AWARD NUMBER: W81XWH-18-1-0724

TITLE: Bacteriophage Therapy Against Multidrug-Resistant Bacteria Causing Serious Wound Infections

PRINCIPAL INVESTIGATOR: Thomas J. Walsh, MD, PhD (Hon), FIDSA, FAAM

CONTRACTING ORGANIZATION: Weill Cornell Medicine, New York, NY

REPORT DATE: JULY 2020

TYPE OF REPORT: Final Report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
JULY 2020	FINAL	**15SEP2018 - 14MAR2020**

4. TITLE AND SUBTITLE

Bacteriophage Therapy Against Multidrug-Resistant Bacteria Causing Serious Wound Infections

5a. CONTRACT NUMBER
W81XWH-18-1-0724

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Thomas Walsh

E-Mail:

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Joan & Sanford I.Weill Medical College of Cornell University
NEW YORK, NY

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT: This project aligns directly with the current FY17 PRMRP topic area antimicrobial resistance. The research focuses on development and preclinical investigation of bacteriophages as novel antimicrobial agents for treatment and prevention of infections caused by multidrug resistant bacteria (MDRB). MDRB cause life-threating infections involving wounds, penetrating abdominal injuries, hospital acquired pneumonia, and sepsis. These infections are important global public health diseases that impact severely on our wounded war-fighters, veterans, and their hospitalized family members. The resulting shortage of safe and effective antibiotics poses a great challenge to the care of wounded military personnel, as well as civilians. Our objective in this study will be to develop a transformational strategy beyond the next logical steps of using bacteriophages for treatment and prevention of MDRB infections. Bacteriophages are emerging experimentally and clinically as important antimicrobial agents in treatment of refractory and potentially lethal MDRB infections Recent developments in the field have suggested that in addition to being potent antibacterial agents, additional efficacy can be obtained by utilization of phages that are able to modulate the severity of bacterial infections by selecting for bacterial mutants that are reduced in virulence or that have increased susceptibility to antibiotics. Among the most prevalent and lethal MDRB are Staphylococcus aureus (MRSA), carbapenemase producing Klebsiella pneumoniae (KPC), Acinetobacter baumannii, and Pseudomonas aeruginosa. We will study KPC as a model MDRB.

15. SUBJECT TERMS

N N LI D

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified		19b. TELEPHONE NUMBER *(include area code)*

TABLE OF CONTENTS

1. **INTRODUCTION:** *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

The research focuses on development and preclinical investigation of bacteriophages as novel antimicrobial agents for treatment and prevention of infections caused by multidrug resistant bacteria (MDRB). Recent developments suggest that in addition to being potent antibacterial agents, additional efficacy can be obtained from bacteriophages by utilization of phages to modulate the severity of bacterial infections by selecting for mutants that are reduced in virulence or that have increased susceptibility to antibiotics.

2. **KEYWORDS:** *Provide a brief list of keywords (limit to 20 words).*

Bacteriophages; multidrug resistant bacteria (MDRB); wound infections; public health; sepsis; reduced susceptibility; reduced virulence

3. **ACCOMPLISHMENTS:** *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

What were the major goals of the project?
List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

1. To study KPC as a model MDRB for the property of bacteriophage-treated KPC altered susceptibility and virulence
2. To conduct *in vitro* susceptibility studies of antibacterial antibiotics on strains of our phage-resistant bacteria library
3. To perform whole genome sequencing (WGS) of these bacteriophage resistant KPC isolates to determine mechanisms of increased susceptibility and lowered virulence.
4. To conduct the critical in vivo studies that will enable us to understand the potential clinical impact of altered susceptibility and virulence by bacteriophages on MDRB infections.

What was accomplished under these goals?
For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

We studied KPC for the property of bacteriophage-treated MDRB altered susceptibility and virulence, allowing access of antimicrobial agents that resulted in lowered minimum inhibitory concentrations (MICs) to antimicrobial agents. We conducted *in vitro* susceptibility studies of antibacterial antibiotics on 12 strains of our phage-resistant bacteria library. When compared to phage-susceptible KPC controls, there were changes of both increased and decreased antimicrobial susceptibility in the bacteriophage-resistant mutants.

Moreover, whenever, a β-lactam antibiotic was paired with one of the β-lactam inhibitors, avibactam or relebactam, no growth was observed at the tested concentrations, consistent with significantly increased susceptibility of the bacteriophage KPC mutants to antimicrobial agents.

WGS studies demonstrated that phage-dependent mutations in bacteriophage-resistant mutant KPC demonstrated significant genomic mutational events in receptor proteins that correlated with significant alterations in the modeled capsular PNAG structure of KPC.

These findings suggest that the altered capsular structure likely permits increased penetration of antimicrobial agents, including the β-lactam inhibitors, avibactam or relebactam, to attack the bacterial cell wall.

We are now poised to conduct the critical in vivo studies that will enable us to understand the clinical potential of these properties of bacteriophages on MDRB infections of our wounded war-fighters.

What opportunities for training and professional development have the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

This study provides training and professional development opportunities for doctoral fellows in key methods, analysis, and data interpretation of MDRB *in vitro* antimicrobial susceptibility methods, whole genome sequencing, molecular modeling of capsular polysaccharide structure, and *in vivo* studies of antimicrobial therapy and virulence.

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Nothing to Report

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

Presentation of data at a major national or international meeting of microbiologists and/or infectious disease experts; i.e., ASM and IDSA meetings

4. **IMPACT:** D*escribe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

The *in vitro* results have important implications for the treatment of MDRB by bacteriophages for reduction of organism burden, increased susceptibility to antibacterial agents, diminished capsular polysaccharide poly-β-(1-alpha 6)-N-acetyl glucosamine (PNAG) production, altered PNAG structure, and increased penetration of antibiotics to the cell wall.
The in vivo studies, which need to be performed, are critical for being able to demonstrate the potential clinical impact of increased susceptibility to antibacterial agents. Should these findings prove to be correlated *in vivo*, there will be added compelling rationale for field use bacteriophage to treat MDRB wound infections and sepsis.

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.
Nothing to Report

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to Report

What was the impact on society beyond science and technology?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Successful treatment of MDRB wound infections and sepsis in injured war fighters carries propound societal and economic implications. By returning war-fighters with out loss life or limb to their families and communities strengthens personal, psychological and emotional bonds that have far reaching impact.

5. CHANGES/PROBLEMS: *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

Changes in approach and reasons for change

Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

Nothing to report

Actual or anticipated problems or delays and actions or plans to resolve them

Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

We suffered the loss of a key post-doctoral fellow, who was to conduct the in vivo studies. This led to a delay in the *in vivo* studies until we recruited another qualified member of the laboratory. Upon recruitment of a doctoral student, who would conduct these *in vivo* studies, the events of COVID-19 further impacted upon our ability to conduct the *in vivo* experiments: [1] all laboratories within our institution were closed for nearly six months and are now only re-opening and [2] the doctoral student was restricted in his return to the US from Greece and only now has received J-1 waiver to return to the US following a 14-day quarantine. Upon his return and with the re-opening of the Laboratory, we will be able to conduct the critical in vivo studies if given a no-cost extension.

Changes that had a significant impact on expenditures

Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

In sustaining the loss of a key post-doctoral fellow, who was to conduct the in vivo studies, this led to a delay in the critical laboratory animal studies until we recruited another qualified member of the laboratory (Panagiotis Zagliotis) as a doctoral student.
Following his recruitment, the events of COVID-19 further impacted upon our ability to conduct the *in vivo* experiments: [1] all laboratories within our institution were closed for nearly six months and are now only re-opening and [2] our doctoral student was restricted in his return to the US from Greece and only now has received J-1 waiver to return to the US following a 14-day quarantine.
Upon his return and with the re-opening of the Laboratory, we will be able to conduct the critical *in vivo* studies if given a no-cost extension, which is <u>essential</u> to translate these in vitro findings into potential clinical therapeutics.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects
Nothing to report

Significant changes in use or care of vertebrate animals
Nothing to report

Significant changes in use of biohazards and/or select agents
Nothing to report

6. PRODUCTS: *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

- **Publications, conference papers, and presentations**
  Nothing to report

  Journal publications.
  Nothing to report

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*
Nothing to report

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

We currently have a draft manuscript of the current data. Upon completion of the in vivo studies, we will then incorporate the data and submit to one of several ASM (AAC or I&I) or IDSA (JID) journals.

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.
  Nothing to report

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

  The integration of our sequential technologies of *in vitro* antimicrobial susceptibility, WGS, capsular polysaccharide modeling, and *in vivo* efficacy and virulence studies will provide a pathway for other laboratories to follow in understanding the effect of anti-MDR bacteriophages in modulating reduced virulence and increased antimicrobial susceptibility.

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.
  Nothing to report

- **Other Products**

  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

  - *data or databases;*
  - *physical collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
  - *clinical interventions;*
  - *new business creation; and*
  - *other.*

  This study has important implications for further support of bacteriophages as anti-MDRB therapeutics in the management of serious wound infections and sepsis. Further adding to the rationale that anti-MDRB bacteriophages can reduce bacterial burden in tissues is the rationale of treatment with this novel modality is those bacteriophage-resistant MDRB may have decreased virulence and increased antimicrobial susceptibility, especially to β-lactamase/β-lactam antimicrobial combinations.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

Name: Thomas J. Walsh, MD
Project Role: Principal Investigator
Nearest person month worked: 6
Contribution to Project: organization, data analysis, experimental design
Funding Support: multiple sources

Name: Jason Gill, PhD
Project Role: Co-Principal Investigator
Nearest person month worked: 6

Contribution to Project: organization, data analysis, experimental design
Funding Support: multiple sources

Name: Jordyn Michalik, PhD candidate
Project Role: Graduate Research Assistant
Nearest person month worked: 6
Contribution to Project: *in vitro* activity, resistance profiles, WGS
Funding Support: multiple sources

Name: Panagiotis Zagliotis, PhD candidate
Project Role: Graduate Research Assistant
Nearest person month worked: 3
Contribution to Project: *in vitro* activity, resistance profiles, *in vivo* studies
Funding Support: multiple sources

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing to Report

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*

- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to* ***https://ers.amedd.army.mil*** *for each unique award.*

QUAD CHARTS: *If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.*

9. APPENDICES: *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.*

Abstract

The research focuses on development and preclinical investigation of bacteriophages as novel antimicrobial agents for treatment and prevention of infections caused by multidrug resistant bacteria (MDRB), which cause life-threating infections involving wounds, penetrating abdominal injuries, hospital acquired pneumonia, and sepsis. MDRB infections are important global public health diseases that impact severely on our wounded war-fighters, veterans, and their hospitalized family members. Bacteriophages are emerging experimentally and clinically as important antimicrobial agents in treatment of refractory and potentially lethal MDRB infections. Recent developments in the field suggest that in addition to being potent antibacterial agents, additional efficacy can be obtained by utilization of phages that are able to modulate the severity of bacterial infections by selecting for bacterial mutants that are reduced in virulence or that have increased susceptibility to antibiotics. We studied KPC as a model MDRB for the property of bacteriophage-treated KPC altered susceptibility and virulence, wherein bacteriophage-

treated KPC may have diminished capsular polysaccharide poly-β-(1-alpha 6)-N-acetyl glucosamine (PNAG) production allowing access of antimicrobial agents that resulted in lowered minimum inhibitory concentrations (MICs) to antimicrobial agents, such as the polymyxins. We hypothesized that mutations in the capsule of *K. pneumoniae* are linked with phage resistance and increased susceptibility to antibiotics. We then performed whole genome sequencing (WGS) of these bacteriophage resistant KPC isolates to determine mechanisms of increased susceptibility and lowered virulence. To address this aim, we conducted *in vitro* susceptibility studies of antibacterial antibiotics on 12 strains of our phage-resistant bacteria library. When compared to phage-susceptible KPC controls, there were changes of both increased and decreased antimicrobial susceptibility in the bacteriophage-resistant mutants. These changes were strain-dependent and antibiotic dependent. Moreover, whenever, a β-lactam antibiotic was paired with one of the β-lactam inhibitors, avibactam or relebactam, no growth was observed at the tested concentrations, consistent with significantly increased susceptibility of the bacteriophage KPC mutants to antimicrobial agents. WGS studies demonstrate that phage-dependent mutations in bacteriophage-resistant mutant KPC demonstrated significant genomic mutational events in receptor proteins that correlated with significant alterations in the modeled capsular structure of KPC. These findings suggest that the altered capsular structure likely permits increased penetration of antimicrobial agents, including the β-lactam inhibitors, avibactam or relebactam, to attack the bacterial cell wall. We are now poised to conduct the critical in vivo studies that will enable us to understand the clinical potential of these properties of bacteriophages on MDRB infections of our wounded war-fighters.

MIC Data

To assess possible phenotypes representing changes in antimicrobial susceptibility among the bacterial strains collected in vivo from the mouse model or generated in vitro by serial bacteriophage passaging we conducted several sets of minimum inhibitory concentration (MIC) assays. The first set of assays was conducted with 10 strains and represents one replicate. Commercial MIC plates from Sensititre were used, and the plate layout as given by Thermo-Fisher can be found here. http://www.trekds.com/products/sensititre/files/ESBL.pdf The MIC plates used for this experiment were designed specifically for diagnostic use on extended beta-lactamase producing bacterial strains and contained concentrations of many antibiotics used to treat partially antibiotic resistant bacterial strains. For all MIC plates, the inoculum of bacteria was standardized to between $1x10^5$ and $1x10^6$ CFU using a 0.5 McFarland turbidity standard and immediate serial dilution. In this first set of MICs, the ESBL plate comes lyophilized. Following the dilution to $1x10^6$ CFU/mL 100uL of each the bacterial strain of interest was added to each well of the ESBL plate, the plate was sealed, and incubated at 37C for 18hours before being read the next day. Each well was given either a positive or negative score for growth, defined as the well having any growth being positive. From these positive/negative scores, the actual MIC was generated with the lowest concentration of antibiotic which inhibited bacterial growth being considered the MIC for that strain/antibiotic combination. We found that there were some minor changes in MIC for Ceftazidime and Cefotaxime when comparing the results of the WT strain and the phage resistant in vitro/ in vivo bacterial mutants. The results have been summarized in Table 1 below. The results, however, were challenging to interpret because the strains used in this study were so antibiotic resistant that they frequently went past the highest MIC measured on the commercial ESBL plate. Some strains had an intermediate phenotype at the highest concentrations but were still considered positive growth for this assay. Without having higher concentrations of antibiotic present on the plate, we could not tell if there was any change in MIC for some of the antibiotics tested. Based on this knowledge, we decided to test the bacterial strains on new MIC plates made in house which contained antibiotics at higher concentrations. The plate was designed as shown in Table 2 below. Six antibiotics were selected based on current clinical applications, and each was tested either alone or with an appropriate beta-lactamase inhibitor at concentrations much higher than normal present on commercial MIC plates. Antibiotics included, Cefotaxime, Ceftazidime, Meropenem, Aztreonam, Imipenem, and Piperacillin. Beta-lactamase inhibitors included, Avibactam, Relebactam, and Tazobactam. The plate design is also shown with the 2019 CLSI guidelines for antibiotic susceptibility marked in green (susceptible), yellow (intermediate), and red (resistant) for context. All antibiotics were mixed in Mueller-Hinton broth and diluted according to the plate map, all numbers represent final ug/mL concentrations in each well. The beta-lactamase inhibitors were added at a fixed rate with a final concentration of 4ug/mL per well. Bacterial samples were again adjusted using a 0.5 McFarland Standard and dilution and a concentration of $5x10^5$ was achieved in each well. Final volumes in wells was 200uL. The plates were covered, and incubated at 37C for 18h before being read as previously described. These assays were conducted in triplicate and the results were averaged. In Table 3 below, the results of 4 of the antibiotics are shown. These 4 MIC results were selected because they showed

differences between strains. For all the remaining antibiotics and antibiotic/beta lactamase inhibitor combinations, there were no differences between strains. All strains were completely resistant to Piperacillin, Piperacillin/Tazobactam, and Aztrenoam. Whenever, an antibiotic was paired with Avibactam or Relebactam, no growth was observed at the tested concentrations. The exception to these data was a control strain of *K.pneumoniae* (1776c) which was susceptible to all antibiotics tested at these concentrations as was expected. From these data, it is possible to see that there are changes in the antibiotic susceptibility of the bacteriophage resistant mutants. These changes, however, appear to be strain dependent and antibiotic dependent and were observed to both increase and decrease antibiotic susceptibility.

Phage & CAZ/AVI Data

To better understand the protective phenotype seen in the in vivo experiments where mice were best protected by a treatment of both bacteriophage and Ceftazidime/Avibactam (CAZ/AVI) we attempted to replicate this synergy in vitro with a plate assay. This assay was designed using the effective concentrations observed in the MIC experiments, as well as previous bacteriophage growth inhibition assays. This “checkerboard” assay represents different combinations of bacteriophage and CAZ/AVI and compares how each combination inhibits growth of a given bacterial strain following an 18 hour incubation. Combinations included, the bacteriophage at 10^7 to 10^3 PFU or No Phage, and CAZ/AVI from 16 to 0.025ug/mL or No Antibiotic. Bacterial cultures were added at $5x10^5$ CFU final concentration as described previously. Final well volumes were 200ul and plates were covered, and incubated 18h at 37C before results were read using a plate reader measuring absorbance at 550nm. Bacterial strains tested were, 39427 WT, 39427 Pharr Resistant Mutant, 39427 EI resistant Mutant, and 39427 JR Resistant Mutant. These were all in vitro generated bacteriophage resistant bacterial mutants. Results shown in Table 4 represent 3 averaged replicates. In general for all tested strains, higher combinations of antibiotics and phage were more successful at inhibiting growth than either the corresponding antibiotic or phage concentration alone. These results indicate that there is benefit to treating with both bacteriophage and antibiotics rather than either group alone.

ɛ)

s in WabI
sp
on Asp,Arg,Tyr,Gly,Asp
6)
cation Asp,Gly,Cys
9

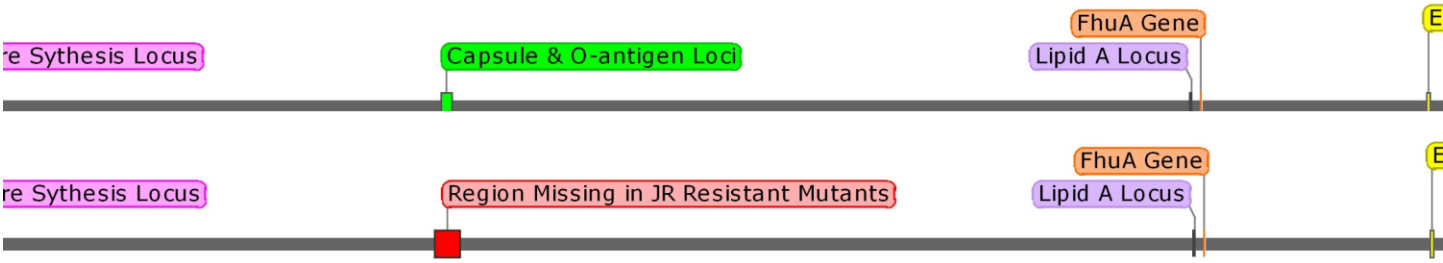
ϕPharr (T3/T7-like)

Mutation in WbaP
R1- +1 frameshift, L29

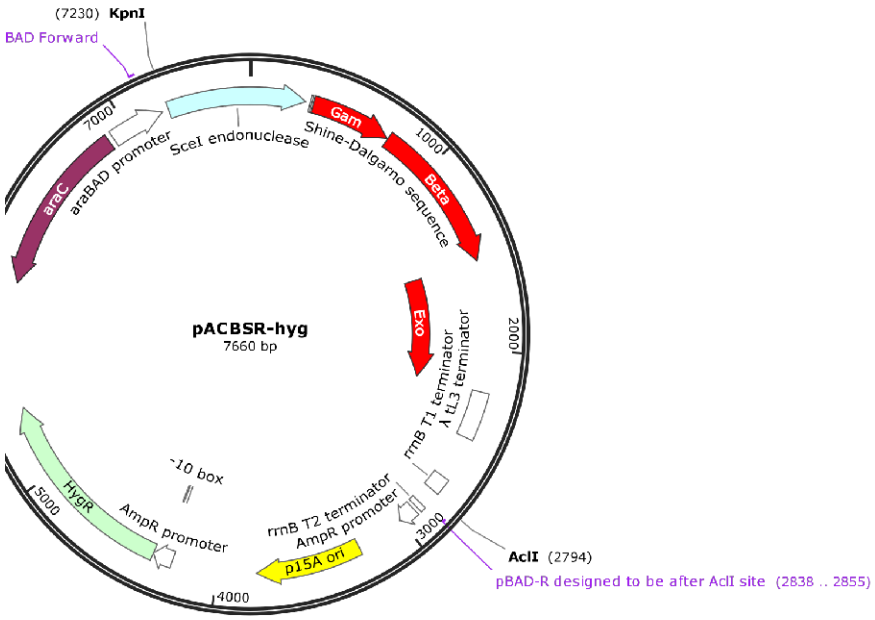
ϕJR (T5-like)

Mutations in F
R1- -1 frameshi
R2- 11 bp delet
R3- +1 framesh

All 3- 86,758 bp



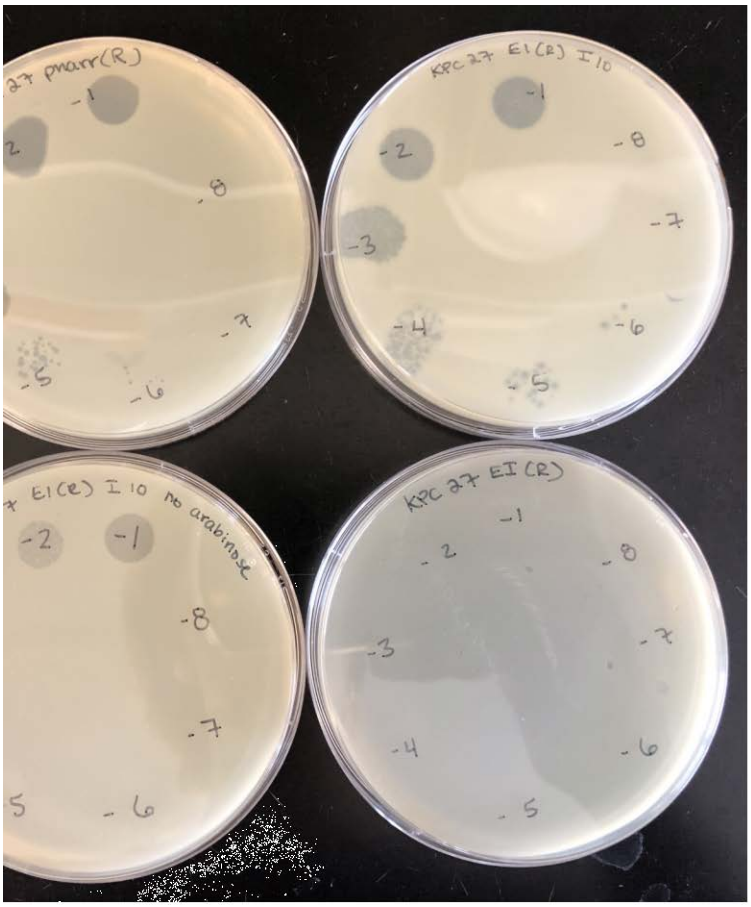
Complementa*on of Phage Receptors



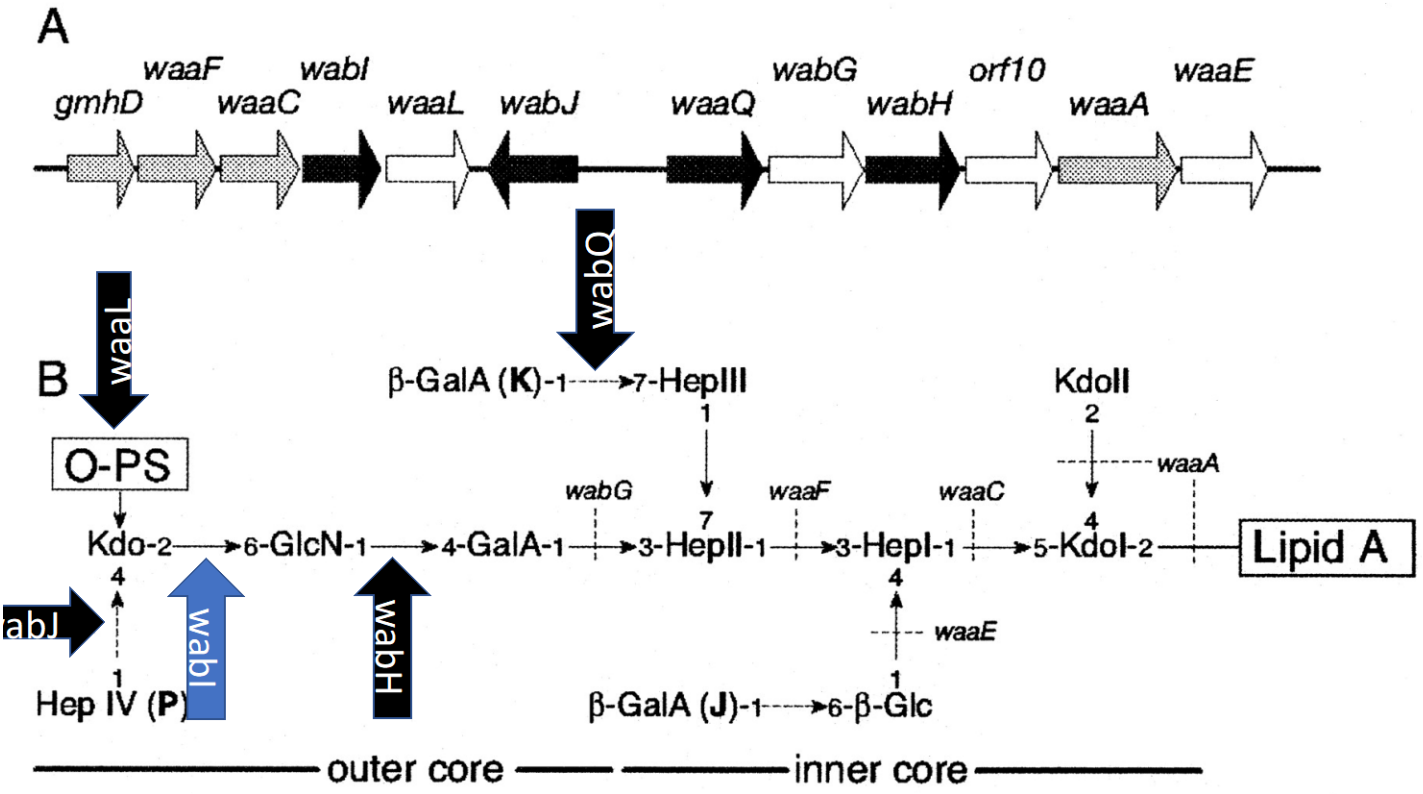
Created with SnapGene®

Vector pACBSR

- pBAD expression
- p15A temperature sensi9ve o
- *K.pneumonaie* op9mized HygF as selec9on marker
- Diges9on of vector with:
  - KpnI 5′...GGTAC C...3′ 3′...C CATGG...5′
  - AclI 5′...AA CGTT...3′ 3′...TTGC AA...5′
- Primers designed for verifica9 flanking insert from colony PC

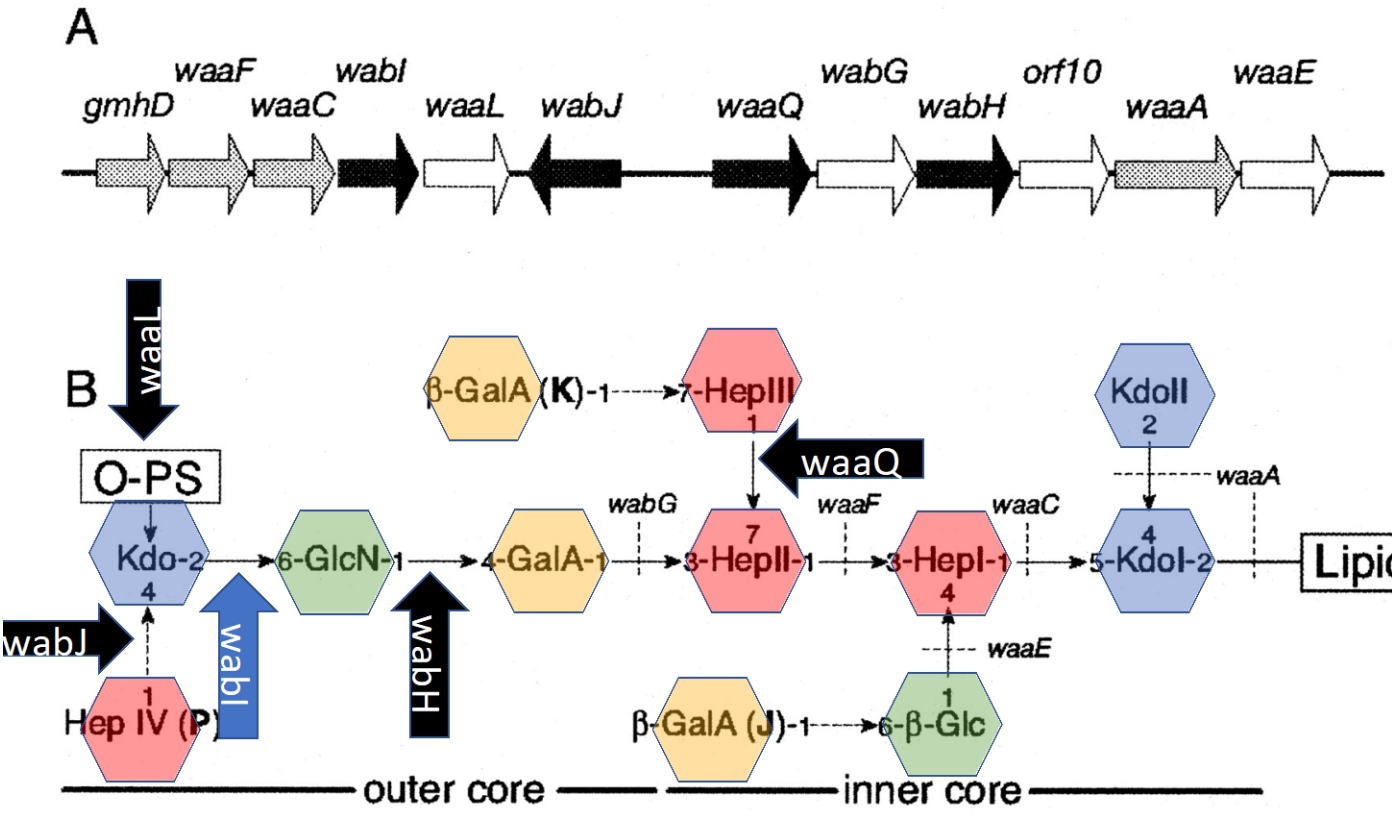


- Host Strain Pharr(R)
- KPC 27 EI(R) I10- Resistan strain with pACBSR:wabI:hyg with .1 arabinose induction
- Previous strain with out induction
- KPC 27 EI(R) Resistant strain no plasmid



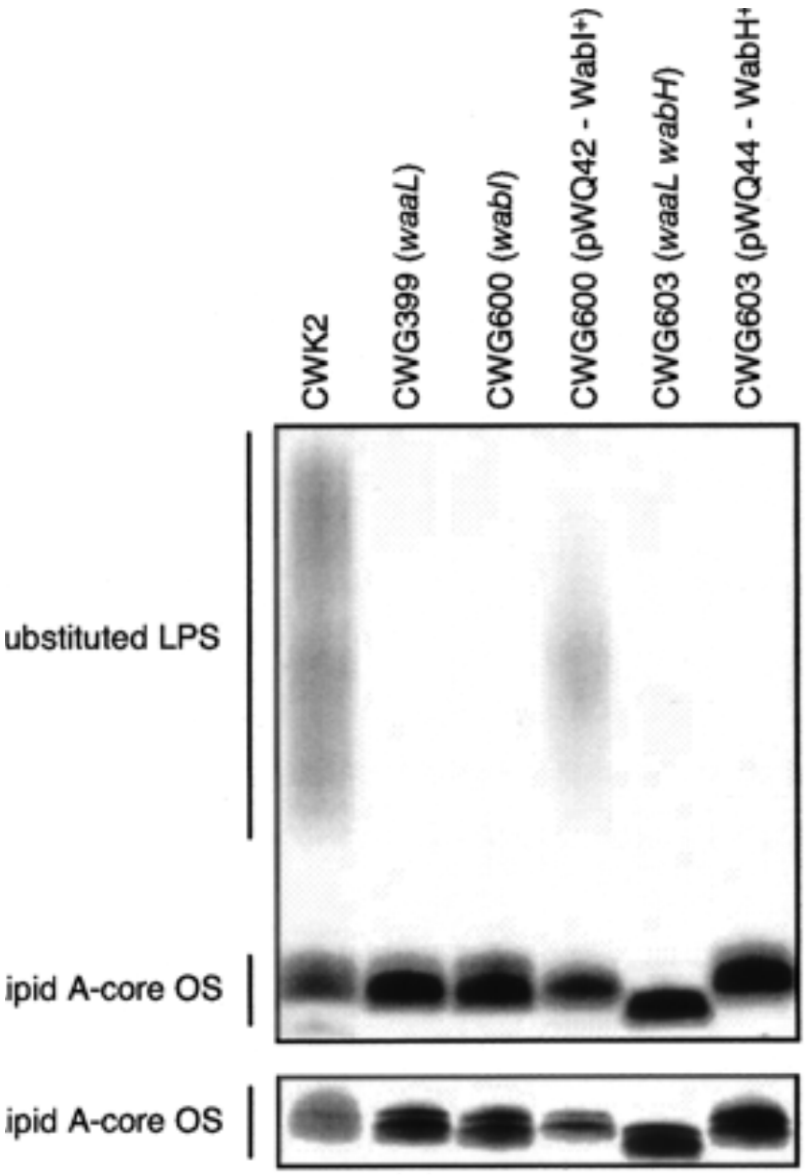
Compound	J	K	P
1	β-GalA	H	Hep
2	β-GalA	H	H
3	H	H	H
4	H	H	Hep
5	β-GalA	β-GalA	Hep

Frirdich, E., Vinogr
Biosynthesis of a n
acid-containing ou
lipopolysaccharide
Biological Chemist



Compound	J	K	P
1	β-GalA	H	Hep
2	β-GalA	H	H
3	H	H	H
4	H	H	Hep
5	β-GalA	β-GalA	Hep

Frirdich, E., V
Biosynthesis
acid-containir
lipopolysacch
Biological Ch



Frirdich, Emilisa, Evgeny Vinog
"Biosynthesis of a novel 3-dec
containing outer core oligosac
of Klebsiella pneumoniae." Jo
279.27 (2004): 27928-27940.

Minimum Inhibitory Concentration Assays of using Commercial ESBL Plates

Antibiotics	Experimental Strain 39427 WT	39427 Pharr(R)in Vitro	39427 Pharr(R) JR (R) in vitro	39427 Pharr (R) El (R) in vitro	OB 6/1/17 Experimental Sample Bacteria only
AXO- Ceftriaxone	>128	>128	>128	>128	>128
CEP- Cephalothin	>16	>16	>16	>16	>16
FOT- Cefotaxime	>64	>64	>64	>64	>64
F/C- Cefotaxime/ clavulanic acid	<64/4	<64/4	<32/4	>64/4	<64/4
TAZ- Ceftazidime	>128	>128	>=128 R	>128	>128
T/C- Cefazidime/ clavulanic acid	>128/4	>128/4	>128/4	>128/4	>128/4
IMI- Imipenem	>16	>16	>16	>16	>16
FEP- Cefepime	>16	>16	>16	>16	>16
POD- Cefpodoxime	>32	>32	>32	>32	>32
FOX- Cefoxitin	>64	>64	>64	>64	>64
P/T4- Piperacillin/ tazobactam	>64/4	>64/4	>64/4	>64/4	>64/4
MERO- Meropenem	>8	>8	>8	>8	>8
GEN- Gentamicin	<4	<4	<4	<4	<4
CIP- Ciprofloxacin	>2	>2	>2	>2	>2
AMP- Ampicillin	>16	>16	>16	>16	>16
FAZ- Cefazolin	>16	>16	>16	>16	>16

Antibiotics	1A 6/2/17 Experimental Bacteria + phage treatment	19-3C 6-30-17 Bacteria/ Phage / 80mg/kg CAZ AVI	20B 6/30/17 bacteria / phage / 40mg/kg CAZ/AVI	20-2C-bacteria / phage / 40mg/kg CAZ/AVI	Antibiotic Sensitive Control Strain 1776c
AXO- Ceftriaxone	>128	>128	>128	>128	<1
CEP- Cephalothin	>16	>16	>16	>16	<8
FOT- Cefotaxime	>64	>64	>64	>64	<.5
F/C- Cefotaxime/ clavulanic acid	>64/4	>64/4	>16/4	>64/4	<.25/4
TAZ- Ceftazidime	>128	>128	<128 R	>128	<.5
T/C- Cefazidime/ clavulanic acid	>128/4	>128/4	>64/4 R	>128/4	<.25/4
IMI- Imipenem	>16	>16	<8 R/I	>16	<.5
FEP- Cefepime	>16	>16	>16	>16	<1
POD- Cefpodoxime	>32	>32	>32	>32	<1
FOX- Cefoxitin	>64	>64	>64	>64	>64
P/T4- Piperacillin/ tazobactam	>64/4	>64/4	>64/4	>64/4	<4/4
MERO- Meropenem	>8	>8	>8	>8	<1
GEN- Gentamicin	<4	<4	<4	<4	<4
CIP- Ciprofloxacin	>2	>2	>2	>2	>2
AMP- Ampicillin	>16	>16	>16	>16	>16
FAZ- Cefazolin	>16	>16	>16	>16	>16

Blue= Intermediate Observed

Yellow= Change Observed

Table 1: Design of High Concentration MIC Plate for Testing Bacterial Mutant Antibiotic Susceptibility

2019 Walsh Model InVivo and InVitro K.pneu Samples						S	I	R				
<>	1	2	3	4	5	6	7	8	9	10	11	12
A	FOT16	F/C16	TAZ8	T/A8	MER8	M/A8	P8	P/T8	AZT2	A/A2	IMI2	I/R2
B	FOT32	F/C32	TAZ16	T/A16	MER16	M/A16	P16	P/T16	AZT4	A/A4	IMI4	I/R4
C	FOT64	F/C64	TAZ32	T/A32	MER32	M/A32	P32	P/T32	AZT8	A/A8	IMI8	I/R8
D	FOT128	F/C128	TAZ64	T/A64	MER64	M/A64	P64	P/T64	AZT16	A/A16	IMI16	I/R16
E	FOT256	F/C256	TAZ128	T/A128	MER128	M/A128	P128	P/T128	AZT32	A/A32	IMI32	I/R32
F	FOT512	F/C512	TAZ256	T/A256	MER256	M/A256	P256	P/T256	AZT64	A/A64	IMI64	I/R64
G	FOT1024	F/C1024	TAZ512	T/A512	MER512	M/A512	P512	P/T512	AZT128	A/A128	IMI128	I/R128
H	POS	POS	POS	NEG	NEG	NEG	P1024	P/T1024	AZT256	A/A256	IMI256	I/R256

		CLSI 2019		
		S	I	R
FOT	Cefotaxime	≤ 1	2	≥ 4
F/C	Cefotaxime/ Avibactam			
TAZ	Ceftazidime	≤ 4	8	≥ 16
T/A	Ceftazidime/ Avibactam	≤ 8/4		≥ 16/4
MERO	Meropenem	≤ 1	2	≥ 4
M/A	Meropenem/ Avibactam	≤ 4/4		≥ 16/4
AZT	Aztreonam	≤ 4	8	≥ 16
A/A	Aztreonam/ Avibactam			
IMI	Imipenem	≤ 1	2	≥ 4
I/R	Imipenem/ Relebactam			
P	Piperacillin	≤ 16	32–64	≥ 128
P/T	Piperacillin/ Tazobactam	≤ 16/4	32/4–64/4	≥ 128/4
NEG	Media			
POS	Positive Control			

Table 2:Selected MIC Results for High Concentration MIC Assay

Antibiotic	Experimental Strain 39427 WT	39427 Pharr(R)in Vitro	39427 Pharr(R) JR (R) in vitro	1A Experimental Bacteria + phage treatment	1B Experimental Bacteria + phage treatment
Cefotaxime	256	>1024	128	1024	1024
Ceftazidime	512	512	128	>512	512
Meropenem	64	128	128	256	64
Imipenem	64	128	128	128	64
Antibiotic	19-3C Bacteria/ Phage / 80mg/kg CAZ AVI	19-4C Bacteria/ Phage / 80mg/kg CAZ AVI	20-2C-bacteria / phage / 40mg/kg CAZ/AVI	20-4C-bacteria / phage / 40mg/kg CAZ/AVI	
Cefotaxime	128	128	512	1024	
Ceftazidime	512	512	256	>512	
Meropenem	64	64	64	256	
Imipenem	64	64	128	128	

Table 3: Bacteriophage & CAZ/AVI Checkerboards

	39427 WT					
	No Phage	10^3	10^4	10^5	10^6	10^7
16ug Caz 4ug Avi	0.24	0.08	0.05	0.04	0.02	0.00
8ug Caz 2ug Avi	0.54	0.34	0.16	0.17	0.07	0.05
4ug Caz 1ug Avi	0.57	0.34	0.30	0.16	0.14	0.04
2ug Caz 0.5ug Avi	0.59	0.35	0.28	0.30	0.07	0.06
1ug Caz 0.25ug Avi	0.62	0.38	0.24	0.19	0.08	0.12
0.5ug Caz 0.125ug Avi	0.59	0.28	0.29	0.14	0.12	0.07
.025ug Caz 0.0625ug Avi	0.53	0.34	0.21	0.20	0.11	0.07
No Antibiotic	0.61	0.28	0.13	0.15	0.12	0.08
	39427 Pharr Resistant					
	No Phage	10^3	10^4	10^5	10^6	10^7
16ug Caz 4ug Avi	0.22	0.09	0.06	0.04	0.02	0.00
8ug Caz 2ug Avi	0.53	0.36	0.14	0.14	0.08	0.05
4ug Caz 1ug Avi	0.45	0.26	0.24	0.12	0.06	0.03
2ug Caz 0.5ug Avi	0.59	0.36	0.26	0.28	0.06	0.06
1ug Caz 0.25ug Avi	0.63	0.39	0.26	0.19	0.11	0.10
0.5ug Caz 0.125ug Avi	0.63	0.32	0.31	0.20	0.08	0.07
.025ug Caz 0.0625ug Avi	0.63	0.35	0.24	0.22	0.09	0.09
No Antibiotic	0.64	0.30	0.17	0.17	0.12	0.07
	39427 EI Resistant					
	No Phage	10^3	10^4	10^5	10^6	10^7
16ug Caz 4ug Avi	0.23	0.11	0.10	0.08	0.04	0.00
8ug Caz 2ug Avi	0.61	0.49	0.31	0.34	0.34	0.33
4ug Caz 1ug Avi	0.50	0.39	0.24	0.25	0.24	0.23
2ug Caz 0.5ug Avi	0.64	0.39	0.33	0.35	0.34	0.36
1ug Caz 0.25ug Avi	0.68	0.39	0.32	0.35	0.35	0.36
0.5ug Caz 0.125ug Avi	0.68	0.37	0.32	0.35	0.34	0.34
.025ug Caz 0.0625ug Avi	0.68	0.44	0.34	0.37	0.35	0.36
No Antibiotic	0.56	0.35	0.34	0.37	0.35	0.35
	39427 JR Resistant					
	No Phage	10^3	10^4	10^5	10^6	10^7
16ug Caz 4ug Avi	0.11	0.09	0.10	0.07	0.05	0.00
8ug Caz 2ug Avi	0.47	0.44	0.42	0.40	0.41	0.41

4ug Caz 1ug Avi	0.36	0.35	0.34	0.33	0.31	0.31
2ug Caz 0.5ug Avi	0.49	0.49	0.46	0.44	0.43	0.44
1ug Caz 0.25ug Avi	0.50	0.49	0.46	0.46	0.46	0.48
0.5ug Caz 0.125ug Avi	0.51	0.49	0.47	0.46	0.45	0.47
.025ug Caz 0.0625ug Avi	0.54	0.50	0.47	0.46	0.45	0.45
No Antibiotic	0.51	0.49	0.46	0.46	0.46	0.46

39427 Bacterial Genome & Proposed Mutations

Following extensive genomic assembly, we currently hypothesize that the 39427 bacterial genome is a circular chromosome of 5,426kb in length with 3 plasmids 168kb, 57kb, and 89kb in length. This is being confirmed by further sequencing for closure. Using this sequence, and the sequence of some of our bacteriophage resistant bacterial mutants, we have identified probable causative mutations for protection against some of our bacteriophage. These genes are currently being complemented to confirm they are responsible for the phage resistant phenotype. Additionally, this closed genome is allowing us to annotate various virulence factors which may affected by the phage treatment.

Bacterial Strain	Type of Mutation	Gene
39427 (Pharr-R)	+1 frameshift, L29	undecaprenyl-phosphate galactose phosphotransferase *wbaP*
39427 (Soft-R)	+1 frameshift, L29	undecaprenyl-phosphate galactose phosphotransferase *wbaP*
39427 (Pharr-R) (Spivey-R1)	-1 frameshift, R316	Ferrichrome transporter *fhuA*
39427 (Pharr-R) (Spivey-R1)	86,758 bp deletion	Region contains CPS and O-Antigen coding regions
39427 (Pharr-R) (Spivey-R2)	11 bp deletion starting in P169	Ferrichrome transporter *fhuA*
39427 (Pharr-R) (Spivey-R2)	86,758 bp deletion	Region contains CPS and O-Antigen coding regions
39427 (Pharr-R) (Spivey-R3)	+1 frameshift, Y106	Ferrichrome transporter *fhuA*
39427 (Pharr-R) (Spivey-R3)	86,758 bp deletion	Region contains CPS and O-Antigen coding regions