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Examining Bacteriophage Reliance on the TolA Protein of Escherichia coli

Ellen Davis
ellend@bgsu.edu

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Ellen Davis

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Dr. Ray Larsen, Department of Biology

Dr. Craig Zirbel, Department of Mathematics & Statistics

Introduction

With an estimated 10^{31} bacteriophage particles in the biosphere, bacteriophage are the most abundant organisms on earth (Hatfull and Hendrix, 2012). Bacteriophage are viruses that cross the bacterial membrane and utilize their protein machinery to reproduce, and ultimately destroy, the host cell. Due to this ability, and the crisis of antibiotic-resistant bacterial strains, bacteriophage research has important implications in public health and medicine. Bacteriophage have been utilized to treat antibiotic-resistant infections and, with further research, provide a possible alternative to antibiotic use in some cases. *Escherichia coli* is a rod-shaped, gram-negative bacterium which is ubiquitous to the intestines of humans and many other mammals (Jang *et al.* 2017). Generally, *E. coli* is a component of healthy intestinal flora, but some strains are pathogenic. Its potential to cause disease gives *E. coli* importance to the field of human health and safety.

The cellular envelope of gram-negative bacteria, including *E. coli*, is composed of an outer membrane, periplasmic layer, and inner membrane. The outer membrane is composed of a lipid bilayer which allows small molecules to permeate the barrier. The outer membrane is asymmetrical, with the outermost portion being composed of lipopolysaccharides and the internal layer being composed of phospholipids (Meng et al. 2021). The periplasm is the space separating the outer and inner membranes and contains a thin layer of peptidoglycan to support cell structure. The periplasm contains many substances and proteins related to the protection of the cell from stress, support of metabolism, and transport of molecules into the cell (Meng et al. 2021).

The cellular envelope also contains important integral membrane proteins which carry out a range of functions including some related to transport, structure, and the cell cycle. The Tol-Pal system is a five-protein complex which is found embedded in the membrane of gram-negative bacteria, including *E. coli.* The Tol-Pal system has a function in providing outer membrane

integrity and in cell envelope remodeling. During cellular division, the Tol-Pal system relies on energy produced by the Proton Motive Force to separate daughter cells and stabilize the outer membrane (Webby et al. 2022). The Tol-Pal system consists of five protein subunits known as Tol-Pal, TolQ, TolR, TolA, and TolB. Three of these proteins, TolA, TolQ, and TolR, are embedded in the phospholipid inner membrane. TolB is found in the periplasmic space, and Tol-Pal is a lipoprotein which is embedded in the outer membrane (Baccelli et al. 2022). The TolQR complex converts chemical energy produced by the Proton Motive Force to movement (Baccelli et al. 2022). This energy is utilized to energize TolA, which in turn interacts with TolB and Tol-Pal in a reaction which is believed to cause the contractile activity associated with cytokinesis (Baccelli et al. 2022).

Despite the importance of these functions, the Tol-Pal system has a sometimes-fatal flaw; certain bacteriophage and bacterial toxins known as colicins take advantage of this system to cross the bacterial cell membrane. Bacteriophage bind to a wide range of bacterial cell receptors, primarily outer membrane proteins, and inject their genetic information into the host cell to be replicated by the host's machinery. The process of active transportation across the membrane requires energy, and bacteriophage do not have the ability to produce the energy required to adsorb into the host cell on their own. The bacteriophage must utilize energy generated by the host cell in order for its genetic materials to be successfully transported across the membrane (Samire et al. 2020).

In addition to the Tol-pal system, there are many other integral membrane protein systems of note. The TonB system is composed of a complex of three proteins, TonB, ExbB, and ExbD, which utilize energy produced by the proton motive force to transport a variety of substances across the cellular envelope. The TonB system provides energy to a variety of receptors including

FhuA and FepA, which transport iron siderophores into the cell (Ferguson 2002). This energy transduction is carried out by the ExbBD protein complex of the TonB system and is homologous to the TolQR complex (Bacceli et al. 2022). A deletion of the TonB system prevents bacteriophage from utilizing a number of TonB dependent proteins, including FhuA and FepA to cross the bacterial membrane. Additionally, a deletion of the FhuA or FepA barrel protein itself prevents certain bacteriophage from crossing the membrane.

Due to the reliance of certain bacteriophage on the Tol-Pal complex, and particularly the central protein TolA, this system is of interest. This project aimed to isolate bacteriophage which seem to be reliant on the TolA protein using genetically engineered *E. coli* strains with relevant gene knockouts. Additionally, over 100 bacteriophage were tested against a few other *E. coli* strains with knockouts for FepA, FhuA, and TonB which are known receptors for bacteriophage binding, and a statistical analysis was performed to determine if there were any clear associations between knockout strains.

Methods

The Larsen laboratory has made it a priority to collect and study bacteriophage which infect a variety of bacterial strains. One important bacterium that is used regularly in the laboratory is *Escherichia coli*. Over the past several years, nearly 200 bacteriophage with a capability to lyse, or destroy, *E. coli* have been identified and isolated from various sources. Most of these bacteriophage strains were isolated from sewage samples and animal feces. Since *E. coli* is a component of normal intestinal flora, these sources often contain bacteriophage which are capable of infiltrating *E. coli.*

Previously, the laboratory has engineered several strains of *E. coli* with single-gene knockouts for the TolA gene, as well as strains containing knockouts of genes associated with the Tol-pal system and the TolQRA complex. In this research, environmentally isolated bacteriophage were spotted on plates of *E. coli* with and without these mutations. The formation of a zone of clearing where no bacteria is present indicates that a bacteriophage effectively lysed the bacteria by preventing growth in that area. The control used for this experiment is an *E. coli* strain without any gene knockouts known as W3110. This strain was the genetic basis from which the knockout strains were developed. If a bacteriophage forms plaques on a control plate but not on a plate with the gene for a TolA removed, this indicates that this bacteriophage utilizes the Tol-pal system to transport its genetic material across the cellular envelope.

Liquid bacterial cultures were created by mixing a small amount of each bacterium with nutrient broth. These cultures were placed in the shaking incubator at 37 degrees Celsius and allowed to grow overnight. The following day, 100 microliters of each bacterial sample were transferred to sterile test tubes. Three milliliters of top agar were combined with each bacterium and vortexed to ensure an even distribution of bacteria throughout the medium. The mixture was poured onto a Tryptic Soy Agar plate and allowed to solidify. Tryptic Soy Agar plates are a general, non-selective media which provides sufficient nutrition for many bacteria, including *E. coli.*

After the media hardened, the plate was labeled with the bacteriophage that would be spotted. 50 microliters of each bacteriophage was spotted onto each plate. The laboratory adapted E. coli strain W3110 was used as a control with no gene knockouts. The strain with a knockout for the TolA protein, RA1038, was created using the W3110 genetic background. This reduces the

chance that other genetic factors will influence whether a phage is capable of infiltrating the bacterial cells.

The plates were carefully transported to the incubator and allowed to incubate for 24 hours at 37 degrees Celsius. After incubation, the plates were observed, and data was recorded. If a plaque appeared, this indicated that a given bacteriophage was reproducing and was therefore not reliant on the associated protein for entry into the bacterial host. If no plaque formed, this indicated that in order for the bacteriophage to enter into the host cell and proliferate, this protein was required. A table summarizing these results is found in Appendix A. A positive result refers to the presence of plaques, while a negative result indicates an absence of plaques.

The spotting technique identified several bacteriophage which appeared to be reliant on the TolA protein for bacterial infiltration. Each of these were spotted on the control strain and the single-gene knockout for the TolA protein. From these samples, a small number of plaques formed on the control plate but not on the knockout of TolA, indicating that these bacteriophage may be reliant on the TolA protein.

From the control plates, individual plaques of each bacteriophage were identified. To further study these bacteriophage, Eppendorf tubes were prepared containing 500 microliters of sterile phage buffer. These tubes were treated by adding a few drops of chloroform to destroy any potential bacterial contaminants. An individual plaque of each bacteriophage was picked and released into each tube. These were left to set at room temperature. The goal of this technique was to ensure that each sample contained only one type of bacteriophage and to create a more concentrated sample. These picked plaques were then tested against the control and TolA knockout strain and results were recorded. These picked samples had a higher bacteriophage concentration and formed a few small plaques on the TolA knockout strain.

The spotting technique was repeated with several genetically engineered *E. coli* strains containing knockouts for other known bacteriophage receptors. These included TonB, FhuA, and FepA. Ultimately, 107 bacteriophage were tested against these three strains and the wild type as a control. Contingency tables were constructed (Figure 4) comparing the three systems and p-values were calculated to determine whether there was a clear relationship between any of the receptors.

Results

After testing over 100 bacteriophage against a single-gene-knockout of the TolA protein in *E. coli,* it was surprising to find none which were clearly reliant on TolA. The initial samples of five bacteriophage appeared to be reliant on the TolA protein because no plaques formed on the TolA knockout plate. However, when individual plaques were picked to create a more concentrated sample, a small number of plaques appeared on the TolA knockout strain. This indicates that although the infiltration of these bacteriophage was limited by the gene knockout, their function was not entirely eliminated.

Additionally, upon creating contingency tables of each possible combination of FepA, FhuA, and TonB, p-values were calculated to determine whether there is a clear relationship between any of the receptors. The relationships between FhuA and FepA, and between TonB and FepA were not statistically significant. However, there was a statistically significant relationship between FhuA and TonB, with a p-value of .0015. The contingency table created for these receptors is found in Appendix A. This indicates that the ability for bacteriophage to utilize these receptors may be related. However, there are some considerations to be made for a small sample size of seemingly FepA reliant bacteriophage. Of the 107 bacteriophage tested across these three receptors, only one seemed to be reliant on the FepA protein.

Discussion

The data collected throughout this project demonstrated a statistically significant relationship between the TonB system and the FhuA protein. This makes sense because FhuA utilizes its contact with TonB to transduce energy which TonB receives from the Proton Motive Force (James et al. 2008). A disruption of TonB would therefore disturb the ability of bacteriophage to parasitize energy in the FhuA protein to cross the bacterial cell membrane. The data showed that, in the absence of TonB, FhuA was unlikely to be infiltrated by bacteriophage. However, a deletion of FhuA did not seem to have the same impact on TonB. This is consistent with the fact that FhuA is reliant on TonB for energy transduction. There is one group of bacteriophage known as T5 bacteriophage, which utilize the FhuA receptor but have been shown to be independent of TonB (Rabsch et al. 2007). The reasons for this independence are not well characterized, but it may explain why six bacteriophage appeared to be reliant only on FhuA.

Through testing bacteriophage against E. coli strains with gene knockouts of the TonB system and FepA and recording whether a zone of clearing occurred, I was able to quantify the relationship between TonB and FepA within my sample. Based on the patterns formed by negative and positive results of bacteriophage testing, the calculated relationship between TonB and FepA in this sample did not appear to be statistically significant. Other studies have demonstrated that comparatively few bacteriophage utilize FepA, and this was consistent in my results. In fact, in my sample of bacteriophage, only one demonstrated reliance on the FepA protein. Therefore, this relationship is likely not representative of the relationship between FepA and TonB as a whole. Since FepA relies on TonB for energy transduction, a larger sample would be expected to demonstrate a statistical relationship like that of TonB and FhuA. There was no statistically

significant relationship shown between FepA and FhuA, although the small number of FepA reliant bacteriophage remains an issue.

Although none of the bacteriophage seemed to be fully reliant on the TolA receptor system, several formed larger, more concentrated plaques when the receptor was present. There are several possibilities for why this occurs. It is possible that there are bacteriophage within the sample which have developed a gain-of-function mutation which allows them to utilize a different receptor to cross the membrane. This would allow these bacteriophage to cross the membrane despite the deletion of a gene which would have otherwise been required. It is also possible that the limited plaque formation is due to a stress-response mechanism carried out by the Rcs System. The Rcs system is a phosphorelay mechanism which is activated in response to a disruption of the outer membrane or peptidoglycan layer of the periplasm (Macquarie 2010). The system is composed of three proteins, RcsD, RcsC, and RcsB. In response to stress, two of these proteins, RscC and RcsD, act as phosphatases to phosphorylate RcsB (Meng et al. 2021). Once phosphorylated, RcsB regulates the transcription of several genes associated with the synthesis of protective secretions which change the surface composition of the cell (Macquarie 2010). These secretions may impact the ability of bacteriophage to utilize the TolA protein to cross the membrane.

Appendix A: Tables

Phage	W3110	ΔTolA	Phage	W3110	ΔTolA	Phage	W3110	ΔTolA
IVA	POS	POS	L1	POS	POS	PB05	POS	POS
STU	POS	POS	L2	POS	POS	PB45	POS	POS
FER	POS	POS	M1	POS	POS	AR3	NEG	NEG
L2	POS	POS	S1	POS	POS	S2	POS	POS
NIC	POS	POS	EA4	POS	POS	C1	POS	POS
L3	POS	POS	EA	POS	POS	C2	POS	POS
R1	POS	POS	ECV55	POS	POS	C3	POS	NEG
L1	POS	POS	UE	POS	POS	C4	POS	POS
DES	POS	POS	EV	POS	POS	C5	POS	NEG
CAJ	POS	POS	PB	POS	NEG	C6	POS	POS
SAI	POS	POS	AB	POS	POS	C7	POS	POS
R4	POS	POS	Ar2	POS	POS	C8	POS	POS
R2	POS	POS	Ar3	POS	NEG	C9	POS	POS
CAL	POS	POS	PC52	POS	POS	C10	POS	POS
BEL	POS	POS	VS52	POS	POS	C11	POS	POS
LIL	POS	POS	AR52	POS	POS	C12	POS	POS
R3	POS	POS	PC4	POS	POS	C13	POS	POS
WES	POS	POS	US35	POS	POS	C14	POS	POS
YZE	POS	POS	US352	POS	POS	C15	NEG	NEG
CAB	POS	POS	US4L	POS	POS	C16	NEG	NEG
VEN	POS	POS	PB4L	POS	POS	C18	POS	POS

Figure 1: TolA Testing Results (POS indicates formation of zone of clearing, NEG indicated no zone of clearing)

Picked Phage	W3110	ΔTolA
3P	POS	POS (Low Concentration)
5P	POS	POS (Low Concentration)
Ar3P	POS	POS

Figure 2: Results of Testing with Picked Plaques

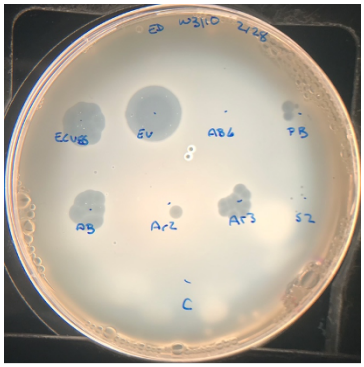
Phage	W3110	FhuA	FepA	TonB	Phage	W3110	FhuA	FepA	TonB	Phage	W3110	FhuA	FepA	TonB	Phage	W3110	FhuA	FepA	TonB
1	POS	NEG	POS	POS	R2	POS	POS	POS	POS	UE	POS	NEG	POS	POS	EC1	POS	POS	POS	POS
2	POS	POS	POS	NEG	CAL	POS	POS	POS	POS	EV	POS	POS	POS	POS	EC2	POS	NEG	POS	NEG
3	POS	POS	POS	POS	BEL	POS	POS	POS	POS	AB6	POS	POS	POS	POS	3ML	POS	NEG	POS	POS
4	POS	POS	POS	POS	LIL	POS	POS	POS	POS	PB	POS	POS	POS	POS	1S5	POS	POS	POS	POS
5	POS	POS	POS	POS	R3	POS	POS	POS	POS	AB	POS	NEG	POS	POS	MU	POS	NEG	POS	NEG
6	POS	NEG	POS	POS	WES	POS	POS	POS	POS	Ar2	POS	POS	POS	POS	TS1	POS	POS	POS	POS
7	POS	POS	POS	POS	YZE	POS	POS	POS	POS	Ar3	POS	POS	POS	POS	EC3	POS	POS	POS	POS
8	POS	POS	POS	POS	CAB	POS	POS	POS	POS	PC52	POS	POS	POS	POS	C1	POS	POS	POS	POS
9	POS	POS	POS	POS	VEN	POS	POS	POS	POS	VS52	POS	POS	POS	POS	EC4	POS	POS	POS	POS
10	POS	POS	POS	POS	BG120	POS	POS	POS	POS	AR52	POS	NEG	POS	POS	PB1 (1)	POS	POS	POS	POS
11	POS	POS	POS	POS	PC120	POS	POS	POS	POS	PC4	POS	NEG	POS	POS	AB1 (2)	POS	POS	POS	POS
12	POS	NEG	POS	NEG	US1/20	POS	POS	POS	POS	US35	POS	NEG	POS	POS	US1 (3)	POS	POS	POS	POS
13	POS	POS	POS	NEG	PC1/20	POS	POS	POS	POS	US352	POS	NEG	POS	POS	PC1 (4)	POS	POS	POS	POS
14	POS	POS	POS	NEG	BG1/9	POS	NEG	POS	NEG	US4L	POS	NEG	POS	POS	5	POS	POS	POS	POS
HL1C	POS	POS	POS	POS	AB1/9	POS	NEG	POS	NEG	PB4L	POS	NEG	POS	POS	6	POS	POS	POS	POS
H8	POS	POS	NEG	POS	PC26	POS	POS	POS	POS	PBO5	POS	POS	POS	NEG	7	POS	POS	POS	POS
HRIE	POS	NEG	POS	POS	PB1/9	POS	POS	POS	POS	PB45	POS	NEG	POS	POS	8	POS	POS	POS	POS
T5	POS	POS	POS	POS	BG1/2	POS	POS	POS	POS	AR3	POS	POS	POS	NEG					
IVA	POS	POS	POS	POS	AC1/2	POS	POS	POS	POS	S2	POS	NEG	POS	NEG					
STU	POS	POS	POS	POS	PB1/2	POS	POS	POS	POS	1ST	POS	POS	POS	POS					
FER	POS	POS	POS	POS	PB12/26	POS	NEG	POS	NEG	2ST	POS	POS	POS	POS					
L2	POS	POS	POS	POS	BG1/18	POS	POS	POS	POS	4SL	POS	NEG	POS	NEG					
NIC	POS	POS	POS	POS	AB12/26	POS	NEG	POS	POS	L	POS	POS	POS	POS					
L3	POS	POS	POS	POS	L1*	POS	POS	POS	POS	L1**	POS	POS	POS	POS					
R1	POS	POS	POS	POS	L2*	POS	POS	POS	POS	1S5	POS	NEG	POS	POS					
L1	POS	POS	POS	POS	M1	POS	POS	POS	POS	1S5	POS	NEG	POS	NEG					
DES	POS	POS	POS	POS	S1	POS	POS	POS	NEG	PW	POS	pOS	POS	pOS					
CAJ	POS	POS	POS	POS	EA4	POS	POS	POS	POS	SBM	POS	NEG	POS	POS					
SAI	POS	POS	POS	POS	EA	POS	POS	POS	POS	BY61	POS	POS	POS	POS					
R4	POS	POS	POS	POS	ECV55	POS	NEG	POS	POS	RW1	POS	POS	POS	POS					

Figure 3: Table of FhuA, FepA, TonB Phage Testing Results

		FhuA		
		Positive	Negative	Total
TonB	Positive	75	17	92
	Negative	6	9	15
	Total	81	26	107

Figure 4: Contingency Table of FhuA and TonB, p = .0015

Appendix B: Photographs



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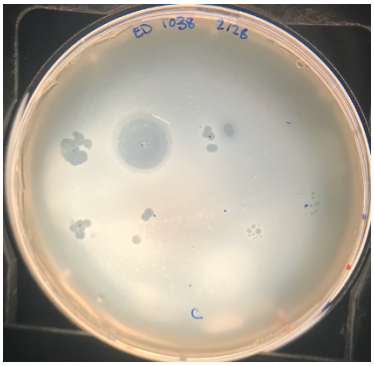
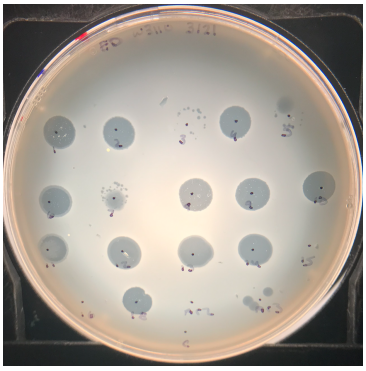


ΔTolA



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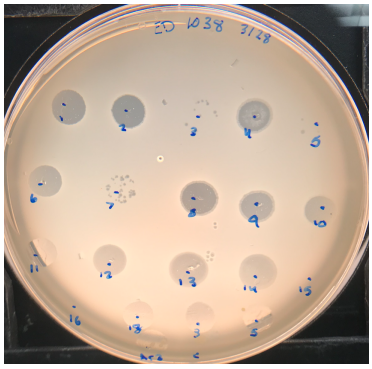

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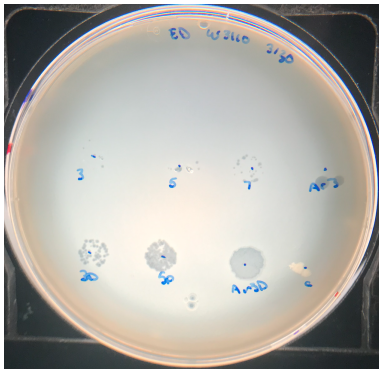

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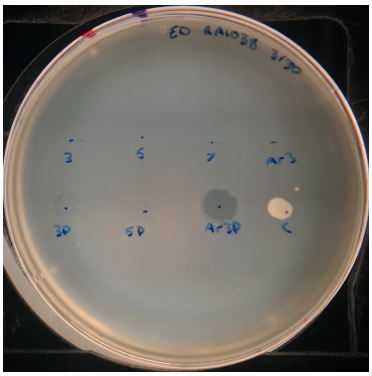

ΔtolA

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