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14. ABSTRACT

During the award period, we successfully developed protocols to extract biofilm EPS associated with ESKAPE organisms (*Enterococcus, Staphylococcus, Klebsiella, Acinetobacter, Pseudomonas*, and *Enterobacter* species). We further performed composition and linkage analysis on these samples and determined that 2 and 3-linked mannose residues constitute the major carbohydrate moiety in the EPS. In parallel, we synthesized, expressed, purified, and characterized 15 putative depolymerase enzymes by a variety of assays against static, dynamic, and mixed biofilm conditions. At least eight enzymes displayed significant anti-biofilm activity and three (NagZ, DspNW, and Betty) were advanced to animal safety testing.

15. SUBJECT TERMS

Bacteriophage, depolymerase, biofilm, extrapolymeric substance, enzyme

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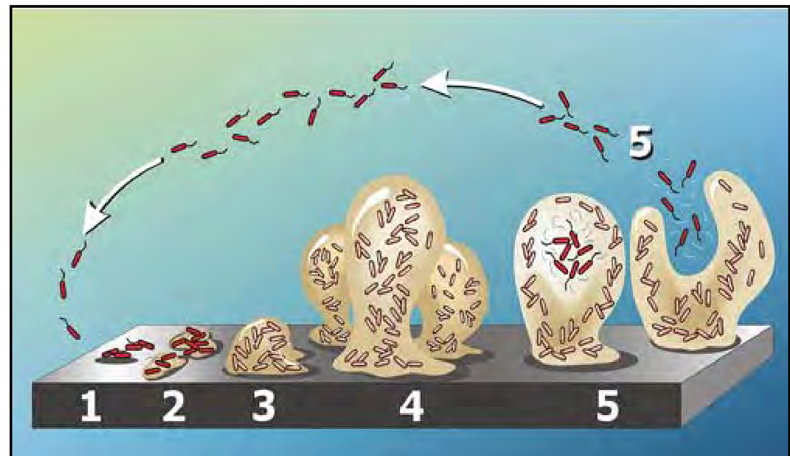
INTRODUCTION:

There is a critical need for basic research to discover new methods that would improve the outcomes of soldiers who incur battlefield wounds. Initially, most war wounds are colonized by Gram-positive bacteria. However, after initial stabilization and surgery, residual infections in open wounds are characterized by predominantly Gram-negative bacteria including *Acinetobacter baumannii-calcoaceticus* complex, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*. Additionally, wounded soldiers have a very high rate of hospital-acquired infections by such pathogens as *Staphylococcus aureus.* Thus, war wounds remain susceptible to infection from the time of injury through subsequent surgery, therapy, and rehabilitation and often contain multiple bacterial species. Moreover, each of these organisms has different resistance patterns to antibiotics, further limiting treatment options. Often, even broad-spectrum antibiotics are not sufficient to eradicate all of the organisms contained within a wound.



While bacterial pathogenesis mechanisms, virulence factors, and antimicrobial resistance vary greatly between pathogens associated with war wounds, one common trait shared by all is the ability to colonize wounds as a biofilm (see figure). Biofilms are formed when planktonic bacteria (i.e., free, individual cells) adsorb onto a wound surface and form multi-cellular colonies (figure, stage 1). Once the colonies become established, phenotypic changes cause them to secrete polysaccharides that serve as the backbone for the biofilm (figure, stage 2 and 3). Non-cellular components and debris, including additional carbohydrates, proteins, lipids, and nucleic acids, become entangled in the polysaccharide backbone and constitute the extrapolymeric substance (EPS) or "slime" layer of a biofilm (figure, stage 4). Significantly, the superstructure of the biofilm is known to protect internal bacteria from antimicrobials, antibodies, and circulating immune cells (figure, stage 5). *Thus, approaches that disrupt or dissolve the biofilm superstructure of polymicrobial infections would offer a therapeutic avenue to reduce the morbidity and mortality associated with war wounds by "re-sensitizing" the bacteria to antibiotics and the soldier's immune system.*

To accomplish this goal, we will use special enzymes called depolymerases. Depolymerases are normally found on the surface of bacterial viruses (i.e. bacteriophage) where they function to dissolve the EPS layer on naturally occurring biofilms allowing the phage to invade the bacterial cell. We plan to identify and test many such depolymerases to find the best enzyme, or cocktail of enzymes, that will dissolve biofilms associated with the bacteria that infect war wounds. Although the depolymerases do not directly kill the bacteria, it is believed that dissolution of the biofilm protective layer will allow common antibiotics or the immune system to clear the infection.

BODY:

This is the final report for DM102823. This award consisted for five specific aims as follows:

Specific Aim 1. Identify, clone, and express potential depolymerases.

For identification, cloning, and expression of potential depolymerase enzymes, we initially synthesized ~8 genes based on bioinformatic analysis as well as distribution over many types of catalytic activity. However, this was an iterative process. Based on whether those initial enzymes showed promising or disappointing activity, additional genes to synthesize were selected. In all, we ended up synthesizing 15 enzymes (10 putative depolymerases and 5 lyases) as shown in the tables below. These tables thus represent the cumulative work of almost 2 ½ years, including gene names of the enzymes, their characteristics/target pathogens, mechanisms of action, mass, whether expression was achieved, information about purification tags (N- or C-terminus), yield, and potential tertiary structure.

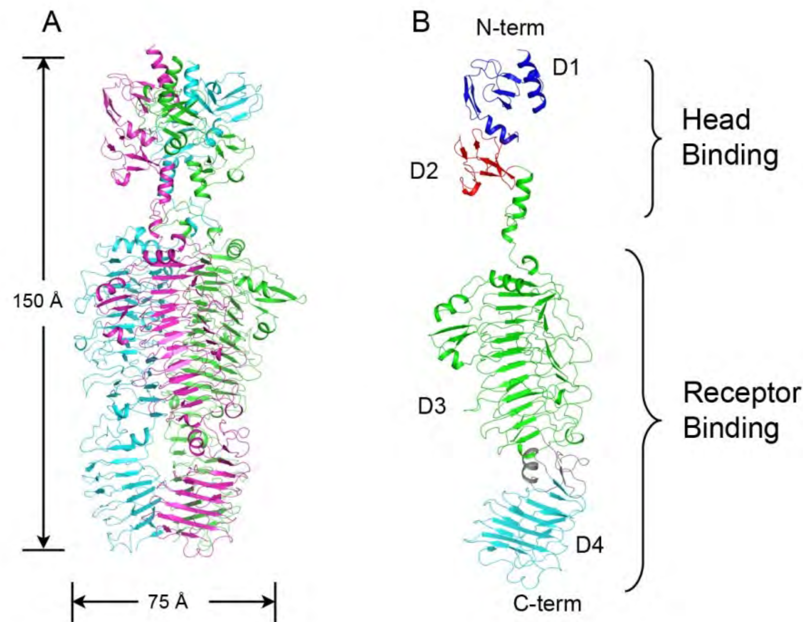
	DspB	SF6	CBA_120_160c	DSP-NW	DSP-AA
GenBank accession #	AAP31025.1	AAQ12204.1	AEM91896.1	ZP_08938922.1	EGY31409.1
Characteristics	Kills *S. epidermidis*	Tail-spike of Sf6, a P22-like phage of *Shigella flexneri*	Putative CBA_120 myophage tail-spike	Related to DspB, from Neisseria sp.	Related to DspB, from Aggregatibacter sp.
Target pathogen	*Staph*	Possibly *E. coli*	*E. coli* O157:H7	*Staph* spp., *E. coli*	*Staph* spp., *E. coli*
Mechanism of action	Beta- (1,6)- N-acetyl glucosaminidase	Endorhamnosidase	Unknown	Unknown. Chosen based on DspB homology	Unknown. Chosen based on DspB homology
Predicted MW (kDa)	42	157.6	82	57.9	46.7
Expression	Y	Y	Y	Y	Y
N-term tag purifiable	Y	Y	N	N	N
C-term tag purifiable	N/A	N/A	Y	Y	N
Yield	Medium	High	High	Medium	Low
4° structure	Monomer	Trimer	Trimer	Possible trimer	Monomer
Induction	37	37	37 or 18	37	37

	Gluc-Eq	Gluc-NH	Gluc-FO	NagZ-AB	Bdm
GenBank accession #	YP_002746062.1	XP_003046865.1	EGU88110.1	ADX02168.1	
Characteristics	Related to E. coli glucuronidase, from S. equi	Related to E. coli glucuronidase, from N. haematocca	Related to E. coli glucuronidase, from F. oxysporum	Related to E. coli NagZ, sequenced from Acinetobacter	Related to biofilm dispersing beta-mannosidase from *Xanthomonas*
Target pathogen	*K. pneumonia*	*K. pneumonia*	*K. pneumonia*	*Acinetobacter baumanii*	ALL OF THEM, except probably less so *E. coli* O157:H7 and *Staph*
Mechanism of action	Putative beta-glucuronidase	Putative beta-glucuronidase	Putative beta-glucuronidase	Putative beta- 1,4- (1,6)-N-acetyl glucosaminidase	Putative exported alpha 1,2 mannosidase
Predicted MW (kDa)	69.2	68.9	69.1	39	85.6
Expression	Y	Y		Y	Y
N-term tag purifiable	Y	Y	Y	Y	N
C-term tag purifiable	N/A	N/A	N/A	N/A	Y
Yield	Low due to insolubility	High	Medium	Low	Medium, possibly due to export
4° structure	Possible tetramer	Possible trimer	Possible hexamer	Probably a monomer	
Induction	37 or 18, higher yield at 37	37	37	37 or 18	37 or 18

In addition to the enzymes above, we sought to evaluate anti-biofilm properties of various alginate lyases. Significantly, biofilms of *Pseudomonas aeruginosa* are known to elaborate alginate, which would be refractory to the cleavage specificities of the depolymerases discovered to date. With this in mind, we sought to specifically discover new enzymes that can degrade alginate. The table below lists the five lyases expressed, purified, and studied.

Protein	AA	MW	ID1	Expressed	Purified	Activity	Description
lyase MB	358	38181	BAJ62034	Yes	Yes	Yes	alginate lyase [Microbulbifer sp. 6532A].
lyase PA	367	40774	YP_001346982	Yes (very low)	Yes (very low)	Yes (very low)	poly(beta-D-mannuronate) lyase [Pseudomonas aeruginosa PA7].
lyase SD	524	56303	YP_527950	No	n/a	n/a	cyclic nucleotide-binding domain-containing protein [Saccharophagus degradans 2-40].
lyase SM	332	36876	YP_004791784	Implied (has activity)	Implied (has activity)	Yes (very low)	poly(beta-D-mannuronate) lyase [Stenotrophomonas maltophilia JV3].
lyase U	323	35410	AEO50363	Yes	Yes	Yes	alginate lyase [uncultured bacterium].

Finally, Prof. Osnat Herzberg, an X-ray crystallographer, expressed interest in crystallizing any of our depolymerases that were derived from bacteriophage tailspike proteins. Our protein "Betty", also called CBA_120, is such an enzyme. Since it is one of our more active depolymerases (see aims 3 and 4), understanding its structural characteristics will allow future bioengineering approaches to optimize its activity. We have now solved the structure of this enzyme (Figure below) and are currently working on the structures of several other depolymerse enzymes. Based on this structure and its associated activity, we have one manuscript submitted and currently under review, and a second manuscript in preparation.



(A) The overall structure of tailspike TSP1 homo-trimer. (B) The structure of CBA_120 monomer showing four distinct structural domains (D1 to D4). The N-terminal head binding domain contains D1 (residues 12-96, colored blue) and D2 (residues 97-154, colored red). The ligand binding domain contains D3 (residues 198-580, colored green) and D4 (residues 581-796, colored grey and cyan). The ligand binding domain assumes primarily alpha-helical structure, but the helical axis is bent by an intervening fragment (residues 581-623) colored grey.

Specific Aim 2. Characterize biofilm polysaccharide composition.

Since we selected strains that readily formed biofilms, establishing appropriate biofilm conditions was fairly simple. However, we spent considerable time developing and validating a novel method to extract the high molecular weight polysaccharide that constitutes the structural backbone of the biofilm EPS. Numerous protocols describe extraction methods for bacterial polysaccharide capsule as well as covalently attached surface polysaccharides, but very few are specific for biofilm EPS. Of those that are published, none allowed for the level of purification we required for our analysis. In the end, we took elements from three prior publications (Liu and Fang (2002); Oliveira, Marques, and Azeredo (1999); Sofia Andersson (2009)) combined with a gel filtration step in order to achieve the purity required. When assessed by gas chromatograph, the composition and linkage analysis revealed a surprisingly high level of 2- and 3-linked mannose residues throughout all biofilms.

Our novel extraction technique for the EPS was recently published. It includes the complete composition and linkage analysis for eight strains covering five of the ESKAPE pathogen species. Please see the following publication in our appendix, which contains the methods and results of Specific Aim 2 in its entirety.

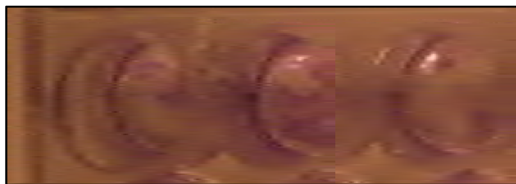
Bales, P.M., Renke, E.M., May, S.L, Shen, Y., & Nelson, D.C. (2013) Purification and characterization of biofilm-associated EPS exopolysaccharides from ESKAPE organisms and other pathogens. PLoS ONE. 8: e67950.

Specific Aim 3. Test anti-biofilm efficacy of depolymerases.

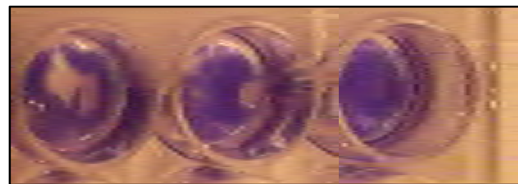
For the majority of these experiments, we utilized static biofilms and three different methods to demonstrate depolymerase activity. For each method, we first grew static biofilms in 96 well plates for 24 hours. Examples of data collected from each method are below.

1. Biomass Eradication Assay. In this assay, biofilm wells are treated with putative depolymerase enzymes or buffer, incubated, and the remaining biofilm biomass is stained with 0.1% crystal violet. The crystal violet can be quantified by extraction in 1% SDS and read on a spectrophotometer at 590 nm. An active enzyme will decrease the crystal violet staining compared to controls. The image below shows the anti-biofilm activity of DspB vs. PBS on biofilms of *Staphylococcus aureus*.

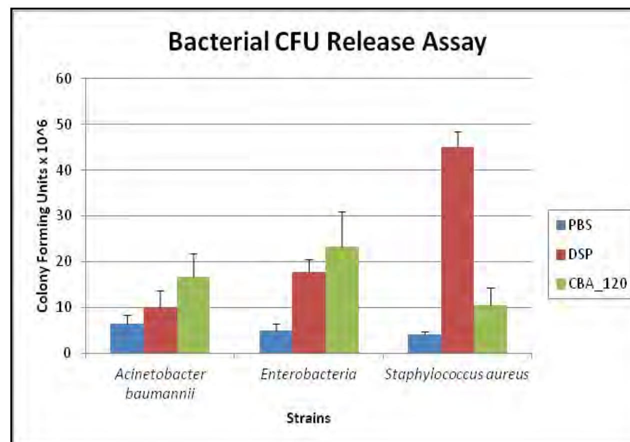
Dsp Treatment



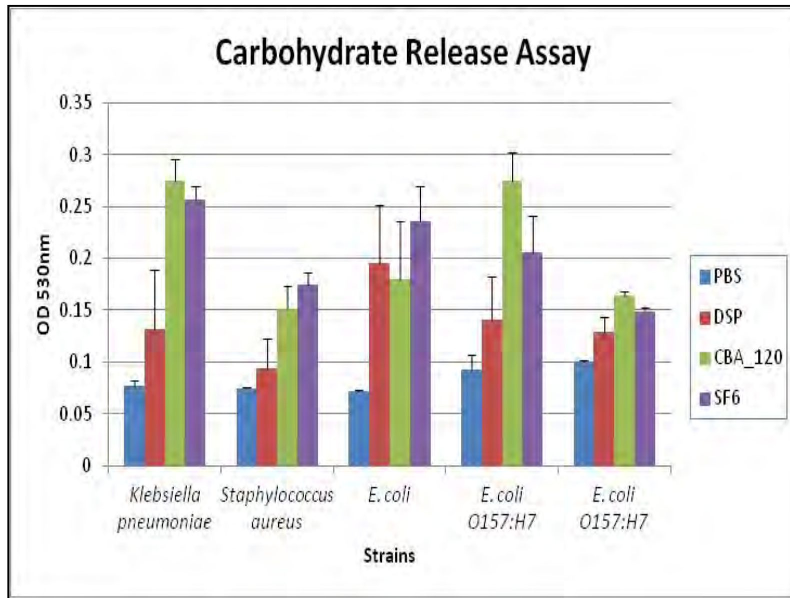
PBS Treatment



2. Bacterial CFU Release Assay. In this assay, biofilm wells are treated with putative depolymerase enzymes or buffer, incubated, and the supernatant is serial diluted and plated to enumerate bacterial colony forming units (CFU) present. An active enzyme will digest the biofilm matrix and increase the CFUs released to the supernatant. The figure below shows the anti-biofilm properties of DspB and CBA_120 compared to PBS on biofilms from three species.



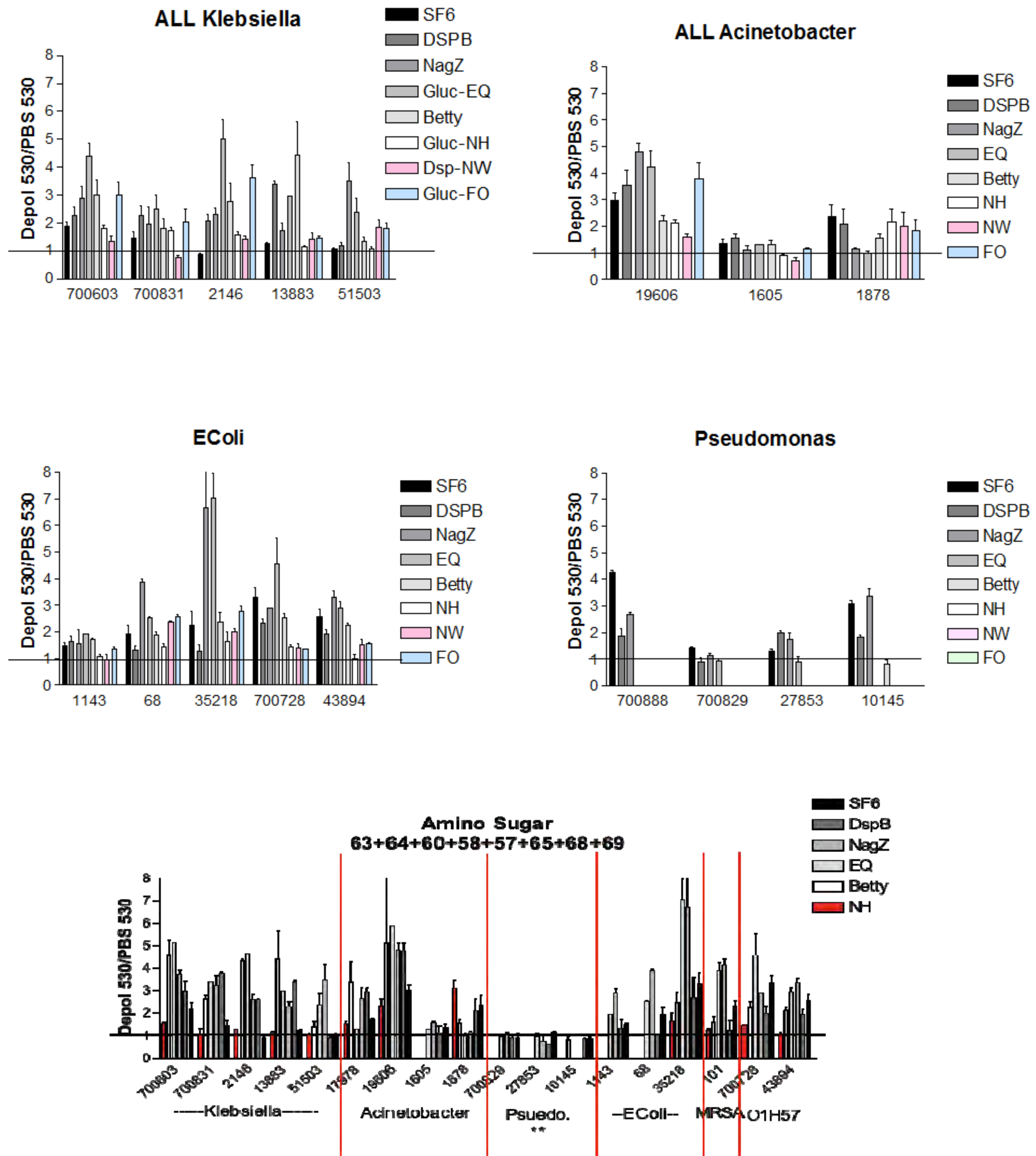
3. <u>Carbohydrate Release Assay.</u> In this assay, biofilm wells are treated with putative depolymerase enzymes or buffer, incubated, and the supernatant is assayed for total carbohydrate content by the phenol sulfuric acid method of Dubois or the reducing sugar method of Morgan-Elson. An active enzyme will digest the biofilm matrix and increase total carbohydrate content of the supernatant, as measured by an increase in absorbance at 530 nm. The figure below shows the anti-biofilm activity of three depolymerases (DspB, CBA_120, and SF6) against multiple species and strains.



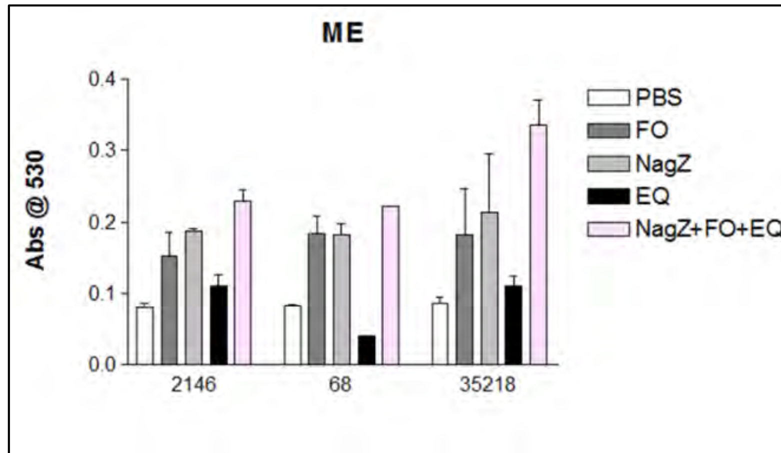
Below is a chart that summarizes our finding for just three of the fifteen depolymerases:

ATCC #	Organism	Biomass Reduction	Bacterial CFU Release	Carbohydrate Reslease
1605	*Acinetobacter baumannii*		DSP	DSP
1878	*Acinetobacter baumannii*		DSP/CBA_120/SF6	
35218	*E.Coli*		DSP/CBA_120	
700728	*E. coli O157:H7*		CBA_120/SF6	DSP/CBA_120/SF6
43894	*E. coli O157:H7*			DSP/CBA_120/SF6
1143	*Enterobacter*		DSP	DSP/CBA_120
68	*Enterobacter*		DSP/CBA_120	CBA_120
700603	*Klebsiella pneumonia*		DSP/CBA_120	DSP/CBA_120/SF6
10145	*Pseudomonas aeruginosa*		DSP/CBA_120	
27853	*Pseudomonas aeruginosa*	DSP/CBA_120/SF6	DSP/Betty/SF6	
700829	*Pseudomonas aeruginosa*		CBA_120	
101	*Staphylococcus aureus*	DSP/CBA_120	DSP/CBA_120	

We have performed dozens and dozens of experiments with all of the enzymes using static, dynamic, and mixed biofilms using the biomass eradication assay, the bacterial CFU release assay, and the carbohydrate release assay, also known as the Elson Morgan assay. We have concluded that the carbohydrate release (Elson Morgan) assay gives us the most reproducible results for depolymerase enzymatic activity. Below is just a brief sample of our results with some of the enzymes using the Elson Morgan assay. Clearly, different enzymes display efficacy for certain ESKAPE strains.

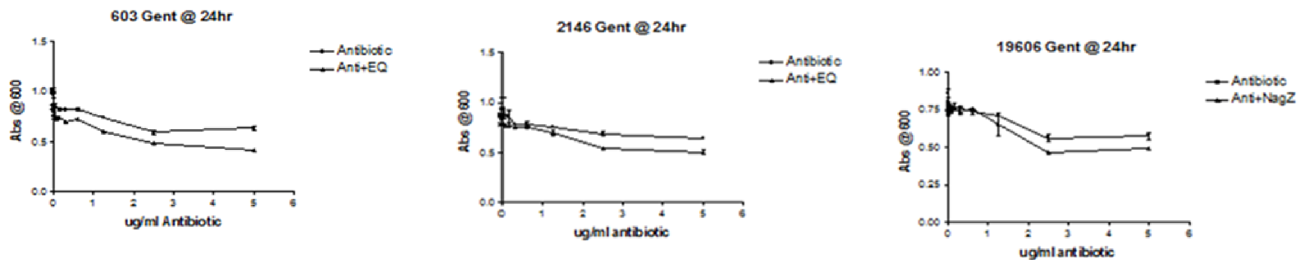


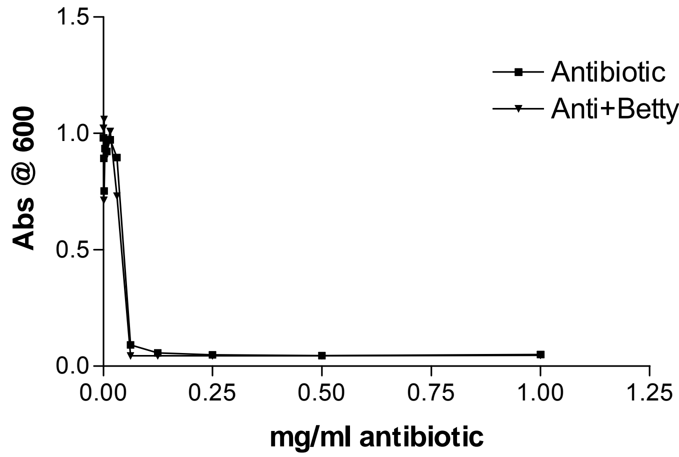
In addition to studies on static, dynamic, and polymicrobial biofilms, we also tested synergy between depolymerase enzymes to see if a “cocktail” approach was better than individual enzymes. As can be seen by the figure below, a combination of three depolymerases had greater activity against biofilms than any individual enzyme.

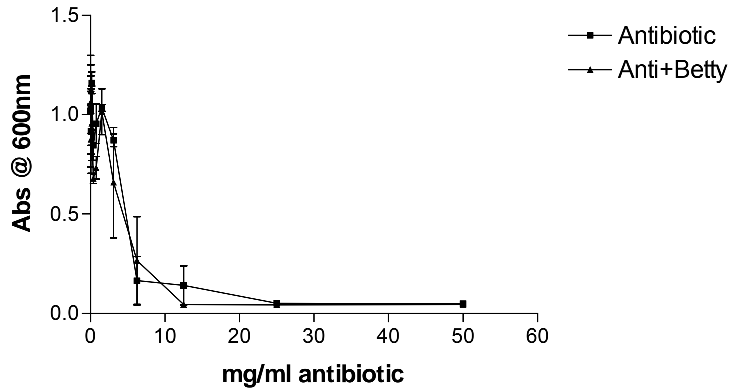


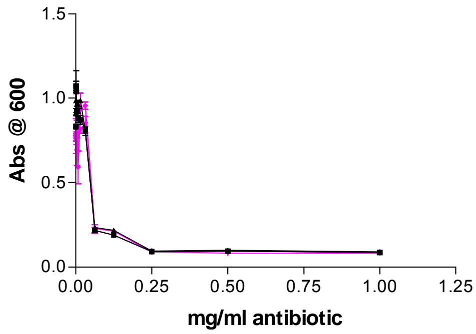
Specific Aim 4. Evaluate ability of depolymerases to re-sensitize bacteria to antibiotics.

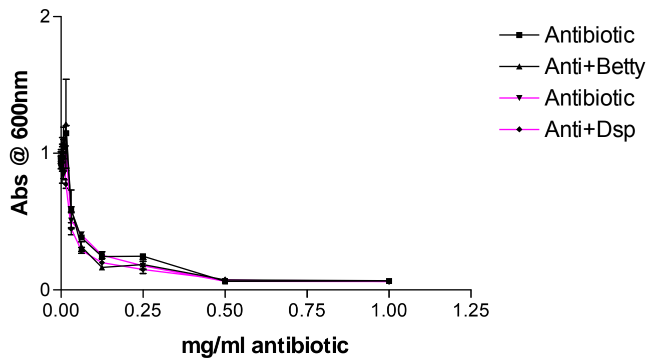
We evaluated the combined or synergistic use of antibiotics (gentamycin, sublactam, ampicillin) with and without depolymerase enzymes against various biofilms (i.e 603, 2146, 19606, 35218, 13883, 700603). Note, these figures are representative samples from dozens of distinct experiments. While there were examples of positive results, overall the results were underwhelming in that addition of depolymerase enzymes did not seem to significantly enhance the efficacy of the antibiotic. This may be due to the experimental design and use of static biofilms. In this case, wells containing biofilms were soaked in antibiotics or antibiotics and depolymerases overnight. It is likely that the antibiotics are able to penetrate the dense biofilm network in these static conditions. Future proposals/studies would be aimed at looking at a catheter flow model or possibly an in vivo biofilm model, but those experiments are beyond the scope of the current proposal.



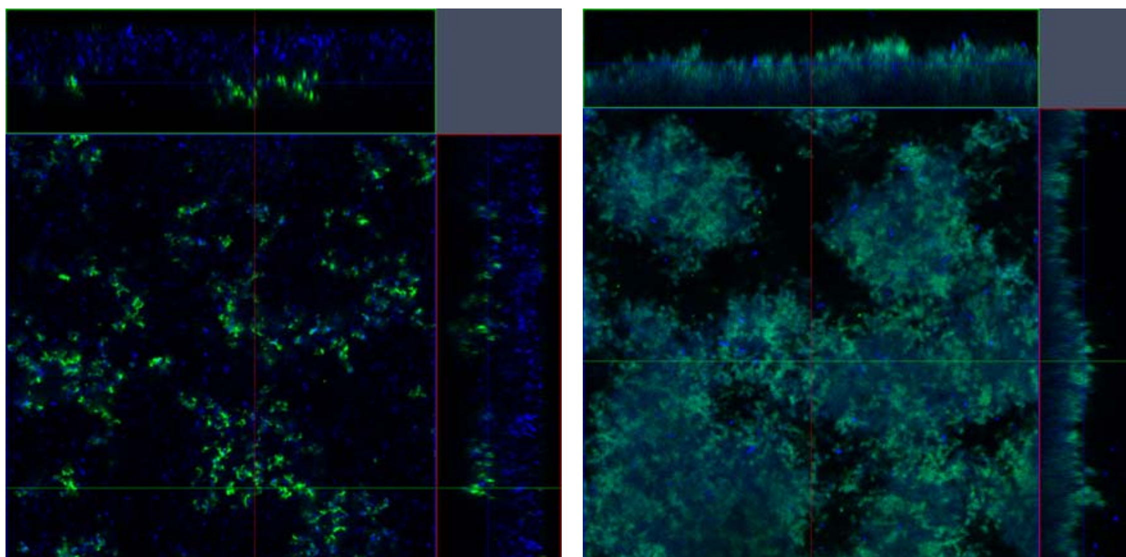

13883 Sulbactam
Antibiotic
Anti+Betty
Abs @ 600
1.5
1.0
0.5
0.0
0.00
0.25
0.50
0.75
1.00
1.25
mg/ml antibiotic


13883 Amp
Antibiotic
Anti+Betty
Abs @ 600nm
1.5
1.0
0.5
0.0
0
10
20
30
40
50
60
mg/ml antibiotic


700603 Sulbactam
Antibiotic
Anti+Betty
Antibiotic
Anti+Dsp
Abs @ 600
1.5
1.0
0.5
0.0
0.00
0.25
0.50
0.75
1.00
1.25
mg/ml antibiotic


700603 Gentamycin
Antibiotic
Anti+Betty
Antibiotic
Anti+Dsp
Abs @ 600nm
2
1
0
0.00
0.25
0.50
0.75
1.00
1.25
mg/ml antibiotic

Finally, we looked at the ability of depolymerases to re-expose the bacteria to antibodies (i.e. through degradation of the biofilm matrix). 3D confocal miscroscopy was used to visualize the thickness of biofilms of *Staphylococcus aureus* without treatment (left) and with depolymerase (NagZ) treatment (right). A nucleic acid dye (blue) was used along an AlexaFluor linked antibody (green) that binds to the cell surface via Protein A on the staphylococcal surface. Round staphylococcal cells can clearly be viewed on the right image as the antibody gains access through the biofilm EPS.



Specific Aim 5. Examine safety and toxicity of depolymerases.

In this Aim, we looked at safety and toxicity of three lead depolymerase enzymes, Betty (i.e. CBA_120), DspNW, and NagZ. All were evaluated for endotoxin contamination during purification using the HEK-Blue™ LPS detection kit and found to be less than 10 EU/ml. Next, basic toxicity was evaluated in a trypan blue dye exclusion assay using Hep-2 cells. None of the three proteins displayed any toxicity. Finally, the three proteins were sent to a vendor (Ethox International) for animal irritation and toxicity testing with IACUC and ACURO approvals. These included a mouse systemic toxicity test, a rabbit skin irritation test, and a rabbit vaginal mucosal membrane test. After discussions with ACURO and the vendor, it was determined that the planned hamster cheek pouch test was redundant with the rabbit vaginal test (i.e. both tested irritation/toxicity on mucous membranes). As such, the hamster cheek pouch test was not performed, but we instead added extra animals to the rabbit vaginal test.

In the attached appendix, we provide the methods and data collected for NagZ in the mouse systemic toxicity test, the rabbit skin irritation test, and the rabbit vaginal mucous membrane test. We have similar data files for Betty and DspNW. None of the enzymes were found to be toxic or cause topical irritation in the tested animal models. It is expected that the safety and toxicity testing will be incorporated into future manuscript(s) describing the anti-biofilm properties of these enzymes.

KEY RESEARCH ACCOMPLISHMENTS:

- Determined that tailspike proteins most likely represent the identity of the bacteriophage-derived depolymerase enzymes associated with anti-biofilm properties
- Experimentally determined the optimal growth conditions for biofilm expression
- Developed a novel method for extraction of biofilm EPS
- Performed composition and linkage analysis for EPS from several ESKAPE organisms
- Successfully synthesized, expressed, and purified 15 putative depolymerase enzymes
- Demonstrated some degree of anti-biofilm activity for the majority of these enzymes
- Demonstrated anti-biofilm in static, dynamic, and polymicrobial biofilms
- Demonstrated synergy between depolymerase enzymes in a cocktail approach
- Demonstrated the ability of depolymerase enzymes to "re-sensitize" the biofilm to the actions of antibodies.
- Showed a positive safety and toxicity profile for the top 3 enzymes in validated animal models.
- Solved the crystal structure of one lead enzyme (Betty/CBA_120).
- Publications: We have already published one article on the isolation and characterization of the EPS from the ESKAPE organisms (see appendix), we have one paper describing the crystal structure of Betty under review with a second paper being written, and a manuscript is being written for one of the lyase enzymes. Notwithstanding these successes, we have a considerable amount of data for 7 or 8 other enzymes. We are currently evaluating whether to package all of the data into one very large manuscript describing all of the enzymes, or break it down into multiple manuscripts, each one focusing on one or two enzymes of a similar class. Either way, it is anticipated that multiple additional publications will be generated based on the data collected during this project.

REPORTABLE OUTCOMES:

Some aspects of the data and/or overall strategy have been presented at the following meetings/symposia:

1. PhageRAST (Rapid Annotation Using Subsystems Technology) Meeting. Tucson, AZ. Symposium speaker. "Bacteriophage Lytic Systems" (January, 2011).
2. Georgia Institute of Technology, Atlanta, GA. Seminar speaker. "Bacteriophage Endolysins: Engineering A Targeted Antimicrobial Approach" (February, 2011).
3. Military Infectious Diseases Research Program, Defense Health Program, Wound Symposium 2011. San Antonio, TX. Symposium speaker. "Rapid Dispersion of Polymicrobial Wound Biofilms with Depolymerase Enzymes" (May, 2011).
4. 19th International Phage Biology Meeting. Olympia, WA. Poster. "Rapid Destruction of Biofilm Matrices by Bacteriophage-Encoded Enzymes" (August, 2011).

5. University of Georgia, Athens, GA. Distinguished Alumni Marquee Speaker, Department of Biochemistry and Molecular Biology. “Structure/Function Studies on Enzybiotics” (October, 2011).
6. The 14th Annual Undergraduate Research Symposium in the Chemical and Biological Sciences. Baltimore, MD. “Utilization of Bacteriophage-Encoded Enzymes for the Vigorous Disruption of Biofilm Matrices” 1st Place Student Poster Award to Emilija Renke. (October, 2011)
7. 3rd Annual Biomedical Sciences Day, The Universities at Shady Grove, Rockville, MD. “Evaluation of Bacteriophage Encoded Enzymes as Novel Therapeutic Agents against Biofilm-associated Infections” 1st Place Poster Presentation Award to Emilija Renke. (November, 2011)
8. National Institute for Standards and Technology, Gaithersburg, MD. Invited Speaker for the Metrology of Microbial Systems Seminar Series. “Exploiting Bacteriophage Endolysins for Therapeutic and Diagnostic Use against Bacterial Pathogens” (November, 2011).
9. Viruses of Microbes International Conference. Brussels, Belgium. Keynote symposium speaker. “X-ray Crystal Structure of PlyC, a Novel Enzybiotic” (July, 2012).
10. Twentieth Evergreen International Phage Biology Meeting. Olympia, WA. Symposium speaker. “Tail Spikes and Biofilm Degradation” (August, 2013)
11. Uniformed Services University of the Health Sciences, Bethesda, MD. Seminar speaker, Department of Biochemistry. “Structural and Functional Studies on the Antimicrobial Properties of Bacteriophage Endolysins” (September, 2013).
12. American Registry of Professional Animal Scientists, Regional Meeting, Rockville, MD. Invited speaker. “Evaluating Bacteriophage-based Strategies for Diagnosis and Treatment of Bacterial Infections” (September, 2013).

CONCLUSION:

Our goal was to study the carbohydrate composition of the EPS matrix associated with biofilms of ESKAPE organisms and identify ~10-15 enzymes that would act upon the bonds in this matrix. We initially focused on “depolymerase” enzymes associated with bacteriophage as phage are known to penetrate biofilms. However, as we expanded our search of suitable enzymes, we included bacterial and fungal enzymes based on bioinformatic analysis. In the end, we successfully cloned, expressed, purified, and studies 15 distinct enzymes. In parallel, we developed methods to isolate and analyze the biofilm carbohydrate from five of the ESKAPE pathogens. The various enzymes were tested for anti-biofilm activity against static, dynamic, and polymicrobial biofilms of all ESKAPE pathogens. In addition, cocktails of depolymerases were tested and showed synergy. Overall, species-specific heterogeneity was noted in the activity profiles, but of enzymes did show broad anti-biofilm activity. In particular, NagZ, DspNW, and Betty became the lead enzymes based on activity as well as host range. These enzymes were further evaluated for their safety and toxicity profiles in hopes that they would have future translational applications. Furthermore, while not directly one of the stated Aims, we solved the crystal structure of Betty, which will aid us in future engineering endeavors since this enzyme is a lead candidate. In summation, we are now armed with several anti-biofilm enzymes that have been biochemically characterized and their spectrum of activity noted. It is anticipated that future experiments would include in vivo biofilms models for further development of these enzymes.

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APPENDICES: Beginning on the next page, the appendices include a 2013 manuscript on the characterization of the biofilm EPS from the ESKAPE organisms and methods/raw data from safety and toxicity testing for the NagZ depolymerase.

Purification and Characterization of Biofilm-Associated EPS Exopolysaccharides from ESKAPE Organisms and Other Pathogens

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Abstract

In bacterial biofilms, high molecular weight, secreted exopolysaccharides can serve as a scaffold to which additional carbohydrates, proteins, lipids, and nucleic acids adhere, forming the matrix of the developing biofilm. Here we report methods to extract and purify high molecular weight (>15 kDa) exopolysaccharides from biofilms of eight human pathogens, including species of *Staphylcococcus*, *Klebsiella*, *Acinetobacter*, *Pseudomonas*, and a toxigenic strain of *Escherichia coli* O157:H7. Glycosyl composition analysis indicated a high total mannose content across all strains with *P. aeruginosa* and *A. baumannii* exopolysaccharides comprised of 80–90% mannose, *K. pneumoniae* and *S. epidermidis* strains containing 40–50% mannose, and *E. coli* with ~10% mannose. Galactose and glucose were also present in all eight strains, usually as the second and third most abundant carbohydrates. N-acetyl-glucosamine and galacturonic acid were found in 6 of 8 strains, while arabinose, fucose, rhamnose, and xylose were found in 5 of 8 strains. For linkage analysis, 33 distinct residue-linkage combinations were detected with the most abundant being mannose-linked moieties, in line with the composition analysis. The exopolysaccharides of two *P. aeruginosa* strains analyzed were consistent with the Psl carbohydrate, but not Pel or alginate. The *S. epidermidis* strain had a composition rich in mannose and glucose, which is consistent with the previously described slime associated antigen (SAA) and the extracellular slime substance (ESS), respectively, but no polysaccharide intracellular adhesion (PIA) was detected. The high molecular weight exopolysaccharides from *E. coli*, *K. pneumoniae*, and A. *baumannii* appear to be novel, based on composition and/or ratio analysis of carbohydrates.

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Introduction

Microorganisms that infect humans differ in mechanisms of pathogenesis, virulence factors, and antimicrobial resistance profiles. However, one common trait shared by most is the ability and propensity to form biofilms [1]. Along with upregulation of adhesins, phenotypic changes cause bacteria to secrete high molecular weight exopolysaccharides during conversion from planktonic to biofilm modes of growth [2–5]. These exopolysaccharides can make up a crucial part of the extracellular polymeric substance (EPS) associated with biofilm development that serves to cement whole bacterial populations to a surface rather than enclosing individual cells [6]. Also included in the EPS are proteins, secreted nucleic acids, humic substances, and metal ions. Together, the EPS protects biofilm bacteria from environmental stress [7,8].

The role of the EPS in pathogenesis has been studied in many organisms where the biofilm mode of growth has been shown to allow for increased resistance to antibiotic treatment, the immune response, and nutrient-limiting conditions within the host, promoting long-term persistence [9]. Biofilm formation on medical implant devices such as catheters and mechanical heart valves is also a major problem that is closely tied to the adhesion- and resistance-related abilities granted them by the ability to synthesize and secrete exopolysaccharides [10–12]. Human pathogens associated with biofilm development include species of *Enterococcus faecalis*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter spp*. These "ESKAPE" pathogens are the leading causes of nosocomial infections [13,14] and are so-named to emphasize their ability to "escape" the effects of antimicrobial treatment due to acquisition of resistance genes as well as formation of biofilms.

While surface-associated exopolysaccharides and capsules play a role in both extracellular and intracellular adherence during the conversion from planktonic to biofilm growth, our interests focus on the secreted exopolysaccharides, particularly the high molecular weight exopolysaccharides that are believed for form the "backbone" of the EPS to which proteins, nucleic acids, and capsular polysaccharides adhere [6,15]. While there are many protocols in the literature for isolation of capsular polysaccharides or for the extraction of total bacterial EPS, only a few have attempted to fractionate exopolysaccharides by size [16] and none

have been specifically tailored for the isolation of high molecular weight backbone exopolysaccharides from biofilms. Bulk EPS extraction requires methods that physically break up the biofilm matrix such as ultrasonication or EDTA, which promotes EPS separation by chelating cations that are thought to crosslink polysaccharide chains within the EPS. The use of glutaraldehyde or formaldehyde is also common to fix bacterial cells to prevent contamination via cell lysis during the extraction steps [17,18]. Once the bulk EPS is extracted, the polysaccharide fraction must be separated from DNA, proteins, and lipids. Oliveira and colleagues successfully utilized 20% trichloroacetic acid (TCA) for the precipitation of proteins from the EPS [19], whereas Sutherland used ethanol to precipitate the polysaccharide fraction [20]. Similar protocols have also included the use of NaOH to promote dissociation of acid groups within the EPS for increased solubility, cation exchange chromatography, differential centrifugation, and selective dialysis [9,21–23]. Our laboratory has combined portions of the above protocols and added a size exclusion chromatography step to produce an effective method of purifying high molecular weight EPS exopolysaccharides. We then apply this methodology to biofilms of eight medically important pathogens, including several representative ESKAPE organisms, as well as a methicillin-resistant *Staphylococcus epidermidis* strain and a toxigenic strain of *Escherichia coli* O157:H7, both of which are known biofilm producers. Finally, we characterized the resulting EPS exopolysaccharides by composition and linkage analysis.

Materials and Methods

Bacterial strains and growth conditions

Unless otherwise indicated, all reagents were purchased from Thermo-Fisher Scientific and were of the highest purity available. *S. epidermidis* strain NRS-101 was obtained from the Network on Antimicrobial Resistance in *S. aureus* (NARSA). All other strains were purchased from the American Type Culture Collection (ATCC). These include two *P. aeruginosa* strains, 700829 and 700888, both known biofilm production strains; two *K. pneumoniae* strains, 700603, a multi-drug resistant strain and 700831, a biofilm strain; two *A. baumannii* strains, BAA-1878 and BAA-1605, a multi-drug resistant strain; and one *E. coli* strain, 43894, a toxigenic O157:H7 serotype. *S. epidermidis* strain NRS101 was grown in brain-heart infusion media, *K. pneumoniae* strain 700603 was grown in Luria broth, and all other strains were grown in tryptic soy broth. Glycerol stocks of all strains were stored at −80°C. Biofilms grown for EPS purification were prepared by inoculating 20 ml of overnight culture into 400 ml of fresh media in a 1.5 L Fernbach flask to provide a large surface area for biofilm adherence. Biofilms were grown at 37°C without shaking for 4–5 days until a thick biofilm "sludge" was observed.

EPS extraction

After development of a mature biofilm, 60 µl of formaldehyde (36.5% solution) was added to each 10 ml of sludge to fix the cells and prevent cell lysis during subsequent steps. The formaldehyde-sludge mixture was incubated at room temperature in a chemical hood with gentle shaking (100 rpm) for 1 hour. Four ml of 1 M NaOH was added for each 10 ml of sludge and incubated at room temperature, with shaking, for 3 hours to extract EPS. Cell suspensions were then centrifuged (16,800×g) for 1 hour at 4°C. The supernatant containing soluble EPS was filtered through a 0.2 µm filter (Corning) and dialyzed against distilled water using a 12–14 kDa molecular weight cut-off (MWCO) membrane for 24 hours at 25°C.

Purification of exopolysaccharides

TCA was added (20% w/v) to extracted EPS solutions on ice to precipitate proteins and nucleic acids. After 30 minutes, the solution was centrifuged (16,800×g) for 1 hour at 4°C, the supernatant was collected, and 1.5 volumes of 95% ethanol was added and the mixture was placed at −20°C for 24 hours to precipitate exopolysaccharides away from lipids. The solution was then centrifuged (16,800×g) for 1 hour at 4°C and the exopolysaccharide pellet was resuspended in Milli-Q water and dialyzed against the same for 24 hours at 4°C using a 12–14 kDa MWCO membrane to remove low molecular weight impurities and the remaining retentate was lyophilized overnight. The lyophilized powder was then resuspended in 5–10 ml of phosphate buffered-saline (pH 7.4) and purified on a 26/60 S-200 gel filtration column (GE Healthcare) using an AKTA FPLC system (GE Healthcare) that had been calibrated with gel filtration standards (Bio-Rad) to generate a standard curve of apparent molecular mass vs. retention volume. Fractions were tested for the presence of carbohydrates by the phenol-sulfuric acid method of DuBois as previously described [24] and only high molecular weight fractions, defined as >15 kDa, containing carbohydrates were pooled, dialyzed against Milli-Q water to remove PBS, and lyophilized a final time for subsequent composition and linkage analysis.

Composition and linkage analysis

Carbohydrate composition and linkage analysis was performed at the Complex Carbohydrate Research Center (Athens, GA) as previously described [25,26]. Briefly, for glycosyl composition analysis, an aliquot (~500 µg) was taken from the purified EPS exopolysaccharide sample and added to a separate tube with 20 µg of inositol as an internal standard. Methyl glycosides were then prepared from the dry sample by methanolysis in 1 M HCl in methanol, followed by re-*N*-acetylation with pyridine and acetic anhydride in methanol for detection of amino sugars. The sample was then per-*O*-trimethylsilylated (TMS) by treatment with Tri-Sil (Pierce). Combined gas chromatography/mass spectrometry (GC/MS) analysis of the TMS methyl glycosides was performed on an Agilent 6890N GC interfaced to a 5975B MSD (mass selective detector), using a Supelco EC-1 fused silica capillary column (30 m×0.25 mm ID). For glycosyl linkage analysis, an aliquot (~500 µg) was taken from the purified EPS exopolysaccharide sample and suspended in ~300 µl of dimethyl sulfoxide and placed on a magnetic stirrer for 2 days. The sample was then permethylated by the method of Ciukanu and Kerek [27], hydrolyzed for 2 hours with 2 M trifluoroacetic acid in a sealed tube at 121°C, reduced with $NaBD_4$, and acetylated using acetic anhydride/pyridine. The resulting partially methylated alditol acetates were analyzed by GC/MS as described above.

Biofilm microscopy

Biofilms were grown in two-well chamber slides (Lab-Tek) with 1 ml of tryptic soy broth for 1 or 3 days. Biofilm wells were washed 2X with PBS and then stained with 5 µg of the FITC-labeled Hippeastrum hybrid lectin (HHA) from Amaryllis (EY Labs) and Hoechst 33342, a nucleic acid stain (Invitrogen), in PBS for 1 hour at room temperature. After incubation, the wells were again washed 2X with PBS, chambers were removed from the glass slide, and biofilms attached to the slides were imaged by an Eclipse 80i fluorescent microscope workstation (Nikon) or an LSM710 laser scanning confocal microscope workstation (Zeiss) as previously described [28]. NIS-Elements (Nikon) or ZEN (Zeiss) software packages were used for image analysis.

Results and Discussion

EPS purification

A schematic of the protocol we developed to purify EPS exopolysaccharides from biofilms is shown in **Figure 1**. This methodology incorporates into one protocol many extraction and purification steps successfully demonstrated by others [17–23], along with selection steps for high molecular weight for exopolysaccharides (i.e.>15 kDa) through use of large pore dialysis and gel filtration. As detailed by specific examples in the sections below, our protocol did not result in isolation of surface-associated or capsular polysaccharides, indicating that the methods were specific for secreted exopolysaccharides. Additionally, the protocol was robust, allowing us to successfully extract EPS exopolysaccharide from all eight bacterial strains representing five species. Our yields ranged from 2–15 mg of purified EPS exopolysaccharide for each strain from 1.2 L of biofilm culture (three Fernbach flasks, each containing 400 ml of bacteria). This is lower than yields reported by other methods, but is most likely due to our selection of only the high molecular weight exopolysaccharide that forms the EPS backbone, which excludes lower molecular weight exopolysaccharides and oligosaccharides that would co-purify with more crude purification methods.

Composition and linkage analysis

The glycosyl composition results are summarized in **Table 1**. Across all samples, a total of 11 sugars and aminosugars were detected. Notably, mannose was found in exopolysaccharides of all strains and was the predominant carbohydrate in every strain except *E. coli*. Indeed, mannose accounted for ~80–90% of the total carbohydrate content in *P. aeruginosa* and *A. baumannii* species and ~40–50% of the total carbohydrate in *K. pneumoniae* and *S. epidermidis* species. Galactose and glucose were also found in all eight strains tested and were often ranked as the second or third most abundant carbohydrate. Other carbohydrates were also found to be well represented in biofilm EPS exopolysaccharides. *N*-acetyl-glucosamine (GlcNAc) and galacturonic acid (GalA) were found in 6 of 8 strains while arabinose, fucose, rhamnose, and xylose were found in 5 of 8 strains. In contrast, *N*-acetyl-galactosamine (GalNAc) was only present in the *E. coli* and *S. epidermidis* strains and glucuronic acid (GlcA) was only found in a single *K. pneumoniae* strain.

The glycosyl linkage analysis for EPS exopolysaccharide of all strains is summarized in **Table 2**. Across all samples, 33 distinct residue-linkage combinations were detected. Consistent with the glycosyl composition results, a substantial proportion of the total linkages involved mannose. 2-linked mannose (2-Man) and terminally-linked mannose (t-Man) residues were detected in all samples, whereas 3-linked mannose (3-Man), 6-linked mannose (6-Man), and 4-linked glucose (4-Glc) were present in the EPS exopolysaccharide of 7 of the 8 strains tested. Mannose is also a common branch point for these complex structures as 2,6-linked mannose (2,6-Man) was present in 6 strains, 2,3-linked mannose (2,3-Man) was present in five strains, 3,6-linked mannose (3,6-Man) was present in three strains, and 2,3,4-linked mannose (2,3,4-Man) was present in one strain.

Strain variability within a species

For each of the three ESKAPE pathogens tested, we analyzed two independent strains, which provide a means to assess strain to strain variability by our methods. In the two *P. aeruginosa* strains, 700829 and 700888, both were found to be >85% mannose by composition, followed by minor constituents of glucose, galactose,

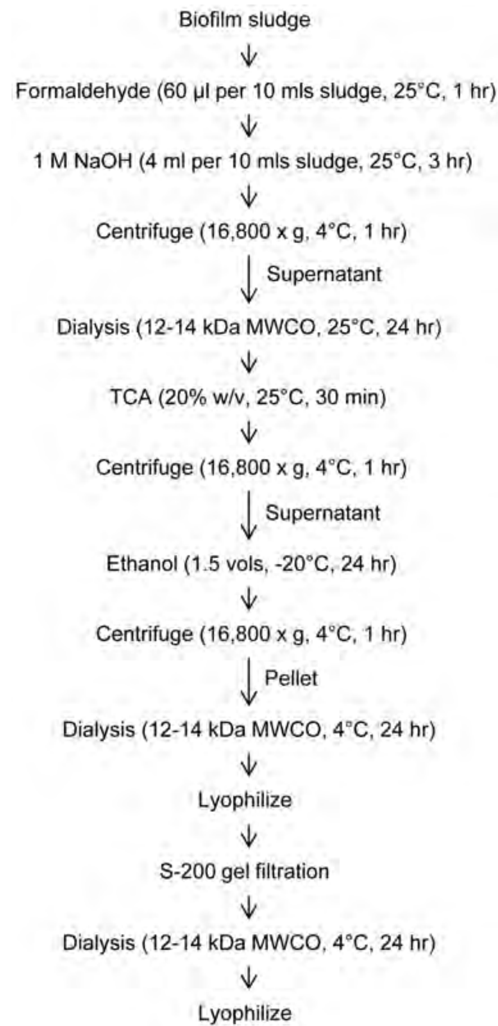

Figure 1. Schematic for extraction and purification of EPS exopolysaccharides. Depending on strain, yields range from 2–15 mg of purified polysaccharide per starting 1.2 L of sludge.
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arabinose, and rhamnose **(Table 1)**. Likewise, the major linkages of EPS exopolysaccharides from both strains in order were 2,6-Man, t-Man, 2-Man, and 3-Man **(Table 2)**. Similar to the *P. aeruginosa* results, both *A. baumannii* strains, BAA-1605 and BAA-1878, had a nearly identical composition and linkage profile not only to each other, but also to the *P. aeruginosa* strains. In sharp contrast, the two *K. pneumoniae* strains differed considerably from each other. While both strains had high mannose content, the next most abundant carbohydrates for strain 700603 were rhamnose, galactose and GlcA. For strain 700831, those residues were replaced by glucose, rhamnose, and GalA in order of importance. 700603 contained 5% GlcA, which was not found in 700831 and the latter contained trace amounts of arabinose, fucose, and xylose, which were all absent in the former. Thus, in instances where our data differ from historical results as described below, we cannot conclude whether these variations are attributable to differences in extraction/purification methods, or simply represent natural strain to strain heterogeneity within a species as displayed by the two *K. pneumoniae* strains we tested.

Table 1. Glycosyl Composition Analysis.

Glycosyl Residue	Abbreviation	*P. aeruginosa*		*K. pneumoniae*		*A. baumannii*		*S. epidermidis*	*E. coli*	Frequency
		700829	**700888**	**700603**	**700831**	**BAA-1605**	**BAA-1878**	**NRS 101**	**43894**	
Arabinose	Ara	1.6	2.4	–	1.5	1.1	0.5	–	–	5
Fucose	Fuc	0.2	0.2	–	0.3	0.1	–	–	22.6	5
Galactose	Gal	3.0	3.7	14.0	7.8	4.0	13.2	1.7	2.1	8
Galacturonic Acid	GalA	0.7	0.8	2.5	9.8	2.0	1.0	–	–	6
Glucose	Glc	3.6	6.8	1.3	31.1	7.9	4.5	35.7	36.8	8
Glucuronic Acid	GlcA	–	–	5.0	–	–	–	–	–	1
Mannose	Man	89.5	84.3	49.4	38.3	84.0	79.3	52.8	9.8	8
N-Acetyl-Galactosamine	GalNAc	–	–	–	–	–	–	6.3	26.8	2
N-Acetyl-Glucosamine	GlcNAc	–	–	0.8	0.7	0.4	1.3	3.5	1.9	6
Rhamnose	Rha	1.0	1.3	27.0	10.0	0.1	–	–	–	5
Xylose	Xyl	0.4	0.5	–	0.5	0.4	0.2	–	–	5
Total:		100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	

Values expressed as mole percent of total carbohydrate.
–, not detected.
doi:10.1371/journal.pone.0067950.t001

Species-specific findings

Of the bacteria studied in this report, biofilms of *P. aeruginosa* are perhaps the best studied. *P. aeruginosa* produces alginate, a high molecular weight, acetylated polysaccharide that is well known for its association with the mucoid phenotype common in cystic fibrosis patients. It is composed of β-1,4 linked L-guluronic and mannuronic acids. In addition, some *P. aeruginosa* species are known to produce Psl, which consists of a repeating pentasaccharide of 3 mannose, 1 rhamnose, and 1 glucose [29], and Pel, whose exact structure is not completely known, but is reported to have a high glucose content [30,31]. Very little data are available on the specific strains we tested, 700829 and 700888, although 700888 has been sequenced. Its genome possesses all of the genes for production of alginate (*alg44*, *alg8*, *algA-algZ*, *mucA-mucC*) and Psl (*pslA-pslM*), but does not contain the genes for Pel. Since we found the EPS exopolysaccharide from both *P. aeruginosa* strains to be predominantly mannose (~85–90%) and the linkages were chiefly 2-Man, 3-Man, 2,6-Man, and t-Man, we conclude that the majority of the EPS exopolysaccharide we observed is consistent with Psl, although we cannot rule out additional structures.

Much is also known about exopolysaccharides of *S. epidermidis*, in particular the NRS-101 strain (a.k.a., RP62A; ATCC 35984) we employed for this study. This strain and similar *S. epidermidis* strains are known to have linear β-1,6-linked GlcNAc polysaccharides, termed the polysaccharide intercellular adhesion (PIA), encoded by the *icaADBC* operon that mediates intercellular interactions during the biofilm mode of growth [32,33]. A separate, galactose-rich capsular polysaccharide adhesion (CPA) is also reported to be associated with this strain [34]. Christensen and colleagues used a mutant of RP62A that lacked the ability to make CPA and isolated a high molecular weight exopolysaccharide, called the slime associated antigen (SAA), which was found to be primarily glucose (~59%) [35]. In contrast, Peters *et al* isolated a mannose-rich exopolysaccharide from a slime layer of *S. epidermidis* strain KH11 and called it the extracellular slime substance (ESS) [36]. We did not observe any evidence of PIA, which would be expected given the methods we used and the surface localization of this polysaccharide. However, our results indicate the high molecular weight EPS exopolysaccharide is 52.8% mannose and 35.7% glucose, suggesting both SAA and ESS may be present in our sample. Further experimentation is required to define the structures of these polysaccharides.

The polysaccharides of *E. coli* associated with the capsular O serogroups and K-antigens have been studied extensively, but comparatively little is known about the non-capsule high molecular weight exopolysaccharides of the EPS [37]. However, most *E. coli* strains are known to secrete colanic acid, which consists of glucose, galactose, fucose, GlcA, acetate, and pyruvate in molar proportions roughly 1:2:2:1:1:1, respectively [38,39]. Moreover, the O157:H7 strain we tested, ATCC 43894 (a.k.a. CDC EDL 932), has been shown to specifically generate colanic acid as its exopolysaccharide [40]. While our data show this same strain produces glucose (36.8% of total carbohydrate), fucose, (22.6%), and galactose (2.1%) as would be expected for colanic acid, the proportions are not consistent with colanic acid and most noticeably, there is a complete absence of detectable GlcA. In addition, the presence of GalNAc (26.8%), mannose (9.8%), and GlcNAc (1.9%) were unexpected findings, suggesting we isolated a previously uncharacterized exopolysaccharide.

Similar to *E. coli*, the surface exopolysaccharides (K-antigens) of *K. pneumoniae*, numbering over 80 serovars, have been studied in detail. However, there have only been a few attempts to isolate the exopolysaccharide associated with biofilm EPS. Rättö and colleagues used an ethanol extraction protocol to isolate EPS exopolysaccharide from two similar *K. pneumoniae* strains and found each contained ~60% mannose, 20% galactose, and 17% GalA [41]. While both of our *K. pneumoniae* strains contained a high percentage of mannose as well as significant amounts of galactose and GalA, they also possessed considerable rhamnose as well as minor fractions of arabinose, fucose, GlcA, GlcNAc, and xylose, depending on strain. This indicates both strains may possess EPS exopolysaccharide structures that have not been previously characterized. Despite the remarkable heterogeneity in EPS exopolysaccharide composition between the two *K. pneumoniae* strains, it is interesting to note that the isolated exopolysaccharides bear no resemblance to the known K-antigens. For example, strain

Table 2. Glycosyl Linkage Analysis.

Glycosyl Linkage	Abbreviation	*P. aeruginosa*		*K. pneumoniae*		*A. baumannii*		*S. epidermidis*	*E. coli*	Frequency
		700829	**700888**	**700603**	**700831**	**BAA-1605**	**BAA-1878**	**NRS 101**	**43894**	
4-linked arabinopyranosyl residue	4-Ara	0.2	0.2	–	0.2	–	0.2	–	–	4
3-linked fucopyranosyl residue	3-Fuc	–	–	–	–	–	–	–	3.6	1
terminally-linked fucopyranosyl residue	t-Fuc	–	–	–	–	–	–	–	0.9	1
3-linked galactopyranosyl residue	3-Gal	–	0.3	18.2	–	–	0.3	–	–	3
3,6-linked galacopyranosyl residue	3,6 Gal	0.3	0.2	–	–	0.8	0.2	–	–	4
4-linked galactopyranosyl residue	4-Gal	1.2	1.6	–	0.2	1.1	1.6	–	1.1	6
terminally-linked galactopyranosyl residue	t-Gal	0.1	0.2	0.3	–	–	0.2	1.5	0.2	6
4-linked *N*-acetyl-galactosamine	4-GalNAc	–	–	–	–	–	–	–	0.1	1
6-linked *N*-acetyl-galactosamine	6-GalNAc	–	–	–	–	–	–	–	2.8	1
terminally-linked *N*-acetyl-galactosamine	t-GalNAc	–	–	–	–	–	–	–	0.4	1
2-linked glucopyranosyl residue	2-Glc	–	–	–	–	–	–	6.5	–	1
2-linked 6-deoxy-4 glucosamine	2-(6-deoxy)-4-GlcN	–	–	–	–	–	–	–	13.0	1
3-linked glucopyranosyl residue	3-Glc	1.2	1.3	–	14.4	–	1.3	4.0	–	5
3,6-linked glucopyranosyl residue	3,6-Glc	–	–	–	–	–	0.7	1.3	–	2
4-linked glucopyranosyl residue	4-Glc	0.2	0.4	0.4	0.4	–	0.4	1.2	39.2	7
4,6-linked glucopyranosyl residue	4,6-Glc	–	–	–	11.4	–	–	–	–	1
6-linked glucopyranosyl residue	6-Glc	0.6	0.7	–	0.4	1.3	0.8	1.3	–	6
terminally-linked glucopyranosyl residue	t-Glc	–	–	0.0	1.2	–	–	3.7	6.3	3
4-linked *N*-acetyl-glucosamine	4-GlcNAc	–	–	–	–	–	–	–	0.2	1
6-linked *N*-acetyl-glucosamine	6-GlcNAc	–	–	–	–	–	–	–	3.3	1
terminally-linked *N*-acetyl-glucosamine	t-GlcNAc	–	–	–	–	–	–	–	0.9	1
2-linked hexafuranosyl residue	2-HexF	–	–	–	12.2	–	–	–	–	1
2-linked mannopyranosyl	2-Man	20.6	19.3	40.1	13.8	20.2	19.2	6.0	2.0	8
2,3-linked mannopyranosyl residue	2,3-Man	1.3	1.2	15.4	0.5	–	1.2	–	–	5
2,3,4-linked mannopyranosyl residue	2,3,4-Man	–	–	–	11.6	–	–	–	–	1
2,6-linked mannopyranosyl residue	2,6-Man	32.3	28.5	–	16.1	32.5	28.5	32.3	–	6
3-linked mannopyranosy lresidue	3-Man	16.7	16.0	15.4	5.2	16.5	15.9	7.5	–	7
3,6-linked mannopyranosyl residue	3,6-Man	0.6	0.7	–	0.4	–	–	–	–	3
4-linked mannopyranosyl residue	4-Man	–	0.4	–	–	–	0.4	1.2	–	3
6-linked mannopyranosyl residue	6-Man	1.4	1.5	–	1.2	2.2	1.6	5.7	0.7	7
terminally-linked mannopyranosyl residue	t-Man	23.3	27.4	1.3	10.5	25.4	27.4	27.8	25.3	8
2-linked rhamnopyranosyl residue	2-Rha	–	–	7.3	–	–	–	–	–	1
terminally-linked rhamnopyranosyl residues	t-Rha	–	–	1.6	0.3	–	–	–	–	2

Table 2. Cont.

Glycosyl Linkage	Abbreviation	*P. aeruginosa*		*K. pneumoniae*		*A. baumannii*		*S. epidermidis*	*E. coli*	Frequency
		700829	**700888**	**700603**	**700831**	**BAA-1605**	**BAA-1878**	**NRS 101**	**43894**	
2-linked xylopyranosyl residue	2-Xyl	–	0.1	–	–	–	0.1	–	–	2
Total:		100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	

Values expressed as mole percent of total carbohydrate.
–, not detected.
doi:10.1371/journal.pone.0067950.t002

700603 has a K6-type capsule composed of a repeating linear polysaccharide of fucose, glucose, mannose and GlcA in equal proportions [42]. In our analysis, the composition of these moieties was 0% fucose, 1.3% glucose, 49.4% mannose, and 5.0% GlcA. Thus, we can conclude that our extraction/purification methods are effective at separating the high molecular weight EPS exopolysaccharide from capsular polysaccharides.

The EPS exopolysaccharide of *A. baumannii* is the least studied of all the pathogens we tested. Cell-associated poly-β-1,6-linked GlcNAc has previously been linked to biofilm development in *A. baumannii* [43], but our protocol should not have isolated this surface polysaccharide, which is confirmed by the composition results for the two *A. baumannii* strains we tested, each of which showed only ~1.0% GlcNAc. Crude extraction of EPS exopolysaccharide has been performed on other species of *Acinetobacter*, including *A. junii* (3 mannose: 1 galactose: 1 arabinose) [44] and *A. calcoaceticus* (4 rhamnose: 1 glucose: 1 glucuronic acid: 1 mannose) [45], but neither of these composition ratios matches the results of our two *A. baumannii* strains (BAA-1605; BAA-1878), indicating our exopolysaccharide may be a new finding.

Mannose contribution to EPS

The most salient finding of our study was the high mannose content of the EPS exopolysaccharide across all species and strains (**Table 1**). As such, we investigated the binding of the Amaryllis HHA lectin to pathogen biofilms. HHA specifically binds α1,3 and α1,6-linked mannose units, linkages that are common to all 8 pathogens tested (**Table 2**). Preliminary fluorescent binding studies indicated HHA adhered to biofilms of all strains (data not shown). We therefore focused on biofilms of *E. coli* strain 43894, since the EPS exopolysaccharide of this strain had the lowest mannose content (9.8%) of all strains tested. As can be seen in **Figure 2A**, HHA binding results in an extracellular cloud around *E. coli* cells in 1 day biofilms. When 3 day biofilms are viewed by confocal microscopy (**Figure 2B**) HHA only binds to the outer surface of the ~40 μm biofilm, presumably limited in diffusion by the density of the biofilm matrix. In contrast to the HHA lectin, which is a globular protein composed of 4 subunits, the small molecular weight nucleic acid stain penetrates the full thickness of the biofilm maxtrix, staining all *E. coli* cells within the matrix. Given the frequent use of mannose by microorganisms as a component of surface antigens or capsule, detection of mannose alone cannot be considered diagnostic for the presence of a biofilm. Nonetheless, mannose was ubiquitous in the biofilm exopolysaccharides of the five species we tested and even the EPS with the lowest mannose content was easily visualized by staining with the HHA lectin. Additional lectins or a lectin-based arrays [46] may be useful in future characterization of EPS composition from a broad range of organisms.

It has long been known that the exopolysaccharide portion of the EPS plays a substantial role in bacterial adherence and resistance [6,7,47] and the mannose results above suggests there may be commonalities between biofilm EPS that can be exploited for diagnostic and/or therapeutic purposes. For example, breaking the most common bonds that connect polysaccharide residues in the EPS could be an effective means of dispersing biofilms and making the subjacent bacteria more susceptible to treatment by antibiotics. Notably, bacteriophage have co-evolved with natural biofilms of host organisms for billions of years and have developed enzymatic domains on tail fiber and tailspike proteins that degrade polysaccharides of the EPS and capsule, allowing the phage access to surface receptors for infection [48–50]. These enzymes, generically termed depolymerases, have been shown to degrade exopolysaccharides even in the absence of phage [51], although

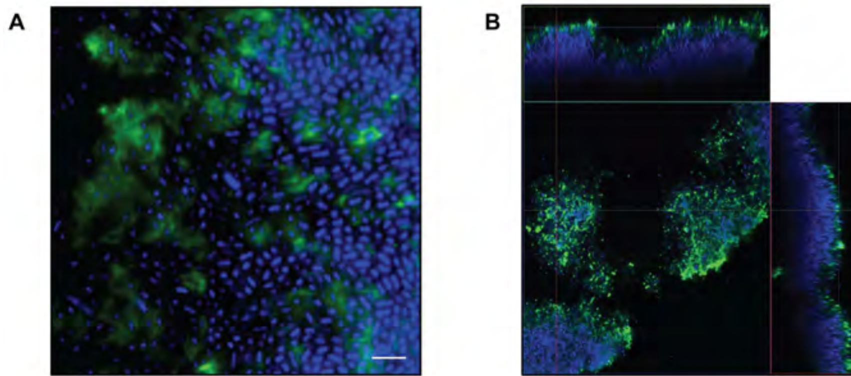

Figure 2. EPS staining of *E. coli* strain 43894 biofilms. The FITC-labeled mannose-specific HHA lectin was used to stain exopolysaccharides (green) and Hoechst 33342 was used to stain the bacterial nucleic acids (blue). (**A.**) Extracellular green staining of the EPS by FITC-HHA can be seen on 1 day old biofilms of *E. coli* at 200X. Scale bar = 5 µm. (**B.**) Confocal image of 3 day old *E. coli* biofilms at 63X. The large square panel is a plan view looking down on the biofilm. The top and right-side rectangular panels are vertical sections representing the XZ plane and YZ plane, respectively, at the positions indicated by the colored lines. The biofilm is 40 µm thick (i.e Z-axis).
doi:10.1371/journal.pone.0067950.g002

extensive analysis of their specificity and utility as anti-biofilm agents has yet to be elucidated.

Another example of a potential anti-biofilm enzyme is dispersin B, a β-1,6-*N*-acetyl-glucosaminidase from the bacterium *Actinobacillus actinomycetemcomitans*. First described in 2003 [52], this enzyme has the ability to degrade EPS from *Actinobacillus* [4] and *S. epidermidis* [53,54] biofilms. While enzymatic digestion of the EPS exopolysaccharide is not expected to directly kill bacterial cells, the dissolution of biofilms or prevention of future biofilm formation should allow bacteria to become re-sensitized to antibiotics and immune system mechanisms (i.e., complement, antibodies, phagocytes, etc.) Alternatively, these agents could be used to prevent biofilm formation. For example, dispersin B has been successfully incorporated into a polyurethane material, showing that materials, such as medical implants, could be engineered with anti-biofilm enzymes to prevent colonization [55].

Obtaining detailed structural characteristics by NMR for the current set of EPS exopolysaccharides in relationship to the composition and linkage data generated here will help validate our extraction and purification methods. Further characterization of ESKAPE biofilm EPS by the methods we employed here may also enable the identification and design of more effective anti-biofilm therapeutic agents.

Acknowledgments

The authors acknowledge Parastoo Azadi and the Complex Carbohydrate Research Center at the University of Georgia, Athens for assistance with sample processing. We also thank Sara Linden and Debra Weinstein for technical assistance.

Author Contributions

Conceived and designed the experiments: PMB DCN. Performed the experiments: PMB EMR SLM YS. Analyzed the data: PMB DCN. Wrote the paper: PBM DCN.

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PHONE (585) 533-1672 FAX (585) 533-1796

Sponsor:	University of Maryland - IBBR 9600 Gudelsky Drive Rockville, MD 20850	Test Number:	T13-1521
		Study Number:	NA
		Date Received:	5/30/13
Attention:	Daniel C. Nelson, Ph.D.	Lot Number:	NA
		Other:	NA
		P.O. Number:	H165217
Test Article(s):	One (1) NagZ		

TEST DATA REPORT FORM
ACUTE SYSTEMIC INJECTION TEST – ISO METHOD

Sample Preparation:

___ $6cm^2/ml$ ___ 0.2g/ml

___ $3cm^2/ml$ ___ 0.1g/ml

___ $1.25cm^2/ml$

√ Other: Tested as received TS 7/11/13

Extraction Media (control)

√ Saline (Mfr. B/Braun / lot J2D652 / exp.10-2014)

___ Vegetable Oil (Type:________________)

___ Other:____________________________

Extraction Time and Temperature NA

___ 121° ± 2°C for 1 hr. ± 6 minutes
___ 70° ± 2°C for 24 hrs. ± 2 hours
___ 50° ± 2°C for 72 hrs. ± 2 hours
___ 37° ± 1°C for 72 hrs. ± 2 hours

Test Animals: Healthy purpose-bred young adult mice, either sex, of known origin and with defined microbiological health status; weight variation within a sex should not exceed ± 20% of the mean weight; if females are used, they shall be nulliparous and non-pregnant.

Test Methodology: Each of five (5) mice/group were injected intravenously (IV) or intraperitoneally (IP) with the test article extract. All mice receiving IV injections were injected at a rate of 0.1 ml/sec. A control group consisting of 5 mice per group was injected in the same manner with each corresponding blank. The animals were observed immediately after injection, again at four (4) hours post injection, and then at least at twenty-four (24), forty-eight (48) and seventy-two (72) hours post injection. Attachment I lists the grading criteria used to score for toxicity of the test articles. Animals were weighed individually on the day the test article was administered and daily thereafter. Results are found in Tables I and II.

Conclusion: The sample evaluated **does** meet the requirements of this test.

Test Released By: Sonia Schaubroeck Date: 7-18-13

TABLE I

SYSTEMIC INJECTION TEST

TEST RESULTS - **TEST EXTRACT**

Injection Solution	Animal ID No.	Start Wt.(g)*	Dose (ml)	0 Hr.	2 Hr.	4 Hr.	24 Hr.		48 Hr.		72 Hr.		Wt. Change (+/- g)
				Obs.	Obs.	Obs.	Obs.	Wt.(g)	Obs.	Wt.(g)	Obs.	Wt.(g)	
Test	1302959	21.4	1.1	X	X	X	X	21.5	X	22.3	X	23.8	+1.6
	1302960	25.3	1.3	X	X	X	X	25.2	X	26.5	X	27.8	+2.5
	1302961	23.6	1.2	X	X	X	X	23.8	X	24.9	X	26.1	+2.5
	1302962	24.1	1.2	X	X	X	X	24.2	X	24.6	X	25.5	+1.4
	1302963	22.6	1.1	X	X	X	X	22.9	X	23.5	X	23.9	+1.3
Tech:		JD	JD	JD	LC	MB	JD		JD		JD		
Date:		7/8/13	7/8/13	7/8/13	7/8/13	7/8/13	7/9/13		7/10/13		7/11/13		

*Mean weight of the test mice = 23.4 g. The weight variation of the animals did not exceed +/- 20% of the mean weight (range is 18.7 g to 28.1 g).

X = Normal

For other symptoms, see page 5 (refer to the grading criteria in Attachment I).

TABLE II

SYSTEMIC INJECTION TEST

TEST RESULTS - **BLANK**

Injection Solution	Animal ID No.	Start Wt.(g)*	Dose (ml)	0 Hr.	2 Hr.	4 Hr.	24 Hr.		48 Hr.		72 Hr.		Wt. Change (+/- g)
				Obs.	Obs.	Obs.	Obs.	Wt.(g)	Obs.	Wt.(g)	Obs.	Wt.(g)	
Saline Blank	1302949	25.2	1.3	X	X	X	X	25.9	X	26.5	X	26.7	+1.5
	1302950	21.5	1.1	X	X	X	X	21.4	X	22.0	X	22.5	+1.0
	1302951	21.5	1.1	X	X	X	X	21.9	X	22.2	X	23.2	+1.7
	1302952	23.3	1.2	X	X	X	X	23.5	X	24.5	X	25.0	+1.7
	1302953	22.9	1.1	X	X	X	X	23.1	X	24.0	X	24.2	+1.3
Tech:		JD	MB	MB	LC	MB	JD		JD		JD		
Date:		7/8/13	7/8/13	7/8/13	7/8/13	7/8/13	7/9/13		7/10/13		7/11/13		

*Mean weight of the control mice = 22.9 g. The weight variation of the animals did not exceed +/- 20% of the mean weight (range is 18.3 g to 27.5 g).

X = Normal

For other symptoms, see page 5 (refer to the grading criteria in Attachment I).

Animal Diet: Standard laboratory rodent food was supplied per rodent vendor recommendations and potable water was supplied *ad libitum*. Feed and water are expected to have reasonable levels of contaminants which should not have the potential to influence the outcome of the study.

Test System Justification: This test system is recommended by ISO 10993-11, Biological Evaluation of Medical Devices – Part 11: Tests for Systemic Toxicity and the current USP/National Formulary. The justification for use of this test system is from ISO 10993-1, Biological Evaluation of Medical Devices - Part 1: Evaluation and Testing. This test is applicable for all external communicating devices, all implant devices, surface devices that have prolonged or permanent contact with a mucosal membrane, and surface devices that have any contact with a breached or compromised surface of the body, per the FDA modified ISO 10993-1 test matrix.

Test Criteria: If during the observation period, none (0%) of the animals treated with the extract of the test article show a significantly greater reaction than the animals treated with the blank, the test article meets the requirements of this test. If two or more mice die, or if abnormal behavior such as convulsions or prostration occurs in two or more mice, or if a body weight loss greater than 10% occurs in three or more mice, the test article does not meet the requirements of this test. If any of the animals treated with the extracts of the test article show only slight signs of biological reactivity, and not more than one (1) animal shows gross symptoms of biological reactivity or dies, a repeat of the test using ten (10) mice for each group is warranted. On the repeat test, the requirements of the test are met if none of the animals treated with the test sample preparation show a significantly greater reaction than that observed in the animals treated with the blank. If the blank mice fail to meet test requirements, the test shall be repeated using 5 mice per group.

The animals used in this study were obtained from the following vendor (check appropriate):
☒ Charles River ☐ Taconic ☐ Jackson ☐ Other: ____________________

Mouse gender:
☒ Female ☐ Male

Check only the following if applicable:

☐ All extracts were clear and free of particulate matter.

☐ Upon decanting, particulate matter was present in the ________________ test extract(s). Therefore, filtration was required, along with the corresponding blank(s), prior to injection using a 0.8µm syringe filter (Mfg/Lot#/Exp:____________________________).

Additional Comments:

NA

Description of abnormal observations:

NA

References:

(1) ANSI/AAMI/ISO 10993-11:2006, Biological Evaluation of Medical Devices-Part 11: Tests for Systemic Toxicity (Identical to ISO 10993-11:2006).

(2) ANSI/AAMI/ISO 10993-1, Biological Evaluation of Medical Devices - Part 1: Evaluation and Testing (Identical to ISO 10993-1:2003 (E)).

(3) CLE-003: Systemic Injection Test (USP, JP and ISO Acute Methods).

SYSTEMIC INJECTION TEST
Grading Criteria

Code	Clinical observation	Observed sign	Involved system(s)
I	Respiratory	Dyspnea (abdominal breathing, gasping), apnea, cyanosis, tachypnea, nostril discharges	CNS, pulmonary, cardiac
II	Motor activities	Decrease/increase somnolence, loss of righting, anesthesia, catalepsy, ataxia, unusual locomotion, prostration, tremors, fasciculation	CNS, somatomotor, sensory, neuromuscular, autonomic
III	Convulsion	Clonic, tonic, tonic-clonic, asphyxial, opisthotonos	CNS, neuromuscular, autonomic, respiratory
IV	Reflexes	Corneal, righting, myotact, light, startle reflex	CNS, sensory, autonomic, neuromuscular
V	Ocular signs	Lacrimation, miosis, mydriasis, exophthalmos, ptosis, opacity, iritis, conjunctivitis, chromodacryorrhea, relaxation of nictitating membrane	Autonomic, irritation
VI	Cardiovascular signs	Bradycardia, tachycardia, arrhythmia, vasodilation, vasoconstriction	CNS, autonomic, cardiac, pulmonary
VII	Salivation	Excessive	Autonomic
VIII	Piloerection	Rough hair	Autonomic
IX	Analgesia	Decrease reaction	CNS, sensory
X	Muscle tone	Hypotonia, hypertonia	Autonomic
XI	Gastrointestinal	Soft stool, diarrhea, emesis, diuresis, rhinorrhea	CNS, autonomic, sensory, GI motility, kidney
XII	Skin	Edema, Erythema	Tissue damage, irritation
XIII	Death	N/A	N/A

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Sponsor: University of Maryland - IBBR
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Attention: Daniel C. Nelson, Ph.D.

Test Number: T13-1519
Study Number: NA
Date Received: 5/30/2013
Lot Number:NA
Other:NA
P.O. Number:H165217

Test Article(s): One (1) NagZ

TEST DATA REPORT FORM
PRIMARY SKIN IRRITATION TEST - ISO METHOD
(Reference SST-002.1)

Extraction Time and Temperature

____ 121°C ± 2°C for 1 hr. ± 6 minutes
____ 70°C ± 2°C for 24 hrs. ± 2 hrs.
____ 50°C ± 2°C for 72 hrs ± 2 hrs.
____ 37°C ± 1°C for 72 hrs. ± 2 hrs.
____ 37°C ± 1°C for 24 hrs. ± 2 hrs.

NA 7-9-13 JD

Sample Preparation/20 ml extract media

____ 120 cm^2 (6 cm^2/ml)
____ 60 cm^2 (3 cm^2/ml)
____ 25 cm^2 (1.25 cm^2/ml)
____ 4g (0.2 g/ml)
____ 2g (0.1 g/ml)

Extract Preparation Vehicle:

____ Sterile Water
____ Saline
____ Vegetable Oil (V.O.)(Type:____)

Sample Application/2.5 x 2.5 cm Gauze Patches

____ 0.5 ml of extract
(pH*: Saline ____ V.O. ____)
____ 0.5 gm of solid test article
____ 2.5x2.5 cm patch moistened with:
☐ Sterile Water ☐ Other: ____
✓ 0.5 ml liquid test article (pH* 7)
____ Other: ____

Control:

✓ Sterile Water:
Mfr./Lot No./Exp.:
BBraun|J3C238|03-2016
____ Extraction Medium
____ ____
____ Other: ____

*pH Strip Mfr./Lot No.: EMD|HC133115|NA

Number of exposure hours: ☐ 4 Hours ☒ 24 Hours ☐ Other: ____

Conclusion:

Based on the Primary Irritation Index (see Attachment I), the irritation response produced by the test article/~~extract~~ was: ☒ Negligible ☐ Slight ☐ Moderate ☐ Severe

Test Released By: Sonia Schaubroeck Date: 7-18-13

Test Animals: Three (3) healthy young adult albino rabbits of either sex from a single strain ((NZW)SPF) or equivalent) from an approved vendor, weighing not less than 2 kg, will be used. If the response in the initial test is equivocal or not clear, additional testing shall be considered. A positive control group consisting of three (3) additional animals is conducted semi-annually independent of this test (see Table II).

Test System Justification: This test system is recommended by ISO 10993-10:2010, Biological Evaluation of Medical Devices – Part 10: Tests for Irritation and Skin Sensitization. The justification for use of this test system is provided in ISO 10993-1, Biological Evaluation of Medical Devices - Part 1: Evaluation and Testing.

Test Methodology: Rabbits are shaved (dorsal region) twenty-four (24) to four (4) hours before testing. Three healthy, young, adult albino rabbits of either sex weighing not less than 2 kg ((NZW)SPF) are used to evaluate each test substance. The test **~~extract~~/article** and corresponding control are placed on patches which are applied to skin of each rabbit as follows: cranial right = control, cranial left = test, caudal right = test, caudal left = control. For the positive control evaluation, the "test" sites consist of Sodium Dodecyl (Lauryl) Sulfate (SLS) and the "control" sites consist of petrolatum or water only (method is based in length of exposure; see Table II). After a minimum of four (4) hours (exposure may be longer based on the end use of the test material), the patches are removed. Residual test material/extract is removed by appropriate means, such as washing with lukewarm water or other suitable non-irritating solvent and careful drying. Rabbits are then scored for erythema and edema at 1 hour ± 6 minutes, 24 ± 2 hours, 48 ± 2 hours and 72 ± 2 hours post patch removal (see Attachment I). Scores are reported in Table I.

Test article description: Clear aqueous solution 7·10·13 JO

Comments: NA

The animals used in this study were obtained from the following vendor (check appropriate):

☐ Charles River ☒ RSI ☐ Covance ☐ Other: ______

Test No.: T13-1519
Study No.: NA

PRIMARY IRRITATION TEST RESULTS

☒ **Direct Application** ☐ **Saline Extract**

Animal No. Weight (kg)	Test Article/ Test Extract	Shaved (X)	Sample Applied (X)	Sample Removed (X) *	1 Hour ± 6 Min Scores[1]	24 ± 2 Hour Scores	48 ± 2 Hour Scores	72 ± 2 Hour Scores	Avg. Score[2]	PIS[3]	Total PIS[4]	PII[5]
130116 3.90	Test	X	X	X	er: 0,0 ed: 0,0	er: 0,0 ed: 0,0	er: 0,0 ed: 0,0	er: 0,0 ed: 0,0	0	0	0	0
	Control	X	X	X	er: 0,0 ed: 0,0	er: 0,0 ed: 0,0	er: 0,0 ed: 0,0	er: 0,0 ed: 0,0	0			
130117 3.75	Test	X	X	X	er: 0,0 ed: 0,0	er: 0,0 ed: 0,0	er: 0,0 ed: 0,0	er: 0,0 ed: 0,0	0	0		
	Control	X	X	X	er: 0,0 ed: 0,0	er: 0,0 ed: 0,0	er: 0,0 ed: 0,0	er: 0,0 ed: 0,0	0			
130118 3.89	Test	X	X	X	er: 0,0 ed: 0,0	er: 0,0 ed: 0,0	er: 0,0 ed: 0,0	er: 0,0 ed: 0,0	0	0		
	Control	X	X	X	er: 0,0 ed: 0,0	er: 0,0 ed: 0,0	er: 0,0 ed: 0,0	er: 0,0 ed: 0,0	0			
Tech:		DO/MB	DO/MB	DO	DO	DO	TC	TC				
Date:		7-9-13	7-10-13	7-11-13	7-11-13	7-12-13	7-13-13	7-14-13				
Time:		3:26pm	9:41am	9:44am	10:41am	11:04am	10:48a	10:43a				

Note - Scores are recorded as left side, right side.

* All test and control sites were rinsed with lukewarm water/ NA to remove any residual matter.

[1]er = erythema, ed = edema
[2]Avg. Score is sum of erythema and edema 24, 48 & 72 hour scores ÷ 6
[3]PIS = Primary Irritation Score which is test score average minus control score average.
[4]Total PIS is PIS added for all three animals.
[5]Primary Irritation Index (PII): Total PIS ÷ 3 animals

Test No.: T13-1519
Study No.: NA

PRIMARY IRRITATION TEST RESULTS

☐ **Direct Application** ☐ **Vegetable Oil Extract**

Animal No. Weight (kg)	Test Article/ Test Extract	Shaved (X)	Sample Applied (X)	Sample Removed (X)	1 Hour ± 6 Min Scores[1]	24 ± 2 Hour Scores	48 ± 2 Hour Scores	72 ± 2 Hour Scores	Avg. Score[2]	PIS[3]	Total PIS[4]	PII[5]
______	Test				er:	er:	er:	er:				
					ed:	ed:	ed:	ed:				
______	Control				er:	er:	er:	er:				
					ed:	ed:	ed:	ed:				
______	Test				er:	er:	er:	er:				
					ed:	ed:	ed:	ed:				
______	Control				er:	er:	er:	er:				
					ed:	ed:	ed:	ed:				
______	Test				er:	er:	er:	er:				
					ed:	ed:	ed:	ed:				
______	Control				er:	er:	er:	er:				
					ed:	ed:	ed:	ed:				
Tech:												
Date:												
Time:												

NA 7-9-13 JO

Note - Scores are recorded as left side, right side.

* All test and control sites were rinsed with lukewarm water/ ______________________ to remove any residual matter.

[1]er = erythema, ed = edema
[2]Avg. Score is sum of erythema and edema 24, 48 & 72 hour scores ÷ 6
[3]PIS = Primary Irritation Score which is test score average minus control score average.
[4]Total PIS is PIS added for all three animals.
[5]Primary Irritation Index (PII): Total PIS ÷ 3 animals

Positive Control Test No.: T13-0340, GLP-2013-0063, Amendment I

Current Test No.: T13-1519

PRIMARY IRRITATION TEST RESULTS
POSITIVE CONTROL EVALUATION
24 hour exposure: 100% SLS
Effective Dates: 2/18/13 – 8/18/13

Animal No. Weight (kg)	Test Article/ Test Extract	Shaved (X)	Sample Applied (X)	Sample Removed (X)	1 Hour ± 6 Min Scores[1]	24 ± 2 Hour Scores	48 ± 2 Hour Scores	72 ± 2 Hour Scores	Avg. Score[2]	PIS[3]	Total PIS[4]	PII[5]
130000 3.23	Test	X	X	X	er: 2,2 ed: 2,1	er: 4,4 ed: 2,1	er: 4,4 ed: 2,1	er: 4,4 ed: 2,2	5.7	5.7	17.7	5.9
	Control	X	X	X	er: 0,0 ed: 0,0	er: 0,0 ed: 0,0	er: 0,0 ed: 0,0	er: 0,0 ed: 0,0	0			
130001 3.01	Test	X	X	X	er: 2,2 ed: 2,1	er: 4,4 ed: 2,2	er: 4,4 ed: 2,2	er: 4,4 ed: 2,2	6.0	6.0		
	Control	X	X	X	er: 0,0 ed: 0,0	er: 0,0 ed: 0,0	er: 0,0 ed: 0,0	er: 0,0 ed: 0,0	0			
130002 3.19	Test	X	X	X	er: 2,3 ed: 2,2	er: 4,4 ed: 2,2	er: 4,4 ed: 2,2	er: 4,4 ed: 2,2	6.0	6.0		
	Control	X	X	X	er: 0,0 ed: 0,0	er: 0,0 ed: 0,0	er: 0,0 ed: 0,0	er: 0,0 ed: 0,0	0			
Tech:		LC	JD/LC	JD/LC	JD/LC	JD	JD	JCC				
Date:		2/11/13	2/12/13	2/13/13	2/13/13	2/14/13	2/15/13	2/16/13				
Time:		3:32 pm	1:25 pm	1:32 pm	2:34 pm	1:04 pm	11:46 ~~pm~~ am (correction JJ 7-16-13)	12:37 pm				

[1]er = erythema, ed = edema
[2]Avg. Score is sum of erythema and edema 24, 48 & 72 hour scores ÷ 6
[3]PIS = Primary Irritation Score which is test score average minus control score average.
[4]Total PIS is PIS added for all three animals.
[5]Primary Irritation Index (PII): Total PIS ÷ 3 animals

Note - Scores are recorded as left side, right side.

Sodium Dodecyl (Lauryl) Sulfate (SLS), Mfr./Lot No./Exp: Sigma 028K0108 06/14 Sterile Water, Mfr./Lot No./Exp: B/Braun J2K159 08/15 Positive Control Prepped By/Date: JD 2/12/13

Based on the Primary Irritation Index (see Attachment I), the irritation response produced by the positive control was:
☐ Negligible ☐ Slight ☐ Moderate ☒ Severe

QA Review/ Date: Brandon Taylor 04/09/13

SCORING CRITERIA

Erythema and Eschar Formation	Score
No erythema	0
Very slight erythema (barely perceptible)	1
Well defined erythema	2
Moderate erythema	3
Severe erythema (beet redness) to eschar formation preventing grading of erythema	4
Edema Formation	**Score**
No edema	0
Very slight edema (barely perceptible)	1
Well defined edema (edges of area well defined by definite raising)	2
Moderate edema (raised approximately 1 mm)	3
Severe edema (raised more than 1 mm and extending beyond the area of exposure)	4

Primary Irritation Index Categories in the Rabbit

RESPONSE CATEGORY	MEAN SCORE (PII)
Negligible	0.0 to 0.4
Slight	0.5 to 1.9
Moderate	2.0 to 4.9
Severe	5.0 to 8.0

Test Criteria: Only the 24, 48 and 72 hour observations are used for calculations. Observations made prior to dosing or after 72 hours, to monitor recovery, are not used in determination.

For each animal calculate the average skin reaction score by adding together the scores for the test material for erythema and edema at the twenty-four (24), forty-eight (48) and seventy-two (72) hour points and divide by six. Calculate the same average for the vehicle controls and then subtract that average score from the test material average score to obtain the Primary Irritation Score (PIS).

Add all the PIS for each animal and then divide by 3 (the number of animals) to obtain the Primary Irritation Index (PII).

MOOG
MEDICAL DEVICES GROUP
Life Science Laboratories - Rush

7500 W. Henrietta Rd., Rush, NY 14543
PHONE (585) 533-1672 FAX (585) 533-1796

Sponsor:	University of Maryland - IBBR	Test Number:	T13-1520
	9600 Gudelsky Drive	Study Number:	NA
	Rockville, MD 20850	Date Received:	5/30/13
Attention:	Daniel C. Nelson, Ph.D.	Lot Number:	NA
Test Article(s):	NagZ	Other:	NA
		P.O. Number:	H165217

TEST DATA REPORT FORM
Vaginal Irritation Test - ISO Method
Extraction Method
(Reference VAP-028)

Number of Days Insertion: ☒ 5 days ☐ Other (please specify): ____________

Test Animals: Healthy young adult female albino rabbits from a single strain ((NZW)SPF) or equivalent) from an approved vendor, weighing not less than 2 kg, will be used. A minimum of three animals will be used initially to evaluate the test material, and three animals as the control group. If the response in the initial test is equivocal or not clear, additional testing shall be considered.

Test System Justification: This test system is recommended by ISO 10993-10:2010, Biological Evaluation of Medical Devices – Part 10: Tests for Irritation and Skin Sensitization. The justification for use of this test system is provided in ISO 10993-1, Biological Evaluation of Medical Devices - Part 1: Evaluation and Testing. This test shall only be considered for materials with intended contact with vaginal tissue and if safety data cannot be obtained by other means.

Test Methodology: Measured amounts of test material and control materials (liquids: 1 ml) is introduced into the vaginas of young adult females (through a lubricated catheter) once daily at 24 ± 2 hour intervals for 5 consecutive days. 24 ± 2 hours after the last dose, the rabbits are euthanized and the tissues are harvested, examined macroscopically, and placed in an appropriate fixative for histological examination.

The scores for microscopic evaluation for all the animals in the test group are added and divided by the number of observations (3 rabbits x 4 evaluation categories = 12) to obtain a test group average. The maximum score is 16 for an individual animal. The same procedure is repeated for the control group(s).

A total score that is greater than nine for the microscopic evaluation in a control animal may indicate trauma at dosing and may require a retest if test and control animals exhibit similar high scores. The control group average is subtracted from the test group average to obtain the Irritation Index. (See Attachment I)

For prolonged repeated exposure, scoring may be modified to accommodate additional tissue responses associated with chronic irritation.

Conclusion: The sample evaluated results in an Irritation Index of 0 causing no vaginal irritation.

Test Released By: Tonia Schaubroeck Date: 7-31-13

Vaginal Irritation
Weight and Insertion Data

Test material was tested as received by sponsor

Animal ID No.:	Day 1 Weight (kg)	Day 2 Weight (kg)	Day 3 Weight (kg)	Day 4 Weight (kg)	Day 5 Weight (kg)	Day 6** Weight (kg)
130059	4.01	3.98	3.94	3.98	3.98	4.01
130061	4.60	4.59	4.56	4.49	4.50	4.55
130087	3.93	3.92	3.94	3.92	3.93	3.93
130088*	4.26	4.28	4.27	4.24	4.29	4.32
130027*	4.07	4.06	4.02	3.96	3.94	3.99
130028*	3.97	3.95	3.98	4.00	4.03	4.05
Dosed By:	JD	JJC	JJC	JD	JD	
Date:	7/12/13	7/13/13	7/14/13	7/15/13	7/16/13	7/17/13
Time:	9:57 am	11:17 am	10:36 am	10:05 am	10:14 am	8:59 am

*Negative Controls (Saline: Mfr./lot #/exp.: B/Braun / J2D652 / 10-2014)
**Test and control material are not dosed on day 6.

pH of extracts:
☒ Test material supplied by sponsor: 7
☒ Saline: Test: NA Control: 5
☐ Vegetable: Test: ____ Control: ____
Mfr./lot # / exp.: EMD / HC133115 / NA

Vaginal Irritation
Macroscopic Observations
of Vaginal Opening and Perineum

	Animal ID No.:	Day 1	Day 2	Day 3	Day 4	Day 5
Test Material	130059	N	N	N	N	N
	130061	N	N	N	N	N
	130087	N	N	N	N	N
Saline Control	130088	N	N	N	N	N
	130027	N	N	N	N	N
	130028	N	N	N	N	N
Technician:		JD	JJC	JJC	JD	JD
Date:		7/12/13	7/13/13	7/14/13	7/15/13	7/16/13

N = Vaginal opening and perineum appear normal.

Comments: NA

Vaginal Irritation Test
Macroscopic Results upon Euthanasia*

Date: 7/17/13

	Animal ID No.:	Observations	Technician
Test Material	130059	NSF	JP
	130061	NSF	JP
	130087	NSF	MB
Saline Control	130088	NSF	JP
	130027	NSF	JP
	130028	NSF	JP

NSF = No significant findings

*Vagina is dissected out upon euthanasia and examined for irritation, injury to the epithelial layer of tissue and necrosis.

Vaginal Irritation Test - Histopathology Results

Saline Extract	Animal ID Number	Total Score	Score Average
Test	130059	4	4.0
	130061	4	
	130087	4	
Control	130088	4	4.0
	130027	4	
	130028	4	

Irritation Index: Test score average (4.0) minus the average of the control scores (4.0) equals Irritation Index of 0 described as none.

Comments: NA

The animals used in this study were obtained from the following vendor (check appropriate):
☐ Charles River ☒ RSI ☐ Other: ____________

Microscopic (Histopathology) classification system for vaginal tissue reaction

Observations	Numerical Grading	Observations	Numerical Grading
1. Epithelium		**3. Vascular Congestion**	
Normal-Intact	0	Absent	0
Cell degeneration or flatting	1	Minimal	1
Metaplasia	2	Mild	2
Focal erosion	3	Moderate	3
Generalized erosion	4	Marked with disruption of vessels	4
2. Leukocyte Infiltration (per high power field)		**4. Edema**	
Absent	0	Absent	0
Minimal - less than 25	1	Minimal	1
Mild - 26- 50	2	Mild	2
Moderate - 51 - 100	3	Moderate	3
Marked - Greater than 100	4	Marked	4

Irritation Index*:	Adjectival Description
0	None
1 to 4	Minimal
5 to 8	Mild
9 to 11	Moderate
12 to 16	Severe

*Irritation Index is the control group average subtracted from the test group average.

NOTE: Other adverse changes of the tissues shall be recorded and included in the assessment of the response.