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TITLE: A Factor H-Fc Fusion Therapy for Methicillin-Resistant Staphylococcus aureus Infection

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CONTRACTING ORGANIZATION: Planet Biotechnology Inc

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6. AUTHOR(S)
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13. SUPPLEMENTARY NOTES

14. ABSTRACT

The purpose of this project is to develop a new drug for methicillin-resistant *S. aureus* (MRSA), engineered from two human proteins, that will undermine the mechanism that *S. aureus* uses to evade detection and destruction by the human immune system. We have produced FH(18-20)/Fc, an engineered protein that combines the *S. aureus*-binding CCPs from human Factor H with immunoglobulin Fc, an antibody fragment that activates complement on surfaces to which it is bound.

In the first reporting period we completed our first aim, which was to use our plant expression system to produce FH(18-20)/Fc along with two control FH/Fc fusions: one with a mutation that eliminates Fc's ability to activate complement and one with modified Fcs designed to enhance complement-activating activity.

In the second reporting period we completed our second aim, showing that FH(18-20)/Fc blocks binding of human FH to *S. aureus*. We also measured how FH(18-20)/Fc bound to *S. aureus* affects complement activation. Finally, we measured the efficacy of FH/Fc fusions in boosting killing of MRSA by human polymorphonuclear cells.

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
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Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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Introduction

The purpose of this research is to develop a novel therapy for methicillin-resistant *Staphylococcus aureus* (MRSA) infections. *S. aureus* has developed immune-evasion strategies to promote survival within the host. One of the ways it does this is by recruiting to its surface the soluble human complement regulator Factor H (FH). Specifically, *S. aureus* binds to FH C-terminal domains 19-20 *via* the surface protein serine-aspartate repeat protein E (SdrE). In a preliminary experiment, we showed that a fusion of human FH domains 18-20 and the Fc of human IgG1, FH(18-20)/Fc, binds to the surface of *S. aureus*. We hypothesized that FH(18-20)/Fc would displace intact FH from the surface of *S. aureus*, increasing complement-mediated opsonization and targeting the bacterium for destruction by phagocytes. It is possible that FH/Fc could become a novel class of potent anti-infectives that can contribute to treating disease caused by MRSA. Using a plant expression system, we produced FH(18-20)/Fc along with three control FH/Fc fusions. We first tested the extent to which FH/Fc competes for binding to *S. aureus* in the presence of intact serum FH. Next, we measured the extent to which *S. aureus*-bound FH/Fc affects complement activation by examining its impact on factor-I mediated cleavage of C3b, the level of C3-fragment deposition, and its effect on C5a generation. Finally, we measured the efficacy of FH/Fc fusions in boosting opsonophagocytosis of MRSA by human polymorphonuclear cells.

Key words

Methicillin-resistant *Staphylococcus aureus* (MRSA)
Complement Factor H (FH)
Immunoglobulin
Fc fusion
Anti-infective
Opsonophagocytosis

ACCOMPLISHMENTS

Major goals of the project:

Goal (Major Task) 1: Produce FH/Fc transiently in *N. benthamiana*

We proposed producing three variants of FH(18-20)/Fc:

- Original: Human FH(18-20) fused to the N-terminus of human IgG1 Fc
- Variant 1: Human FH(18-20) fused to the N-terminus of human IgG1 Fc with two Fc mutations (D270A/K322A): lacks ability activate complement
- Variant 2: Human FH(18-20) fused to the N-terminus of human IgG3 Fc: enhanced ability to activate complement

Milestone: Purify at least 10 mg of each variant of FH(18-20)/Fc. Demonstrate purity of >95%. Quantify success of labeling.

In addition to the three proposed FH(18-20)/Fc variants, we expressed and purified two additional variants:

- Variant 2R: Human FH(18-20) fused to the C-terminus of human IgG1 Fc (reverse of Original)
- Variant 5: The same as Variant 2R with the addition of an EFT modification in the Fc region that has been shown to improve binding to C1q (the initiating protein of the classical pathway of complement).

We estimated the % purity of these five FH(18-20)/Fc Variants by densitometry using SDS-PAGE BioRad Criterion StainFree gels.

Purity is calculated as follows:
(sum of signal ≥ main band) / (total lane signal - blank lane signal)
Main band = ~50 kDa under reducing conditions and 90-105 kDa under non-reducing conditions (depending on construct)

Lane	Sample	Calc. Purity by StainFree
1	BioRad Protein Standard	
2	Original (reduced)	95.0%
3	Variant 1 (reduced)	90.7%
4	Variant 2 (reduced)	96.6%
5	Variant 2R (reduced)	99.8%
6	Variant 5 (reduced)	94.0%
7	Blank	
8	Original (non-reduced)	87.5%
9	Variant 1 (non-reduced)	88.4%
10	Variant 2 (non-reduced)	91.9%
11	Variant 2R (non-reduced)	79.4%
12	Variant 5 (non-reduced)	74.7%

Note: It appears that the 50 kDa band observed in the non-reduced samples is monomeric FH-Fc or Fc-FH. Because this 50 kDa band is still present even after diafiltration using a 50 kDa membrane, it is likely that it is part of a dimeric product that is not disulfide bonded (held together by non-covalent interactions). Since it is part of a homodimer of FH-Fc, it is most likely active and should be included in the purity calculation.

Labeling was performed using the Thermo Fisher SiteClick Antibody Azido Modification Kit and Biotium CF Dye BCN 405M. Click chemistry was used to specifically modify terminal galactose residues and label with a fluorescent dye. Targeting the CH2 region of the Fc portion of the fusion proteins with a low molecular weight fluorescent dye is not expected to interfere with either FH(18-20) or Fc interaction with SdrE or spA (protein A), respectively.

Due to the propensity of FH(18-20)-Fc to aggregate, the first modification attempt yielded no protein. We substituted spin filters with dialysis to circumvent this difficulty. Labeling of FH(18-20)-Fc was performed twice to yield enough labeled FH(18-20)-Fc for Aim 2.1 (competition assay detecting FH(18-20)-Fc bound to intact cells). For a negative control protein, DAF-Fc (decay accelerating factor-Fc) was chosen due to similar N-glycosylation sites to FH(18-20)-Fc. Another negative control Fc fusion, DPP4-Fc (dipeptidyl peptidase 4), has significantly more terminal galactose sites compared to FH(18-20)-Fc, making it an unsuitable protein for labeling.

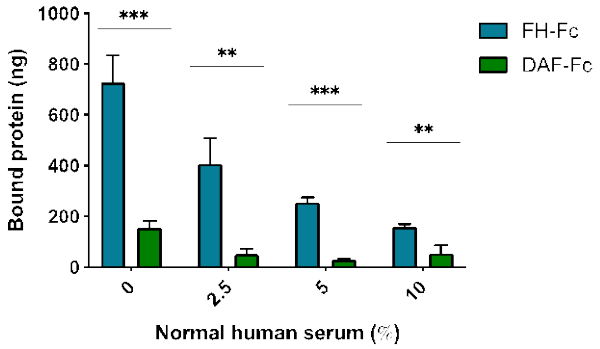
Target date for completion: End of month 4

Actual completion date: Mid-month 7

Goal (Major Task) 2: Examine potency of FH/Fc by measuring its effect on the binding of serum FH by *S. aureus*

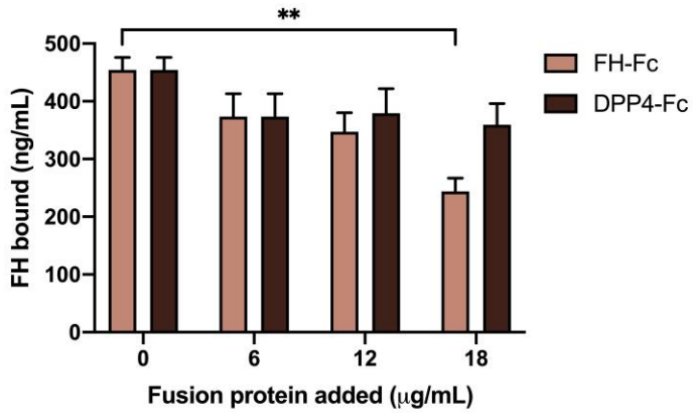
Subtasks 1 and 2: Completed the HRPO review/approval process and isolated serum from healthy volunteers for use in subsequent experiments involving complement.

Subtask 3: Assess the level of FH(18-20)-Fc binding to MRSA in the presence of serum FH (NHS, normal human serum). *S. aureus* strain R7 was incubated with 3 µg fusion protein with various amounts of NHS. After washing, bound fusion protein was measured using a fluorescent plate reader. A standard curve of purified labeled fusion protein was used for quantitation purposes. Binding of FH(18-20)-Fc was compared to DAF-Fc.

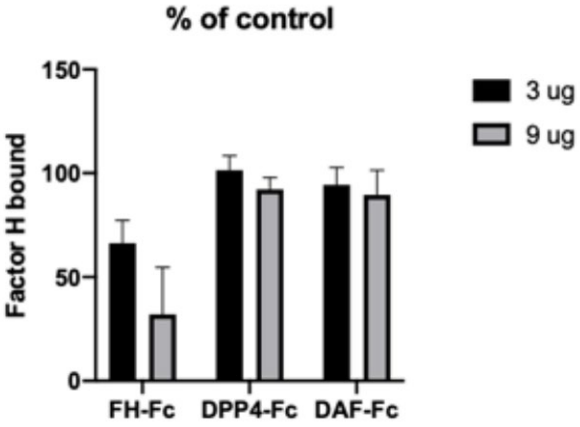


Using this data (Figure 1), we extrapolated that NHS inhibits the binding of 3 µg of FH(18-20)-Fc by 50% at a concentration of 3.269% serum.

Subtask 4: Evaluate recruitment of FH by *S. aureus* in the presence of FH/Fc variants using normal human serum. This was accomplished by a competition assay: assessing serum FH bound in the presence of increasing amounts of non-labeled fusion protein (2.5% Normal Human Serum). ELISA probing with anti-CCP1 mAb (Quidel); this mAb binds to an epitope found on serum FH but not on FH(18-20)-Fc.



The reduction of serum FH binding in the presence of 18 µg/mL FH(18-20)-Fc compared to control (no fusion protein) was significant, indicating that FH(18-20)-Fc competes with serum FH for SdrE binding under these conditions (Figure 2).



Extracts were also evaluated via anti-FH western blot (WB), with FH bands compared to % of control to determine effect. Due the semi-quantitative nature of a WB, these results are not as tight (Figure 3); however, they confirm the ability of FH(18-20)-Fc to compete with serum FH for *S. aureus* strain R7 recruitment in a specific manner (i.e.: DPP4-Fc and DAF-Fc did not compete).

Milestone Achieved: Optimize concentration of FH(18-20)-Fc that competitively reduces serum FH binding by MRSA. This concentration was 18 µg/ml.

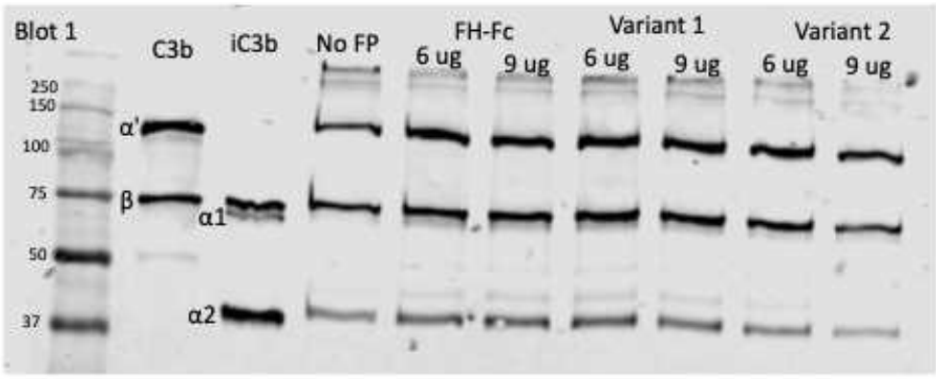
Target date for completion: End of month 8

Actual completion date: End of month 9

Goal (Major Task) 3: Examine potency of FH(18-20)-Fc by measuring its effect on complement-mediated opsonization of *S. aureus*

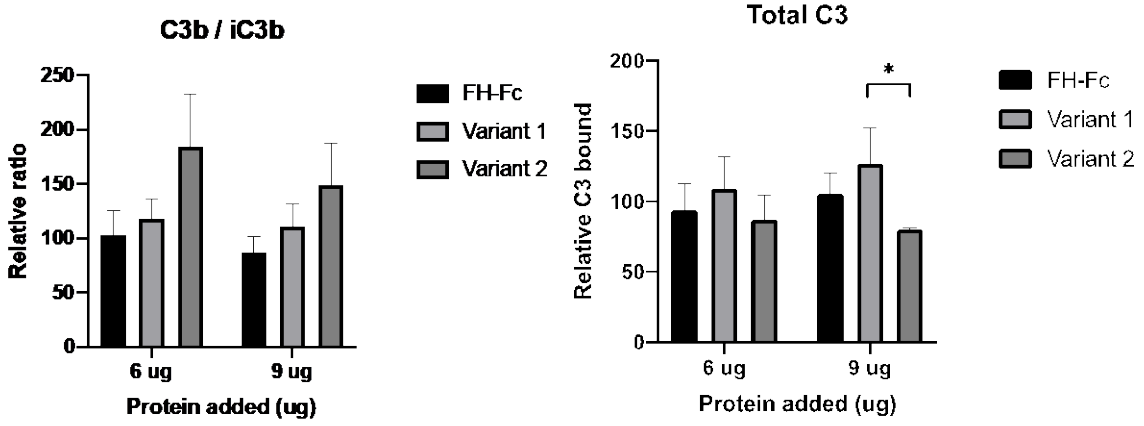
Subtask 1: Investigate effect of *S. aureus*-bound FH(18-20)-Fc on C3-fragment deposition

S. aureus strain R7 was incubated in 2.5% NHS and various amounts of fusion protein. To assess the level of C3-fragment deposition, bound C3 was stripped with 25 mM methylamine. We tested FH(18-20)-Fc as well as DAF-Fc; however, since DAF-Fc retains functionality, it was found to be an unsuitable control when measuring effect of FP on C3-fragment deposition (complement activation).



An anti-C3 WB verified C3-fragment presence and identity (iC3b (α1 + α2), C3b (α'), total C3 (β)). Figure 4 (left) is data from the WB using 6 µg vs 9 µg of FH(18-20)-Fc.

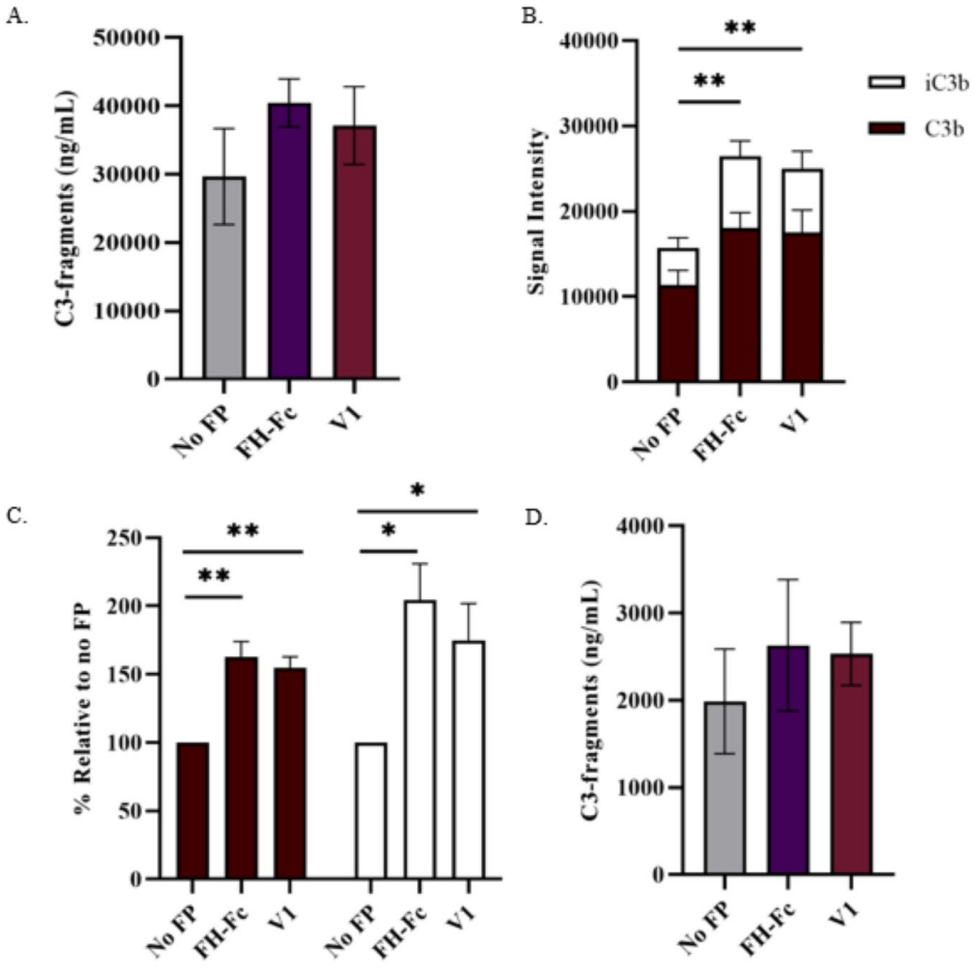
Optical densitometry of the WB resulted in Figure 5:



Unexpectedly, treatment with Variant 2 leads to less overall C3-fragment deposition, but with a higher proportion of C3b vs iC3b. In Variant 2, the CH2 and CH3 domains of IgG1 have been replaced with the corresponding domains of IgG3. IgG3 activates complement- and FcγR-mediated functions more effectively than other subclasses, so this was a surprising result.

Based on this result, we varied the method by doubling the reaction volume and using 30 µg of FH(18-20)-Fc in 1.25% NHS vs 45 µg of FH(18-20)-Fc and 2.5% NHS, keeping the amount of *S. aureus* strain R7 constant; however, these conditions resulted in a high degree of variability and therefore, were considered unsuitable. To minimize potential overcrowding of reaction components, we increased the volume to 1 mL and tested 1.25% and 2.5% NHS with 5×10^7 bacteria with and without fusion protein (FH(18-20)-Fc or Variant 1). Conditions permitting the alternative pathway (AP) only (using EGTA) were also evaluated.

Figure 6. FH-Fc and Variant 1 increase C3-fragment deposition of CA-MRSA R7. Bacteria (5×10^7 CFU) were incubated with or without 4.5µg fusion protein (FP) in 2.5% NHS for 30 minutes. Deposited C3-fragments were extracted and analyzed. A, C3-ELISA analysis of total C3-fragment deposition for all complement pathways. B, Western blot quantification of samples tested in (A) as measured by optical densitometry of C3b α' band and iC3b α1 band. C, Data from (B) presented as a percent relative to no-treatment control (no FP). D, C3-ELISA analysis of total C3-fragment deposition by the AP only. Data represent mean ± SEM of 4 independent experiments: * $p < 0.05$, ** $p \leq 0.004$.



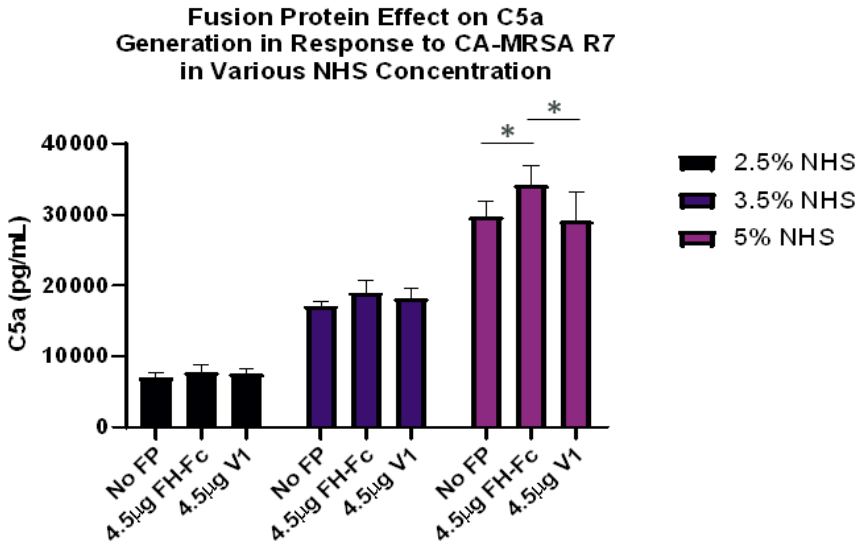
Total C3-fragment deposition for both treatment groups was increased compared to the no-treatment control, as analyzed via ELISA; however, these results were not statistically significant (Figure 6A). An examination of bands for C3b and iC3b via optical densitometry revealed a significant increase for C3b and iC3b for both FH(18-20)-Fc or Variant 1 treatment groups compared to no-treatment control (Figure 6B and C). In conditions that permitted activation of the alternative pathway only, total C3-fragment deposition was increased for both treatment groups, but this result was not significant. Western blot analysis of the fragments complementary to those analyzed in Fig 6D were too low to confidently examine by optical densitometry (data not shown).

Target date for completion of Subtask 1: End of month 11

Actual completion date: End of month 14

Subtask 2: Determine how FH/Fc variants affect the generation of the anaphylatoxin C5a

To investigate the extent to which FH-Fc affected the generation of the anaphylatoxin C5a, we varied the concentration of serum, incubation time, number of bacteria, and reaction volume.
Mid-log R7 (5×10^7 cfu) were incubated with 2.5%, 3.5%, or 5% normal human serum (NHS) ± 4.5 µg fusion protein in a total volume of 500µL for 15 minutes at 37C, after which EDTA was added to the reaction tube to stop complement. To determine the quantity/presence of C5a generated under these conditions, we analyzed the supernatant via ELISA (C5a DuoSet ELISA kit from R&D Systems). In 5% NHS, 4.5µg of FH-Fc resulted in significantly more C5a generation than the no fusion protein control or Variant 1 ($p < 0.03$, student's t-test).



Milestone Achieved: Optimize FH/Fc effect on complement-mediated opsonization of MRSA

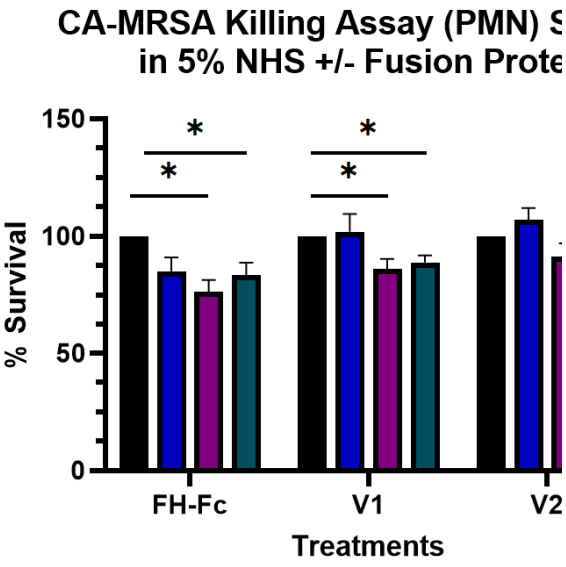
Target date for completion of Subtask 2: End of month 14

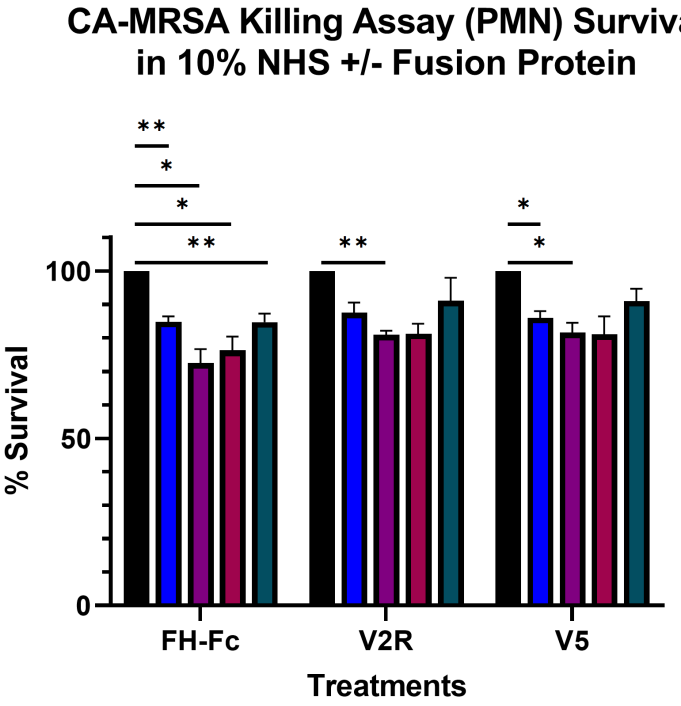
Actual completion date: End of month 18

Goal (Major Task) 4: Measure the potency of FH/Fc in boosting opsonophagocytosis of MRSA by human polymorphonuclear cells (PMNs)

Subtask 1: Test concentrations of normal human serum for optimal phagocytosis of *S. aureus*

To establish optimal conditions for complement-mediated opsonophagocytosis/killing of MRSA, we tested a range of NHS with a constant ratio of bacteria to PMN (10:1), as well as examined whether pre-incubation with NHS prior to PMN addition affected the outcome. It was determined that a pre-incubation of 15-minutes with bacteria and serum, prior to phagocyte (PMN) addition, produced a condition with the best reproducibility and was theoretically more physiological than a simultaneous addition of all reaction components (bacteria, serum, PMNs). Further, to generate optimal plating conditions and reliable bacterial counts, we established a dilution scheme using sterile water and sterile saline to protect the viability of bacteria during plating and produce a final colony count that was typically between 50 and 100 colonies. Optimal concentrations of bacteria and PMNs were determined to be 2×10^7 cfu *S. aureus* and 1×10^7 PMNs.





Subtask 2: Performing phagocytosis assay using various concentrations of FH/Fc with MRSA and normal human serum

To determine the effect of fusion proteins on the survival of R7 bacteria when challenged with PMNs, mid-log R7 (2×10^7 cfu) ± fusion protein were incubated with serum in 500μL total volume at 37C. After 15 minutes, to allow for complement mediated opsonization of bacteria, PMNs were added at a ratio of 1:10 (PMN to bacteria). Samples were then rotated for 75 minutes at 37C to permit phagocytosis. Mid-log R7 challenged with serum and PMNs (without fusion protein) were used as controls and represented 100% survival of bacteria for all assays.

Following the incubation with PMNs, samples were serially diluted then plated onto Columbia 2% NaCl plates (at least two plates per sample) and incubated overnight. The following day, colonies were counted as a measure of bacterial survival. Percent survival was calculated by comparing colony counts from fusion-protein treated samples to control plates.

For 2.5% NHS, FH-Fc showed the greatest reduction in survival (17.5% reduction), however, this result was not significant (data not shown).

In 5% NHS, both FH-Fc and Variant 1 reduced survival of R7 compared to control. Although the reduction was slight (14-21% reduction in survival), these results were statistically significant ($p \leq 0.01$).

As FH-Fc demonstrated the greatest reduction in *S. aureus* survival using 5% NHS, we next tested the effect of a range of FH-Fc on *S. aureus* survival using 10% NHS and introduced two new variants, Variant 2R and Variant 5.

Again, FH-Fc showed the greatest reduction in survival (6μg FH-Fc, 27.4% reduction in survival) compared to control (one-way ANOVA, mixed-effects analysis, *, $p < 0.03$,**, $p < 0.01$).

Taken together, FH-Fc treatment resulted in reduced survival of CA-MRSA R7, a clone of USA300 which is the predominant CA-MRSA type in the US. FH-Fc competitively binds to the *S. aureus* surface protein SdrE thus blocking serum FH binding and augmenting complement activation and Fc-receptor mediated phagocytosis via the exposed Fc region of FH-Fc.

As indicated by a 27% reduction in survival, FH-Fc treatment was successful in increasing phagocytosis of *S. aureus*; however, a large proportion of *S. aureus* were able to persist. In addition to panton-valentine leukocidin, a toxin expressed by USA300 strains that kills phagocytes, *S. aureus* has other mechanisms by which it can survive phagocytosis. While FH-Fc treatment negatively affects *S. aureus* survival, which is encouraging, it does not address these additional survival mechanisms. Future studies will focus on developing a supplemental or modified component to target *S. aureus*' ability to withstand the caustic environment within the phagocyte.

Milestone Achieved: Identify FH/Fc variant and dosage that results in optimal opsonophagocytosis.

Target date for completion of Subtask 3: End of month 18

Actual completion date: End of month 24

- **What opportunities for training and professional development has the project provided?**
  - *This project has provided an opportunity for the training of a master's-level graduate student with respect to experimental design, technical competence, and data analysis.*
- **How were the results disseminated to communities of interest?**
  - *Some of this data was presented at the American Association of Immunologists' (AAI) Virtual Immunology Symposium in May 2021 as a poster presentation.*
- **What do you plan to do during the next reporting period to accomplish the goals?**
  - *Nothing to Report (this is the final report)*

IMPACT:

- **What was the impact on the development of the principal discipline(s) of the project?**
  - *Nothing to Report*
- **What was the impact on other disciplines?**
  - *Nothing to Report*
- **What was the impact on technology transfer?**
  - *Nothing to Report*
- **What was the impact on society beyond science and technology?**
  - *Nothing to Report*

CHANGES/PROBLEMS:

- *Nothing to Report*

PRODUCTS:

International Application No. PCT/US21/50533 entitled **"**FACTOR H FRAGMENT Fc FUSIONS WITH IMPROVED POTENCY AND MANUFACTURABILITY" was filed 15 September 2021**.** Inventors: Keith Lynn WYCOFF and Y TRAN. Applicant: Planet Biotechnology, Inc.

This application claims priority from US provisional patent application 63/204,194, filed September 16, 2020, US provisional patent application 63/258,022 filed April 5, 2021, and US

provisional patent application 63/259,003 filed June 11, 2021. All three provisionals have the title **"**FACTOR H FRAGMENT Fc FUSIONS WITH IMPROVED POTENCY AND MANUFACTURABILITY"**,** nventors: Keith Lynn WYCOFF and Y TRAN and applicant: Planet Biotechnology, Inc.

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Name:	*Keith Wycoff*
Project Role:	*Principal Investigator*
Researcher Identifier (e.g. ORCID ID):	0000-0002-5343-5329
Nearest person month worked:	1
Contribution to Project:	*Dr Wycoff was the Principal Investigator of this project.*
Funding Support:	

Name:	*Julia A. Sharp, Ph.D.*
Project Role:	*Co-PI (PI at EVMS)*
Researcher Identifier (e.g. ORCID ID):	*0000-0001-7473-1058*
Nearest person month worked:	*4*
Contribution to Project:	*Dr. Sharp oversees the grant at EVMS including experimental design and performance, data analysis, reporting, and training staff.*
Funding Support:	

Name:	*James Maclean*
Project Role:	*Molecular biology*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	0.6
Contribution to Project:	*Dr Maclean*
Funding Support:	

Name:	*Y Tran*
Project Role:	*Protein purification*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	3
Contribution to Project:	*Mr Tran expressed, purified and performed biophysical characterization of protiens*
Funding Support:	

Name:	*Megan Golliher*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*3*
Contribution to Project:	*Ms. Golliher performed experiments associated with Aim 2 of the grant, specifically factor H competition assays and data analysis. She also fluorescently labeled fusion proteins (Aim 1) for use in Aim 2.*
Funding Support:	*Effort not monetarily compensated*

Name:	*Michele Semeraro, M.S.*
Project Role:	*Research Assistant*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*5*
Contribution to Project:	*Ms. Semeraro performed experiments associated with Aim 2 of the grant, specifically fusion protein binding comparisons (including optimization). She also contributed to data analysis, protocol troubleshooting, and assisted with technical issues.*
Funding Support:	

Name:	*Katelyn Cranmer, M.S.*
Project Role:	*Research Assistant*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*5*
Contribution to Project:	*Ms. Cranmer performed experiments associated with Aim 2 and 3 of the grant, specifically C3-fragment deposition assays, C5a assays, and opsonophagocytosis/killing assays. She also contributed to data analysis, protocol troubleshooting, and assisted with technical issues.*
Funding Support:	

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

- **Changes to active other support since the grant started is provided below**

Updated Previous/Current/Pending Support (PCPS)

Nothing to report

What other organizations were involved as partners?

- **Organization Name: Eastern Virginia Medical School (EVMS)**
- **Location of Organization:** Norfolk, VA
- **Partner's contribution to the project**
  - **Collaboration**