Duquesne University

Annual Progress Report: 2011 Formula Grant

Reporting Period

July 1, 2014 – December 31, 2014

Formula Grant Overview

Duquesne University received $107,464 in formula funds for the grant award period January 1, 2012 through December 31, 2014. Accomplishments for the reporting period are described below.

Research Project 1: Project Title and Purpose

A Biomaterial Approach to Inhibit Melanoma Growth and Metastasis in Mice – The purpose of this project is to test a novel strategy that aims to neutralize transforming growth factor-beta (TGFβ), a cytokine implicated in cancer immune escape and metastasis. Antibodies have been developed to inhibit TGFβ functions in cancer patients. It was thought that anti-TGFβ antibodies could be more effective when administered directly into tumors, but studies have shown that even locally injected antibodies do not accumulate in cancer lesions. We have designed and characterized a peptide-based injectable system that can circumvent this problem. In essence, we are proposing a novel way to use antibodies to inhibit dissemination of cancer cells by decreasing local TGFβ concentration.

Duration of Project

1/1/2012 – 12/31/2014

Project Overview

Carcinoma in mice and humans produce supra-physiological concentrations of TGFβ. In progressive cancers, elevated TGFβ enhances tumor cell invasiveness; attenuation of TGFβ signaling suppresses metastasis in mouse models of colon, skin and mammary cancers. Cancer progression driven by TGFβ is also attributed to the cytokine's ability to expand regulatory T (Treg) cells, a tumor-promoting lymphocyte, and its potent suppressive effects on cytotoxic T cells (CTLs), a type of tumor-killing lymphocytes. Thus, effective local neutralization of TGFβ should impede the growth and spread of cancer cells. The antibody displaying system is an injectable by which exceptionally high concentrations of anti-TGFβ antibodies can be retained in tumors.

Note: This project is funded, in part, under a grant with the Pennsylvania Department of Health. The Department specifically disclaims responsibility for any analyses, interpretations or conclusions.

The objective of the project is to demonstrate in vivo functions of a novel multivalent anti-TGFβ antibody assembly. Underpinning the research is the engineering of self-assembling peptides to form a gel-like substance to display IgG molecules in cluster. The amphiphilic peptide AEAEAKAKAEAEAKAK (single amino acid code; hereafter "EAK") self-assembles to form stable β-sheet fibrous networks in vitro and in vivo. We have previously reported a design in which the EAK network is modified to bind and orient multiple immunoglobulin-gamma (IgG) molecules through consecutive histidines (his-tag). Recent preliminary data show that IgG molecules form clusters in tumors for at least 24 hours, at which time free antibodies were no longer detectable. These data unequivocally demonstrate the ability of the system to enhance local concentration of antibodies in tumors. What is not known is the extent to which anti-TGFβ clusters can impede TGFβ functions in tumors.

The hypothesis is that clustering of anti-TGFβ IgG enhances the neutralization of the cytokine in vivo. The study entails injecting anti-TGFβ clusters into established syngeneic melanoma tumors in mice. We will investigate in tumors TGFβ concentration and signaling, frequency of Tregs recovered from tumors, and metastasis of cancer cells to draining lymph nodes. Changes in these outcomes will be measured with respect to dose and frequency of administration.

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Other Participating Researchers

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Expected Research Outcomes and Benefits

Tumor-derived TGFβ is a critical mediator in cancer growth and metastasis. Currently, no anti-TGFβ therapies have been approved in the U.S. This project seeks to advance a tool by which drug delivery barriers in solid tumors can be circumvented. Systemically injected antibodies do not accumulate in tumors to a great extent. Intratumoral injection can bypass the vascular barriers, but nonetheless drug molecules rapidly leave from the site of injection. We have devised a peptide-based scaffold on which IgG molecules can be concentrated and spatially oriented. Preliminary studies show that antibodies displayed by the system remain in living tumors much longer than those injected without the system. The research will generate data to determine the extent to which the displayed antibodies can neutralize TGFβ in the tumor microenvironment. We will identify the optimal dosing regimens and benchmark against conventional intravenous administration. The clinical significance of the research is that it can lead to new treatment modalities for suppressing dissemination of localized breast and skin

tumors. The injectable anti-TGFβ clusters can reduce the metastatic potential of cancer cells in patients awaiting surgery. It can be applied after tumor resection to generate a local antitumor immunity to decrease the likelihood of relapse. It may provide an option for patients with non-resectable tumors. The anti-TGFβ clusters can be used as an adjuvant for cancer vaccines by diminishing Treg cells and unleashing tumor-specific CTLs. The potential impact is high because the system can be adapted to antibodies already in clinical development.

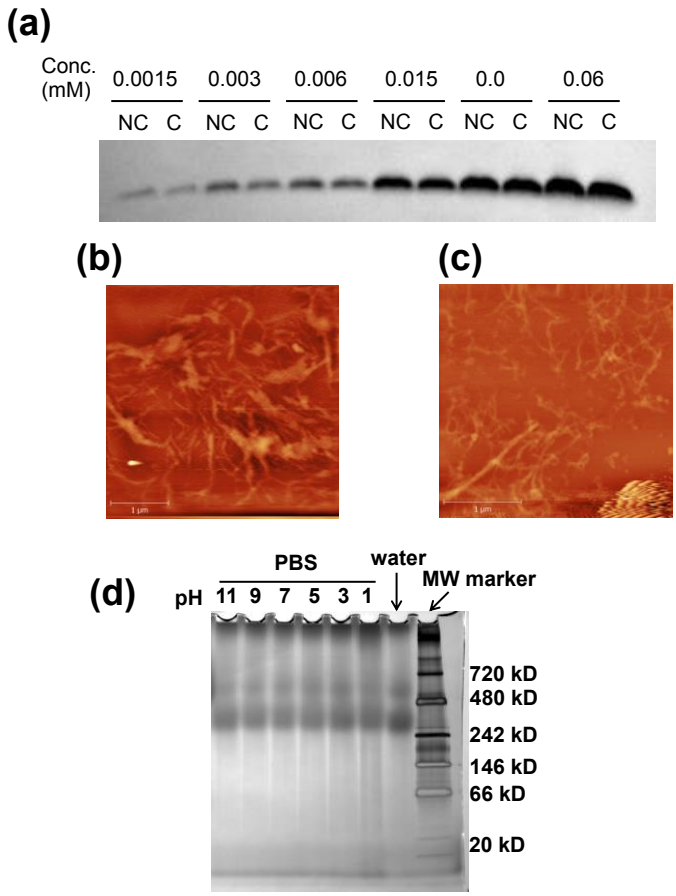
Summary of Research Completed

We conducted studies to understand the molecular mechanisms of the hydrogel assembly. The purpose is to optimize display of antibodies. EAK16-II (AEAEAKAKAEAEAKAK) is one of the first building blocks of environmentally responsive materials. This self-assembling peptide undergoes solution-to-gel transition when transferred from a low to high ionic strength environment. Previously we have demonstrated the histidinylated analogue EAKIIH6 (AEAEAKAKAEAEAKAKHHHHHH) coassembles with the parent peptide to render His-tags as a functionalization mechanism *in vitro* and *in vivo*. The present study aimed to understand the pathways by which the analogue coassembles with EAK16-II. The results presented herein suggested two competing but not mutually exclusive events in the coassembly. Atomic force microscopic and gel electrophoretic data showed that EAKIIH6 self-sorted to high molecular weight species without EAK16-II. Self-sorting of EAKIIH6 was inhibited by the parent peptide in a concentration dependent manner. Injecting mixtures containing EAKIIH6 subcutaneously rendered His-tags detectable in live mice for at least 312 hours, despite diluting the histidinylated analogue by 10 to 50 folds compared to a previous formulation. The study provided a formulation by which *in vivo* display of His-tags was attained without excess amphiphilic peptides. By increasing coassembling efficiency, the likelihood of generating immunogenic aggregates outside the main fibrils could be minimized. These findings provide insights for rational functionalization of *in situ* self-gelling materials.

Self-sorting of EAKIIH6

Understanding the competing events of coassembly and self-sorting is imperative for rational design of multi-component fibrillar materials. As expected, high-density materials were not found in EAKIIH6 below the critical assembling concentration (CAC) of EAK16-II; the peptide dissolved at 1.5-60 μM in PBS remained in supernatants after centrifugation (Fig. 1a). However, EAKIIH6 (30 μM) self-sorted into higher-ordered structures as evidenced in AFM. Similar to EAK16-II (Fig. 1b), EAKIIH6 deposited on mica exhibited fibrillar morphologies (Fig. 1c). Raising the concentration of EAKIIH6 to 0.3 mM resulted in formation of oligomers in acidic, neutral and alkaline buffers (Fig. 1d); multiple species weighing between 240 to 480 kDa and beyond 720 kDa were found in non-denaturing gels. These data revealed the intrinsic propensity of EAKIIH6 to self-sort into multimeric structures.

Figure 1



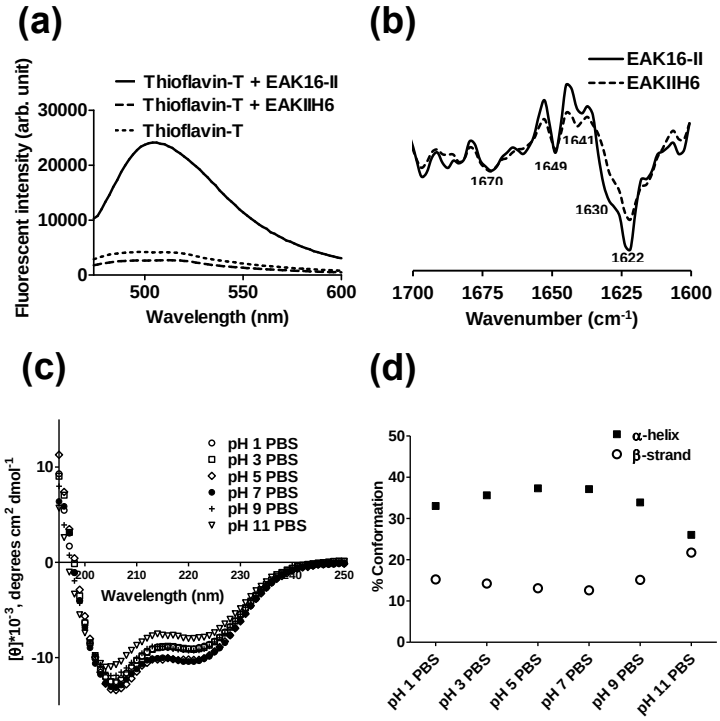
EAKIIH6 conformational ensemble

We next determined if the EAKIIH6 species revealed in AFM and native PAGE were β-fibrils. We have previously shown that the amyloid-specific chromogenic dye Congo red fails to bind EAKIIH6. Herein we used a fluorescent dye that also binds β-amyloids to probe the peptide samples. Addition of EAKIIH6 (0.3 mM) to solutions of thioflavin T (ThT) failed to elicit fluorescence enhancement typically observed with EAK16-II (Fig. 2a) and other β-fibrils. This suggested a lack of □-fibril-like structures in solutions of EAKIIH6. The mica surface could induce fibrillization of EAKIIH6, but that remains to be determined.

To probe the nature of these higher-ordered structures, ATR-FTIR was used to determine the peptide conformational ensemble in air-dried samples of EAKIIH6 dissolved in PBS. Second derivatives revealed the presence of α-helix structure as indicated by the peak at 1649 cm^{-1}. The peaks at 1630 cm^{-1} and 1622 cm^{-1} revealed the presence of β-strands and β-sheets (Fig. 2b). These spectral features resembled those found in the spectra of EAK16-II, but the peak at 1622 cm^{-1} was weaker in the EAKIIH6 spectrum. The more intense peaks at 1641 cm^{-1} and 1670 cm^{-1} in the EAKIIH6 spectrum suggested the presence of unordered structures and β-turns, respectively. The intensity ratio of EAKIIH6 to EAK16-II at 1622 cm^{-1} was determined to be 0.7, thus it was deduced that β-strand conformation was relatively less prominent in EAKIIH6. The dominance of α-helix in EAKIIH6 was supported by circular dichroism in which spectrum

of the peptide showed double minima at 208 and 222 nm, typical of α-helix conformation. Intensities of these transitions changed at varying pH (Fig. 2c). Deconvolution revealed dominant α-helix (~35%) between pH 1 to pH 7 (Fig. 2d). The pattern reversed when pH rose above 9, at which β-strand increased to 22%, apparently at the expense of α-helix (26%). The α-helix to β-sheet transition coincided with deionization of the histidines. Most of the imidazoles (assumed histidine $pK_a = 6.10$) would be unionized at pH 9. These data suggested a plasticity of EAKIIH6 conformational ensemble in which □-helix and β-strand coexisted. The findings supported a previous postulation that while EAKIIH6 generally adopts a □-helix conformation, it could be forced into β-strands.

Figure 2



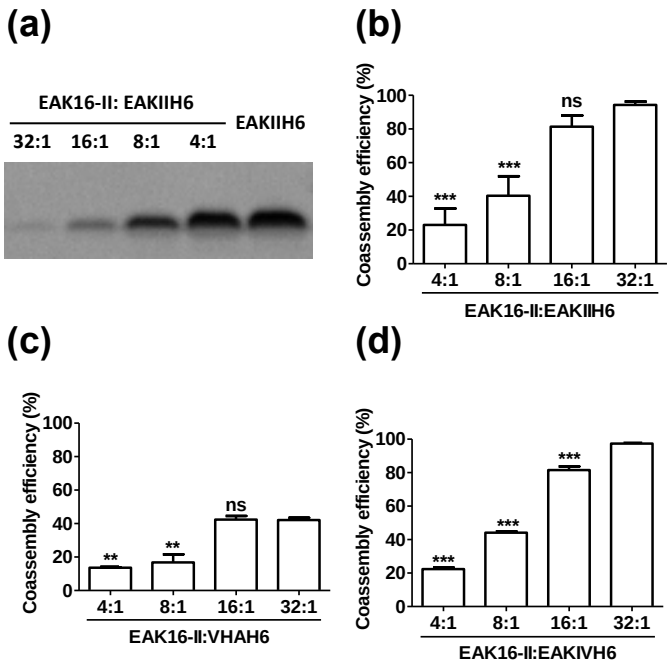
Dilution of EAKIIH6 in EAK16-II

The observation that EAKIIH6 self-sorted into higher-ordered species instructed how the peptides were formulated for *in vivo* applications. We postulated that at above a certain relative amount, EAK16-II inhibits self-sorting of EAKIIH6, providing a rationale for maximizing coassembly efficiency. To test this, the extent of coassembly was examined by mixing the two at different molar ratios. The fraction of EAKIIH6 was held constant while varying EAK16-II, with the latter added at 4, 8, 16 and 32-fold higher concentrations. Coassembled peptides would precipitate and un-incorporated peptides would remain in supernatant. Using SDS-PAGE it was found that at all ratios tested, no EAK16-II was detected in the supernatant and therefore precipitated completely (data not shown). Diluting EAKIIH6 with EAK16-II increased the efficiency of the coassembly (Fig. 3a). At relatively low excess of EAK16-II (4:1 and 8:1), less than 40% of EAKIIH6 co-precipitated into fibrils. Raising EAK16-II amount to 32:1 resulted in

close to 95% of EAKIIH6 precipitating (Fig. 3b). These data indicated that the presence of abundant EAK16-II inhibited self-sorting of EAKIIH6. Increasing the relative amount of EAK16-II increased the efficiency of coassembling. Taken together, excess EAKIIH6 in the low efficient mixtures (4:1 and 8:1) most likely diverted into aggregating byproducts outside the main fibrils generated from coassembly.

Two other His-tagged peptides were studied for comparison. When mixed with EAK16-II, VHAH6 co-precipitated poorly (Fig. 3c). VHAH6 was shown in circular dichroism adopting random coils in neutral phosphate buffers. Conversely, EAKIVH6 incorporated efficiently with EAK16-II (Fig. 3d). This peptide adopted a weak α-helix conformation (supplemental data: Fig. S2), although the parent EAK16-IV adopts a hairpin/β-turn configuration, owing to intra-peptide charge pairing. The offset in charge patterns in the amphiphilc segment between EAKII-16 (--++--++) and EAKIVH6 (----++++) did not preclude their co-precipitation (Fig. 3c). These data indicated that peptides containing amphiphilic domain in α-helical conformation could participate in fibrillization. Ionic pairing among β-sheets in the fibrils appeared flexible. EAKIVH6 could slide along the EAK16-II β-sheet horizontal plane to locate lateral hydrogen bonds while minimizing charge repulsion.

Figure 3

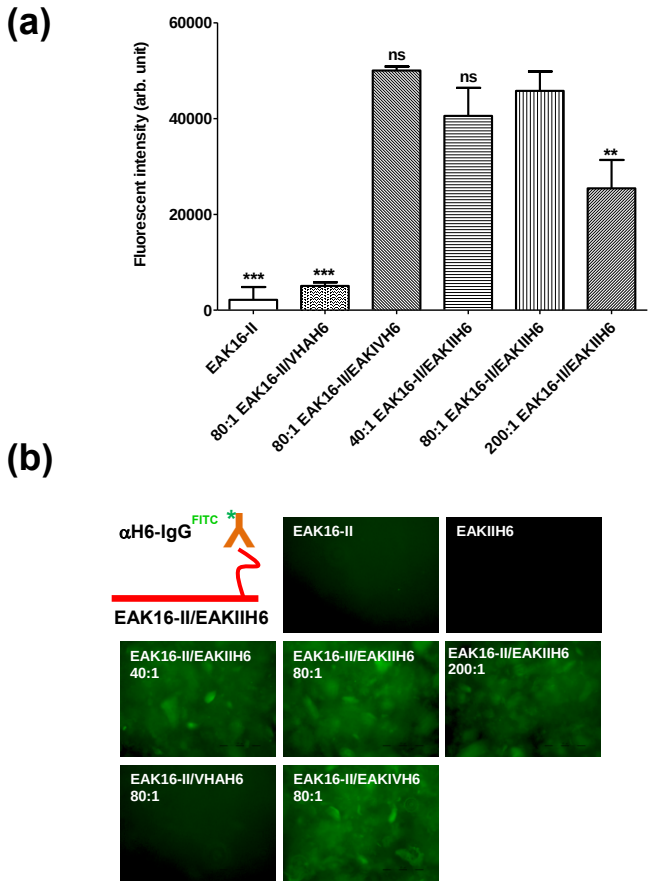


In vitro His-tag accessibility

Having established the threshold (32:1) beyond which coassembly occurred at above 95% efficiency, we next studied His-tag accessibility. Near complete incorporation could be assumed in these systems of EAK16-II-diluted EAKIIH6 (40:1, 80:1 and 200:1). Accessible His-tags were detected using a fluorescein-conjugated anti-His-tag antibody (αH6-IgG^{FITC}). In these and studies described hereafter, the amount of EAK16-II was held constant while varying EAKIIH6

in the mixtures. This was done to maintain non-specific binding relatively constant. All groups containing EAKII-16 and EAKIIH6 exhibited fluorescence. No significant difference was observed in co-precipitates prepared at ratios 40:1 and 80:1, but the fluorescence in 200:1 was reduced significantly (Fig. 4a). Direct imaging of the precipitates confirmed the differential binding of αH6-IgG^{FITC} (Figure 4b). His-tag detection was specific; only near background fluorescence was observed in fibrils formed with EAK16-II without EAKIIH6 (Fig. 4b: "EAK16-II"). Weak fluorescence was observed in precipitates formed by admixing EAK16-II with the non-complementary VHAH6. On the other hand, EAKIVH6 incorporated efficiently with EAK16-II, rendering strong fluorescence in the co-precipitates. These data recapitulated results of the coassembly studies (Fig. 3) and demonstrated that higher amounts of EAKIIH6 would not necessarily generate more accessible His-tags.

Figure 4



Research Project 2: Project Title and Purpose

Determination of the Role of pilin glycosylation in Pseudomonas aeruginosa Infections – The purpose of this project is to discover new knowledge concerning the distribution, among clinical isolates, of *Pseudomonas aeruginosa* strains producing glycosylated pili. This organism is a major cause of hospital-acquired pneumonia. We have found that *P. aeruginosa* strains producing glycosylated pili are associated with acute pneumonia. Respiratory tract damage caused by smoking includes a greatly increased susceptibility to pneumonia. The information gained from this project can be applied to the prevention or treatment of acute pneumonias caused by this organism. Prevention would be through vaccine design based on pilus structure. Treatment would be through the development of chemotherapeutic agents that interfere with pilin glycosylation.

Duration of Project

1/1/2012 – 12/31/2014

Project Overview

The broad objective of this research project is to establish the relationship between the group I pilin glycosylation state in *P. aeruginosa* and specific diseases caused by this organism. A specific aim of this project will be to do a comprehensive survey of the group I pilin glycosylation state of *P. aeruginosa* clinical isolates representing acute disease (such as pneumonias, urinary tract infections, burn infections, keratitis, ear infections, folliculitis and septicemias), as well as chronic infections such as those occurring in patients with cystic fibrosis or COPD (chronic pulmonary obstructive disease). This effort will first require the establishment of a comprehensive collection of clinical *P. aeruginosa* isolates with representative numbers of each of the diseases mentioned. These will be obtained from collaborators at local hospitals and institutes in Pittsburgh as well as elsewhere. The determination of group I pilin distribution will be accomplished by Western blot using a group I pilin-specific monoclonal antibody. A second aim is to do a complete survey of the O-serotype of all isolates collected. This will be done by serotyping and will allow the determination of O-antigen frequency among strains producing group I pilins. Finally, the laboratory results obtained will be subjected to a rigorous statistical analysis frequency of group I glycosylation and non-glycosylation will be compared between various infections sites. P values will obtained using 2x2 contingency tables and employing The Fisher's exact test. A two tailed P-value calculation will be used.

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Other Participating Researchers

Tara Kennedy, BS – employed by Duquesne University

Expected Research Outcomes and Benefits

My hypothesis is that the pilin glycan is important to the outcome of specific diseases caused by *P. aeruginosa*. Preliminary evidence suggests that this is the case with acute respiratory disease for which this organism is the etiological agent. Work presented will confirm or reject this hypothesis and will also examine other diseases caused by this organism to determine whether group I pilin glycosylation influences the distribution and frequency of this trait. This information could have practical value. Determination of a tropism caused by a particular pilin type and by an O-antigen serotype will allow for prophylaxis or chemotherapy.

Group I pili are very homogeneous in sequence and are extremely immunogenic. It has previously been shown that *P. aeruginosa* pili are protective against challenge from whole cells by a respiratory route. The lipopolysaccharide O-antigen of this organism has been shown to be an excellent vaccine target. While the O-antigen of *P. aeruginosa* is much more structurally variable than the pilin of this organism, three common serotypes predominate. In addition, we have shown that the glycan of group I pilin produces a protection which targets the O-antigen. A group I pilin vaccine preparation containing major O-antigen representatives would be expected to provide broad protection.

While active immunization of individuals at risk for these respiratory infections is an important goal, passive immunization of individuals in critical condition is also a consideration. Future work in this area would require human monoclonal antibodies directed against group I pilin or against the glycan. Antibodies directed against the pilin glycan would inhibit twitching motility, a trait required for virulence, and also stimulate opsonization.

Summary of Research Completed

<u>Influence of pilin glycan R-type on pilus functionality:</u>
Since the distribution of R-type among the group I pilin strains varies so dramatically as compared to the non-group I frequencies, I suggested experiments to see if this disparity was reflected in pilus functionality. I posited that group I pili are not found in strains producing R2 and R9 O-antigen because such strains are selected against due to their production less effective pili. Any difference in response could translate into a difference in resistant to respiratory-specific host defense mechanisms. These could include the response of alveolar macrophages or complement fixation in the respiratory environment.

I proposed to test this hypothesis during the last six months of support by expressing a functional cloned *pilA* gene or *pilAO* operon in *P. aeruginosa* strains producing the R2 or the R9 oligosaccharide (PAO1 and PA103, respectively). These experiments would require expression in mutants defective in their *pilA* gene. These experiments were not carried out because, on

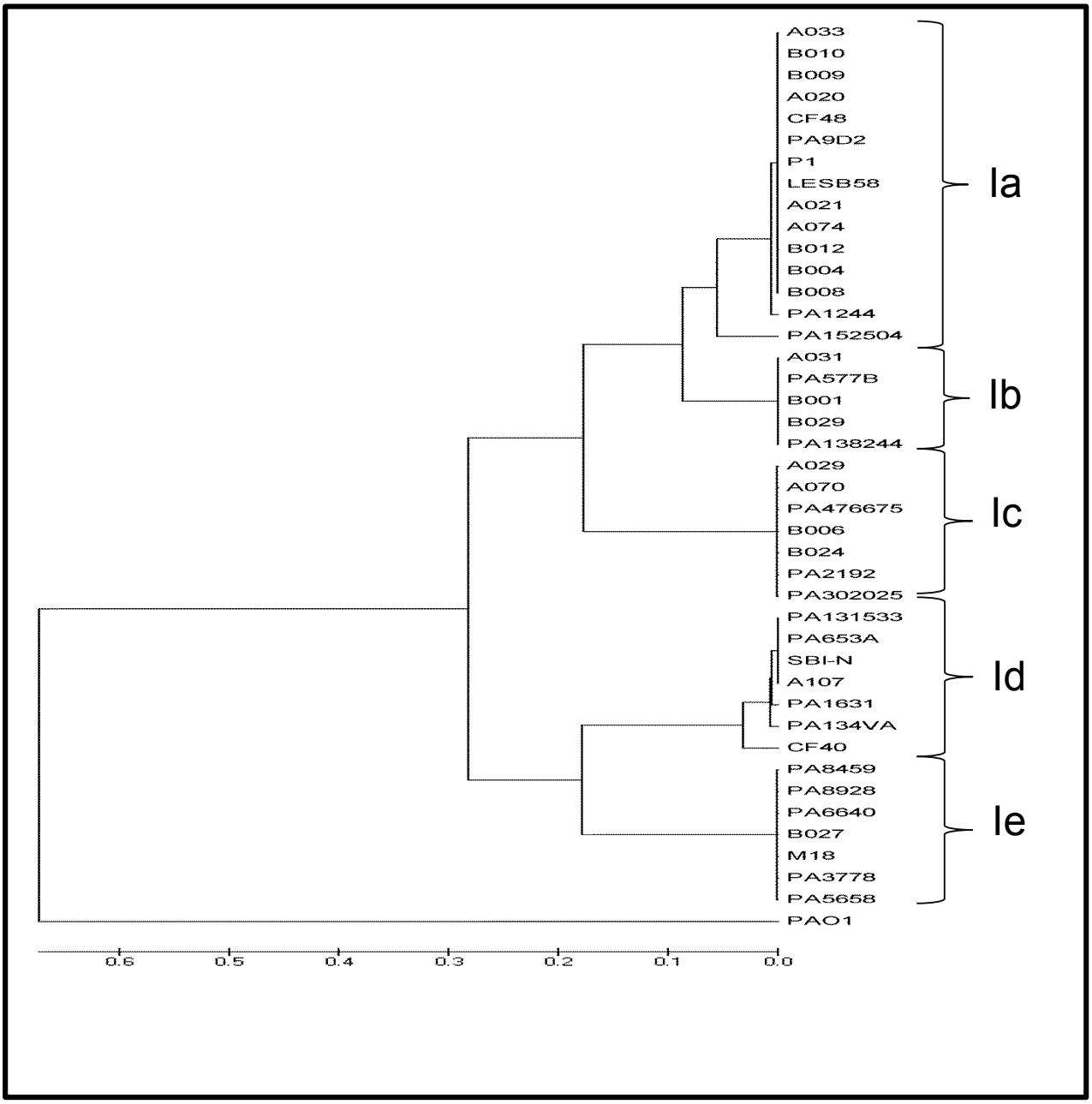
reconsideration, I felt that they would be difficult to interpret due to the potential effect of the mutation on other aspects of piliation. I still think that these experiments are important. However, future studies would benefit by taking an alternate approach. Here strains that were defective in O-antigen production, but produced well characterized and functional group I pili, would be employed. Cloned O-antigen biosynthetic gene clusters of different R-types would be expressed in these strains where features of pilus function could be measured. The influence of glycosylation using this system could be used to determine if there was a glycan-specific effect on host defense.

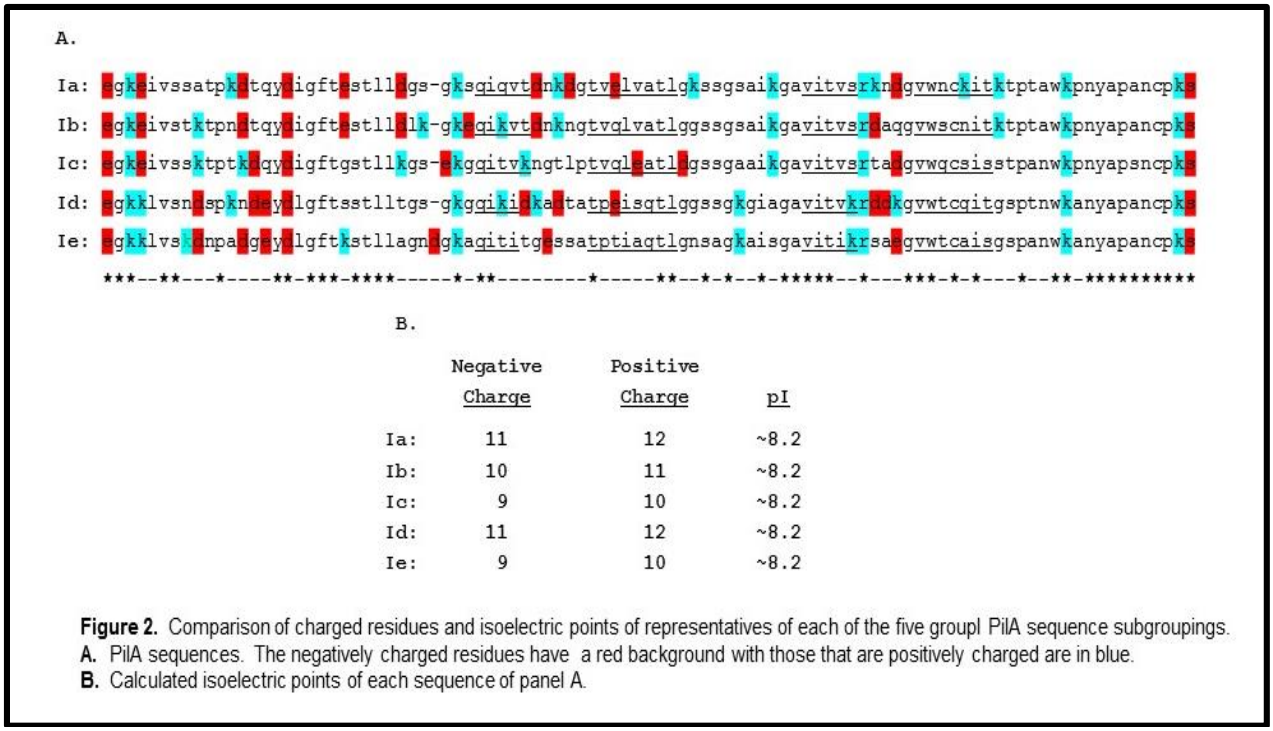
Influence of pilin glycan charge on pilin isoelectric point:

In the final months of this project we also further examined the influence of the negative glycan charge on the pilin subunit. Figure 1 shows the relationship of forty-one group I pilin sequences indicating that they occupy five homology groups. While there is very little variation within groups, there are clear differences among groups. Figure 2 compares the variable region of representatives of each of these groups emphasizing the ionizable residues. While there are conserved regions, much variation among charged amino acids is seen. Even with this variation, the predicted pI remains constant at approximately pH 8.2. This suggests that, in the absence of the glycan, the pilus has an overall net positive charge at neutral pH. This situation is unusual since most bacterial surface proteins display a net negative charge. It is also different from the group II and III pilins (which make up a majority of the remaining *P. aeruginosa* pilin types) the pI's of which average approximately 6.5 and 5.1, respectively. However, the presence of a negatively charged glycan reduces the actual measured group I pilin pI to approximately 6.5 (Figure 3). In this situation, the plus would display a net negative charge at pH 7.0. My overall interpretation of these results is that the pilin glycan functions as a protective molecule which prevents fouling of these pili in part through electrostatic repulsion due to the negative surface (See final grant report).

Publications:

A manuscript describing the R-typing of the clinical isolate library is currently being prepared for submission. The work (alluded to in the progress report of last summer) which concerns the role of glycosylation state on piliation has been completed and will be prepared for publication. CURE support will be acknowledged in all manuscript submitted.


A033
B010
B009
A020
CF48
PA9D2
P1
LESB58
A021
A074
B012
B004
B008
PA1244
PA152504
A031
PA577B
B001
B029
PA138244
A029
A070
PA476675
B006
B024
PA2192
PA302025
PA131533
PA653A
SBI-N
A107
PA1631
PA134VA
CF40
PA8459
PA8928
PA6640
B027
M18
PA3778
PA5658
PAO1
Ia
Ib
Ic
Id
Ie
0.6
0.5
0.4
0.3
0.2
0.1
0.0



A.

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Ia: egkeivssatpktqyligftestlldgs-gksgiqvtinkdgtvelvatlgkssgsaikgavitvsrkndgvwnckitktptawkpnyapancpks
Ib: egkeivstktpntqyligftestlllk-gkegqikvtinkngtvqlvatlggssgsaikgavitvsrdaqgvwscnitktptawkpnyapancpks
Ic: egkeivssktptkdqyligftgstlldgs-ekggitvkngtlptvqlgatldgssgaaikgavitvsrtadgvwqcsisstpanwkpnyapsncpks
Id: egkklvsnlspknneyllgftsstlltgs-gkggikidkadtatpeisqtlggssgkgiagavitvkrndkgvwtcqitgsptnwkanyapancpks
Ie: egkklvsidnpadgeyllgftkstllagndgkaqititgessatptiaqtlgnsagkaisgavitikrsadgvwtcaisgspanwkanyapancpks
    ***--**---*----**-***-****-----*-**--------*-----**--*-*--*-*****--*---***-*-*---*--**-**********
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B.

	Negative Charge	Positive Charge	pI
Ia:	11	12	~8.2
Ib:	10	11	~8.2
Ic:	9	10	~8.2
Id:	11	12	~8.2
Ie:	9	10	~8.2

Figure 2. Comparison of charged residues and isoelectric points of representatives of each of the five groupI PilA sequence subgroupings.
A. PilA sequences. The negatively charged residues have a red background with those that are positively charged are in blue.
B. Calculated isoelectric points of each sequence of panel A.

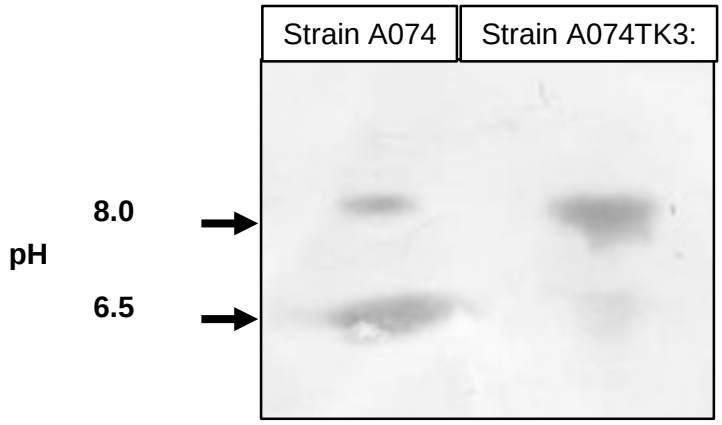

Figure 3. Isoelectric focusing blot of pilin showing the **i**nfluence of glycan charge on pilin electrophoretic mobility. This blot was probed with a monoclonal antibody (5.44) which is specific for group I pilin. Strain A074 belongs to pilin subgroup 1b and R-type 5 (which is predicted to provide a single negative charge). Strain A074TK3 is a mutant in which nearly all of the *pilO* gene has been deleted. This results in the inability to attach the O-antigen repeating unit to pilin. The band at pH 8.2 likely represents a minor fraction of non-glycosylated pilin.