypochlorite, 100 mg/L NaOH and 0.5 M urea. All treatments were applied for a maximum time period of two hours. Of these agents, only sodium hypochlorite and sodium hydroxide rapidly and consistently dispersed biofilm. These preliminary results indicate that it is important to evaluate dispersion in multiple biofilm models, including a flow model, to determine robust activities. Additional agents are under investigation.

Dispersal of methicillin-resistant *Staphylococcus aureus* (MRSA) biofilms

Presenter: Alex Horswill, Associate Professor, Microbiology
Affiliation: Carver College of Medicine, University of Iowa, Iowa City, IA 52242

Staphylococcus aureus is an adaptable bacterial pathogen that can cause a diverse range of acute and chronic infections. To persist in the host, *S. aureus* attaches to tissue or medical implant material and develops a biofilm community of cells that resist immune clearance and antibiotic therapy. Antibiotic resistant strains, such as methicillin-resistant *S. aureus* (MRSA), are increasingly being identified in biofilm-related infections in the hospital setting. We previously determined that the *S. aureus agr* quorum-sensing system regulates biofilm formation and dispersal, and we sought to test whether recent MRSA clinical isolates would behave in a similar manner. Using community MRSA isolates of the USA300 pulse field gel type, we demonstrated that these strains have robust biofilm capacity using a flow cell apparatus. Known mutations in the *sigB* and *sarA* loci that limit biofilm formation in *S. aureus* laboratory strains also prevented biofilm formation in the MRSA isolate. Similar to the laboratory strains, the addition of the quorum-sensing signal (also called an autoinducing peptide or AIP) activated the *agr* system and disassembled the MRSA biofilm. To test the generality of this phenomenon, we developed a flow cell apparatus with titanium coupons and determined that MRSA formed a biofilm on titanium with similar properties to biofilms formed on other surfaces, and these titanium-based biofilms could be detached with AIP addition. Notably, these released cells regained susceptibility to antibiotics that approached the levels of planktonic bacteria, suggesting that combination therapy could be a potential route of treatment. In further studies on the MRSA isolate, we determined that biofilm matrix material is proteinaceous in nature and the formation of MRSA biofilms is dependent on proper regulatory control over the production of secreted proteases. Finally, we demonstrated that extracellular DNA (eDNA) levels and the presence of a secreted nuclease enzyme encoded in all *S. aureus* strains also modulate the capacity of MRSA to develop a biofilm. Altogether, these findings indicate that clinical MRSA isolates form biofilms with an intact *agr* dispersal mechanism, and their matrix is proteinaceous and eDNA-based. The development of strategies that exploit this dispersal mechanism could improve MRSA biofilm treatments.

Immersed boundary model of biofilm deformation and detachment in response to fluid flow

Presenter: Jeff Heys, Assistant Professor, Chemical & Biological Engineering and CBE
Affiliation: Center for Biofilm Engineering, Montana State University, Bozeman, MT

Biofilms are complex mixtures of microorganisms and extracellular matrix that exist on many wetted surfaces. A mathematical model based on the immersed boundary method has been developed to examine the physical interaction between a biofilm and a moving fluid in a capillary. This model has been validated previously by comparing the predicted biofilm deformation to experimental measurements. The model is currently being used to study two different biofilm-fluid interaction problems.

The first problem is from the measurement of fluid velocities near a biofilm using magnetic resonance microscopy (MRM). These velocity measurements showed unexpectedly high secondary velocities (*i.e.*, high velocity magnitudes perpendicular to the direction of bulk flow and perpendicular to the surface on which the biofilm is attached). The simulation predicts the formation of a recirculation downstream of a biofilm, and this recirculation deforms and lifts the biofilm upward from the surface to which the biofilm is attached. Changing the mechanical properties (*i.e.*, stiffness) of the biofilm impacts both the lifting of the biofilm and the magnitude of the secondary velocities. The maximum lifting of the biofilm occurs when the biofilm properties are similar to previous experimental measurements, which indicates that the mechanical properties of the biofilm may be tuned for the generation of maximum secondary velocity magnitude and transport of substrates to the biofilm.

The second problem examines the different factors that impact biofilm detachment and dispersion. One of the advantages of a mathematical model is that different factors can be varied independently and in a controlled manner. The specific model parameters that are varied here are biofilm shape, mechanical

stiffness, biofilm density, and the maximum strain before failure. All of these parameters impacted biofilm detachment, and the talk will quantitatively describe their impact.

SESSION 2: Metals, Microbes & Microbially Influenced Corrosion (MIC)

Methods for the study of extracellular electron transfer in electrode-associated biofilms using *Geobacter sulfurreducens* as a model

Presenter: Ashley Franks, Research Assistant Professor
Co-authors: Nikhil Malvankar, Kengo Inoue, Kelly P. Nevin and Derek R. Lovley
Affiliation: Department of Microbiology, University of Massachusetts, Amherst, MA

Microbial fuel cells (MFC) have attracted interest due to their ability to convert environmental wastes and renewable biomass into electricity in a carbon neutral fashion. Fundamental to power production in an MFC is the formation of specialized biofilms on an anode surface. A greater understanding of these biofilms and the development of techniques for their study are required for further improvements to MFC technology. The well studied electricigen *Geobacter sulfurreducens* has been a model to study high current density biofilms due to: a current density equal to or greater than all other reported mixed or pure cultures; a genetic system; and the fact that it is the most commonly recovered microbe from MFC-associated biofilms with environmental inoculants. *G. sulfurreducens* current-producing biofilms are up to 50 μm thick and spatially heterologous. A real-time imaging MFC was created to allow nondestructive spatial and temporal imaging of the current-producing biofilms in situ. Metabolic staining indicated that the entire biofilm was metabolically active, demonstrating the ability to transfer electrons up to 50 μm. Studies using an MFC modified to measure biofilm conductivity indicated high conductivity of the *G. sulfurreducens* biofilm—greater than that of a conductive polymer and unique when transferring electrons extracellularly. A pH sensitive fluorometric dye demonstrated that while all members of the biofilm are metabolically active, a potentially inhibitory proton gradient formed within the biofilm due to extracellular electron transfer. Due to the heterologous nature of the biofilm, it was speculated that large differences occur in gene transcription between the cells closest to the anode surface and those farthest away. To further investigate differences within these biofilms, a novel strategy was developed to effect spatial separation of the biofilm into an inner and outer layer in order to extract RNA. A sufficient quantity of high quality RNA was extracted such that the first spatial transcriptional analysis within a single biofilm was conducted. Analysis of the transcriptional differences revealed a large number of genes with roles in cell metabolism and growth down-regulated in the outer layer. These results led to the creation of short half-life green fluorescent protein reporter plasmids for spatial and temporal analysis of gene expression within the current-producing biofilms, indicating expression patterns throughout the formation of the biofilm. To examine differences between expression and protein localization patterns, gold-labeled antibodies were used to probe embedded current-producing biofilms. Differences in distribution were observed for proteins thought to be essential for power production. These results highlight the unique and highly heterologous nature of current-producing biofilms and are expected to lead to better strategies for optimizing the power output of MFCs, broadening their applications.

Chromium (VI) reduction by environmental microbes—Influence of common soil constituents and carbon sources on chromium (VI) reduction and toxicity

Presenter: Robin Gerlach, Associate Professor, Chemical & Biological Engineering
Co-authors: Erin Field, Mike VanEngelen, Brent Peyton
Affiliation: Center for Biofilm Engineering, Montana State University, Bozeman, MT

Chromium is a common contaminant in soil environments, especially at Department of Energy sites. The fate of chromium in the environment is influenced by a number of factors including microbial processes and microbial interactions with soil constituents such as carbon sources, iron minerals, and electron shuttling compounds.

Clone library, PhyloChip, and quantitative PCR analyses of the bacterial community within a simulated low-level waste site at the Idaho National Laboratory suggested that the presence of cellulosic waste influenced the bacterial community structure with respect to soil depth at the site.

We isolated and characterized a number of microbes with potential roles in Cr(VI) transformation in soil environments. Studies with *Cellulomonas* sp. strain ES6 and an *Arthrobacter* sp. strain EF01 demonstrated that Cr(VI) reduction by these organisms was influenced by the carbon source present as well as by the addition of electron shuttles and iron minerals.

Consequently, the influence of potential cellulose degradation products on Cr(VI) toxicity and reduction by *Arthrobacter* spp. was assessed. Results indicated that Cr(VI) toxicity was linked to carbon metabolism.

Overall, the results provide insight into the potential interactions between microorganisms, soil constituents, and chromium in situ and support our previous observations on carbon source-dependent uranium reduction and toxicity with other environmental isolates obtained from the same site.

In situ microbial reduction of selenium in backfilled phosphate mine overburden, S.E. Idaho

Presenter: Lisa Kirk, PhD, Land Resources & Environmental Science
Affiliation: Center for Biofilm Engineering, Montana State University, Bozeman, MT

Recent biogeochemical research characterizing the microbial community and chemistry of mined rock, tailing, and post-mine pit lakes has improved our understanding of the significant influence of microbial ecology on metal cycling in mine waste and mining affected water resources. The overall understanding of microbial influences on geochemical cycling and speciation of metals has grown exponentially in recent years as well, yet standard mining industry testing protocols integrate microbial influences on metal cycling only marginally and make limited use of molecular biology methods. These tools represent important new resources for operational characterization and management of mine waste throughout mine life, as demonstrated in the following case study from phosphate mine waste deposi ts in southeast Idaho.

Microbial reduction of Se is controlled by oxygen and lithology within mixed deposits of shale, chert, and mudstone mined from the Meade Peak member of the Phosphoria Formation in southeast Idaho. Waste rock and groundwater from backfilled mine pits—which have been studied using geochemical, microbial cultivation, and cultivation-independent molecular methods—host indigenous populations of Se-reducing bacteria (including Se^{VI} respiring *Dechloromonas* and Se^{IV} detoxifying heterotrophs such as *Stenotrophomonas*) that can rapidly and near-quantitatively reduce soluble Se to insoluble minerals. Reduction occurs within a consortium of psychrophilic NO_3- Fe^{III} -Mn^{IV} -reducing *Polaromonas*, *Rhodoferax, Brevundimonas*, and *Sphingomonas* organisms capable of degrading naturally occurring hydrocarbons. Most-probable number estimates of Se^{VI} reducers are highest in well sediments and in shale, and very low in chert and mudstone. Se reduction rates were studied in microaerophilic, saturated native chert and shale

sediments inoculated with live groundwater cultures under controlled oxygen and temperature conditions. The biogeochemical Se reduction pathway described in chert differs from that in shale, proceeding most rapidly to production of Se^{IV} and Se^{o} end products. In shale, Se reduction is slower but produces more reduced SeM, Se^{o}, and Se^{-II} mineral phases. Operational design strategies that construct facilities to manage moisture and oxygen can promote in situ Se reduction by indigenous organisms using native carbon, thus offering a sustainable approach to natural attenuation of Se in mined rock.

Advances in biological treatment for the mining industry

Presenters: Caroline Dale, Biological Principal Engineer, Veolia Water
Scott Benowitz, P.E., President, Water Engineering Technologies, Inc.

Process water and wastewater from mining activities can contain nitrogen, cyanide, and metals. Waters recycled for use in the mining or milling process often require treatment so as not to impede product recovery. Water for discharge to the environment is managed under global environmental regulatory requirements and often requires treatment. The origins of the nitrogen, cyanide and metals will be discussed together with typical characteristics found in mining effluent.

Veolia Water Solutions & Technologies (VWS) offers biological treatment solutions to the mining industry. A focus on the MBBR (Moving Bed Biofilm Reactor) Technology will be made as it is particularly adapted to the mining industry. Case studies will be presented in which MBBR technology is used to remove nitrogen and cyanide from mine waters. Ongoing research for selenium removal and enhancements to the MBBR Technology will also be presented.

Investigating biofilm-influenced corrosion using molecular tools

Presenter: Iwona Beech, Visiting Professor, Department of Botany & Microbiology
Affiliation: University of Oklahoma, Norman, OK

Investigations were undertaken to determine the cause of unexpected accelerated marine corrosion of 70/30 Cu-Ni alloy at three different geographical locations. Sites coded T1 and T2 represented corroding systems at an average temperature of 24°C, while site V, at an average temperature of 10°C, was a non-corroding location. To determine whether reported field failures were due to biofilm-influenced corrosion, Cu-Ni specimens, as-received and pre-conditioned with fresh water and iron sulphate to gain a protective oxide layer, were exposed to complex prokaryotic communities recovered from T1, T2 and V systems. For each system, aerobic and anoxic enrichments, selective for slime- and acid-producing and sulphide-generating microorganisms, respectively, were obtained from fouling layers. Combined enrichments representing each system served as inocula for continuous flow bioreactors operating for 6 months with filter-sterilized natural seawater at two different temperatures (10°C and 24°C for systems T1 and V and 24°C for system T2). Controls consisted of specimens exposed in sterile bioreactors. Field emission scanning electron microscopy and digital light microscopy imaging of Cu-Ni specimens exposed in inoculated bioreactors revealed abundant biofilms. Varying levels of pitting attack were observed on specimen surfaces after biofilm removal. Each system had produced a distinct pitting morphology. Localized pitting similar to that reported in field failures was observed in the V-24 °C and T1-, T2 -24 °C bioreactors. The deepest, but not the most abundant, pits were measured in the former bioreactor. When developed at 10°C, the biofilm in the V bioreactor did not cause appreciable pitting. In contrast, biofilms in the T1 bioreactor demonstrated increased corrosion at 10°C, thus indicating that temperature was not the major factor controlling biocorrosion. Micro pits detected on Cu-Ni surfaces of control specimens did not exceed depths measured for Cu-Ni alloys in marine environments. Molecular characterization of inocula and resulting biofilms were carried out using 16S rRNA and functional genes approaches. DGGE, sequencing and functional gene microarray (GeoChip, 4th generation) revealed similarities between enriched microbial communities obtained from different geographical locations. Preliminary analysis of the GeoChip results confirmed a high abundance of Cu-resistant genes in biofilm populations, irrespective of

their origins. The frequency of genes representing S-redox pathways and methanogenesis was considerable. Functional genes detected in biofilms using GeoChip correlated with bacterial and archaeal taxa identified through 16S rRNA sequencing. The study has demonstrated that biocorrosion was the cause of the failure of Cu-Ni alloys. The results strongly imply that in the case of Cu-Ni, material and not the geographic location influences biofilm ecology. It is concluded that DNA-microarrays comprising functional genes coding for metabolic pathways pertinent to corrosion can be used as tools for rapid biocorrosion risk assessment and that better understanding of co-operative microbial metabolism is key to the development of effective biocorrosion mitigation strategies.

Special Presentation

Bacteria in the food industry—biofilm, survival, control

Presenter: Trond Møretrø, Research Scientist
Affiliation: Nofima Food, Tromsø, Norway

Bacteria may survive and grow on surfaces in the food industry depending on the type of bacteria and parameters such as humidity, temperature, availability of nutrients, type of surface, and cleaning regime. Bacteria on surfaces may cross-contaminate food during production, which may have implications for safety and quality of food.

The bacteria dominating on surfaces in the food industry are non-pathogenic isolates of *Staphylococcus, Pseudomonas, Serratia,* and *Acinetobacter.*

Listeria monocytogenes is a foodborne pathogen that can cause, for example, meningitis, abortion, and stillbirths. The route to infection of food is normally not through raw materials, but through cross-contamination of food in the later stages of food production. Specific clones of *L. monocytogenes* have been shown to persist in food production plants for years and are very difficult to eradicate. It has been suggested that biofilm formation is important for persistence of *Listeria*, but the hypothesis has been difficult to prove.

Many disinfectants have limited effects against biofilms. In order to control biofilm, the bulk of the biofilm needs to be removed during the cleaning process so that the remaining bacteria on the surface can be killed by the subsequent disinfection step. Different types of cleaning agents and disinfectants may have different effects against biofilms. Build-up of bacteria and biofilm formation can be a problem on surfaces or equipment that is difficult to clean and on places that are seldom cleaned.

Competent cleaning personnel and good hygienic design are important to reduce the risk of biofilm formation and cross-contamination of food.

SESSION 3: Systems Biology

Transcriptome analysis of biofilm physiology

Presenter: Phil Stewart, Director
Affiliation: Center for Biofilm Engineering, Montana State University, Bozeman, MT

The physiological activities and growth status of *P. aeruginosa* in biofilms were investigated via transcriptional profiling. Biofilms of strain PAO1 were grown in vitro for three days in drip-flow reactors using a glucose-minimal medium. RNA was harvested from six replicate biofilms and the transcriptome

was determined using Affymetrix® microarrays. To gain insight into the priorities of the biofilm population, the MAS5 scaled signal intensity of each transcript was ranked. Similar rankings were obtained from data sets published in the GEO database (www.ncbi.nlm.nih.gov/geo). By comparing the rank of genes selected as markers for particular physiological responses between the biofilm and comparator data sets, it was possible to infer qualitative features of the physiological state of the biofilm bacteria. These biofilms appeared, from their transcriptome, to be glucose nourished, iron replete, oxygen limited, and growing slowly or exhibiting stationary phase character. In eleven published data sets, specific growth rate correlated with the difference in rank of the *rpoS* and *fis* transcripts. Using this measure, the average specific growth rate of the biofilm cells was between 0.03 h^{-1} and 0.12 h^{-1} —much less than the maximum specific growth rate of the microorganism in this medium of 0.74 h^{-1}. The biofilm population did not indicate oxidative stress, but did exhibit copper stress. Six of seven indicator genes for homoserine lactone-mediated quorum sensing were expressed only at low levels. Efflux pumps were not up-regulated in the biofilm. Of extracellular polysaccharide synthetic loci, only the *pel* genes were moderately more highly ranked than in the comparator data sets. Genes associated with the elaboration of pili were strongly expressed by the biofilm cells. As the database of published transcriptomes grows, comparisons based on internally ranked sets can provide insight into the activities of a given specimen. The transcriptome of drip-flow biofilm underscores the oxygen-limited, slow-growing nature of the population.

Characterization of biofilm heterogeneity using transcriptomic approaches

Presenter: Mike Franklin[1], Associate Professor, Microbiology
Co-authors: Kerry S. Williamson[1], Philip S. Stewart[1], Ailyn C. Pérez-Osorio[2], Kate Mcinnerney[1]
Affiliation: [1]Montana State University, Bozeman, MT;
[2]Washington State Department of Health, Shoreline, WA

Bacteria growing in biofilms are heterogeneous with respect to cell physiology. As nutrients diffuse into the biofilm and are utilized by the bacteria, chemical concentration gradients of nutrients, waste products, and signaling compounds are established. These gradients may intersect, creating many unique microenvironments within biofilms. Transcriptomics and proteomics profiling of microbial biofilms have been used to characterize adaptation of bacteria to growth in biofilms. For these global approaches it is often necessary to harvest the entire biofilm community to obtain sufficient quantities of RNA or protein for analysis. Therefore, these approaches, while providing an average value for transcription or translation throughout the biofilms, may not be adequate for discovering differences in gene expression that may occur at localized sites within the biofilms. In this study, we used laser capture microdissection (LCM) to isolate bacteria from the aerobic periphery and from the base of *Pseudomonas aeruginosa* biofilms. Following RNA amplification, Affymetrix® microarrays were used to characterize gene expression heterogeneity in these regions of the biofilms. The microarray results showed that most genes had higher mRNA abundances at the top compared to the base of the biofilms. Among the genes showing the highest mRNA levels at the biofilm top but very little at the bottom were genes involved in general cell metabolism, including ATP biosynthesis, cell division, and lipid production. These results suggest that cells at the top of the biofilm are actively growing, while cells at the base of the biofilm are in a slow growth state. Similarly, mRNA for genes involved in stationary phase growth and quorum sensing (QS) were highly abundant at the top but not at the bottom of the biofilms. Genes regulated by Anr and oxygen limitation stress were also highly abundant in cells at the top of the biofilm, suggesting that these cells may be in a transition state from oxygen sufficient to hypoxic conditions. Cells deeper in the biofilms showed little mRNA for Anr regulated genes, and have likely experienced long-term anoxia. Ribosomal RNAs were highly abundant throughout the biofilms, but mRNA for ribosomal proteins were only observed at the top of the biofilms, suggesting that de novo ribosome synthesis occurs in cells near the air-biofilm interface, but ribosomes are stably maintained throughout the biofilm. Consistent with these results was the identification of mRNAs for ribosome hibernation factors, which were highly abundant at both the top and bottom of the biofilms. The results suggest that in thick *P. aeruginosa* biofilms, cells are physiologically distinct spatially. The cells near the air-biofilm interface are actively involved in quorum sensing, but possibly entering stationary phase

state. Cells deep in the biofilm are in a slow growth state and may be dormant, possibly due to long-term oxygen starvation.

Acknowledgments: This work was supported by Public Health Service grant AI-065906 from the NIAID.

Proteomic and physiological support for the stoichiogenomic analysis of metabolic networks under nutrient stress

Presenter: Reed Taffs, PhD candidate, Chemical & Biological Engineering
Affiliation: Center for Biofilm Engineering, Montana State University, Bozeman, MT

The carbon conversions and energy conservation necessary to support life form a complex network of reactions catalyzed by an array of enzymes. It was hypothesized that natural selection favors efficient metabolic networks, regulated to optimize tradeoffs between overall metabolic yields and the investment of potentially scarce resources into the protein machinery required to obtain those yields. Based on this hypothesis, a metabolic network for *E. coli* was decomposed *in silico* into a complete set of simpler subunits (~5.2 million elementary flux modes). These subunits were analyzed in terms of overall conversion efficiency, as well as the various resources required for protein infrastructure. The analysis provides predictions about competitive steady-state metabolism under a multidimensional continuum of nutrient scarcities. *E. coli* chemostat cultures were grown under a range of carbon, nitrogen, and iron stresses, providing physiological and proteomic datasets for comparison with those predictions. The study highlights a fundamental design paradigm for competitive metabolic network structure and control, with applications to bioprocess engineering, environmental microbiology, and treatment of microbial pathogens.

Medical devices: The changing FDA landscape

Presenter: Craig Coombs, RAC; President,
Affiliation: Coombs Medical Device Consulting

The FDA is charged with promoting device innovation AND ensuring the safety and efficacy of medical devices in commercial distribution in the US.

We are currently observing an unprecedented level of frustration with the FDA in review times and in the unpredictability of requirements necessary for an FDA approval.

The three areas that are observed as most problematic with the FDA, both inside and outside the Agency, are:

1. Burdensome device classification system
2. Unreasonable or unexpected supporting data requirements
3. FDA has few usable databases for monitoring or regulating devices

The Root Causes of each of these problems are, in respective order:

1. A classification dependent upon substantial equivalence to a specific device, rather than substantial equivalence to a similar group of devices.
2. Reviewers with short tenure in position; little or no access to experts or to substantive training.
3. Lack of investment in usable databases, resistance by industry

Unless the Root Causes of these problems are addressed, the problem will not go away.

Upcoming changes that will address some aspects of the Root Cause problems:

1. Simplify the de novo process

2. Development of the Science Council
3. Development of the UDC

The proposed changes do little to attack the Root Causes of their problems. They are likely to persist to a great degree. Larger changes will probably require input from Congress.

SESSION 4: Biofilm Methods

Standard biofilm methods: A roadmap to anti-biofilm claims for medical devices

Presenter: Nick Allan, Contract Research Manager
Affiliation: Innovotech, Inc.

It has been well established that indwelling or perpherally inserted medical devices can be readily colonized by microbial biofilms of many pathogenic bacteria and yeast, and that this colonization leads to device failures and potentially much more serious device related infections. To prevent this, many implant medical device manufacturers offer antimicrobial coated or impregnated or even anti-adherent versions of these products. The fact that not all microorganisms can rapidly or successfully colonize all types of surfaces (due to the specifics of their individual life cycles) and the complex variety of materials, antimicrobials, coatings, and surface configurations of the various implant medical devices, makes this a difficult and often cumbersome situation to effectively model in vitro. This poses real problems for researchers, device manufacutrers, and, more important, regulatory bodies that ultimately need to approve any antimicrobial or antibiofilm claim these devices may carry. To address this issue, researchers at the University of Calgary developed the Biofilm Eradication Surface Test (or BEST™) assay. This is an adaptation of the MBEC™ assay (which just recently became an ASTM standard test method). The BEST™ assay utilizes a versatile, multi-well plate technology that allows biofilm growth on a wide variety of implant medical devices that can be conveniently transferred to serial challenge, rinse, neutralization, and recovery steps. The potential results of this simple, rapid assay include substrate-specific determination of biofilm growth curves, biofilm morphologies, efficacy break points and log reductions of biofilm growth due to chemical/antibiotic treatments, antimicrobial or anti-adherent coatings or polymers. Multiple concentrations, combinations, and treatments can be simultaneously evaluated in a replicated format. This presentation outlines antimicrobial medical device claims performed in support of several recent 510(k) applications by Innovotech utilizing the BEST™ assay as an example of how the system can be employed for antimicrobial claim applications. More important, the presentation outlines recent regulatory agency feedback on the test method as well as future directions on the potential requirements for an antibiofilm claim for an implant medical device. These directions will almost certainly involve the use of the BEST™ assay (or similar device) in a standardized test method. This presentation endeavours to elaborate on this approach.

Statistical considerations in equivalence testing

Presenter: Al Parker, Statistician & Research Engineer
Affiliation: Center for Biofilm Engineering, Montana State University, Bozeman, MT

Equivalence testing is becoming increasingly common in microbiology. This talk will demonstrate how statistical tests of equivalence can be applied to data from properly designed experiments to establish that two different processes produce results with differences which are considered negligibly small on the average. For example, for years the FDA has required that premarketing (510(k)) submissions demonstrate "substantial equivalence" between a new medical device and a legally marketed (predicate) one with respect to applicable characteristics, such as growth of inoculated organisms on surfaces treated with an

antimicrobial. In methods development, equivalence testing can be used to establish that a new method is equivalent to an existing "gold standard" with respect to such desirable attributes as responsiveness and reproducibility. Equivalence testing can also be applied to the neutralization step when testing disinfectants, where it is crucial to show that a neutralized disinfectant has a negligibly different anti-microbial effect when compared to the neutralizer itself.

SESSION 5: Medical Biofilms

Selective killing of biofilm pathogens

Presenter: Peter Suci, Plant Sciences & Plant Pathology
Affiliation: Center for Biofilm Engineering, Montana State University, Bozeman, MT

Abstract not available.

Oxygen, wound healing, and biofilms

Presenter: Garth James, CBE Medical Projects Manager, Associate Research Professor
Co-authors: Hung Duc Nguyen[2], Haluk Beyenal[2], Alice Ge Zhao[3], Alessandra Agostinho[1], Elinor deLancey Pulcini[1], Marcia Usui[3], Bob Underwood[3], Philip Fleckman[3], John Olerud[3], and Philip Stewart[1]
Affiliation: [1]Center for Biofilm Engineering, Montana State University, Bozeman, MT
[2]The Gene and Linda Voiland School of Chemical Engineering and Bioengineering, Washington State University, Pullman, WA
[3]Department of Medicine/Dermatology, University of Washington, Seattle, WA

Local oxygen (O_2) concentration is a critical parameter in wound healing. Tissue injury, in some cases combined with pre-existing ischemia, creates hypoxic niches in wounds. The presence of biofilms in chronic wounds has been demonstrated, but their role in delayed wound healing is unclear. We hypothesized that bacterial biofilms exploit hypoxic niches in wounds and function as an O_2 sink, perpetuating anoxia and preventing O_2-dependent wound healing processes. We used microelectrodes to measure O_2 concentration profiles of biofilms grown in vitro using bacteria isolated from human chronic wounds, as well as wound scabs and wound beds in a murine model for delayed healing of biofilm-infected wounds. Biofilms formed in vitro by *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Enterococcus faecalis*, as well as mixtures of these species, were all capable of depleting O_2 to less than 10% of air saturation within distances of a fraction of a millimeter. Mice challenged with *P. aeruginosa* biofilm had the largest populations of bacteria associated with the wound scabs. O_2 profiling was performed in situ using scabs on both live and euthanized mice as well as in excised scabs. O_2 profiling demonstrated steep oxygen gradients similar to those measured in the in vitro models in the all of the scabs. O_2 profiles from scabs were more complex than from in vitro biofilms, which may correlate with the heterogeneous ultrastructure of the scab. These O_2 gradients were eliminated by heat killing. In contrast, a wound bed probed after removal of the scab did not have a significant O_2 gradient. These results demonstrate that bacterial biofilms function as an oxygen sink in both the in vitro and murine models. Perpetuation of anoxic conditions by biofilms may be an important barrier to wound healing.

Intracellular pathogens and host cytoskeletal structures

Presenter: Kelly R. Kirker[1], Research Scientist
Co-Authors: Kevin Hybiske[2], Garth A. James[1]
Affiliation: [1]Center for Biofilm Engineering, Montana State University, Bozeman, MT
[2] Public Health and Infectious Diseases, University of California-Berkeley, Berkeley, CA

Exiting the cell is a vital process for intracellular pathogen dissemination and transmission to new hosts, yet it is poorly understood. The goal of this research is to develop an understanding of the mechanisms used by intracellular pathogens to exit host cells. Specifically, the unique mechanisms used by *Chlamydia* to manipulate host cell signaling pathways and promote cellular escape were investigated. *Chlamydiae* have evolved two distinct strategies for exiting host cells: (i) lysis, a destructive protease-dependent process that releases free bacteria into the extracellular milieu; and (ii) extrusion, a packaged release of bacteria in which they exit the cell in a double-membrane encased compartment; this leaves the original host cell intact, often with a residual chlamydial inclusion. A key advantage of the extrusion exit pathway is that it confers upon *Chlamydia* unique abilities for immune evasion, cell-to-cell spread, dissemination to distal tissues, transmission to new hosts, persistence, and chronic infection. Experimental evidence indicates that cellular cytoskeletal structures are recruited to aid in the extrusion process. This investigation employed scanning electron microscopy to visualize the extrusion process and the cellular structures that may be involved.

Effects of direct current application on biofilms

Presenter: Elizabeth Sandvik, PhD candidate, Chemical & Biological Engineering
Affiliation: Center for Biofilm Engineering, Montana State University, Bozeman, MT

Biofilm related infections on artificial devices remain a challenging problem in medicine. One proposed strategy is the use of direct current to enhance or replace existing antibiotic regiments. Low levels of direct current (DC) are reported to increase killing, induce bacterial detachment, and prevent bacterial establishment on surfaces. Studies show that while biocides or direct current alone have little or no effect on bacterial survival, the combination of direct current and biocide significantly increases killing efficacy. It is thought that this synergistic phenomenon, termed the bioelectric effect, could be optimized to enhance antibiotic treatment at clinically relevant concentrations that would otherwise typically have little effect on biofilms. In other studies, direct current used alone is reported to have killing efficacy and to affect bacterial adherence. Many of these studies used a minimal salts medium, excluding chloride-containing compounds, to minimize electrolysis effects related to the generation of chlorine species. However, a minimal salts medium does not reflect the physiologic salt concentrations (0.9% NaCl) found in the body.

Our research investigates the application of varying low levels of direct current to target staphylococcal infections of orthopedic implants in a dilute nutrient solution and a salt concentration of physiologic saline to approximate the conditions in an artificial joint. The effect of direct current in conjunction with ciprofloxacin was also examined.

S. epidermidis RP62A biofilms were grown at 37ºC on polycarbonate disks in a CDC biofilm reactor. The disks were then transferred to polycarbonate wells with platinum wire electrodes for treatment. The treatment wells contained $1/10^{th}$ strength tryptic soy broth with 9 g/L total NaCl with the addition of 2.5 µg/mL ciprofloxacin when applicable. Significant log reductions were observed with the application of direct current for 24 hours at 37ºC at all four levels (0.7, 1.1, 1.4, and 1.8 mA/cm^2) both in the presence and absence of ciprofloxacin. There were no significant differences in log reductions for wells containing ciprofloxacin compared to those without the antibiotic at the same current levels. When the experiments were repeated without biofilm or the organics in the media, significant generation of free chlorine was measured over the treatment time after application of current to the buffered saline solution. In a third variation, free chlorine concentrations equivalent to the 24-hour endpoint concentration for each current

level were used to treat biofilms in place of the direct current. Reductions in cell density were not statistically different from those observed with current. Electrolysis reaction related to chloride is likely a main contributor to the efficacy of direct current application in these experiments. A physiologically relevant NaCl concentration is thus a critical parameter in experimental design if direct current is to be investigated for medical applications.

Laboratory Demonstration Descriptions

Tuesday, July 12: 3 sessions, 1:50pm–3:10pm

EPS West Hall

EPS 301 Standard Methods
The Standard Methods Lab will highlight several customized reactor systems used to mimic specific industrial or consumer products environments. The design considerations of these reactors will be explained, as will the operational variables and sampling strategies. We'll describe how we modify our standard methods to isolate the variables of interest in each custom experimental system and the type of data that each system delivers. (**12 per session**)

EPS 302 Biomineralization Demonstration
The Biomineralization demonstration will focus on applying ureolytically inspired biomineralization using *Sporosarcina pasteurii*. We will first explain briefly how the biomineralization process works, then demonstrate its application in several visual porous media reactor systems. We will show you how the deposition of biomineralization crystals (*i.e.*, calcium carbonate) substantially alters the flow, transport and structural environment of porous media. (**12 per session**)

EPS 312 Biofilm Control
This demonstration will show different methods for testing the antimicrobial efficacy of cutting boards containing silver or triclosan. (**10 per session**)

EPS 316 Medical Biofilms
Medical Biofilms Lab team members will demonstrate
in vitro catheter model systems, which include both intraluminal and extraluminal central venous catheter model systems as well as a model for urinary catheters. We will demonstrate set-up and maintenance of the systems to establish biofilms, treatment strategies, and sampling including viable plate counts and microscopy. These models have been successfully used in the MBM for a number of company sponsored testing projects. (**12 per session**)

EPS North Hall

EPS 323 Lecture: Statistical methods for biofilm research
CBE statisticians Marty Hamilton and Al Parker will present this module. There will be an opportunity for participants to ask questions about statistics in biofilm work. It will begin with a very brief summary of statistical methods in biofilm research and an introduction to CBE's series of Knowledge Sharing Articles about statistics: (*www.biofilm.montana.edu/resources/knowledge_sharing_articles*) Then, as time allows, Marty and Al will discuss statistical issues put forward by participants. (**15 per session**)

EPS 326 Microscopy
The demo session will focus on application of time-lapse confocal microscopy to the observation of dynamic processes in capillary flowcell biofilms. Participants will observe *S. aureus* biofilms as treatments are applied and time lapse images are collected. Discussion will include: variations on flow cell design, the range of effects and events which can be imaged this way; and treatment application and imaging conditions. (**8 per session**)

First Floor, EPS

EPS 115 Algal Biofilms
The algal biofuels group will demonstrate the photo-bioreactor systems we use to optimize lipid production from novel algal strains. The demo will highlight algal isolation, culturing, and growth conditions, and will show the methods used to stimulate and assess lipid production (as a precursor to biodiesel). (**8 per session**)

Cobleigh Hall

Cob 321 Magnetic Resonance
The Magnetic Resonance (MR) Lab applies MR imaging and molecular dynamics measurements to biofilms and other complex systems, *e.g*., colloids, gels and porous media. Since MR is noninvasive, it provides unique 3D image and transport data. Topics discussed will be: basics of MRI, what is measured in MR experiments, and how this can be applied to a range of biofilm issues. The diversity of MR equipment and probes available in the MSU lab will be shown. (**12 per session**)

EPS East Hall

EPS 334 Microbial Fuel Cell
Assembly of a microbial fuel operated under potentiostat control will be demonstrated. While microbial fuel cells are often studied with the goal of optimizing power output, different anode or cathode materials can be tested for processes like corrosion and cathodic protection of metals. Controlling surface potentials with a potentiostat allows us to poise materials at particular states to gain a greater understanding of biofilm physical features and signals associated with such processes, as well as electron transfer within cells and biofilms. (**8 per session**)

EPS 336 Ecology and Physiology

EPS 337 Environmental Biofilms: Wetlands
The wetlands group in the Environmental Biofilms lab will be displaying experimental mesocosms that highlight biofilms in natural treatment systems. We will be discussing how we utilize our mesocosms to investigate the mechanics of pathogen removal and the relationships between plant species, temperature, and carbon loading on complete denitrification in treatment wetlands. (**10 per session**)

EPS 339 Image & Chemical Analysis (ICAL

Poster Abstracts

Center for Biofilm Engineering posters

CBE Poster #393

Date: 07/2007
Title: **Cost-benefit analysis of microbial resource allocation: Implications for intracellular pathogens**
Authors: ***Ross P. Carlson***, Taffs RL, Folsom J
Affiliation: Center for Biofilm Engineering, Montana State University, Bozeman, MT
Sponsored by: National Institutes of Health

Background and Objective: Persisting intracellular pathogens must adapt their metabolic functioning to cope with harsh environments, including low pH and high oxidative stress, while utilizing limited resource pools. Robust metabolic networks possess a wide range of options, which complicates prediction of pathogen stress response. This study uses systems biology to predict and interpret competitive pathogen resource allocation strategies that are likely necessary for intracellular colonization.

Methods: A cost-benefit analysis of an in silico *Escherichia coli* network model was performed using ecologically relevant resource allocation strategies. The strategies were identified by decomposing the metabolism into mathematically defined biochemical pathways (elementary flux modes) and assessing the resource investment cost-benefit properties for each pathway.

Results: The cost-benefit analysis revealed competitive molecular-level relationships between pathway enzyme investment, pathway efficiency, and enzyme functionality. The study identifies novel competitive network design principles which can be used to counter microbial strategies by accounting for the inherent trade-offs of investing finite resources like iron into different enzymes.

Discussion and Conclusions: The interpretation of bioinformatics data in terms of cellular function is a major challenge facing systems biology. The current study establishes a competitive relationship between resource allocation and metabolic fitness. This relationship is likely essential for pathogen adaptation to low nutrient environments like the phagosome. Understanding competitive strategies provides a rational basis for countering intracellular pathogens.

CBE Poster #504

Date: 08/2009
Title: **Analysis of methane producing communities within underground coal beds**
Authors: ***Elliott Barnhart***[1], Wheaton J, Cunningham A, and Fields M
Affiliation: Center for Biofilm Engineering, Montana State University, Bozeman, MT
Sponsored by: US Department of Energy

We have conducted initial phylogenetic diversity studies using inoculated coal from methane producing wells in the Powder River Basin (PRB) of southeastern Montana and northeastern Wyoming. Methane generating enrichments were grown with coal as the only energy source and compared to enrichments with acetate. Preliminary data revealed an extremely diverse bacterial community established in coal cultures compared to enrichments without coal. DNA sequences indicative of methanogens (methane-producing archaea) were detected in both enrichments. These findings offer a compelling motive for further investigations of the biogeochemical processes controlling coal bed methane (CBM) production. The research is aimed at enhancing the fundamental understanding of the ecology and physiology of methane producing communities with the intent of identifying strategies for enhancement of in situ CBM production.

CBE Poster #521

Date: 07/2010
Title: **In situ microbial reduction of selenium as source control in phosphate mine waste**
Authors: ***Lisa Bithell Kirk***[1], Peyton B[1], Childers S[2], and Gerlach R[1]
Affiliation: [1]Center for Biofilm Engineering, Montana State University, Bozeman, MT
[2]Dept. of Geological Sciences, University of Idaho, Moscow ID
Sponsored by: Inland Northwest Research Alliance, EPA Science to Achieve Results, Montana Water Center, and Idaho Mining Association

This study of subsurface microbial ecology investigates selenate reduction by indigenous microorganisms, using naturally available carbon in backfilled phosphate mine waste at sites in southeast Idaho, with an ultimate goal to define how backfilled mine pits can be ecologically engineered to reduce toxic and mobile selenate to insoluble and non-toxic elemental selenium. Several *Dechloromonas*-like, indigenous facultative ß-proteobacteria rapidly reduce selenate within a consortium of cold-tolerant hydrocarbon-degrading microbes. Temperature, lithology, and oxygen availability influence extent and rate of selenate reduction. More selenate-reducing organisms live in anaerobic shale than chert or mudstone, and almost no selenate reduction occurs when oxygen is present. Microbial reduction is distinguished from abiotic processes by evidence of biotic stable isotope fractionation and comparison with killed controls. Operational waste management strategies that promote Se(VI)-reduction by indigenous organisms using native carbon offer a sustainable, design-based approach to natural attenuation of selenium in mined rock.

CBE Poster #524

Date: 07/2010
Title: **Colocalization of syntrophs in a methanogenic biofilm**
Authors: ***Kristen A. Brileya***[1], Hatzenpichler R[2], Arkin AP[3], Hazen TC, and Fields MW[1]
Affiliation: [1]Center for Biofilm Engineering, Montana State University, Bozeman, MT
[2] Universitat Wien, Vienna, Austria
[3]Lawrence Berkeley National Lab, Berkley, CA
Sponsored by: NIH, INBRE

Transfer of reduced carbon and electrons between microbial community members is of interest in methanogenic systems that represent natural mediators of atmospheric carbon flux. The current work uses a dual-culture approach to examine the structure of syntrophic biofilm formed by the sulfate-reducing bacterium *Desulfovibrio vulgaris* Hildenborough and the methanogenic archaeon *Methanococcus maripaludi*s. We hypothesized that biofilm structure would reflect the energetic benefits of living in close association; the aim of the study is to visualize intact 3-dimensional biofilm structure to make testable predictions of structure-function relationships. Biofilm was grown in a continuously stirred biofilm reactor where cells could attach to a silica surface or remain suspended. Intact biofilm was fixed for fluorescence in situ hybridization (FISH) and embedded in agarose to maintain 3-dimensional structure. FISH revealed a framework of *D. vulgaris* with both single cells and large micro-colonies of *M. maripaludis* interspersed within the biofilm. FISH also confirmed steady-state biofilm irregularity, with ridge, valley, and spire macro-architecture. SYBR® Green counterstaining confirmed the presence of extracellular material. Colorimetric assays indicated cell-associated carbohydrate was composed of .035 µg hexose/µg protein, .017 µg pentose/µg protein and .011 µg uronic acid/µg protein, similar to *D. vulgaris* mono-culture biofilm and approximately 5 times less than *M. maripaludis* biofilm. Filaments presumed to be protein have been observed in dual-culture biofilm matrix with electron and atomic force microscopy, and matrix was sensitive to proteinase K treatment during preliminary work with Catalyzed Reporter Deposition FISH. Syntrophic biofilm 3-D structure appears to be driven by *D. vulgaris* while providing an advantageous situation for *M. maripaludis* to establish presumably active micro-colonies throughout the *D. vulgaris* scaffold.

CBE Poster #527

Date: 07/2010
Title: **Microbial diversity in a humic-free environment on the Cotton Glacier, Antarctica**
Authors: ***Heidi Smith***[1], ***Christine Foreman***[1], Sattler B[2], Chin Y-P[3], and McKnight D[4]
Affiliation: [1]Center for Biofilm Engineering, Montana State University, Bozeman, MT
[2]University of Innsbruck, Austria
[3]The Ohio State University
[4]University of Colorado–Boulder
Sponsored by: National Science Foundation OPP-0838970

A supraglacial stream forms annually on the Cotton Glacier, Antarctica. Analysis of dissolved organic matter (DOM) from this stream in 2004–05 and again in 2009–10 showed that the concentration was low (44–48 μM C), and lacked humic signatures, unlike typical DOM of microbially based ecosystems. Our results indicate that DOM in this system is seasonally formed from soluble microbial products and that a reservoir of recalcitrant humified DOM does not pre-exist. In most aquatic ecosystems, humic DOM acts as a natural sunscreen; the absence of humics may thus represent an additional stressor influencing the microbial community. Nonetheless, the stream contained an active microbial assemblage with bacterial cell abundances from 2.94×10^4–4.97×10^5 cells ml^{-1}, and bacterial production ranging from 58.8–293.2 ng C l^{-1} d^{-1}. Chlorophyll-a concentrations ranged from 0.3 to 0.53 μg l^{-1}, indicating that algal phototrophs were the probable source of the DOM. Microbial isolates produced a rainbow of pigment colors, suggesting adaptation to UV stress, and were similar to those from other cryogenic systems (Cytophagales and β-Proteobacteria lineages). Clone library analysis of the microbial assemblages from the stream water, ice, sediments, and aeolian communities were significantly different, but still related to organisms from other cold temperature environments. Taken together, these results suggest that the occurrence of related phylotypes from diverse glacial environs is due to similar survival strategies and that UV stress due to the absence of humics is important in supraglacial streams. Supraglacial streams provide an example of contemporary microbial processes on the glacier surface and a natural laboratory for studying the microbial adaptation to the absence of humics, as well as chemical processes controlling the eventual genesis of humic DOM.

CBE Poster #531

Date: 03/2010
Title: **EFRI-HyBi: Fungal processes for direct bioconversion of cellulose to hydrocarbons**
Authors: ***Natasha Mallette***[1,2], Peyton B[1,2], Carlson R[1,2], Strobel G[3], Hunt K[1,2], Strobel S[4], Smooke M[4]
Affiliation: [1]Center for Biofilm Engineering, Montana State University, Bozeman, MT
[2]Dept. of Chemical and Biological Engineering, Montana State University, Bozeman, MT
[3]Dept. of Plant Sciences, Montana State University, Bozeman, MT
[4]Yale University, New Haven, CT
Sponsored by: NSF Emerging Frontiers in Research & Innovation (EFRI)

While considerable national effort has been focused on ethanol production, very little research—beyond characterization of cellulolytic fungal enzymes—has examined the potential role of fungi in renewable fuel production. *Ascocoryne sarcoides* (NRRL 50072) is an endophytic fungus recently isolated from Northern Patagonia by Gary Strobel (MSU). *A. sarcoides* produces and excretes "mycodiesel," an extensive series of straight chained and branched medium chain-length hydrocarbons including heptane, octane, undecane, dodecane, and hexadecane (Strobel et al., 2008). This organism has the potential to produce petroleum directly using a cellulose fermentation process that is essentially carbon neutral. The goal of this research is to determine kinetic parameters of optimal fungal growth and hydrocarbon production through fermentation experiments. Experimental results from shake flask and 5 L reactor runs have verified

hydrocarbon compound production under many different growth conditions. Biomass yields have improved from 0.05 g/L to 4.8 g/L. The pH tolerance of *A. sarcoides* is in the acidic range, and optimal temperature is between 16–23°C. These preliminary results confirm the ability of *A. sarcoides* to produce valuable fuel compounds. Future research will focus on product chemistry and yields, and completing the mass balance for the system.

CBE Poster #532

Date: 01/2011
Title: **Imaging biofilm and microbially induced $CaCO_3$ precipitation in 2D porous media reactors**
Authors: ***James Connolly***, Phillips A, Bugni S, and Gerlach R
Affiliation: Center for Biofilm Engineering, Montana State University, Bozeman, MT
Sponsored by: National Science Foundation and the United States Department of Energy

Biological processes in the subsurface environment are important to understand in relation to many engineering fields including, but not limited to: groundwater remediation, geologic carbon sequestration, and petroleum production. Two biological processes studied here are biofilm formation and microbially induced calcium carbonate precipitation. Many analytical tools are available to researchers to study these processes, but supplemental microscopic imaging provides additional information and validation to these data sets.

Confocal scanning laser microscopy (CSLM), field emission scanning electron microscopy (FEM), and visible light stereoscopy were used to study processes in two-dimensional reactors with regular etched pore structures. Two kinds of reactors were used. The first (Fig. 1A and Fig. 2A, B) has uniform 1.0mm square pore structures and is designed for direct observation under a stereoscope or destructive sampling

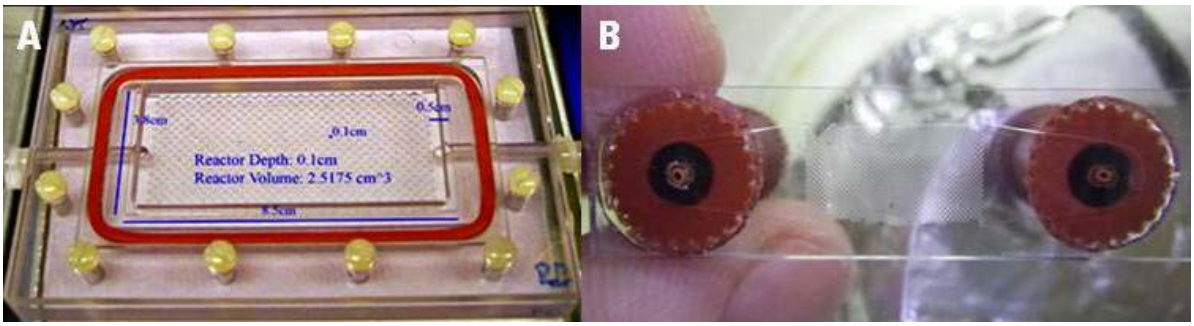

Figure 1 - (A) 2D porous media reactor with 1mm pore structures. (B) 2D porous media reactor with 100µm pore structures designed for CSLM imaging.

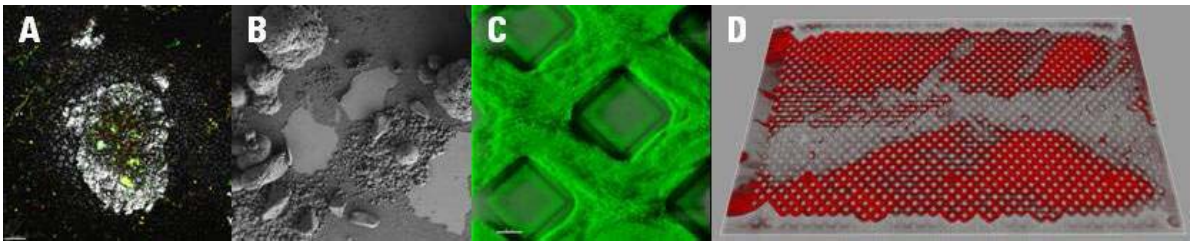

Figure 2 – (A) CSLM image of a microbially induced $CaCO_3$ crystal (gray) with cells attached (green and red). (B) FEM micrograph of microbially induced $CaCO_3$ precipitation. (C) Living bacterial biofilm grown in a porous media reactor with 100µm pore structures. The bacterial strain is expressing green fluorescent protein, so no staining was required. (D) 3D CSLM reconstruction of a stained bacterial biofilm grown in the reactor pictured in Fig 1B.

and imaging with other techniques. The second reactor (Fig. 1B and Fig. 2C, D) has 100μm pore structures and is specifically designed for CSLM imaging. Samples imaged under CSLM are generally prepared by staining the biofilm with various fluorescent stains. However, since staining may cause deleterious changes to metabolic processes, organisms with fluorescent protein are also imaged with CSLM so as to study basic biofilm functions. Finally, CSLM and FEM imaging are used in conjunction to obtain the most complete sets of images and data from a sample.

CBE Poster #533

Date: 01/2011
Title: **Using synthetic biology to engineer microbial consortia based on syntrophic metabolite exchange**
Authors: ***Hans C. Bernstein***, Paulson SD, Carlson RP
Affiliation: Center for Biofilm Engineering, Montana State University, Bozeman, MT
Sponsored by: National Institutes of Health

A combination of synthetic biology and metabolic engineering was used to construct artificial microbial consortia comprised of engineered *Escherichia coli* strains. The design was based on biomimicry of key ecological roles found in stable, naturally occurring microbial consortia. The constructed consortia were demonstrated to partition resources based on design and were then studied under batch, chemostat, and biofilm growth conditions. The consortium culturing strategy enabled an increased biomass yield, as compared to traditional mono-culturing, for all three experimental systems. The artificial community metabolic interactions dampened chemostat oscillations associated with the production of inhibitory compounds like acetate, highlighting ecological and bioprocess implications of consortia interactions. The engineered community, when cultured as a biofilm, self-assembled into micron-scale spatial regions, highlighting a new tool for engineering multi-reaction bioprocess systems.

CBE Poster #535

Date: 02/2011
Title: **Time-lapse imaging at the Center for Biofilm Engineering**
Author: ***Betsey Pitts***
Affiliation: Center for Biofilm Engineering, Montana State University Bozeman MT

Much of the optical microscopy at the Center for Biofilm Engineering is performed on live, fully hydrated biofilm samples. In the ideal live-cell imaging situation, the imaging process has as little impact on the biofilm as possible, so that time-lapse imaging can be a means of watching biofilm bacteria interact with each other and the environment. Time-lapse microscopy of biofilms has been a strength at the CBE since the center's inception, and some of our most stunning and insightful biofilm observations have come from time-lapse microscopy. The CBE's earliest transmitted light movies showed streamer development and surface migration, and allowed for investigation of biofilm rheology and detachment processes. With the development of fluorescent proteins and fluorescent probes for bacterial activity in the last ten years, the breadth of application of time-lapse microscopy has increased dramatically. Some of the processes that time-lapse is used to capture include: biofilm accumulation and development beginning with initial attachment of cells; detachment of clusters in response to treatment; pathogen capture by existing biofilms; diffusion of antimicrobials into a biofilm; the impact of an antimicrobial on a single cell; motility of biofilm cells; the viscoelasticity of biofilm clusters and how they can be chemically altered; and the impact of biofilm as compared to planktonic bacteria on human tissue culture. This poster summarizes some recent highlights of the application time-lapse microscopy to biofilm science at the CBE.

CBE Poster #536

Date: 05/2011
Title: **Antimicrobial penetration and efficacy in an in vitro oral biofilm model**
Authors: Audrey Corbin[1], Pitts B, Parker A and ***Philip S. Stewart***[2]
Affiliation: Center for Biofilm Engineering and [2]Department of Chemical and Biological Engineering, Montana State University Bozeman MT
[1]Current Address: ALCIMED, 75016 Paris, France
Sponsored by: This work was sponsored in part by Colgate-Palmolive Company.

The penetration and overall efficacy of six mouthrinse actives were evaluated using an in vitro flow cell oral biofilm model. The technique involved pre-loading biofilm cells with a green fluorescent dye that leaked out as the cells were permeabilized by a treatment. The loss of green color, and of biomass, was observed by time-lapse microscopy during 60 min of treatment under continuous flow conditions. The six actives analyzed were: ethanol, sodium lauryl sulfate (SLS), triclosan (TRN), chlorhexidine digluconate (CHX), cetyl pyridinium chloride (CPC), and nisin. Each of these agents effected loss of green fluorescence throughout biofilm cell clusters, with faster action at the edge of a cell cluster and slower action in the cluster center. The time to reach half of the initial fluorescent intensity at the center of a cell cluster, which can be viewed as a combined penetration and biological action time, ranged from 0.6 min to 19 min for the various agents. These times are much longer than the predicted penetration time based on diffusion alone, suggesting that anti-biofilm action was controlled more by the biological action time than by the penetration time of the active. None of the agents tested caused any removal of the biofilm. The extent of fluorescence loss after 1 h of exposure to an active ranged from 87% to 99.5%, with CHX being the most effective. Extent of fluorescence loss in vitro, but not penetration and action time, correlated well with relative efficacy data from published clinical trials.

CBE Poster #537

Date: 02/2011
Title: **SSU rDNA gene sequence region and quality-checking are essential for species richness and diversity estimates via pyrosequencing**
Authors: ***Kara B De León*** and Fields MW
Affiliation: Center for Biofilm Engineering, Thermal Biology Institute, and Department of Microbiology, Montana State University, Bozeman, MT

Due to errors during sequencing, pyrosequencing can overestimate the diversity of a system. The traditional sequence refinement method of removing sequences that contain ambiguous nucleotides, primer errors, and sequences that are less than one standard deviation from the mean length is not sufficient to account for this overestimation. Recent in silico and single organism studies have revealed the importance of SSU rDNA region selection and sequence quality score cutoffs in the estimation of diversity, respectively. This is the first study to validate these findings with an *in situ* environmental sample via the comparison of species richness and diversity estimates to a corresponding clone library. A clone library (1,113 sequences) and pyrosequencing library (18,628 sequences) were generated for two regions of the SSU rDNA, one that slightly overestimates (V4) and one that underestimates (V6) the diversity of a sample. Sequence refinement included the traditional refinement method as mentioned above; all sequences were trimmed to the mean length and checked for chimeric sequences. Additionally, the pyrosequences were subjected to varying quality score cutoffs ranging from 20 to 32, corresponding to an error probability rate of 0.063% to 1%. At each quality score cutoff either 10% or 15% of the nucleotides were allowed to be below the cutoff, the minimum and maximum allowable as suggested by Pyrotagger, an online program for sequence refinement (hereafter designated as a subscript of the quality score)(Kunin and Hugenholtz, 2010). The additional refinement resulted in 30.1–95.1% of total sequences removed. Sequences were clustered at 97% and rarefaction data and Chao1 diversity estimates were generated to compare the species richness and diversity at each quality score cutoff to the clone library data. For both the V4 and V6

SSU rDNA regions, a quality score cutoff of less than 25 resulted in an overestimation of species richness and diversity. The most stringent quality cutoff of $32_{10\%}$ for the V4 region was comparable to the clone data for species richness and diversity. A quality cutoff of $27_{10\%}$ for the V6 region corresponded best to the species richness and diversity estimates for the clone library data. The species richness and diversity estimates were underestimated for the V6 region when quality score cutoffs of $30_{10\%}$ and $32_{10\%}$ were used. These results indicate that pyrosequencing data must be thoroughly filtered and that a quality score cutoff is not universal across the SSU rDNA gene, likely due to differing proportions of conserved and variable regions. Using an environmental sample, our results further stress the importance of quality-checking pyrosequencing data in a region-dependent manner for the estimation of species richness and diversity.

CBE Poster #542

Date: 02/2011
Title: **Evaluation of bulk soap dispenser washing procedures**
Authors: ***Lindsey Lorenz[1]***, Goeres D[1], and Zapka C[2]
Affiliation: [1]Center for Biofilm Engineering, Montana State University, Bozeman, MT
[2]GOJO Industries, Inc. Akron, OH
Sponsored by: GOJO Industries, Inc.

A field study of soap dispensers demonstrated that up to 25% of open refillable bulk hand soap dispensers are contaminated with approximately 6 LOG_{10}(CFU/mL) heterotrophic bacteria based upon samples collected from the bulk soap[1]. In 2009 the CBE completed a research project to determine if biofilm growth within the dispensers contributed to bulk soap contamination. Plastic counter-mounted, plastic wall-mounted, and stainless steel wall-mounted dispensers collected from various locations in Ohio were analyzed for suspended and biofilm bacteria using heterotrophic and coliform viable plate counts and total cell counts. Bacterial identifications from the plate counts were performed using biochemical profiling of isolated colonies. Results indicated that the bulk soap was contaminated with 4–7 LOG_{10}(CFU/mL) bacteria and 4–7 LOG_{10}(CFU/cm^2) biofilm bacteria from the inside of the dispensers (n=6), independent of dispenser type or construction material. Overall the biochemical profiling identified 14 unique bacterial species and 11 different genera from all the dispensers tested. Bacterial populations were also identified using 16s SSU rRNA gene sequencing for the plastic and stainless steel wall-mounted dispensers to confirm organism identifications. No significant differences in bacterial genera were observed. All microorganisms identified are considered opportunistic pathogens.

The goal of the current project was to determine how the presence of biofilm impacts the ability to clean and sanitize the dispensers. Two dispenser washing experiments were performed.

Three washing procedures were evaluated for plastic wall-mounted dispensers:

1) a simple rinsing technique,
2) a rinse and scrubbing technique, and
3) a rinse, scrub, 5,000 mg/L bleach treatment, rinse combination.

Three additional washing procedures were evaluated for stainless steel wall-mounted dispensers:

4) a rinse, scrub, 5,000 mg/L bleach treatment with 10 minute soak, rinse combination,
5) a rinse, scrub, 8 mL/L Quat treatment with 10 minute soak, rinse combination, and
6) a rinse, scrub, full strength mildew remover treatment with 10 minute soak, rinse combination.

The washing study results showed that bacterial counts in the bulk soap returned to pre-wash levels within two weeks of cleaning a dispenser, then treating it with any of the methods tested.

These studies showed that dispensers contaminated with bacteria in the bulk soap also had high levels of biofilm bacteria that would be available to re-contaminate a dispenser, even if the old soap is emptied and the dispenser washed and treated with bleach/quat/mildew remover before new soap is added.

[1]Gerba CP, and Maxwell SL, "Bacterial contamination of liquid hand soaps used in public restrooms," Poster Presentation at NEHA 71st Annual Educational Conference & Exhibition, Atlantic City, NJ, 2007.

CBE Poster #543

Date: 07/2010
Title: **Isolation of bacterial alginate from *Pseudomonas aeruginosa* biofilms**
Authors: ***Matthew L. Sherick***[1,2], Fabich HT[1,2], Pitts B[2], Franklin MJ[2], Codd SL[2,3], and Seymour JD[1,2]
Affiliation: [1]Chemical and Biological Engineering, Montana State University, Bozeman, MT
[2]Center for Biofilm Engineering, Montana State University, Bozeman, MT
[3]Mechanical and Industrial Engineering, Montana State University, Bozeman, MT
Sponsored by: Undergraduate Scholars Program
Montana Space Grant Consortium (MSGC)

Certain strains of *Pseudomonas aeruginosa* bacteria produce the extracellular polysaccharide alginate, which forms a biopolymer gel in the presence of a divalent cation.[1,2] Using isolation processes outlined in publications, bacterial alginate can be isolated from *P. aeruginosa* biofilms and made to form a gel by introduction of a divalent cation.[3] Gelation of algal alginate has been thoroughly examined using Nuclear Magnetic Resonance (NMR)[4], and a point of interest is to compare the properties of bacterial alginate with those of the previously characterized algal alginate. The formation of the gel under diffusion reaction conditions produces a capillary system within the structure. By examining gels from both acetylated and non-acetylated bacterial alginate, the process by which alginate binds water can be analyzed using NMR. The capillary system is of particular interest in the field of biotechnology, where these gels can potentially be formed to provide the structure for artificial tissue development.[5] This system is also of interest for cystic fibrosis (CF) patients, who are vulnerable to chronic *P. aeruginosa* infections. Studying bacterial alginate formation and gelation will provide a greater insight into the molecular dynamics of these infections.[2]

CBE Poster #548

Date: 2011
Title: **Construction and characterization of metabolically engineered *Escherichia coli* biofilm communities**
Authors: ***Steve Paulson***, Bernstein HC, Carlson RP
Affiliation: Center for Biofilm Engineering, Montana State University, Bozeman, MT

Every year biofilms cause thousands of infections by contaminating medical devices such as implants, prosthetics, and catheters and often become a problem in the treatment of chronic wounds. In addition, damage to industrial equipment and massive power plant energy losses can be attributed to biofilm growth. In order to find new and innovative solutions to these problems, the morphological and physiological characteristics of biofilms must be thoroughly understood. This study examined and compared engineered *Escherichia coli* biofilms through enumeration, microscopy, and oxygen microsensor analysis to gain more knowledge about the mutualistic relationships found in biofilms. The results showed that community biofilm cultures engineered to mimic the common fermenter-oxidizer ecological theme generally exhibited higher growth and substrate productivity than their respective mono-cultures. Heterogeneous spatial partitioning effects have been observed and are reproducible. Nutrient availability of oxygen and glucose, coupled with mass transfer limitations, was found to induce strain specific laminations in community biofilm structure. This work increases our understanding of syntrophic biofilm characteristics, which are a common occurrence in biofilms. Future studies will focus on characterizing new strains that are currently being constructed, including an oxygen-negative strain that uses alternative terminal electron acceptors.

CBE Poster #549

Date: 04/2011
Title: **Controlling well bore leakage of CO_2 using engineered biomineralization barriers**
Authors: ***Alfred Cunningham*[1]**, Spangler L[2], Mitchell AC, Phillips A, Gerlach R
Affiliation: [1]Center for Biofilm Engineering, Montana State University, Bozeman, MT
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Sponsored by: US DOE EPSCoR; ZERT; DURIP; and the NASA Exobiology Program

If CO_2 is injected in deep geological formations, it is important that the receiving aquifer have sufficient porosity and permeability for storage and transmission and be overlain by a suitable low-permeability cap rock formation. When the resulting CO_2 plume encounters a well bore, leakage may occur through various pathways in the "disturbed zone" surrounding the well casing. To be effective, leakage mitigation methods must effectively and permanently block leakage pathways, be able to be delivered to desired locations in the aquifer without causing well screen plugging, and be resistant to challenge by supercritical CO_2 ($ScCO_2$). Technologies that can be delivered via low viscosity fluids and that can effectively plug small aperture pathways, or even the porous rock surrounding the well, could have significant advantages for some leakage scenarios. We propose a microbially mediated method for plugging preferential leakage pathways and/or porous media, thereby reducing well bore permeability and lowering risk of unwanted upward migration of CO_2. We examine the concept of using engineered microbial biofilms capable of precipitating crystalline calcium carbonate using the process of ureolysis. The resulting combination of biofilm plus mineral deposits, if targeted near points of CO_2 injection, may result in the long-term sealing of preferential leakage pathways. Successful development of these biologically based concepts could result in a CO_2 leakage mitigation technology that can be applied either before CO_2 injection or as a remedial measure. Laboratory results will be presented that illustrate how biomineralization deposits can be developed to plug porous media over a wide range of pore sizes (including fractured media) under both uniform and radial flow regimes. An experimental workplan will be presented for scaling up from laboratory results to field applications.

CBE Poster #550

Date: 04/2011
Title: **Transcriptome analysis of *Pseudomonas aeruginosa* biofilm subpopulations**
Authors: ***Michael Franklin*[1]**, Williamson KS[1], Stewart PS[1], Perez-Osorio AC[2], McInnerney K[1]
Affiliation: [1]Center for Biofilm Engineering, Montana State University, Bozeman, MT
[2]Washington State Department of Health, Shoreline, WA
Sponsored by: This work was supported by Public Health Service grant AI-065906 from the NIAID.

Bacteria in biofilms are heterogeneous with respect to cell physiology. As nutrients diffuse into biofilm and are utilized by the bacteria, chemical concentration gradients of nutrients, waste products, and signaling compounds are established. These gradients may intersect, creating many unique microenvironments within biofilms. In this study, we used laser capture microdissection (LCM) and Affymetrix® microarrays to characterize bacterial adaption to local environmental conditions within biofilms. RNA was purified from cells isolated from the top and bottom 30 μm of *P. aeruginosa* biofilms. As controls, eight genes with differing expression levels were also assayed by LCM and qRT-PCR. The microarray results showed that most genes had higher mRNA abundances at the top compared to the base of the biofilms. Among the genes showing highest mRNA levels at the biofilm top were genes involved in general cell metabolism, including ATP biosynthesis, cell division, and lipid production, suggesting that cells at the top of the biofilm are involved in cell growth. mRNA for genes regulated by Anr and oxygen limitation stress were highly abundant in cells at the top of the biofilm, suggesting that these cells may be in a transition state from oxygen-sufficient to hypoxic conditions. Cells deeper in the biofilms showed little mRNA for Anr-regulated genes, and have likely experienced long-term anoxia. Other transcripts that were highly abundant at the top of the biofilms, but below detection at the bottom of the biofilms, were for genes involved in stationary

phase growth and quorum sensing. Ribosomal RNAs were highly abundant throughout the biofilms, but mRNA for ribosomal proteins was only observed at the top of the biofilms, suggesting that de novo ribosome synthesis occurs in cells near the air-biofilm interface, but ribosomes are stably maintained throughout the biofilm. Consistent with these results was the identification of mRNAs for ribosome hibernation factors, which were highly abundant at both the top and bottom of the biofilms. The results suggest that in thick *P. aeruginosa* biofilms, cells are physiologically distinct spatially, with cells near the air-biofilm interface in a transition state from exponential to stationary phase, while cells deep in the biofilm may be dormant, possibly due to long-term oxygen starvation.

CBE Poster # 551

Date: 05/2011
Title: **A chemical trigger for inducing triacylglycerol accumulation in algae**
Authors: ***Rob Gardner***, Macur R, Mus F, Moll K, Eustance E, Carlson RP, Gerlach R, Peyton BM, Cooksey KE
Affiliation: Chemical and Biological Engineering, Montana State University, Bozeman MT
Microbiology, Montana State University, Bozeman MT
Sponsored by: DOD, DOE, NSF-IGERT

Background:
One of the main goals of the DOE Aquatic Species Program in the 1980s was the identification of the so-called "lipid trigger"—a set of circumstances or a signaling molecule that mediates lipid synthesis or accumulation. No new definitive answers were found (growth medium N-limitation was already known), but there were indications that interference of the algal cell cycle by monofluoroacetic acid or elevated medium pH were possible candidates, albeit for a single species of alga (*i.e.*, *Chlorella* Chlor-1). Here the results of a pH study on cellular lipid accumulation on two more Chlorophytes: *Coelastrella sapiensis* PC-3 (similar to *Chlorella* Chlor-1,) and *Scenedesmus obliquus* WC-1 are presented.

Methods:
Triacylglycerol (TAG) accumulation, nitrate remaining in the growth medium, and pH were monitored in biologically buffered and unbuffered cultures.

Results:
This presentation documents the differential effects of pH and nitrate depletion on TAG accumulation (monitored by Nile Red fluorescence and confirmed by gas chromatography) and separates the causal effects of this phenomenon (*i.e.*, N-depletion in the growth medium versus pH changes). A control point for TAG accumulation was suggested, and further focus directed at elucidating this control point resulted in discovery of a chemical trigger that stops cellular replication in Chlorophytes and induces TAG accumulation. The chemical trigger has been shown to work on multiple Chlorophytes and diatoms, both marine and freshwater, demonstrating induction of TAG accumulation across a broad spectrum of algae. The rate and concentrations of TAG accumulation are strain-specific but in general, total culturing time to reach optimal TAG accumulation (or higher) is reduced by half.

Conclusion:
Results here suggest an industrially relevant technique to metabolically trigger algal cultures from high growth to high lipid content scenarios with decreased total culturing times.

CBE Poster #552

Date: 07/2011
Title: **Exploration and optimization of hydrocarbon production by *Ascocoryne sarcoides***
Authors: ***Elle Pankratz***, Mallette N, Peyton B, Carlson R
Affiliation: Center for Biofilm Engineering, Montana State University, Bozeman, MT
Sponsored by: NSF Emerging Frontiers in Research & Innovation (EFRI), Montana State University Undergraduate Scholars Program

While a great deal of effort has been focused on ethanol and algal renewable fuel production, very little research has examined the role of fungi as a possible energy source. *Ascocoryne sarcoides* is an endophytic fungus isolated from Northern Patagonia by MSU scientist Dr. Gary Strobel. *A. sarcoides* produces a widespread series of straight chained and branched medium chain-length hydrocarbons including heptane, octane, undecane, dodecane, and hexadecane and has the potential to produce petroleum from a cellulose fermentation process. The goal of this research is to determine optimal growth kinetic parameters and hydrocarbon production through fermentation experiments. Results from shake flask and limited oxygen serum bottles have shown optimal growth rates in a lower acidic range (~ pH 3.5), but optimal carbon source yields in a higher acidic range (~ pH 6.5). The optimal temperature range for growth consistently proves to be 20°C–25°C. Growth on cellulose has demonstrated production of a range of hydrocarbon compounds including branched and straight-chained undecane, branched benzenes, and 5-6 carbon alcohols. Future research will focus on product chemistry and screening other fungi as potential producers of fuel compounds.

CBE Poster #553

Date: 07/2011
Title: **Evaluation of human hand bacterial communities with different techniques**
Authors: ***Carrie A. Zapka***[1], Ramsay B[2], Rackaityte E[1], Lauber C[3], Weimer BC[4], Desai P[4], Fierer N[3], Macinga DR[1], Fields MW[2]
Affiliation: [1]GOJO Industries, Inc., Akron, OH
[2]Montana State University, Center for Biofilm Engineering, Bozeman, MT
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Sponsored by: GOJO Industries, Inc.

Skin of the human hand, one of many microbial habitats of the human body, is of particular interest because it plays important roles in the transmission of pathogens due to constant interaction with the external surroundings and other parts of the body. Previous studies have shown that the skin microbiota can be diverse, dependent upon body location, skin type, and subject. The objective of our study was to compare culture-dependent to culture-independent methods (clonal sequences, pyrosequences, and PhyloChip) for the assessment of hand skin microbiota for 3 test subjects. In addition, we also assessed the bacterial communities post-treatment. A whole-hand sampling method was used on both hands of 6 participants who had not used antimicrobials for 2 days and who had not washed for at least two hours prior to sampling. For the post-sanitizing treatment, hands were re-sampled under the same conditions two days later, immediately after treatment with sanitizing solution. When different cultivation media were used, 7 to 17 different genera were isolated from three individuals and the cultivated populations were present at levels between <500 and 3,000,000 CFU/hand. Viable colonies of *Corynebacterium, Propionibacterium*, and *Staphylococcus* sp. were isolated from all three individuals. Cultivated isolates differed between individuals and represented a subset of the sampled diversity observed with sequence-based techniques. As expected, clonal sequence libraries underestimated the community diversity (between 11 to 29 genera), but differentiated microbial communities for the tested subjects. Pyrosequencing libraries were compared at

different depths of coverage (approximately 1,000 versus 10,000) and gave varying results with estimated OTU levels ranging from 70 to 1,000 per individual. Predominant sequences were detected at similar relative distribution levels (*e.g*., *Propionibacterium*, *Staphylococcus*, and *Streptococcus*), while less predominant populations (*e.g*., *Moraxella*, *Haemophilus*, *Kocuria*, and *Eubacterium*) were only observed with deeper coverage. The PhyloChip detected similar sequences, but estimated the number of predicted OTUs between 165 and 585 OTUs. As expected, pyrosequencing and PhyloChip analyses provided improved community characterization and indicated that individuals displayed distinct communities as well as variation between left and right hands. All bacterial communities were altered post-treatment wash to varying degrees, and the response was subject-dependent.

CBE Poster #554

Date: 07/2011
Title: **Optimization of cell yield and triacylglycerol accumulation for a Yellowstone diatom**
Authors: ***Karen Moll***, Gardner R, Eustance E, Macur R, Gerlach R, Peyton B, Cooksey K
Affiliation: Center for Biofilm Engineering, Montana State University, Bozeman, MT

With increasing global demand for petroleum, microalgae may soon become a viable biodiesel source due to their higher oil yield per hectare compared to other biofuels. Optimizing the accumulation of lipids as triacylglycerol (TAG) for biodiesel production is critical to decreasing production costs. Some algal strains are capable of producing large quantities of TAGs under stressed conditions, such as nutrient limitation or other factors (*e.g*., pH). This study investigated the role of varying silica concentrations on cell yield, growth kinetics, and TAG accumulation for a pinnate diatom isolated from Yellowstone National Park. Additional results are presented on the ability of $NaHCO_3$ to increase the rate of production and accumulation of TAG. Silica concentration was varied to increase diatom cell density. Growth was monitored using direct cell counts; pH, chlorophyll, nitrate, and silica utilization were quantified. TAG measurements were monitored by Nile Red fluorescence. Increasing the silica concentration in the growth medium resulted in higher diatom cell yield and cellular dry weight, but decreased diatom growth rate. This indicates an optimum silica concentration for growth. At silica depletion, lipid accumulation was promoted. It was found that cultures with added $NaHCO_3$ enhanced the rate of lipid production and total TAG accumulation. Following $NaHCO_3$ addition, diatoms reached the same TAG concentration in a shorter time. When silica is fully utilized, cells redirect energy and CO_2 into storage molecules (TAGs). The addition of $NaHCO_3$ increased the rate of TAG accumulation. The combination of increased cell yield and specific TAG accumulation rate significantly increased total lipid production. Results are industrially relevant because they demonstrate decreased time and cost for biodiesel production, improving algal biofuel viability as an alternative energy source.

CBE Poster #555

Date: 07/2011
Title: **Microbial ecology of biofilms on two types of water distribution pipe materials**
Authors: ***Gem Encarnacion***, Camper AK
Affiliation: Center for Biofilm Engineering, Montana State University, Bozeman, MT

This study analyzed differences between bacterial populations in biofilms that formed on polyvinyl chloride (PVC) and copper coupons in reactors that simulate premise plumbing. Here we used modified CDC reactors that had been actively nitrifying for six years. Biofilms were collected for carbohydrate and protein analysis as well as for DNA extraction. Polymerase Chain Reaction (PCR) targeting the 16S ribosomal RNA gene followed by denaturing gradient gel electrophoresis (DGGE) was performed. Carbohydrate to protein ratio of the biofilms from the copper and PVC were found to differ, suggesting a difference in the assemblage of organisms. This result is also reflected in the molecular analysis. DGGE

profiles of the inoculum (Bozeman tap water) biofilm from the copper and PVC coupons were compared and were found to significantly differ from each other. This demonstrated that pipe material affects bacterial diversity, since the reactors with the copper and PVC coupons were given the same inoculua, and microbial assemblages distinct from the source developed in each. To further assess the diversity, bands were excised from the denaturing gel from both copper and PVC samples and were re-amplified, cloned, and sequenced. Sequencing revealed the presence of nitrite oxidizing bacteria (NOB), but not ammonia oxidizing bacteria (AOB) in these actively nitrifying systems. As AOB are the commonly implicated organisms in ammonia oxidation in drinking water distribution systems, results may mean that the techniques used (*i.e.*, DNA extraction, PCR primers), failed to detect their presence or that another group of organisms is responsible for this step in the nitrification process in this specific system.

CBE Poster #556

Date: 6/15/2011
Title: **Toward standardized methods for the analysis of algal lipids: Solvent extraction and direct transesterification**
Authors: ***Richard E. Macur***, Gardner R, Gerlach R, Cooksey KE, Peyton B
Affiliation: Center for Biofilm Engineering, Montana State University, Bozeman, MT
Sponsored by: US Department of Energy Office of Biomass Programs

Accurately evaluating the potential of microalgae to produce lipids is critical if a microalgal-based biodiesel market is to be created. Laboratories currently use a wide variety of methods to extract and characterize lipids produced by algae, and this may be responsible in part for the broad range of reported lipid contents. It is well known that different methods provide different values for lipid content and quality, and consequently, the use of diverse methods has created a situation where direct comparison of data generated by different laboratories is often challenging, if not impossible. A thorough review of published information on algal lipid extraction techniques as well as data recently acquired at our laboratory provides insights into the most efficient methods. Laboratory results support the use of bead beating in a triple solvent system (chloroform/hexane/tetrahydrofuran) as a relatively simple and effective means for extracting triacylglycerides and other lipid metabolites. The most effective direct transesterification (DT) method involves several steps using sodium methoxide and BF_3-methanol as catalysts for transesterification. A simpler and nearly as effective DT method that utilizes H_2SO_4 as the catalyst was also identified. The information reported here may serve as the basis for the development of standardized protocols for research and industry.

CBE Poster # 557

Date: 05/2011
Title: **Differences in bacterial transfer and fluid path colonization through needlefree connector-catheter systems in vitro**
Authors: ***Garth James***[1], Ryder M[2], deLancey Pulcini E[1], Bickle L[1], Parker AE[1]
Affiliation: [1]Center for Biofilm Engineering, Montana State University, Bozeman, MT
[2] Ryder Science, San Marcos, CA
Sponsored by: ICU Medical, Inc.

The use of mechanical valve connectors, as opposed to split septum connectors, has been implicated as an infection risk. Differences exist in the transfer of bacteria through the various connectors (C), but the impact of repeated injections of bacteria into the bloodstream and colonization of the catheter hub (CH) and internal lumen (IL) is unknown. The primary objectives of this study were to evaluate differences in transfer of bacteria through connector-catheter systems and biofilm formation in the C, CH, and IL. Clearlink™, SmartSite®, InVision-Plus®, MaxPlus®, Q-Syte® and MicroCLAVE® connectors were evaluated using an in vitro model designed to simulate clinical use. Connectors were evaluated in three groups of

three experiments, with 2–3 connectors run side-by-side in each group. The connector septum was inoculated with 10^6 CFU *Staphylococcus aureus*, dried for 30 minutes, and then attached to a catheter and flushed with 3 ml sterile saline (SS), which was collected and plated. The catheter-connector sets were flushed 2x and locked with sterile Brain Heart Infusion Broth (BHI) for 1 hour. The sets were flushed again 3x with 3 ml SS. This was repeated again 3x for a total of 15 flushes and a BHI lock after the 1st, 3rd, and 4th set of flushes, for a total of 18 accesses. The last flush was collected and plated. The entire procedure was repeated daily for 5 days. At 72 and 96 hours, 2 connector-catheter sets for each connector type were destructively sampled for bacterial counts and microscopy.

The MicroCLAVE® connector had a significantly smaller mean log density (MLD) of bacteria ($p<0.0001$) when averaged over all flushes, inoculations, days, and runs than any other connector. In contrast, the Q-Syte®, a split-septum connector, had a significantly larger MLD ($p<0.0001$) than the other 5 connectors. In side-by-side comparisons, MLD of the CH and IL was also significantly smaller for the MicroCLAVE® than for the other connectors, except the Clearlink™. At the end of the experiments MicroCLAVE® MLD on the CH was less than SmartSite® ($p=0.0042$), InVision-Plus® ($p=0.001$), MaxPlus® ($p=0.0258$), and Q-Syte® ($p<0.0001$). The MLD on the CH for the Clearlink™ was less than SmartSite® ($p=0.0199$), while the MaxPlus® was less than Q-Syte® ($p=0.0002$). The MLD of bacteria on IL was also less for MicroCLAVE® than SmartSite® ($p=0.0012$), InVision-Plus® ($p=0.0031$), MaxPlus® ($p=0.0196$), and Q-Syte® ($p=0.0004$). The MLD on the IL for the Clearlink™ was less than SmartSite® ($p=0.0012$). Thus, significant differences were observed among connectors for bacterial transfer and colonization of the CH and IL. In general, the split septum connector performed poorly compared to the other connectors, while the MicroCLAVE® performed better than the other connectors. Overall the results suggest that connector selection may impact both bacterial transfer to the bloodstream and bacterial colonization of CH and IL.

CBE Poster # 558

Date: 05/2011

Title: **The effect of chlorhexidine antimicrobial coating on the reduction of intraluminal catheter biofilm formation in a clinically simulated ovine model (Pilot study)**

Authors: ***Laura Bickle***[1], Ryder M[2], Gunther RA[3], Breznock EM[4], James G[1], deLancey Pulcini E[1]

Affiliation: [1]Center for Biofilm Engineering, Montana State University, Bozeman, MT
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[4] BioSurg, Inc., Winters, CA

Sponsored by: Teleflex

The hub and intraluminal surfaces of vascular catheters are a major source of catheter-related bloodstream infection (CRBSI), particularly in catheters indwelling for greater than 5–7 days. The use of PICC catheters with an antimicrobial coating on the intraluminal catheter surface and within the catheter hub may reduce infection risk. The purpose of this pilot study was to determine the effect of a chlorhexidine (CH) coated PICC on the reduction of intraluminal biofilm formation (*i.e.*, colonization) compared to a non-coated PICC catheter in an ovine model. Each of eight sheep was randomized to control (non-coated catheter) or treatment group (CH coated catheter). Each sheep received one catheter inserted into the jugular vein. Post-operatively catheters were locked with a 10^6 *Staphylococcus aureus* inoculum for 3 hours and the inoculum was then removed. Catheters were infused daily with Lactated Ringer's solution over 8 hours for 7 days and removed on day 8. Outcome measures included temperature, white blood cell count, catheter blood culture, intraluminal catheter segment colonization (including connector, hub, catheter extension tubing, and catheter segments), scanning electron microscopy, and confocal laser microscopy of intraluminal catheter surfaces. One control catheter was removed due to the sheep experiencing fever of >105°F for two days. All blood cultures from the treatment catheters returned to, and remained, negative after the positive post-inoculation catheter blood culture. All catheter blood cultures (except for one sheep) in the control group increased to 10^5 CFU/ml within 8 days. No chlorhexidine-coated catheters had to be

removed from sheep due to infection. Average log bacterial colonization counts of control non-coated catheter samples were 3.74 log CFU/cm^2 at the tip, 1.43 log CFU/cm^2 in the middle, 1.57 log CFU/cm^2 in the extension tubing and 2.05 log CFU per hub. Average log bacterial colonization counts of chlorhexidine-coated catheter samples were 0.30 log CFU/cm^2 at the tip, middle, and extension tubing, while the hubs had an average of 0.57 log CFU. The data were pooled for all segments sampled for each catheter and the results indicated an overall 2.91 log reduction with a p value of 0.02. Thus, the chlorhexidine-coated PICC catheter resulted in a significant reduction in intraluminal colonization and infection compared to the non-coated PICC catheter.

CBE Poster # 559

Date: 05/2011

Title: **Reduction of extraluminal bacterial colonization using chlorhexidine antimicrobial-coated PICC catheters in a clinically simulated ovine model (Pilot study)**

Authors: ***Marcia Ryder***[2], Gunther RA[3], Breznock EM[4], James G[1], deLancey Pulcini E[1], Bickle L[1]

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Sponsored by: Teleflex

The risk of catheter-related bloodstream infection (CRBSI) with use of the peripherally inserted central venous catheter (PICC) in hospitalized patients is reported to be similar to conventional central venous catheters. Both the internal and external surfaces of vascular catheters are vulnerable to microbial attachment and biofilm formation. The skin is the primary source of microorganisms colonizing the extraluminal surface. Attachment of bacteria occurs during the insertion of the catheter through the skin or with post-insertion migration through the subcutaneous tract. The use of antimicrobial-coated catheters is among the recommended strategies to reduce catheter colonization and CRBSI. The purpose of this pilot study was to determine the effect of a chlorhexidine (CH)-coated PICC compared to a non-coated PICC catheter on the reduction of extraluminal colonization in an ovine model. Each of eight sheep was randomized to control (non-coated catheter) or treatment group (CH-coated catheter). Each sheep received 2 catheters, one in each jugular vein. All catheters were inserted through disinfected skin inoculated with 10^6 *Staphylococcus aureus*. Catheters were heparin locked for the 10-day study. Sheep were euthanized and catheters were removed within the vessel by enbloc dissection at 10 days or after fever of <105° F for 2 days. Outcome measures included temperature, white blood cell count, peripheral blood culture, insertion site skin cultures, colonization of the skin and subcutaneous tissue, catheter segment colonization, and scanning electron microscopy and confocal laser microscopy of extraluminal catheter surfaces. Six catheters in the control group were removed due to fever, and no catheters were removed for fever in the treatment group. On gross examination, extensive inflammation of the vessel wall, cellulitis of the surrounding tissue, and catheter-related thrombus was observed in the control catheters while all vessels and tissue appeared normal in the treatment group. Mean log bacterial colonization counts of the control catheter were: tip segment 3.42 CFU/cm^2, vessel entry segment 4.22 CFU/cm^2, and segment within the skin 4.42 CFU/cm^2. Mean subcutaneous tissue counts were 5.47 CFU/g and mean skin counts were 6.55 CFU/g. Mean log counts of CH-coated catheter segments were: tip segment 2.58 CFU/cm^2, vessel entry segment 1.68 CFU/cm^2, and skin segment 2.16 CFU/cm^2. CH-coated catheter subcutaneous tissue counts were 4.12 CFU/g and skin 3.92 CFU/g. The results of pooling the data for all samples indicated a 1.64 log reduction for the CH-coated catheter ($p = 0.01$) and a 1.78 log reduction for the tissue ($p = 0.01$). Thus, the chlorhexidine-coated PICC catheter resulted in a significant reduction in intraluminal colonization and infection compared to the non-coated PICC catheter.

Other Academic Posters

Date: 06 / 2011
Title: **Effects of tannic acid on *Pseudomonas aeruginosa* biofilm development**
Authors: ***Che O'May***, Ciobanu A, Lam H, Tufenkji N
Affiliation: Department of Chemical Engineering, McGill University, Montreal, Canada

Bacterial motility plays a key role in surface colonization and formation of biofilms. In particular, the surface-associated swarming motility is intimately linked to biofilm development and antibiotic resistance. Recently, we demonstrated that cranberry fruit derivatives and other tannin containing plant extracts completely blocked *Pseudomonas aeruginosa* swarming motility. This project investigated how the tannin-induced blocking of swarming motility affected biofilm formation in a range of different models. Given that *P. aeruginosa* is implicated in infections under reduced oxygen conditions, select experiments were conducted under both aerobic and anaerobic atmospheres. As the plant extracts contain different mixtures of tannins, initial investigations focused on well-defined, commercially available tannic acid (TA).
As reported with many other tannin extracts, aqueous extracts of TA blocked swarming motility in a dose-dependent manner. Interestingly, TA enhanced aerobic biofilm formation in a high nutrient medium (polystyrene microtitre plate model). However, TA did not enhance biofilm formation in glass or polystyrene culture tubes, and actually impaired biofilm formation in minimal M9 medium (microtitre plate). Under anaerobic conditions, high concentrations of TA caused a reduction in anaerobic growth and subsequent biofilm formation (microtitre plate). This inhibition could be reversed by supplementing the medium with iron, but not calcium or magnesium, suggesting that TA's effects under anaerobic conditions are iron-related. Taken together, these data emphasize the use of multiple model systems when investigating potential anti-biofilm compounds *in vitro.*

Date: 06 / 2011
Title: **Investigating combination treatments with antibiotic and nutrient dispersion compounds against *Pseudomonas aeruginosa* biofilms**
Authors: ***Stacy Sommerfeld Ross***[1], Fiegel J[1,2]
Affiliation: [1]University of Iowa, College of Pharmacy, Department of Pharmaceutical Sciences and Experimental Therapeutics, Iowa City, IA;
[2]University of Iowa, College of Engineering, Department of Chemical and Biochemical Engineering, Iowa City, IA.

Pseudomonas aeruginosa causes serious respiratory infections in patients who have chronic lung disease or who are otherwise immunosuppressed. Once established, *P. aeruginosa* infections persist for decades in the patient, resulting in serious inflammation in the lungs and a high mortality rate. Current antibiotic therapy in patients colonized with this opportunistic pathogen, including inhaled tobramycin therapy, alleviates some acute symptoms caused by the lung infection. However, despite the often aggressive use of antibiotics, these opportunistic infections are not completely eradicated. Researchers have shown that even two and three antibiotic combinations are minimally effective against laboratory and clinical strains of the bacterium. A new approach under development is focused on the use of dispersion compounds to entice the bacteria out of the biofilm. We tested the hypothesis that a combination therapy containing both an antibiotic and a dispersion compound would provide a synergistic effect in breaking up and eradicating biofilms.

Screening studies were conducted to evaluate the ability of a single dose co-treatment of antibiotic and dispersion compounds to eradicate in vitro cultures of a biofilm-forming, mucoid strain of *Pseudomonas aeruginosa* (BAA-47; ATCC). Biofilms were inoculated with approximately 10^8 CFU/mL in Lab-Tek

Chambered Coverglass. The bacteria were exposed to various combinations of 5 antibiotics and 4 dispersion compounds. The antibiotics were amikacin disulfate (1 mg/mL), polymyxinB sulfate (1 mg/mL), colistin sulfate (2 mg/mL), erythromycin (2.54 mg/mL), and tobramycin sulfate (6.26 mg/mL). The nutrient dispersion compounds were sodium citrate (2.94 mg/mL), succinic acid (1.18 mg/mL), xylitol (11.55 mg/mL), and glutamic acid (94 µg/mL). Twenty-four hours after treatment, biofilms were stained using the BacLight Live/Dead assay and visualized via confocal microscopy to acquire 3D image stacks. Images were quantified using COMSTAT to determine the percent of live and dead bacteria remaining after treatment.

Untreated controls contained a mix of live and dead bacteria (83.2 ± 8.0% live). Dispersion compounds tested individually resulted in biofilm growth, without a statistically significant change in percent of live bacteria after treatment. Synergy was found between three antibiotic compounds and various dispersion compounds. While PolymyxinB sulfate (15.8 ± 5.2 % live) and colistin sulfate (15.2 ± 2.9% live) were the most effective antibiotic treatments alone, their effectiveness as co-treatments with dispersion compounds was dramatically different. PolymyxinB sulfate co-treatments were antagonistic. However, colistin sulfate was synergistic with sodium citrate (3.7 ± 1.6% live), succinic acid (9.1 ± 3.2% live), and xylitol (10.5 ± 3.1% live). One other combination, erythromycin with sodium citrate, exhibited comparable effectiveness (12.2 ± 8.8% live). The remaining combinations of antibiotics and dispersion compounds were less effective at eradicating the biofilms.

Results from these studies suggest that co-treatment with antibiotic and dispersion compounds is a promising strategy that should be further explored for potential in vivo application. Currently we are developing dry powder aerosols to efficiently deliver multiple synergistic compounds at the site of infection in the respiratory tract.

Industry Posters

Date: 06 / 2011
Title: **DispersinB® enzyme-based product for treating CF-associated infection**
Authors: ***Karen LoVetri***, Gawande PV[1], Yakandawala N[1], Cardona ST[2], Madhyastha S[1]
Affiliation: [1]Kane Biotech Inc. 5-1250 Waverley Street, Winnipeg, MB R3T 6C6, Canada
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Cystic Fibrosis (CF) is characterized by a plugging up of airways leading to recurrent respiratory tract infection by bacteria and hence a gradual decline in the cardiovascular and pulmonary functions. Since CF infection-associated bacteria in biofilms are resistant to current antibiotic therapy, there is an unmet medical need for developing a combination product with both the antimicrobial and antibiofilm activity. We have developed an antibiofilm-antimicrobial composition comprising DispersinB®, DNase I and tobramycin, which shows a synergistic in vitro antibiofilm-antimicrobial activity against CF-associated bacterial pathogens such as *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Burkholderia* spp. Under in vivo conditions, DispersinB® and DNase I alone or together in combination with tobramycin showed a synergistic antibiofilm- antimicrobial activity against *S. aureus* in *Caenorhabditis elegans* model of infection over seven days. This composition may have potential applications in treating CF patients with lung infections.

Date: 06 / 2011
Title: **In vitro and in vivo efficacy of DispersinB® wound spray**
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With an epidemic increase in obesity and diabetes, combined with an aging population, chronic wounds such as pressure ulcers, diabetic foot ulcers and venous leg ulcers are an increasing clinical concern. Recent studies have shown that bacterial biofilms are a major contributor to wound bioburden and interfere with the normal wound healing process. As the traditional methods of treatment have proven ineffective against chronic wounds involving biofilms, there is an unmet clinical need for developing products with an antibiofilm component that inhibits and/or disrupts biofilms and thus make the biofilm-embedded bacteria more susceptible to antimicrobial therapy. We have developed a DispersinB® antibiofilm enzyme-based wound spray for treating chronic wounds in conjunction with an antimicrobial. Furthermore, we have tested the in vivo efficacy of combination of nanocrystalline silver containing Acticoat™ wound dressing and wound spray, and Acticoat™ alone using chronic wound mouse model of methicillin-resistant Staphylococcus aureus (MRSA) infection. DispersinB® wound spray alone showed antibiofilm activity against wound-associated pathogens such as *S. aureus*, *S. epidermidis*, Coagulase-negative staphylococci (CoNS), *Klebsiella pneumoniae*, and *Acinetobacter baumannii*. DispersinB® and Acticoat™ combination performed significantly better ($P < 0.05$) than Acticoat™ alone, indicating the synergy between the two compounds due to DispersinB® enhancing the antimicrobial activity of Acticoat™. Thus, this combination of DispersinB® with Acticoat™/an antimicrobial dressing or gel prompts clinical evaluation of such an innovative approach.

abstracts

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Title: **A rat subcutaneous implant model demonstrates that bacteria that are "sensitive" or "resistant" to antibiotics in vitro are equally resistant to antibiotic therapy when growing on an implanted medical device**

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Hospitals often use in vitro methods to assess the susceptibility of bacterial strains and use the information collected to guide physicians in the selection of suitable antibiotics to treat infected patients. Much research has already shown that in vitro methods can lead to false conclusions of bacterial susceptibility, since bacteria grown planktonically are hundreds or thousands of times more sensitive to antibiotic treatment compared to bacteria growing in a biofilm. For this study, we used a rat subcutaneous implant model to test this concept by directly challenging "sensitive" and "resistant" *Staphylococcus* strains, first in vitro and then in vivo. As anticipated, both "sensitive" and "resistant" bacteria were immune to extremely high levels of antibiotic therapy when they have contaminated a device implant. These results confirm the necessity of using in vivo animal models, such as this rat subcutaneous implant model, for testing the efficacy of new antibiotics, particularly when those antibiotics are to being developed to treat device-related infections and/or antibiotic resistant organisms.