Infection with Bacteriophage Led to Loss-of-Function Mutations in FhuA Gene Locus

Isabella Castellano-Moen¹ and Zachary Pratt#

¹University of Wisconsin-Madison, USA
#Advisor

ABSTRACT

Bacteriophages, also known as phages, are viruses that solely target bacteria for infection and replication. To initiate infection, a phage must bind to a specific receptor on the surface of bacteria. Genetic changes to the genes that encode these surface proteins may block phage attachment, thus, rendering the bacteria resistant to the phage. We hypothesized that when infected with a phage that targets FhuA, *Escherichia coli* would acquire point mutations, thus allowing the clones to retain the protein’s function while inhibiting infection (Endriss et al., 2003). To test our hypothesis, we isolated bacteriophages from filtered sewage water and generated phage-resistant clones of *E. coli*. We then isolated genomic DNA from the resistant clones, amplified the *fhuA* gene by PCR, and Sanger sequenced the DNA for analysis. We discovered that all but one of the phage resistance clones contained frameshift mutations, which eliminates the protein. This finding indicates that *E. coli* produced non-functional FhuA protein to maintain resistance to phage infection. Since FhuA is an important ferrichrome transporter, we hypothesized that *E. coli* uses other iron transporters, such as FepA and TonB, to compensate for the loss of FhuA.

Introduction

Bacteriophages are viruses that infect and replicate only in bacteria. Phages are thought to be the most abundant biological agent on the planet and are ubiquitous on Earth (Clokie et al., 2011). All bacteriophages contain a capsid protein that protects their genomic material and mediates delivery into infected host cells (Kasman and Porter, 2022). To attack and infect bacteria, phages require a surface protein for attachment. If bacteria lack this required receptor, then there will be no phage infection.

FhuA is an outer membrane protein that transports the ferric siderophore ferrichrome, which aids in the uptake and regulation of iron availability in *Escherichia coli* (Bonhivers et al., 1998). We wondered if bacterial clones infected with bacteriophages that target FhuA would contain loss-of-function mutations, where the altered gene product lacks the wild-type gene function, to gain resistance to phages. Iron is essential for the survival and proliferation of organisms. Yet, because of its lethal effect through the Fenton reaction, its uptake must be regulated. In the Fenton reaction, oxidizing agents are produced, which can react with organic and inorganic substances and degrade them (Touati, 2000). In bacteria, iron plays a role in amino acid biosynthesis, DNA synthesis, and many other critical functions. Therefore, we hypothesized that in the presence of a phage that targets FhuA, *E. coli* clones would be selected with substitution mutations that prevent the binding of phage but do not abolish the function of FhuA.

To test our hypothesis, we generated and confirmed clones resistant to phage infection. Their genomic DNA was isolated, and the *fhuA* gene was amplified and sequenced. We analyzed the sequence to determine the location of genetic changes in *fhuA* and the effect these changes had on the structure of FhuA.

Methods
Bacteriophage Isolation

Bacteriophages were isolated from sewage water from North East Water (NEW, Green Bay, WI) and the Madison Metropolitan Sewerage District (MMSD, Madison, WI). Bacteriophage K4-1 was isolated from NEW in 2015, while P15 and P29 were isolated from MMSD in June 2017. Purification of plaques was performed as described by Kropinski et al. (2009). In brief, sewage water was centrifuged at 1000 g for 6 minutes to remove particulates and then filter-sterilized with a 0.45 μm cellulose acetate syringe filter. Filtered sewage water was incubated with a stationary-phase culture of *Escherichia coli* K-12 substrain MG1655 for 10 minutes at room temperature in a volumetric ratio of 1:1 volume. After incubation, the culture of bacteriophage and *E. coli* was mixed with Luria Bertani (LB) soft agar and overlaid on LB agar plates. After overnight incubation at 30°C, plaques were picked off agar plates with sterile wooden sticks, transferred to a solution of phage buffer with chloroform, and vortexed for 1 minute. This solution was serially diluted and each dilution was incubated with *E. coli* in order to obtain isolated plaques that were homogeneous in morphology. Phages producing homogeneous plaques were frozen at -80°C or 20°C for long-term storage and short-term storage, respectively in 37.5% glycerol and 50% LB broth or phage buffer.

Culturing Conditions and Media

*E. coli* K-12 substrain MG1655 was cultured in LB broth with shaking at 150 rpm at 30°C, or on LB agar plates at 30°C. Stocks of bacteriophages P15, P29, and K4-1 were kept frozen in a phage buffer supplemented with 37.5% glycerol. To generate bacteriophages, a culture of *E. coli* was grown to the mid-log phase (OD600=0.3) at 30°C, and bacteriophages from a frozen stock were added to the culture. After overnight incubation, the culture was centrifuged at 6000 rpm for 10 minutes to pellet the bacteria, and the supernatant was filter-sterilized through a 0.45 μm cellulose acetate syringe filter into a sterile test tube. These cultures of bacteriophages were serially diluted and titered as described (JoVE Science Education Database, 2022).

Generating Mutant Clones

*E. coli* K-12 substrain MG1655 was cultured in LB broth overnight at 30°C with shaking. The culture was diluted 1:100 in fresh LB broth and then aliquoted into wells of a 96-well plate. The *E. coli* was incubated, without shaking, for 2 hours at 30°C, after which bacteriophages were added to each culture at a final concentration of 2.5x10^5 PFU/mL. These plates were incubated overnight at 30°C.

Wells of the 96-well plate containing *E. coli* that were not inoculated with bacteriophage were compared to wells inoculated with bacteriophage to observe growth inhibition. If growth inhibition was observed, a culture of the well was T-streaked onto LB agar plates and grown overnight at 30°C. Clones that grew on these plates were presumed to be resistant to bacteriophages; one clone was picked from each plate and tested further.

In order to confirm whether clones were resistant to a particular bacteriophage, patches of clones were cultured on LB agar or LB agar supplemented with 2x10^8 PFU/mL. Clones capable of growth on LB agar in the absence and presence of bacteriophages were determined to be phage-resistant clones and were stored at -20°C in 15% glycerol for further analysis.

Sequencing Mutant Clones

Genomic DNA was isolated from phage-resistant clones of *E. coli* per the manufacturer’s instructions (BioVision, Milpitas, CA). The *fhuA* gene locus was amplified from genomic DNA using FhuA FWD and FhuA REV primers.
(Table 1) and Phusion Hot Start Flex DNA Polymerase (New England Biolabs, Ipswich, MA) following the manufacturer’s instructions. The conditions for PCR were as follows: Initial denaturation at 98°C for 30 seconds, 35 cycles of amplification (98°C for 10 s, 51.5°C for 10 sec, 72°C for 1.5 min), and final extension at 72°C for 10 min.

PCR amplification was confirmed by performing gel electrophoresis. In brief, PCR products were separated in a 1% TAE agarose gel, and the gel was stained with 0.3 μg/mL of ethidium bromide. Products at the size of the expected PCR product (2.5 kb) or larger were sequenced.

PCR products were treated with exo-CIP (New England Biolabs) following the manufacturer’s instructions prior to sequencing. The exonuclease degrades the PCR primers and calf intestinal phosphatase (CIP) dephosphorylates dNTPs.

Sanger sequencing of mutants was performed by the University of Wisconsin Biotechnology Center (Madison, WI). 500 ng of PCR product and 0.2 μM primer were used for each sequencing reaction. Primers FhuA FWD, FhuA FWD1, FhuA FWD2, FhuA FWD3, and FhuA REV were used to sequence the full fhuA gene locus (Table 1).

Table 1. Primers used in this study.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>FhuA FWD</td>
<td>5’ AGGAACGCTCAGATTGCG 3’</td>
</tr>
<tr>
<td>FhuA REV</td>
<td>5’ CGTGTATTCTGCAATAACAGCC 3’</td>
</tr>
<tr>
<td>FhuA FWD Seq 1</td>
<td>5’ CCTGTTGAATATGGTCTAGC 3’</td>
</tr>
<tr>
<td>FhuA FWD Seq 2</td>
<td>5’ CTTACAGCAAAACAGTGTCG 3’</td>
</tr>
<tr>
<td>FhuA FWD Seq 3</td>
<td>5’ GTGTAACACTTTACTTCAGC 3’</td>
</tr>
</tbody>
</table>

Sequence Analysis

Sequences of PCR products, from the fhuA gene locus, of phage-resistant clones were compared to the sequence in E. coli strain K-12 substrain MG1655 (NCBI ID 944856).

Data Availability

The partially sequenced fhuA gene locus from phage-resistant E. coli clones was deposited in Genbank under the accession numbers listed in Table 2.

Results

E. coli was incubated, without shaking, in a 96-well plate for 2 hours at 30°C. Bacteriophage-resistant clones were generated by adding phage P14 at a final concentration of 2.5x 10^5 PFU/mL in each well and incubating them overnight. After generating clones of E. coli that were resistant to bacteriophage P14 in 96-well plates, we confirmed their resistance on LB agar (Figure 1). First, we plated each clone using a patching technique on LB agar without the presence of the phage. On the second LB agar plate, we supplemented it with bacteriophage P14 and plated each presumptive phage-resistant E. coli following the same patching technique as on the first plate. After incubation overnight, we found that the majority of the clones tested grew both in the presence and the absence of the bacteriophages, indicating they were resistant to the bacteriophage. These clones were banked and analyzed further. The few clones that were not able to grow in the presence of the bacteriophages were excluded from additional analysis.
Figure 1. Clones generated from E. coli strain K12 were plated on LB agar without phage (left) and on LB agar containing 200 μL of bacteriophage P14 at a concentration of 2x10^8 PFU/mL (right). The growth of clones on both plates indicated phage-resistant bacteria. The white boxes represent clones of E. coli that could grow in LB agar plates but could not reproduce in the presence of bacteriophage P14; these clones were considered phage-sensitive and were not further analyzed.

Phages P29 and K4-1, like P14, bind FhuA. Therefore, we wanted to determine if resistance to one of these phages conferred resistance to all three phages. Patching phage-resistant clones on LB agar or LB agar supplemented with one of the three phages revealed that resistance to one phage led to resistance to all three phages. For example, all clones that were resistant to phage P14 were also resistant to bacteriophages P29 and K4-1, indicating that each phage uses the same receptor to facilitate entry into the host cell (Figure 2).
Clones of *E. coli* that were resistant to phage P14 were plated on LB agar containing no phage, or 200 μL of phage P14, phage P29, or phage K4-1 at 1x10⁹ - 1x10¹⁰ PFU/mL. All clones tested were resistant to each phage. The parental K12 *E. coli* was sensitive to each phage (data not shown). The data is a representative image of a subset of phage-resistant clones.

Next, we wanted to amplify and sequence the *fhuA* locus from each clone in order to determine if mutations in *fhuA* could explain resistance to P14. The amplified product was predicted to be 2.5 kb. Gel electrophoresis was performed in order to determine if PCR amplification was successful. Though we anticipated PCR would yield products of 2.5 kb, in some cases, we found amplified products that were greater than 3 kb (Figure 3). PCR products within the 2-3 kb range and some of the larger products were prepared for Sanger sequencing. In total, 20 clones were sequenced across the full length of the *fhuA* locus and yielded results for analysis.
The *fhuA* gene locus was amplified by PCR from the genomic DNA of clones resistant to phage P29. In this representative image, Clones 2, 3, 5, and 6 were positive for a PCR product of approximately the correct size (~2500 bp). Primers consistently produced a single product. Similar analyses were performed with genomic DNA from clones that were resistant to phage P14 or K4-1 (data not shown).

Once we were able to fully analyze the sequences, it became clear that the most common mutations were transposons, although insertions and deletions occurred, too (Table 2). Out of the 20 mutations found in the 20 clones, 25% of them were deletions. For example, clone D4 had a deletion of a guanine at position 168 in the *fhuA* coding sequence, which resulted in a frameshift of the codon sequence. We found that 20% of the mutations were insertions. Of those mutations that were insertions, 75% included the insertion of one nucleotide, while one of the mutations (25%) included the insertion of 8 nucleotides at nucleotide position 373, in clone BJM G2. 50% of the 20 mutations contained large stretches of sequence that were not homologous to *fhuA*. We performed BLAST analysis on these non-homologous sequences and found them to be transposons. 5% of the mutations were point mutations. In clone BE F2, there was a mutation of thymine to guanine at the nucleotide position 2132. This changed the amino acid sequence from Leucine to Arginine at amino acid number 711 and would potentially change the structure of the protein, as Leucine is a hydrophobic molecule and Arginine is a hydrophilic one.

Table 2. Identification of the mutation type and location in phage-resistant *E. coli* clones.

<table>
<thead>
<tr>
<th>Accession Number</th>
<th>Clone Name</th>
<th>Location of Mutation (bp)</th>
<th>Type of mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>OR322054</td>
<td>BE D4</td>
<td>168</td>
<td>Frameshift (Deletion)</td>
</tr>
<tr>
<td>OR322055</td>
<td>BE B2</td>
<td>348</td>
<td>Frameshift (Insertion)</td>
</tr>
<tr>
<td>OR322056</td>
<td>BE C5</td>
<td>348</td>
<td>Frameshift (Insertion)</td>
</tr>
<tr>
<td>OR322057</td>
<td>BE D2</td>
<td>348</td>
<td>Frameshift (Insertion)</td>
</tr>
</tbody>
</table>
**Discussion**

In these experiments, we attempted to identify mutations that led to the resistance of *E. coli* from infection by bacteriophages that bound the FhuA protein. We hypothesized that these mutations would allow us to determine where on the FhuA each of the phages bound the protein. We anticipated we would find substitution mutations, resulting in the change of single amino acids. Point mutations do not abolish the structural transitions that occur in FhuA in response to the TonB system, thus retaining the function of the protein while still preventing phage binding (Endriss et al., 2003). Such mutations would prevent the phages from binding but retain the shape and function of the protein. We anticipated that these mutations would allow the protein to still function in some capacity as an iron transporter.

We observed that most mutations were indels or transposons that were integrated into the *fhuA* gene locus. We first generated clones of *E. coli* that were resistant to one of three bacteriophages that bound FhuA. The picking of these clones was random. We anticipated finding mutations in the *fhuA* gene when clones grew on LB agar plates with phages, confirming phage resistance (Figure 1).

Clones were sequenced to determine the location and identity of mutations that rendered clones resistant to infection (Table 2). Clones that were chosen to be sequenced contained visible products after PCR of the *fhuA* locus. Because of time constraints, we could not follow up and sequence clones that were unable to generate PCR products. This may have skewed our percentages of mutations that were indels, substitutions, and transposon mutations. We also chose products of specific sizes, which biased our results to indels. Shockingly, transposon and deletion mutations were the most commonly found. This tells us that instead of selecting point mutations that disrupted the site where the phage binds, phage selection disrupted the open reading frame.

We hypothesized that the mutations led to the production of non-functional proteins that cannot be appropriately expressed on the outer membrane. Insertion and deletion mutations change the reading frame, which could cause truncated polypeptides. Polypeptides undergo a large structural transformation due to codon disruption when residues are deleted (Endriss et al., 2003). Transposons would disrupt the codon code of mRNAs. The effect of a transposon insertion on a gene has been determined to affect the expression of the gene significantly (de Ruiter et al., 2017). This hypothesis is consistent with our observations in Figure 2; resistance to one phage led to resistance to all three phages. Phages may bind to the same extracellular domain on FhuA. Still, it is unlikely that these three phages are identical because they were isolated at different times from two different locations. Sequencing of the phages would need to be performed to determine if they were similar or unique.
Our results show that *E. coli* can grow and reproduce without FhuA when cultured in an LB medium. The majority of mutations were ones that likely prevented the production of a functional FhuA protein. Clones that were isolated were resistant to phages that bound FhuA, suggesting they must have lacked functional FhuA. This data argues that FhuA is not required for growth in LB broth at 30°C. Point mutations that retain the protein’s function but prevent the bacteriophages’ attachment would most likely be selected in environments where the FhuA protein is necessary for growth. However, bacteria have evolved different methods to acquire iron when put under limiting conditions, that do not include FhuA. Studies have shown that *E. coli* synthesizes and excretes different chelators and siderophores that can use relatively low concentrations of iron and move it into the cell at normal speeds (Foster and Hall, 1992). Since FhuA is not the only iron transporter in *E. coli*, disrupting the expression of this protein to prevent infection may not affect iron transportation enough to create selective pressure. Other iron transporters in *E. coli* include FepA, an outer membrane receptor for ferrienterchelin, and TonB, which works with the *FhuA* gene and also mediates substrate-specific transport across the outer membrane (Braun & Braun, 2002).

Of course, further research would be required to understand if the mutations to FhuA affect the ability of phage-resistant clones to grow in iron-limiting conditions. Results might have differed if we tested for mutants with strains of *E. coli* that were lacking the other iron transporters or the ability to move transposons. Phage selection, in strains such as these, could have favored substitution mutations rather than indels and transposons. There would also be a selective advantage for these point mutations when grown in iron-limiting conditions in the presence of bacteriophages. Further analysis of bacteriophages that can render bacteria sensitive to antibiotics would be another follow-up experiment. Recently, bacteriophages that use multidrug-resistant (MDR) pumps as their required receptors have been shown to elicit an evolutionary trade-off. One such bacteriophage, OMKO1, infects *Pseudomonas aeruginosa* by binding to OprM, an outer membrane protein, and an MDR pump (Chan et al., 2016). Chan et al. observed that clones would gain resistance to OMKO1 when producing a non-functional version of the MDR pump which, consequently, led to antibiotic sensitivity. *E. coli* similarly had an antibiotic efflux pump, called ToIC. This efflux pump plays a common role in the removal of molecules from the cell, including different protein toxins and antibacterial drugs. ToIC offers an exit duct to large and/or small substrates (Koronakis et al., 2004). Moreover, we could isolate the bacteriophages that bind ToIC and determine if mutations that render *E. coli* resistant to the bacteriophages increase sensitivity to antibiotics.

**Conclusion**

Thus, *E. coli* clones resistant to bacteriophage P14 were found to also be resistant to bacteriophages K4-1 and P29, indicating that all three bacteriophages utilize FhuA. Bacteriophage resistant clones were sequenced, and analysis showed that 50% of mutant clones contained a transposon. Further experiments include testing resistant clones for functional FhuA.

**Limitations**

Sequence analysis was taken from PCR products, which were limited to products of certain sizes, as well as those that we were able to amplify.

**Acknowledgments**

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References


