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Development of an EcI5 Group IIB Intron mutagenesis system for use in Chlamydia trachomatis

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A thesis submitted to the University Honors Program for partial fulfillment of the requirements for the Honor's Certificate with Thesis

Approved by

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I. Acknowledgements

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II. Introduction

Chlamydia trachomatis is an obligate, intracellular pathogen that causes the ocular infection known as trachoma and the Sexually Transmitted Infection (STI) known as chlamydia. *C. trachomatis* is the leading cause of preventable blindness, primarily in developing nations, and is also the most common cause of reportable, bacterial sexually transmitted infections both in the United States and world-wide [1]. *C. trachomatis* caused over 1.7 million reported cases in the United States in 2018, but including unreported cases, it is estimated that the total amount of infections reached over 2.86 million [1]. Importantly, two-thirds of chlamydia cases are asymptomatic, making it difficult to reduce transmission and to prevent people from developing the sequalae associated with chlamydia. When chlamydial infections go untreated in women, the severity of the effects increase and can lead to serious diseases such as pelvic inflammatory disease, cervical cancer in conjunction with HPV infection, ectopic pregnancies, and infertility [1]. The large number of asymptomatic infections and overall known disease burden creates an urgent need for prevention methods such as vaccination. Development of vaccines requires a detailed understanding of how a pathogen causes disease [11]. However, *Chlamydia's* pathogenic mechanisms are poorly understood, and genetic methods to discover these processes are limited and cumbersome [4].

C. trachomatis has a unique developmental cycle in which it converts between two structurally and functionally different forms (Fig 1)[3]. When an infection first begins, *C. trachomatis* is in an infectious form known as the Elementary Body (EB). The EB form utilizes a type 3 secretion system that allows for the bacterium to enter the epithelial cell

leading to formation of a vacuole, called an inclusion, derived from the host's cellular membrane. Within the inclusion, *C. trachomatis* then converts into the larger Reticulate Body (RB) form. The RB form is the replicative form that allows for bacterial growth and division. Within the inclusion, RBs will differentiate into the EB form and be released to infect more cells. If the bacteria are introduced to stressful conditions such as antibiotic treatment or host immune factors such as IFN-γ, they can enter a stage with low activity called persistence to ensure survival within the cell [12]. The methods and regulatory functions necessary to covert between forms and to allow for persistence to occur is not well known. In addition, growth in the host requires *Chlamydia* to manipulate the host cell to provide nutrients for the growing bacterial population while attempting to subvert the host's immune response to avoid bacterial clearance [14]. As these collective steps are critical for pathogenesis, a molecular understanding of these processes could give rise to improved therapeutics or construction of live-attenuated vaccine strains.

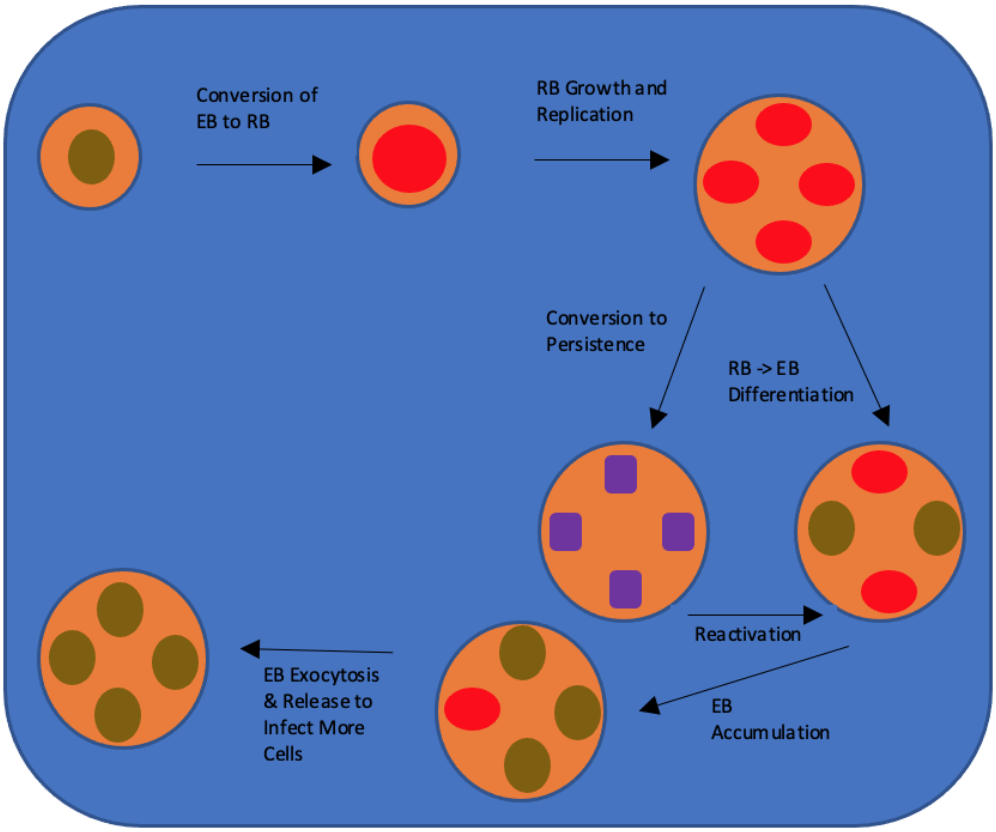

Figure 1. The Biphasic Developmental Cycle of *Chlamydia trachomatis*. *C. trachomatis* differentiates between two distinct forms. The Elementary Body (EB, red) constitutes the infectious form and allows for the initial infection of a cell and formation of the inclusion. Within the inclusion, the EB form coverts into the Reticulate Body (RB, green) which allows for replication within the inclusion. After replications occur, the RBs will convert back to EBs to exit the cell and continue infecting new cells and new hosts.

A classical approach to delineate pathogenic mechanisms important for infections caused by *C. trachomatis* and other pathogens is to genetically modify the

bacterium through gene inactivation. By removing the ability of a gene to function, we can discover whether the gene is essential for disease and/or its role in bacterial growth and virulence, and thus determine which genes would be good candidates for vaccine or antibiotic development. However, for *C. trachomatis*, only a limited and difficult to employ genetic tool kit exists, hindering our ability to study pathogenesis [4].

Thus, a goal of my project was to develop an improved mutagenesis system that creates gene-insertion mutants by utilizing a piece of DNA known as an intron. The current TargeTron mutagenesis system used in the field is based on the Ll.LtrB group II intron from *Lactococcus lacti*s [5][6]. It was adapted by our lab for use in *C. trachomatis* [7]. Unfortunately, the mutation efficiency of the system is very low, making it difficult to create mutants in a timely manner. At its best, the Ll.LtrB system has an efficiency of 10^{-6}% when measured in *Escherichia coli* (Fisher lab, unpublished). My project focused on developing an alternative intron mutagenesis system for *Chlamydia* based on the EcI5 intron from *E. coli* (Fig 2)[2]. We hypothesized that this system would be an improvement in *Chlamydia* over the Ll.LtrB system because EcI5 functions best at 37°C, the optimal growth temperature for *C. trachomatis*, and because preliminary data from our lab has shown an increase in efficiency using the EcI5 intron in comparison to the Ll.LtrB system in *E. coli*. The inability to grow *Chlamydia* under axenic conditions makes it difficult to calculate efficiency, so we use *E. coli* as a surrogate system. My main aim was to test whether the EcI5 intron will continue to function better than the Ll.LtrB intron in *E. coli* after insertion of antibiotic resistance cassettes into the intron, and whether the EcI5 intron will be more efficient than the Ll.LtrB intron in *C. trachomatis.* Antibiotic selection is often essential for identifying mutant bacteria if the

system is of low efficiency or the bacterium cannot be grown under axenic conditions, which hinders isolation of clones. A minor aim of my project was to determine the “rules” of EcI5 cargo carrying to aid in use of the EcI5 system with *C. trachomatis* and other bacteria.

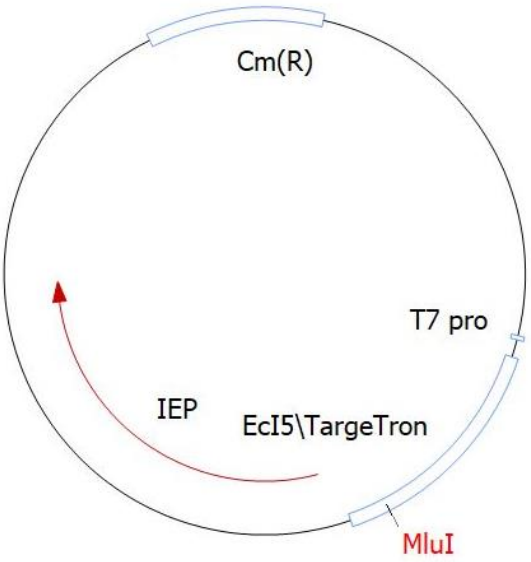

Figure 2. EcI5 Vector Map. The EcI5 intron is shown on a plasmid with intron expression controlled by the T7 promoter. The IEP region is located downstream of the intron. Resistance genes were placed at the MluI restriction enzyme site, which is where the IEP would be present in the wild type intron isolated from *E. coli*.

The EcI5 intron is able to find targeted sequences within a genome by utilizing the associated Intron Encoded Protein (IEP) in conjunction with sequence information in the 5’ region of the intron. It is the 5’ “targeting” sequence that researchers modify to target the intron to different genes. Once the EcI5 intron and its IEP have been transcribed and the IEP open reading frame has been translated, a lariat is formed that

contains a protein part and an RNA part, known as a ribonucleoprotein [9]. The RNA portion is used to scan for targeted insertion sites within the genome via RNA-DNA base pairing. Once the binding site is found, the endonuclease activity of the IEP cuts both strands of the double stranded DNA target. Two more enzyme activities of the IEP, RNase and reverse transcriptase activities, allow for the insertion of the intron RNA into the cut area followed by conversion of the RNA to DNA. Once the intron is in the single stranded DNA form, the host cell's replication system can replicate the intron DNA generating double stranded DNA containing the intron-disrupted gene (Fig 3).

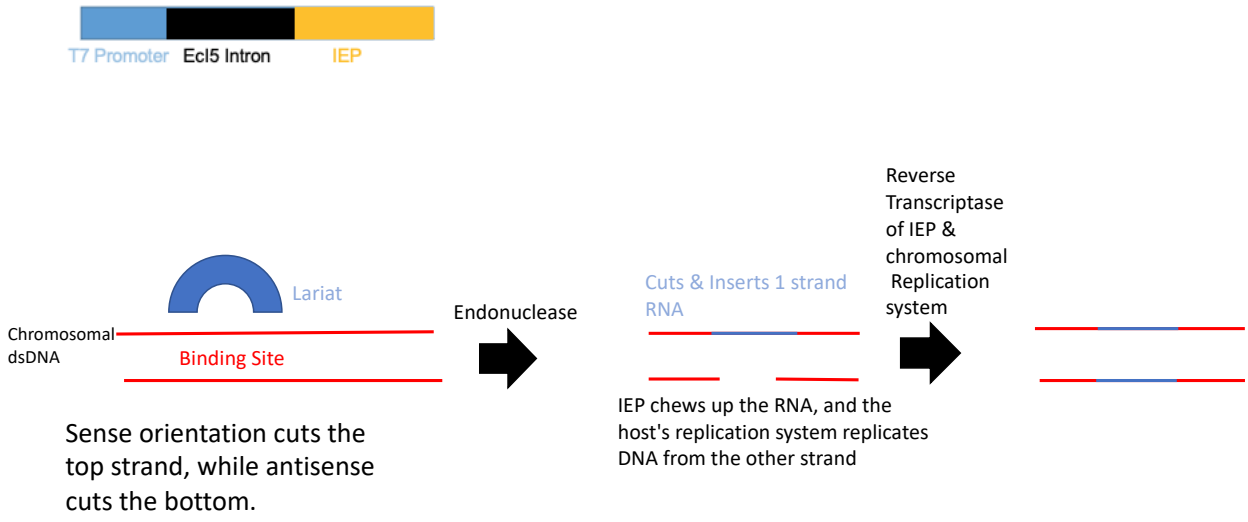

Figure 3. EcI5 mobility. EcI5 intron transcription is induced and EcI5 and intron encoded protein (IEP) transcripts are produced. The IEP transcript is then translated, and the IEP protein processes the EcI5 RNA to form a ribonucleoprotein lariat. The RNA portion has a 5' located targeting sequence that scans the host chromosome for its unique insertion site. The IEP then provides endonuclease, reverse transcriptase, and RNase activities to mediate intron insertion.

III. Results

We first confirmed that the wild-type EcI5 system performed at the high efficiencies reported by Zhuang *et al*. [2]. The intron was targeted to the *lacZ* gene (encodes β-galactosidase) to allow for blue (wild type) -white (insertion mutant) colony screening on LB agar plates with the β-galactosidase colorimetric substrate X-gal. The 1806-1807 *lacZ* insertion site was chosen for targeting, which should give rise to a sense intron insertion (http://www.targetrons.com/lacZ-EcI5-results.txt). Insertion efficiencies determined by counting white versus blue colonies were routinely above 70% (Table 1). Insertions were confirmed by performing PCR on randomly selected white colonies (Fig 4).

Next, we sought to determine the "rules" of the EcI5 mutagenesis system through the use of alternative intron designs and induction protocols. We selected the *aadA* (spectinomycin resistance), *bla* (ampicillin resistance), and *cat* (chloramphenicol resistance) genes for our study due to previous validation of these markers in the Ll.LtrB system with *C. trachomatis* or *Chlamydia caviae* [7][8][15]. All markers were either restriction digested or PCR-amplified from the pDFTT-Ll.LtrB vectors and ligated into the MluI digested lacZ-targeted EcI5 vector. Plasmids were selected that carried the genes in sense or anti-sense orientations to the intron and transformed into the *E. coli* BL21(de3) expression strain for intron mobility studies. The vector constructs are shown in Fig 5.

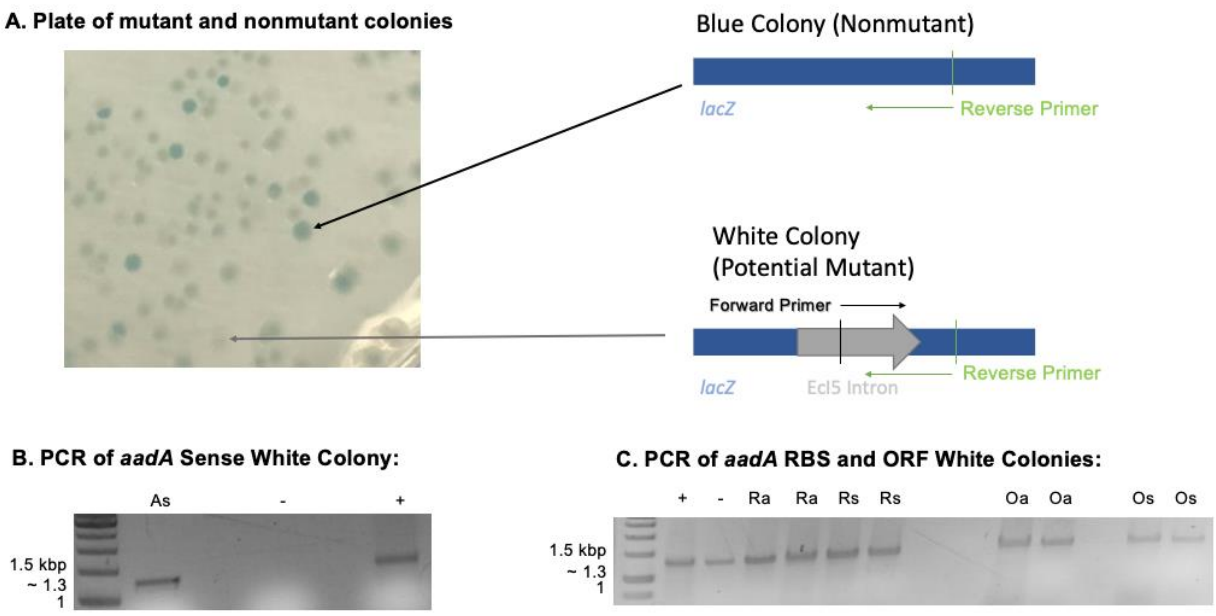

Figure 4: PCR analysis of potential mutant colonies. PCR was performed using a *lacZ* primer and an intron primer to assess intron insertion. PCR products were resolved on 0.8% agarose gels, stained with ethidium bromide, and visualized using UV light. **(A)** A plate from the EcI5 positive control group was checked for white and blue colonies. White colonies have the intron insertion, while blue colonies have an uninterrupted *lacZ* gene. **(B)** One white colony from the *aadA* sense construct grown under the 3 hour + 1.0 mM IPTG condition was tested for EcI5 intron insertion. The 1.3 kbp product supported that intron insertion occurred. **(C)** White colonies from the *aadA* RBS and ORF constructs grown under the 3 hour + 0.1mM IPTG were also tested for EcI5 intron insertion. The results came back positive with bands at 1.3 kbp, matching the expected size of the insertion product. Molecular weight markers are shown to the left of each gel in **(B)** and **(C)**.

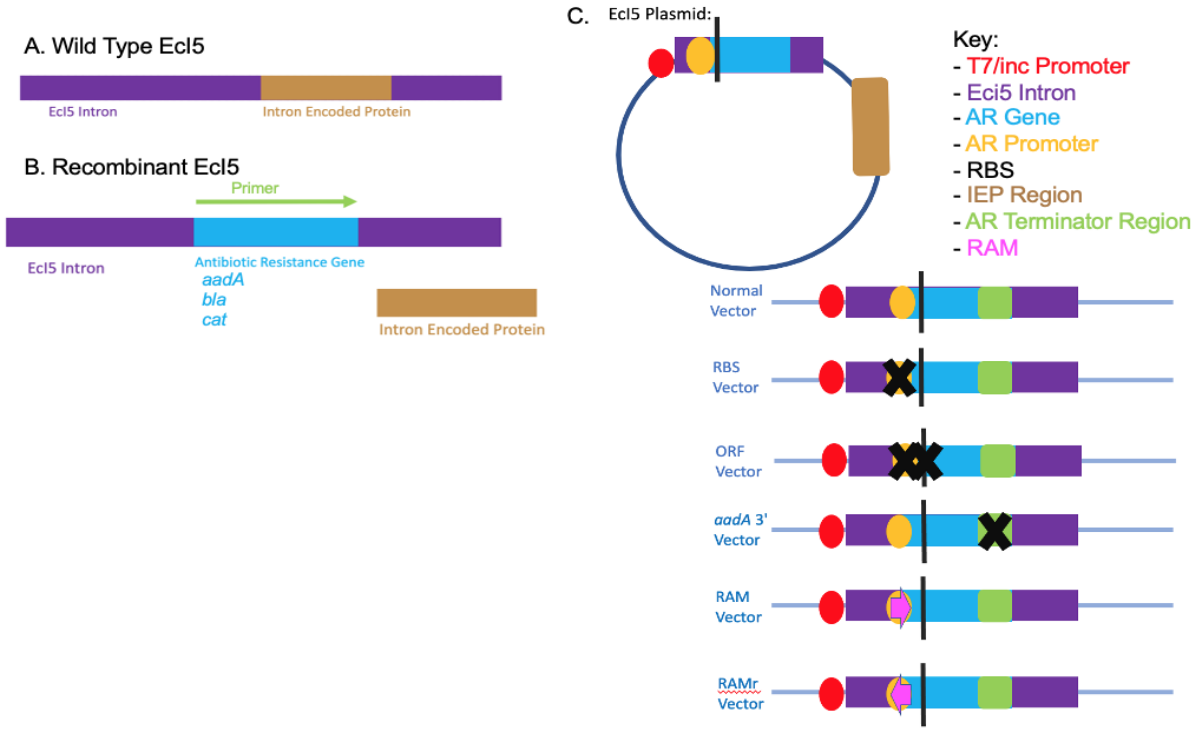

Figure 5. EcI5 constructs. (A) Diagram of the wild type EcI5 intron with the IEP encoded within the intron. **(B)** The recombinant EcI5 mutants encoding an antibiotic resistance gene in place of the IEP region. The IEP is cis encoded downstream of the intron. **(C)** This figure illustrates the *aadA* RBS, *aadA* ORF, the *aadA*-3', RAM, and RAMr constructs. The ORF construct is promoter-less and does not contain an RBS. The RBS construct is promoter-less but contains the RBS.

We found that the EcI5 vectors encoding the full *aadA* and *bla* antibiotic resistance genes (promoter, ribosomal binding site, open reading frame, and terminator sequence) within the intron at the MluI site produced *lacZ*-insertion efficiency rates that were under 1% or were below our limit of detection of 0.01% (Table 1). These efficiencies were well below the non-antibiotic carrying intron, which inserted at an efficiency of ~70% (Table 1). Note that the MluI site corresponds to where the EcI5 intron would have carried the

intron-encoded protein (IEP, Fig 2). For mutagenesis purposes, the IEP is encoded downstream from the intron. Note that the IEP would not possess a promoter sequence when encoded within the EcI5 as expression would rely on the intron promoter. For the mutagenesis system, the intron promoter is lost upon gene intron insertion into the chromosome and any cargo within the intron would need its own promoter for expression.

Since insertion efficiencies were low with the resistance cassette constructs, we next varied the incubation intervals and inducer concentrations to determine if growth and induction conditions affected insertion efficiency. In one trial, we produced one mutant colony from the *aadA* sense construct after a 3-hour incubation condition with 1.0 mM IPTG (inducer), suggesting that the sense orientation might be preferred (Table 1, Fig 4). This result also supports that the efficiency rates of the EcI5 mutagenesis system could represent a large improvement over the previous Ll.LtrB system, which had a maximum insertion efficiency of 10^{-6}%. We also tested a smaller antibiotic cassette, the *cat* gene, to see if cargo size (975 bp *cat*, 1.2 kbp *bla*, 1.3 kbp *aadA*) was negatively impacting mobility. This construct did not make a difference in insertion efficiency as rates were still less than 2.9^{-2}%. During these experiments, we observed that growth of the recombinant *E. coli* strains was reduced with high levels of IPTG (1.0 mM IPTG) and that all of the strains tested seemed to grow best with the 3-hour incubation time, so we made the 3 hour incubation standard for the rest of the trials using the modified resistance cassettes.

Table 1. Insertion efficiency assay results for *aadA* (sense and antisense), *bla* (sense), and *cat* (sense and antisense) vectors

3hr + 0.1mM IPTG[a]

Resistance Marker	Test Colonies (white/total)	Insertion efficiency (Antibiotic Resistance Marker)
bla (sense)	<1/1460	<0.0685%
aadA (anti)	<1/3615	<0.0277%
aadA (sense)	<1/1872	<0.0534%
cat (anti)	<1/2458	<0.0407%
cat (sense)	<1/4074	<0.0245%
EcI5	1397/1948	71.7%

3hr +1.0mM IPTG

Resistance Marker	Test Colonies (white/total)	Insertion efficiency (Antibiotic Resistance Marker)
bla (sense)	<1/1488	<0.0672%
aadA (anti)	<1/3224	<0.0310%
aadA (sense)	1/743	0.135%
cat (anti)	<1/3686	<0.0271%
cat (sense)	<1/3678	<0.0272%
EcI5	357/1292	27.6%

6hr + 0.1mM IPTG

Resistance Marker	Colonies (white/total)	Insertion Efficiency
EcI5	333/347	96.0%
bla (sense)	<1/1219	<0.0820%
aadA (sense)	<1/1129	<0.0886%

6hr + 1.0mM IPTG

Resistance Marker	Trial 1 (white/total)	Insertion Efficiency
EcI5	17/18	94.4%
bla (sense)	<1/78	<1.28%
aadA (sense)	<1/621	<0.16%

Overnight + 0.1mM IPTG

Resistance Marker	Colonies (white/total)	Insertion Efficiency
EcI5	407/410	99.3%
bla (sense)	<1/1272	<0.0786%
aadA (sense)	<1/2293	<0.0436%

[a]White and blue colonies were counted after 24 hours of incubation. Experiments were performed at least 3 times. Data reflects the sum of all colonies from the multiple experiments. Experiments are grouped by induction conditions. Only a single white colony was obtained using the recombinant introns.

As the IEP would not possess an internal promoter in the wild type intron, we hypothesized that the resistance gene promoters might be interfering with intron mobility. To test our hypothesis, we developed new constructs lacking the promoter (*aadA* RBS), both the promoter and RBS (*aadA* ORF), or the 3' region (*aadA*-3, data not shown) of the antibiotic resistance gene. We used the sense orientation of the *aadA* resistance gene because we had seen one positive mutant colony in our previous results (Fig 4). All constructs were grown for 3 hours with 0.1 mM IPTG or 1.0 mM IPTG. The ORF and RBS constructs produced mutant colonies (Fig 4), and the insertion efficiency levels began to approach the efficiency levels of the wild type EcI5 intron

lacking a resistance gene insert (Fig 6). The *aadA*-3 construct, which still encoded the promoter and RBS, yielded no white colonies (efficiency <0.24%, personal communication from Derek Fisher). These results suggested that there might be promoter competition between the intron promoter and the antibiotic resistance gene promoter leading to low expression of the full-length intron.

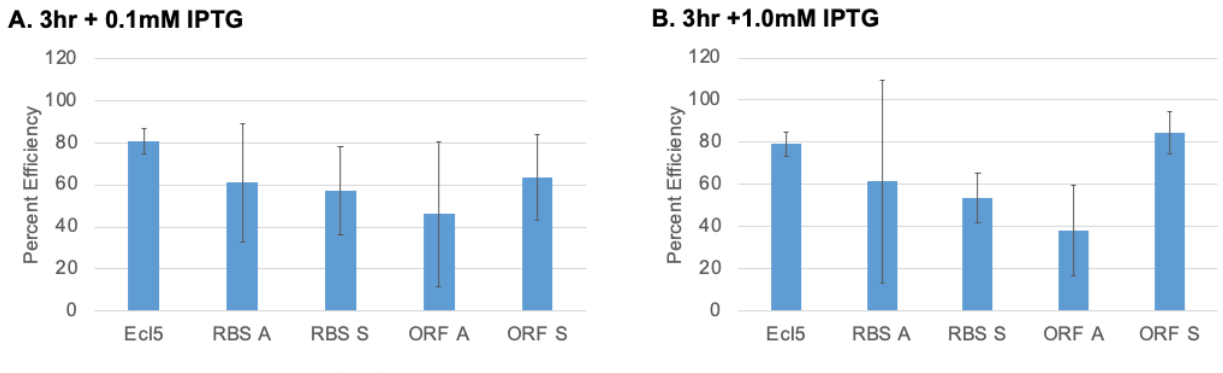

Figure 6. Insertion efficiency assays of *aadA* RBS (sense and antisense) and *aadA* ORF (sense and antisense). (A) White colonies were seen for each construct under 3 hours + 0.1mM IPTG. The number of white colonies per total colonies was used to calculate insertion efficiency. **(B)** White colonies were also observed for each construct under 3 hours + 1.0 mM IPTG.

To attempt to block promoter competition, we decided to insert a Retrotransposable Activated Marker (RAM) within the promoter region. A RAM cassette is a 400-base pair, type I intron that has the ability to self-splice out of a region of RNA upon transcription [10]. In our system, we placed the RAM cassette between the -35 and -10 regions of the *bla* antibiotic resistance gene promoter. The *bla* gene was selected rather than the *aadA* gene as the *bla* promoter is well characterized. We hypothesized that the RAM

intron would cause the promoter region to be unrecognizable within the DNA allowing for unfettered transcription from the intron promoter. After transcription, the RAM should self-splice reconstituting function of the antibiotic resistance gene. We also wanted to determine if the orientation of the antibiotic resistance gene in reference to the RAM cassette played a role in insertion efficiency. We utilized the *bla* antibiotic resistance gene in the antisense orientation with the RAM cassette inserted into its respective promoter region. Neither use of the RAM cassette or RAM orientation rescued intron insertion efficiency (Tables 2 and 3).

Table 2. Insertion efficiency assay results for RAM vector.

A. 3hr + 0.1mM IPTG

Resistance Marker	Trial #1 (white/ total)	Trial #2 (white/ total)	Average Insertion Efficiency
RAM	<1/1362	<1/1113	<0.0404%
EcI5	118/223	106/192	54.0%

B. 3hr +1.0mM IPTG

Resistance Marker	Trial #1 (white/ total)	Trial #2 (white/ total)	Average Insertion Efficiency
RAM	<1/27	<1/131	<0.633%
EcI5	18/29	15/19	68.8%

Table 3. Insertion efficiency assay results for RAMr vector.

A. 3hr + 0.1mM IPTG

Resistance Marker	Trial #1 (white/ total)	Trial #2 (white/ total)	Average Insertion Efficiency
RAMr	<1/1941	<1/2112	<0.0247%
EcI5	73/111	195/283	68.0%

B. 3hr +1.0mM IPTG

Resistance Marker	Trial #1 (white/ total)	Trial #2 (white/ total)	Average Insertion Efficiency
RAMr	<1/218	<1/197	<0.0241%
EcI5	22/22	25/25	100%

IV. Discussion

Current methods for mutagenesis in *C. trachomatis* include our intron approach, transposon mutagenesis, allelic exchange, chemical mutagenesis, and TILLING [7][13] [16][17][18]. While useful, all of these approaches have limitations, and none have been uniformly adopted across the field. In this study, our goal was to create a new intron mutagenesis system for *Chlamydia* based on the EcI5 intron from *E. coli*. Although we were not able to create a system that was functional and provided a means of selection within *Chlamydia*, we were able to discover rules associated with the EcI5 system.

The study began by developing the *bla* (sense), *aadA* (sense & antisense), and *cat* (sense & antisense) vectors by utilizing restriction enzyme digestion at the MluI site on the plasmid. We then tested these vectors for intron mobility at various induction periods and concentrations of the inducing agent, IPTG, within *E. coli*. We found that the constructs with antibiotic resistance markers had insertion efficiency rates that were significantly lower than the construct with the wild type intron, as we were unable to detect mutants with the exception of one trial. This one success in *E. coli* was still a drastic improvement over the LI.LtrB system. The results also show an ~10^6-fold improvement in insertion efficiency with the marker-less EcI5 over the LI.LtrB intron in *E. coli*. Upon the addition of resistance genes, the insertion efficiency was significantly reduced, regardless of orientation. This suggests that there could be competition between the resistance gene promoter and the intron promoter. Alternatively, placing genes within the IEP region of the intron might cause issues with lariat folding, also resulting in decreased efficiency.

In order to determine the cause of the decrease in efficiency, we created constructs that removed either the promoter regions, ribosomal binding site, or both areas within the antibiotic resistance gene in the intron. Like the previous constructs, these were also made by utilizing restriction enzyme digestion at the MluI site on the plasmid. Vectors were then tested at the two testing conditions that worked best in the original experiments, 3hr + 0.1mM IPTG & 3hr + 1.0 mM IPTG. We found that the promoter-less construct (*aadA* RBS) and a construct without a promoter or RBS (*aadA* ORF) had efficiency rates close to the wild type levels suggesting that a smaller insert and/or lack of a promoter within the IEP region was preferred. We also assessed the impact on mobility of sequences 3' to the antibiotic gene ORF. Removal of the 3' sequence did not result in improved efficiency. Note that the 3' constructs still encoded the antibiotic gene promoter and RBS. Overall, the results suggest that including the promoter region significantly reduces insertion efficiency. Although the EcI5 intron system appears more efficient than LI.LtrB with the *aadA* ORF and *aadA* RBS constructs, lacking a promoter for the antibiotic resistance gene would cause problems in the future when attempting to select for mutants in *C. trachomatis*.

Finally, we attempted to transiently inactivate the promoter region of the antibiotic resistance gene until after transcription of the intron and IEP has occurred. To do this, we utilized a Retrotransposable Activated Marker that was inserted to interrupt the promoter region of the resistance gene, making it unrecognizable to the RNA polymerase during transcription from the intron-carrying plasmid. Following transcription, the RAM cassette should self-splice out of the RNA and allow for the

promoter to be recognizable again after the intron inserts into the targeted gene, enabling the selection of mutants. However, our results showed that we were unable to detect any mutants with this construct, suggesting that either the promoter being present following transcription still posed a problem or that the RAM cassette was not functioning as predicted.

Based on the results of this study, we can conclude that the EcI5 group IIB intron mutagenesis system does provide a higher insertion efficiency than the previously used Ll.LtrB *L. lactis* system when used in *E. coli*. However, insertion of a second promoter within the intron drastically decreases insertion efficiency. It should be noted that the Ll.LtrB intron used for comparison to the EcI5 intron carries a kan-RAM cassette, which could impact insertion efficieny. Future work should focus on further developing and improving upon the RAM system to resolve the promoter competition issue. We should also utilize the ORF construct previously made to create mutations by placing it under the control of the targeted gene's promoter to transcribe and translate the selection marker within the intron, thus giving us selectable mutants at a higher efficiency than the previous Ll.LtrB system.

V. Methods

Strains and growth conditions:

E. coli NEB10 (New England BioLabs) was used for cloning steps and was routinely cultured at 37°C in LB broth or on LB agar plates in the presence of 20 µg/mL chloramphenicol when carrying vectors (Fig 5). Vectors were transformed into the *E. coli* BL21(de3) expression strain and grown as described in the results section for mobility experiments. LB agar plates containing 40 µg/mL X-gal and 20 µg/mL chloramphenicol were used for blue-white screening. Plates were incubated at 37°C for 24 hours prior to blue-white assessment.

Vector Construction:

The EcI5 Group IIB intron from *E. coli* is able to splice and insert itself into specific, targeted locations within bacterial chromosome with the help of an Intron-Encoded Protein (Fig 3). Our EcI5 intron was targeted to the *lacZ* gene (encoding beta-galactosidase) using the gBlock approach described in http://www.targetrons.com/lacZ-EcI5-results.txt. Targeting to this gene enabled a screening method to help identify the desired mutants. The *lacZ* encoded β-galactosidase is utilized to break down lactose, and in our case, the colorimetric substance X-gal, which leads to the production of a blue color within the colonies. Colonies that grew blue on the plate had a normal *lacZ* gene and β-galactosidase activity and could breakdown the X-gal within the media. Colonies that grew white had the EcI5 intron interrupting the *lacZ* gene and thus could not utilize the X-gal. White colonies were PCR verified to ensure that the intron had inserted into the correct location. Primers for the *lacZ*-EcI5 PCR are listed in Table 4.

Thermo Scientific PCR Master Mix was used for colony PCR reactions and the primers were used at 0.5 µM (primers were ordered from Integrated DNA Technologies).

Within the intron, we placed various antibiotic resistance markers to allow for selection of mutants. Clones were designed using CloneManager software. Cloning PCR reactions used Phusion polymerase (Thermo Scientific). Resistance markers were chosen based on their success within the *L. lactis* intron [7][8][15]. Genes or PCR products for the *bla* (sense), *aadA* (sense & antisense), *cat* (sense & antisense), *aadA* RBS (sense and antisense), *aadA* ORF (sense and antisense), and *aadA*-3 were digested with MluI and ligated into similarly digested plasmids. Ligation products were transformed into *E. coli* NEB10. To construct the *bla* RAM vector, the RAM intron was PCR amplified from pACD4-K and the *bla* vector was PCR amplified using primers with 20 bp overlaps with the RAM cassette. Both PCR products were then linked using the NEB HiFi DNA assembly as directed by the manufacturer (New England BioLabs). *E. coli* transformants were screened by PCR for insert presence and orientation relative to the intron (primers in Table 4) and plasmids were then isolated for Sanger DNA sequencing (performed by PSOMAGEN). Sequence-verified vectors were then transformed into *E. coli* BL21(de3) for intron mobility testing.

Table 4. *lacZ* detection and gene cloning primers

Gene	Forward primer	Reverse Primer	Notes
aadA	TCT*ACGCGT*TGCCTGACGATG CGTGGAG	GTA*ACGCGT*CCCGGGCCTGA TAGTTTGGCTGTGAG	MluI site in italics
bla	TCGC*ACGCGT*AGGTTAATGTC ATGATAATAATGG	GAC*ACGCGT*GTGGAACGAAA ACTCACG	MluI site in italics
cat	GCG*ACGCGT*GCTATAAATTTTT AAAAATAGCAG	CCCA*ACGCGT*AAAAGGATGG TCGTAAG	MluI site in italics
***aadA* ORF**	CAA*ACGCGT*ATGCGCTCACGC AACTGG	GTG*ACGCGT*TTATTTGCCGAC TACCTTG	MluI site in italics
***aadA* RBS**	CAA*ACGCGT*CTGTAATGCAAGT AGCGTATGCGCTCAC	GTA*ACGCGT*CCCGGGCCTGA TAGTTTGGCTGTGAG	MluI site in italics
***aadA* -3**	TCT*ACGCGT*TGCCTGACGATG CGTGGAG	GTG*ACGCGT*TTATTTGCCGAC TACCTTG	
***bla* vector**	*GACCTTATCTGAACATAATG*CT CATGAGACAATAACC	*GGCCTCAATTAACCCAAGAA*A TTTGAATGTATTTAG	overlap in italics
RAM	*TTCTTGGGTTAATTGAGGCC*TG AG	*CATTATGTTCAGATAAGGTC*G TTAATCTTAC	overlap in italics
***lacZ*-EcI5**	TGGTCTGCTGCTGCTGAACG	ATCCGGTCCATTACAGACTGG CATTCG	
UnivEcI5F	CCCCTCTAGAAGAATTCCCATG CCAAA		insert orientation PCR matched with gene primer

Mobility Assay:

The recombinant BL21(de3) strains were grown overnight in LB with 20 µg/mL chloramphenicol at 37°C. 50 µL of bacteria were then subcultured into 5 ml LB with 20 µg/mL chloramphenicol and grown until an optical density at 600nm of 0.2-0.3 was reached. 200 µL of bacteria were then subcultured in 5 mL LB with 20 µg/mL chloramphenicol and the test amount of IPTG. The subculture was grown for different time periods prior to dilution in PBS to ~1,000 CFU/ml and plating of 100 µl on LB agar plates containing 40 µg/mL X-gal and 20 µg/mL chloramphenicol. OD 600nm readings were used to estimate bacterial numbers prior to dilution (OD 600nm 1 = $5x10^8$ CFU/ml). Plates were incubated at 37°C for 24 hours prior to analysis. Colonies containing mutant bacteria would remain white while nonmutants would have no interruption of the *lacZ* gene and would be able to break down the X-gal, giving rise to blue colonies. For any white colonies, orientation PCR was performed using the forward primer, LacZ F2, and the reverse primer, UnivEcIR, to confirm the presence of the EcI5 intron. The PCR samples were run on a 0.8% agarose gel for 30 minutes, stained with ethidium bromide, and visualized with UV transillumination.

VI. References

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