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14. ABSTRACT

During the performance period, we successfully screened >700 bacteriophage genomes bioinformatically for putative depolymerase enzymes, selected ~15 initial enzymes for gene synthesis, purified several of these enzymes that displayed proper folding and favorable solubility properties, and began characterizing their anti-biofilm potentials. In parallel, we evaluated dozens of ESKAPE (Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumanii, Pseudomonas aeruginosa, and Enterobacter species) organisms for production of biofilm and we extracted and purified biofilm polysaccharide from several of these species.

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
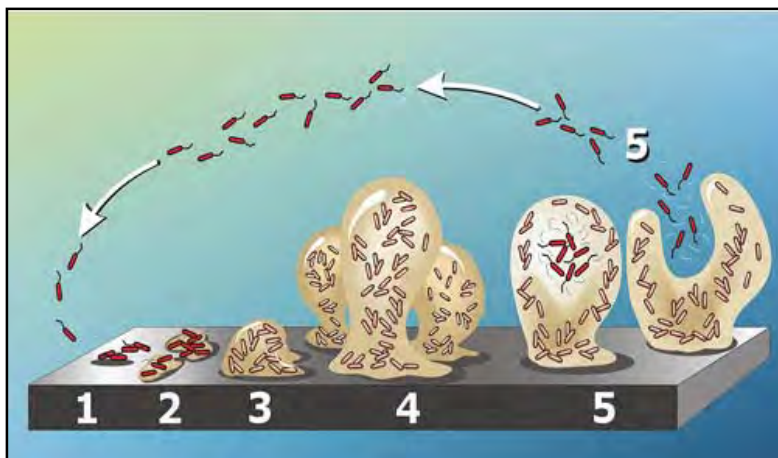
become established, phenotypic changes cause them to secrete polysaccharides that serve as the backbone for the biofilm (figure, stage 2 and 3). Non-cellular components and debris, including additional carbohydrates, proteins, lipids, and nucleic acids, become entangled in the polysaccharide backbone 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:

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

Task 1. IACUC and USAMRMC ACURO review and approval of animal regulatory documents (months 1-4)

I submitted my paperwork to complete this task, but it was placed in the deferred review queue by Ms. Teresa Kuykendal, Administrative Assistant, Animal Care and Use Review Office (ACURO). Because the animal experiments do not take place until year 3 of the award, ACURO did not want to review my request as it is possible that protocols could expire or change during the time between review and actual animal experimentation. Thus, this task remains under deferred review. I will resubmit the paperwork around the 24th month of the project, which should be enough time for proper review before any animal experiments begin.

Task 2. Bioinformatic analysis. (months 1-3)

The bioinformatic component was more difficult than we thought and actually took much of the first year to complete. It began by manually analyzing each and every gene from ~700 fully sequenced phage genomes. Below is just an example for illustrative purposes of one phage that infects *Pseudomonas* species:

ID: Pseudomonas phage 201phi2-1
Genes: 461

We used hierarchical clustering algorithms (i.e. DomClust) as well as online biofinformatics tools such as the Clusters of Orthologous Groups (COGs) of proteins available through the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov) to analyze the phage genomes. For this genome, we identified 20 unique domain clusters as follows:

CLSP34044- cd00209(DHFR)
CLSP31425-cl00053(PTP)
CLSP32345- cl11591(RNA_pol_Rpb1_2)
CLSP32347- COG0420(SbcD)
CLSP31196- TIGR02386(rpoC_TIGR) , cl09967(Hint), pfam05203(Hom_end_hint)
CLSP32161- COG1061(SSL2), cl09099(P-loop NTPase) , cl09967(Hint)
CLSP34080- COG3879(COG3879)
CLSP31578- cd02901(Macro_Poa1p_like)
CLSP32357- cl11427(Polysacc_deac_1)
CLSP32363- cl00243(RuvC_resolvase)
CLSP31092- COG3409(COG3409), pfam01471(PG_binding_1)
CLSP31242- pfam01391(Collagen)
CLSP34105- cd06222(RnaseH)
CLSP31732- cl01733(DUF2345)

CLSP31137- COG0419(SbcC), TIGR02168(SMC_prok_B), cl09099(P-loop NTPase)
CLSP30732- cd00254(LT_GEWL)
CLSP32374- COG0125(Tmk), cd01672(TMPK)
CLSP31752- COG0553(HepA), COG1061(SSL2), cd00079(HELICc), cl09099(P-loop NTPase)
CLSP41345- cl01644(Thg1)
CLSP34140- TIGR01453(grpIintron_endo)
CLSP34135- COG0317(SpoT), cl00076(HDc)
CLSP34129- cd07557(trimeric_dUTPase), cl00493(trimeric_dUTPase)
CLSP31420- COG0272(Lig), cd00027(BRCT), cd00114(LIGANc), cl00038(BRCT), cl03295(LIGANc), pfam03120(DNA_ligase_OB)
CLSP34133- cl00358(TS_Pyrimidine_HMase) PHA2142- cl09660(RNA_ligase)
CLSP37055- cd04301(GNAT)
CLSP41384- cl10028(Radical_SAM)
CLSP32360- cl04270(Glyco_transf_WecG_TagA)
CLSP31018- cd01049(RNRR2), cl00264(Ferritin_like)
CLSP34145- TIGR02506(NrdE_NrdA), cl09939(RNR_PFL)

Of these 29 protein domain clusters, the following were identified as potential domains that might contain a depolymerase-like activity:

1.   CLSP32357- cl11427(Polysacc_deac_1), a putative polysaccharide deacetylase with putative alpha-mannosidase activity
2.   CLSP32360- cl04270(Glyco_transf_WecG_TagA), a putative UDP-N-acetyl-D-mannosaminuronic acid transferase
3.   CLSP30732- cd00254(LT_GEWL), a putative lytic transglycosylase or goose egg white lysozyme

Of the three, only CLSP32357 appeared to contain an activity that would work against polysaccharides known to be present in a *Pseudomonas* biofilm.

Despite the successful identification of one putative depolymerase in the example above, the bioinformatic analysis was cumbersome and yielded relatively few candidate enzymes. This is due in part to the relatively low numbers of known depolymerase genes. However, I then attended a "Phage RAST (Rapid Annotation using Subsystem Technology) Jamboree" session in Tuscon, Arizona at the end of January 2011. This meeting brought together the top bioinformatics scientists in the nation and paired them with phage experts in order to rapidly identify and annotate unedited sequences in the databases that belong to phage genomes. I was invited as a "phage expert" for my knowledge about phage lytic enzymes. However, while I was there, I was able to tap the expertise of the bioinformaticists and search for novel depolymerase enzymes.

In a significant breakthrough, our bioinformatics analysis revealed a class of phage-derived enzymes that may be the "archetypical" depolymerase. This protein, also called a "tailspike" protein, is present on a family of bacteriophage called *Podoviridae*, or phage with short, non-contractile tails. In the cryo-electron image below (taken from Casjens and Thuman-Commike, 2011), the tailspike protein is shown as gp9. Notably, tailspike proteins self assemble into active

trimers that are resistant to detergent and heat, which is favorable for a therapeutic protein. These enzymes cleave the extracellular polysaccharide of the host bacterial species, which form the backbone of the extrapolymeric substance (EPS) associated with biofilm development.

Below is a figure of the unrooted neighbor-joining tree of the P22-like tailspike proteins (adapted from Casjens and Thuman-Commike, 2011). The phage names are colored according to their host species as follows: *Escherichia*, red; *Salmonella*, black; *Shigella*, blue; *Providencia*, orange; *Pectobacterium*, purple; *Hamiltonella*, green; *Sodalis*, gray. Outside of the tree, the polysaccharide repeats of the host are displayed

where known (Glc, glucose; Man, mannose; Rha, rhamnose; Gal, galactose; Abeq, abequose; Tyvel, tyvelose; Para, paratose; Col, colitose; GlcNAc, N-acetyl-glucosamine; ManOAc, O-acetyl-mannose; GalOAc, O-acetyl-galactose).

As can be seen, there is a broad diversity in the polysaccharides that are cleaved by these proteins. One challenge we face is narrowing down the list of potential depolymerases that will presumably cleave the polysaccharides of our target organisms associated with war wounds (i.e., *Acinetobacter baumannii-calcoaceticus* complex, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*). Therefore, we developed a two-pronged approach. First, because many of these polysaccharides are likely to occur in biofilms of war wound pathogens, we selected 10 tailspike proteins from the above figure for gene synthesis and evaluation. Second, we re-focused our bioinformatic search to phage belonging to the *Podoviridae* family that infect war wound pathogens. Below is a complete list of *Podoviridae* phage for *Psuedomonas*, *Klebsiella*, and *Staphylococcus* species as identified by our bioinformatic analysis. Any tailspike proteins contained in these phage will be synthesized after we complete a near neighbor tree to rule out any overlapping tailspikes.

	RefSeq	GenBank	Phage Name
Psuedomonas	NC 004665	AF493143	GH1
	NC_013638	FN594518	phi-2
	NC_015208	FR823298	phi 15
	NC_015264	GU583987	philBB-PF7A
	NC_012418	FN263372	phikF77
	NC_011107	EU236438	PT2
	NC_011105	EU056923	PT5
	NC_005884	AY575774	PaP2
	NC_004466	AY078382	PaP3
	NC_007807	DQ163914	119X
	NC_006552	AY625898	F116
	NC_013692	FN422399	LIT1
	NC_009935	AM265638	LKD16
	NC_010326	AM910651	LUZ19
	NC_010325	AM910650	LUZ24
	NC_013691	FN422398	LUZ7
Klebsiella	NC_011043	EU734173	K11
	NC_013647	GQ413937	KP32
	NC_013649	GQ413938	KP34
Staphylococcus	NC_004679	AF513033	phiP68
	NC_009875	EU136189	SAP-2
	NC_004678	AF513032	44AHJD
	NC_007046	AY954949	66

Task 3. Synthesize depolymerase genes identified by bioinformatic analysis. (months 2-6)

Based on our two-pronged bioinformatics approach, we have already synthesized 10 genes and are currently synthesizing 5 more. We are waiting on preliminary characterization of the first 10 genes to determine which enzymes possess the best anti-biofilm properties. If we find one or two that are significantly better than the rest, we will then synthesize ~5 additional genes based on othrologs that have already been identified by bioinformatic analysis. For illustration purposes, a representative sample of 4 genes is shown below. Each gene sequence was optimized for codon-usage and expression in an *E. coli* host. Additionally, 6X-His tags were added to the 5-prime ends for purification purposes. All gene inserts were cloned into a pBAD24 expression vector between the XbaI and EcoRI restriction sites. If these sites were present in the insert, we mutated them based on alternative codons in order to remove the restriction site from the insert.
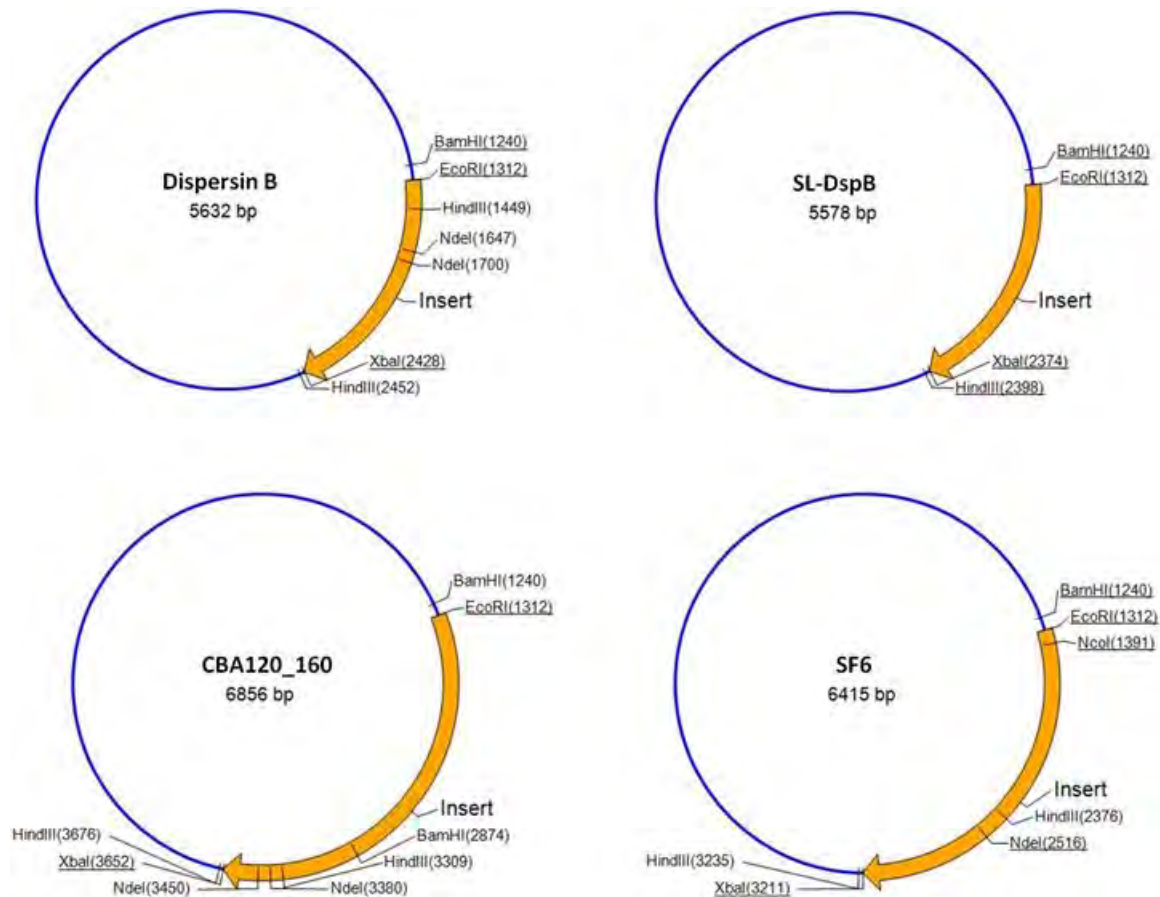
Task 4. Obtain phage from international collections for depolymerase screening. (months 1-3)

We have assembled a limited phage collection from various repositories for depolymerase screening. In addition, we possess a fairly large collection of our own phage. However, we believe the majority of novel depolymerases will be found from bioinformatic analysis of databases rather than screening individual phages.

*Task 5. Clone depolymerases from phage. (months 2-6)*

Because most, if not all, of the depolymerases were identified by bioinformatic analysis, we have not cloned any depolymerases from our current repertoire of phage. As an aside, the phage we have on hand are all sequenced and as such, are already represented in the bioinformatic analysis.

The tailspike proteins, which we identified as the most likely candidate for the depolymerase activity we seek, are only associated with phage that belong to the *Podoviridae* family. While we have identified numerous *Podoviridae* phage and tailspike proteins affiliated with *Pseudomonas*, *Klebsiella*, *Escherichia*, and *Staphylococcus* species, there are no known *Acinetobacter* phage that belong to the *Podoviridae* family (the known *Acinetobacter* phage are members of the *Myoviridae* or *Siphoviridae* families, which typically do not contain tailspike proteins). Nonetheless, we are confident that the extraction and characterization of the *Acinetobacter* polysaccharide (i.e. Aim 2) will reveal that many components and linkages that are identical to many of the other Gram-negative organisms, for which there are *Podoviridae* phage and tailspike proteins. If this is not the case, we will screen sewage and other environmental sources for novel *Acinetobacter* phage that are members of the *Podoviridae* family.

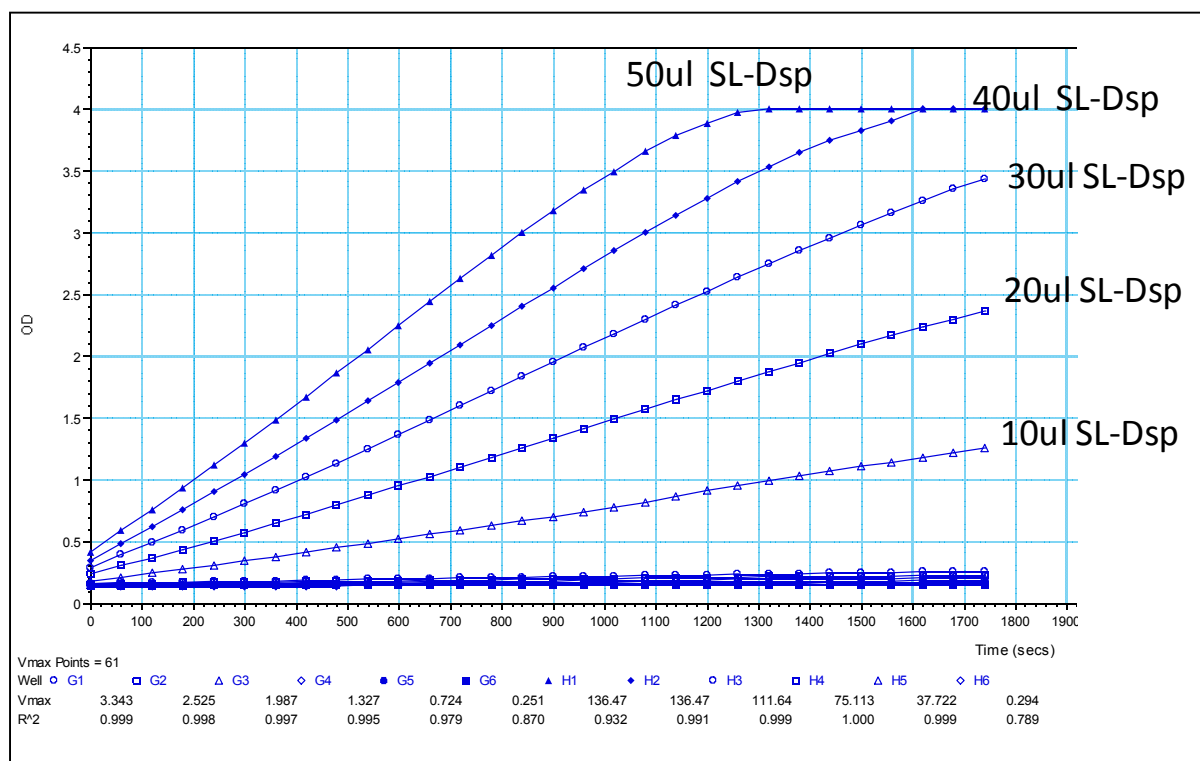*Task 6. Express and purify all assembled depolymerase enzymes. (months 3-14)*

We currently have 10 genes cloned and another 5 being synthesized. Of the 10 clones, 4 of them formed insoluble inclusion bodies. Attempts to refold these proteins (i.e. denature in 8 M urea and refold on an anion exchange column) did not prove successful. The other six proteins were expressed at high levels and displayed good solubility properties. The purification of three of them (Dsp, top; Sf6, middle; and Betty, bottom) on a nickel column are shown on the right. For all gels: Lane 1 – markers; lane 2 – extract; lane 3 – column flow through; lanes 4,5 – wash; lanes 6,7,8 – imidizole elution.

For one protein, CBA_120, the enzyme was soluble, but did not bind the column. We denatured the protein in 6M urea and showed that it did bind to the column, so it had the proper 6XHis tag. Therefore, we surmised that the His tag was buried in a globular fold of the protein.
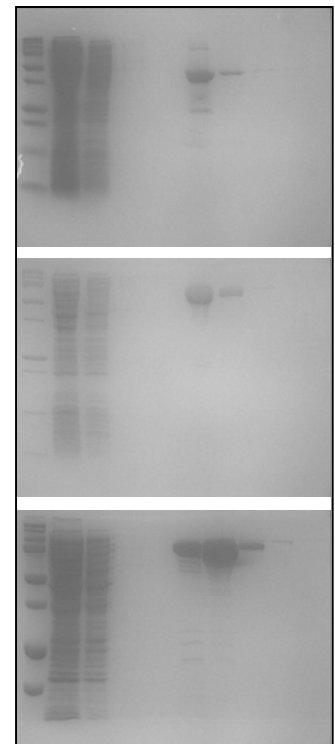
To address this problem, we removed the N-terminal His tag and placed a C-terminal His tag though point mutagenesis techniques. The new protein remained soluble, bound to the nickel column, and was purified to homogeneity similar to the other proteins.

Although characterization of the depolymerases is not scheduled to occur until months 18-24, we nonetheless wanted to assay our enzymes for activity as soon as we purified them. As an example, the enzyme SL-Dsp is a putative glucoasminidase. The synthetic substrate, 4-Nitrophenyl N-acetyl-b-D-glucosaminide, releases the nitrophenyl chromophore when cleaved by a glucosaminidase which can easily be monitored by a spectrophotometer by as an increase in yellow color over time. The image below shows the activity of this enzyme in increasing concentration.

Vmax Points = 61											
Well	○ G1	□ G2	△ G3	◇ G4	● G5	■ G6	▲ H1	◆ H2	○ H3	□ H4	△ H5	◇ H6
Vmax	3.343	2.525	1.987	1.327	0.724	0.251	136.47	136.47	111.64	75.113	37.722	0.294
R^2	0.999	0.998	0.997	0.995	0.979	0.870	0.932	0.991	0.999	1.000	0.999	0.789

Specific Aim 2. Characterize biofilm polysaccharide composition.

*Task 1. Collect biofilm EPS. (months 6-12)*

Much of this Task involved obtaining the bacterial strains and determining the best growth conditions for biofilm expression. Toward this end, we acquired the following strains:

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Organism	Strain	Notes
Acinetobacter baumannii	BAA-1605	Afghanistan isolate, MDR, ESBL
Acinetobacter baumannii	BAA-1878	sepsis, burn patient
Acinetobacter baumannii	19606	Type strain, known biofilm producer
Acinetobacter baumannii	17978	Sequenced strain
Klebsiella pneumoniae	13883	Type strain
Klebsiella pneumoniae	BAA-2146	NDM-1 positive
Klebsiella pneumoniae	700831	known biofilm producer
Klebsiella pneumoniae	51503	MDR, ESBL (TEM-10 and TEM-12)
Klebsiella pneumoniae	700603	MDR, used as positive control for ESBL
Pseudomonas aeruginosa	700829	known biofilm producer
Pseudomonas aeruginosa	700888	known biofilm producer
Pseudomonas aeruginosa	10145	type strain
Pseudomonas aeruginosa	27853	reference strain
Enteric group 137	BAA-68	ESBL
Escherichia coli	BAA-196	ESBL (TEM-10)
Escherichia coli	35218	used as a positive control for B-lactamase, contains TEM1
Enterobacter cloacae	BAA-1143	AmpC beta-lactamase
Staphylococcus aureus	VRS-1	Van^r, MRSA
Staphylococcus aureus	VRS-2	Van^r, MRSA
Staphylococcus aureus	NRS-1	ATCC700699
Staphylococcus aureus	NRS-6	$Van^{intermediate}$, Met^r
Staphylococcus aureus	NRS-8	$Van^{intermediate}$, Met^r
Staphylococcus aureus	NRS-14	$Van^{intermediate}$
Staphylococcus aureus	NRS-71	Epedemic MRSA, Sanger 252
Staphylococcus aureus	NRS-101	Met^r, biofilm producer, ATCC 35984
Staphylococcus aureus	NRS-123	CA-MRSA, USA400
Staphylococcus aureus	NRS-271	MRSA, $Linezolid^r$
Staphylococcus aureus	NRS-382	MRSA, multiple classes, USA100
Staphylococcus aureus	NRS-383	MRSA, multiple classes, USA200
Staphylococcus aureus	NRS-384	CA-MRSA, multiple, USA300
Staphylococcus aureus	NRS-385	MRSA, multiple classes, USA500

As can be seen, many of these strains have multiple drug resistance (MDR), expanded spectrum beta-lactamase (ESBL) resistance, vancomycin resistance (Van^r), or are methicillan resistant *Staphylococcus aureus* (MRSA). In addition, several are military isolates, known to be associated with epidemic outbreaks, or are known biofilm producers.

Once the collection was assembled, we had to determine which strains yield the most abundant biofilms, as we need as much biomass as possible in order to extract and analyze the biofilm

polysaccharide backbone. We have tested both static and dynamic biofilms as well as different types of media, additives, and pH. Below is just one representative experiment where static biofilms made in tryptic soy broth were compared. As can be seen, several strains generated significant biofilms. Surprisingly, some strains that are known biofilm producers did not produce biofilms under these experimental conditions (See #5, #11, and #13 below). However, under different conditions, they did produce copious biofilms.
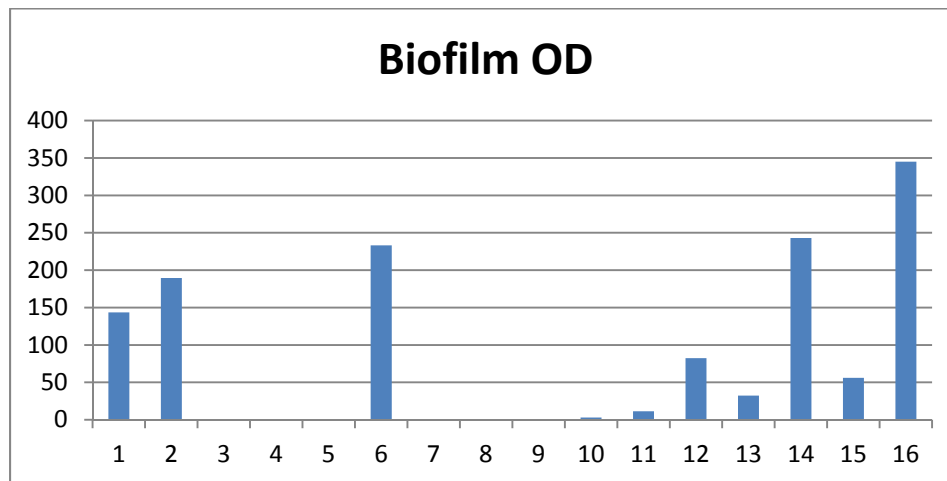
1	35218	*E. coli*; + control for beta-lactamases; contains Tem-1
2	BAA68	Enteric group 137; ESBL
3	BAA1143	*Enterobacter*; AmpC beta-lactamase
4	27853	*Pseudomonas*; reference strain
5	700888	*Pseudomonas*; known biofilm
6	700829	*Pseudomonas*; known biofilm
7	10145	*Pseudomonas*; type strain
8	51503	*Klebsiella*; MDR, ESBL (tem-10 and tem-12)
9	BAA2146	*Klebsiella*; NDM-1 positive
10	13883	*Klebsiella*; type strain
11	700831	*Klebsiella*; known biofilm
12	700603	*Klebsiella*; ESBL
13	19606	*Acinetobacter*; type strain, known biofilm
14	BAA1605	*Acinetobacter*; MDR, ESBL
15	17978	*Acinetobacter*; sequenced strain
16	BAA1878	*Acinetobacter*; sepsis, burn patient

We ended up selecting the top 2 biofilms producing strains for each organism for extraction. Next, we select a method for EPS extraction. The traditional method (going back to the 1950's) is based on phenol/chloroform and heat. However, more recent methods have showed advantages such as higher yields and a more homogeneous preparation. These include the method of Liu and Fang (2002) that employs NaOH extraction and the method of Oliveira,

Marques, and Azeredo (1999) that uses a 20% solution of trichloroacetic acid. In our hands, the NaOH extraction gave the highest yield of carbohydrate but the trichloroacetic acid method gave the least protein contamination. We chose the trichloroacetic acid method followed by neutralization with NaOH and then gel filtration for final purification. All biofilm polysaccharides were lyophilized for composition (Task 2) and linkage (Task 3) analysis to be accomplished during months 12-18. Polysaccharide yields were ~50 to 100 mg/L depending on strain.

KEY RESEARCH ACCOMPLISHMENTS:

- Determined that phage tailspike proteins most likely represent the identity of the depolymerase enzymes associated with anti-biofilm properties

- Successfully synthesized, expressed, and purified 6 putative depolymerase enzymes

- Demonstrated enzymatic activity of one of these enzymes

- Experimentally determined the optimal growth conditions for biofilm expression

- Experimentally determined the optimal method for extraction of biofilm EPS

REPORTABLE OUTCOMES:

Some aspects of our preliminary data and/or overall strategy have been presented at the following meetings/symposia during the past year:

1. 19th International Phage Biology Meeting. Olympia, WA. Poster. "Rapid Destruction of Biofilm Matrices by Bacteriophage-Encoded Enzymes" (August, 2011).

2. 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).

3. Georgia Institute of Technology, Atlanta, GA. Seminar speaker. "Bacteriophage Endolysins: Engineering A Targeted Antimicrobial Approach" (February, 2011).

4. PhageRAST (Rapid Annotation Using Subsystems Technology) Meeting. Tucson, AZ. Symposium speaker. "Bacteriophage Lytic Systems" (January, 2011).

CONCLUSION:

The bioinformatics section was the bottleneck of the entire project and it took much longer than expected. However, the project evolved along with the bioinformatics hurdles and we developed a two-pronged approach to "identifying" novel depolymerase enzymes based on traditional bioinformatics as well as exploring a class of bacteriophage proteins, known as tailspikes, that may hold the key to the identity of many elusive depolymerases. We had hoped to have ~20 candidate enzymes synthesized, cloned, expressed and purified by the end of the first year. In reality, we have 6 at this final stage, 4 more that are formed inclusion bodies when expressed (i.e. misfolded), and 5 more that are currently being synthesized and purified. While it would appear that we are behind our goals, for those proteins we have purified, we have already begun biochemical characterization and testing these enzymes for anti-biofilm properties, which was not envisioned until months 18-24. Indeed, one enzyme has already shown activity on a synthetic substrate as well as on a biofilm. Thus, we are ahead of schedule for those enzymes. In the end, this is an iterative process whereby enzymes are discovered, expressed, purified, and characterized. These processes don't happen simultaneously to all 20 candidate enzymes at the same time and at any given time, we will have different numbers of enzymes at different stages of this continuum. By the end of the second year, all enzymes will have been characterized and the top lead candidates will be advanced for further study in year 3 in accordance with the statement of work. Additionally, the parallel work on characterization of the biolfim EPS is proceeding exactly according to scheduled. Composition and linkage analysis will begin soon for this aspect of the project. In conclusion, we are more or less at a point where we hoped we would be at the end of the first year and we already have evidence that at least one of these enzymes displays true depolymerase activity.

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APPENDICES:

The following abstract was presented as a poster at the Nineteenth Evergreen International Phage Biology Meeting in August, 2011 in Olympia, Washington.

Rapid Destruction of Biofilm Matrices by Bacteriophage-Encoded Enzymes

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While bacterial host-evasion mechanisms, virulence factors, and antimicrobial resistance vary greatly between pathogens, one common trait shared by all is the ability to colonize as biofilms. Biofilms are formed when planktonic bacteria (i.e., free, individual cells) adsorb onto a surface (human tissue, mucous membrane, implanted medical device, etc.) and form multi-cellular colonies. Once the colonies become established, phenotypic changes cause them to secrete a polysaccharide matrix that serves as the backbone for the biofilm. 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. Significantly, the superstructure of the biofilm is known to protect internal bacteria from antimicrobials, antibodies, and circulating immune cells. Thus, methods that disrupt or dissolve the biofilm superstructure are sought. Toward this end, we are evaluating two approaches based on bacteriophage-encoded enzymes. The first approach uses phage peptidoglycan hydrolases, or endolysins. While these enzymes have been investigated as therapeutic agents against bacterial pathogens due to their ability to lyse the bacterial cell wall, they have not previously been evaluated against biofilms. Experiments reveal that planktonic group A streptococci (GAS) were susceptible to both penicillin (minimal inhibitory concentration (MIC) = 0.016 µg/ml) and PlyC, a streptococcal-specific endolysin (MIC = 0.02 µg/ml). However, static GAS biofilms were resistant to penicillin (minimal biofilm eradication concentration (MBEC) = 25 µg/ml) whereas treatment with PlyC (MBEC= 0.1 µg/ml) eliminated 99.9% of the biofilm biomass as supported by quantitative fluorescence staining. Furthermore, the action of PlyC against dynamic GAS biofilms was visualized in a flow cell system by time-lapse microscopy. In a second approach, we are identifying phage-encoded depolymerases, enzymes that can cleave the various glycosidic bonds in the biofilm backbone. Bioinformatic analysis suggests tailspikes and some domains on tail fibers possess the ability to cleave biofilm EPS. Unlike the endolysin approach, depolymerases are not expected to directly kill the bacteria. However, dissolution of the biofilm matrix may "re-sensitize" these organisms to antibiotics, antibodies, and innate immune defenses. Taken together, bacteriophage offer several different classes of enzymes that may possess anti-biofilm activity.

SUPPORTING DATA:

Figures and figure legends are contained within the body of the text above.