BioA and *LysA*: Possible metabolic Requirements for Pathogenicity of
Shigella flexneri

by

Laura Ann Coughlin, B.S.

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Approved by
Supervising Committee:

Shelley Payne

Terry O' Halloran

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Abstract

BioA and *LysA*: Possible metabolic Requirements for Pathogenicity of *Shigella flexneri*

Laura Ann Coughlin, M.A.

The University of Texas at Austin, 2009

Supervisor: Shelley Payne

Shigella flexneri is a Gram negative facultative anaerobe that infects millions world-wide each year. The route for infection of a host is through the intestinal and rectal epithelium layers, but it also can survive in the environment. Different genes have been found to be up regulated depending upon its presence in the intracellular or extracellular environment, as shown in previous work in the lab. This thesis seeks to examine the role these upregulated genes, *bioA* and *lysA*, play in the intracellular activity of *S. flexneri*. Knock-out mutations in the *bioA* and *lysA* genes were created using P1 transduction. To test the effects of these mutations on *S. flexneri*, plaque, invasion, and attachment assays were performed. It was found that the *bioA* mutation resulted in fewer plaques being formed, while the *lysA* mutation resulted in slower forming and incompletely lysed plaques being formed.

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Introduction and Background

Shigella flexneri is a Gram negative facultative anaerobe that can invade and
replicate inside the cytosol of human colonic epithelial cells (Jennison 2003). *S. flexneri*
causes about 165 million cases of shigellosis or bacterial dysentery annually, resulting in
over a million deaths world-wide (Jennison 2003). *S. flexneri* can survive in both the
environment and the human gut. Differences between its physiology in the outside
environment versus inside the host has been an area of interest.

Pathogenesis of *S. flexneri*

S. flexneri is passed between hosts via the fecal-oral route. Infection is usually
acquired through contaminated water supplies or through hand-to-hand contact. The
pathogenesis of *S. flexneri* is linked to the damage it causes to the host by its ability to
invade and replicate inside colonic and rectal epithelial cells (Jennison 2003). This
invasion causes inflammation of the tissues and lysis of the epithelial cells lining the
colon and rectum leading to ulceration and abscess. The damage done to the colorectal
epithelial cells leads to the symptoms of shigellosis, including the passage of bloody
mucoid stools. Figure 1 diagrams the route that *S. flexneri* takes in the initial invasion of
an epithelial cell.

S. flexneri that are ingested by the host are able to survive the acidic conditions of
the stomach by regulating the expression of certain genes based on pH. An expression
profiling study was done on *S. flexneri* in acidic conditions and it was found that among
the acid resistance genes are genes that regulate energy metabolism, the RpoS-dependent

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Figure 1: Schematic diagram of the process of infection of epithelial cells with *S. flexneri* (figure from N. Davies).

S. flexneri invades epithelial cells via the basolateral membrane. In order to reach the basolateral membrane *S. flexneri* can pass across the epithelial cell layer in three ways: through membranous cells in the monolayer, by maneuvering the tight junctions between epithelial cells, or by through the junctions made by polymorphonuclear neutrophil cells as they are moving to the site of inflammation. *S. flexneri*, shown as red bars, are taken up by the membranous epithelial cells, shown in blue. The bacteria are then transcytosed to the antigen presenting cells on the basolateral side. The bacteria are phagocytozed by macrophages present in the cavity provided by the membranous epithelial cells, shown in pink. When *S. flexneri* are taken up by the macrophages, they are able to induce apoptosis of the macrophage and escape. They can then enter the epithelial cells via the basal lateral membrane and move across the cell layer intracellularly through pseudo-pinocytosis from one neighboring cell to the next (Jennison 2004).

Basolateral
Membrane

Lumen
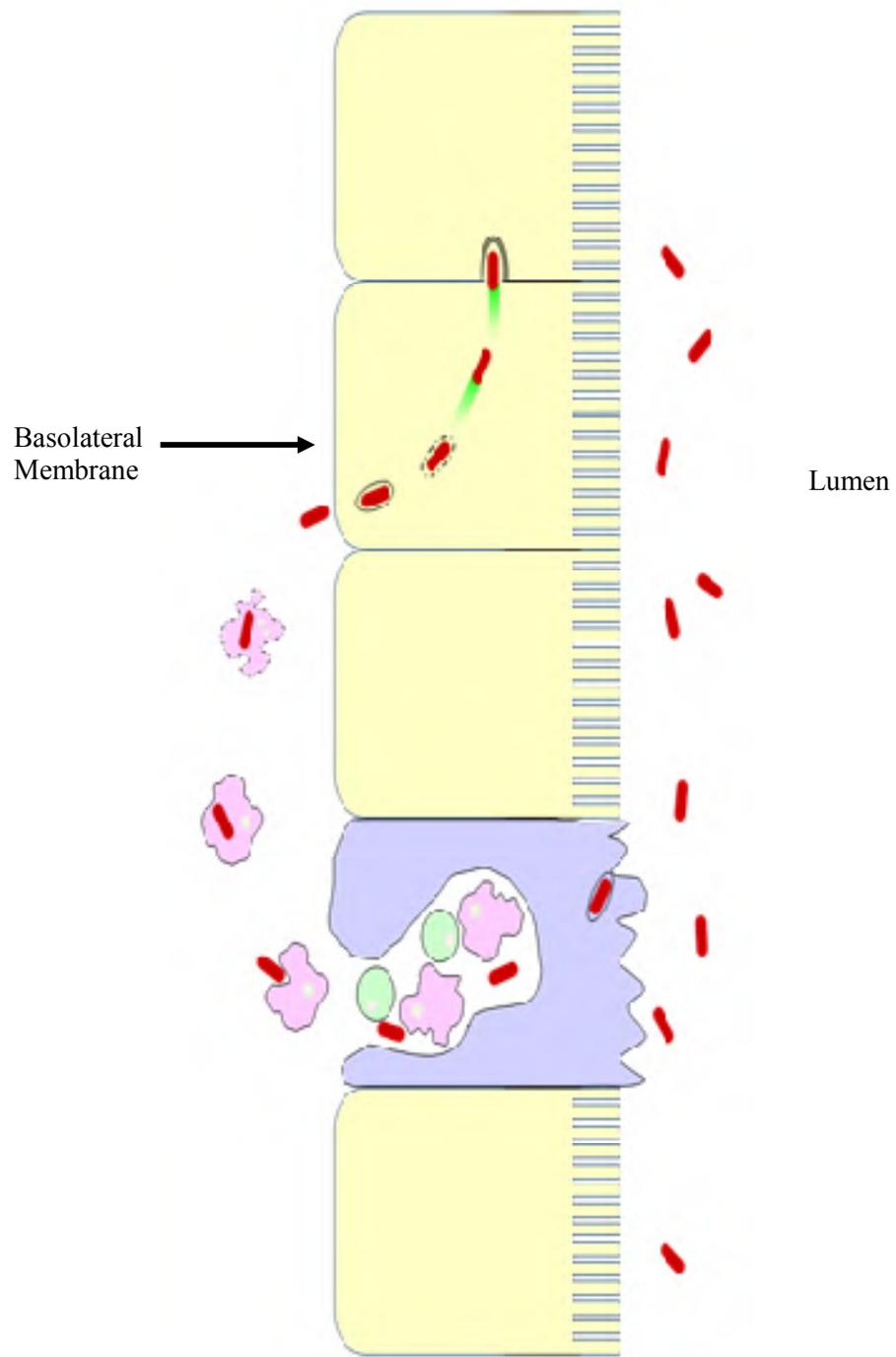
acid-resistance genes, and several stress response genes. It was also found that many virulence associated genes were down-regulated, consistent with *S. flexneri* infecting the lower gut (Cheng, et al. 2007).

Infection by *S. flexneri* requires an infectious dose as low as 100 cells partly due to the up-regulation of acid resistance genes when it reaches the host stomach (Jennison 2003). Those gene products allow *S. flexneri* to survive the acidic conditions of the host stomach in order to reach the intestinal epithelial cells. The host immune response against infection by *S. flexneri* is mediated primarily by inflammation. The inflammation response, however, can be used to the advantage of the pathogen during infection in order to spread to additional epithelial cells.

S. flexneri Invasion and Intracellular Growth

The intracellular lifecycle of *S. flexneri* has been studied and some mechanisms of infection and replication have been elucidated. *S. flexneri* is able to be taken up by the epithelial cells through clathrin-dependent endocytosis. *S. flexneri* induces endocytosis not though binding to a specific cellular receptor, but by use of a type III secretion system. The type III secretion system allows the bacteria to inject its effector proteins directly into the epithelial cell cytosol. The effector protein IpaC from *S. flexneri* interacts with the host actin cytoskeleton by inducing actin polymerization either directly or in conjunction with small GTPases. The result of this polymerization is the formation of membrane extensions that are loosely attached to the bacteria. IpaC is also able to

activate the host Abl kinase required to produce filopodial-like formation of the epithelial cell membrane around the bacteria by interaction of IpaC with Cdc24 (Veiga 2006).

S. flexneri then gains entry to the cytoplasm of the epithelial cell by escaping from the vacuole it formed during internalization. In order to break out of the vacuoles, *S. flexneri* disrupts the vacuole membrane by use of the type III secretion system in order to escape into the cytosol. The IpaB effector protein assembles with IpaC to form a pore complex that binds cholesterol. It is thought that this complex is able to bind the vacuolar membrane and disrupt the membrane through insertion, resulting in the bacterial escape into the cytosol (Ray 2008). The cycle of *S. flexneri* invading and spreading among epithelial cells is shown in Figures 2.

Figure 2: *S. flexneri* invasion and infection cycle.

A. The cycle of *S. flexneri* invasion of epithelial cells. *S. flexneri*, shown as red bars in the figure, enters the host by using their type III secretion system to induce uptake. They then secrete IpaB and IpaC shown as green circles which together form a pore complex, shown as green circles, that allows the disruption of the vacuole membrane. Once free in the cytosol, the *S. flexneri* replicates and utilizes host actin, shown as orange lines, to become motile. (Adapted from Ray 2009)

B. The cycle of *S. flexneri* spread to neighboring cells. Intracellular spread occurs when the *S. flexneri* induces the formation of a secondary vacuole. *S. flexneri* secretes the Ipa protein to form the pore complex and disrupt the vacuole membrane. The bacteria can then replicate and continue spreading between epithelial cells. (Adapted from Ray 2009)

A.
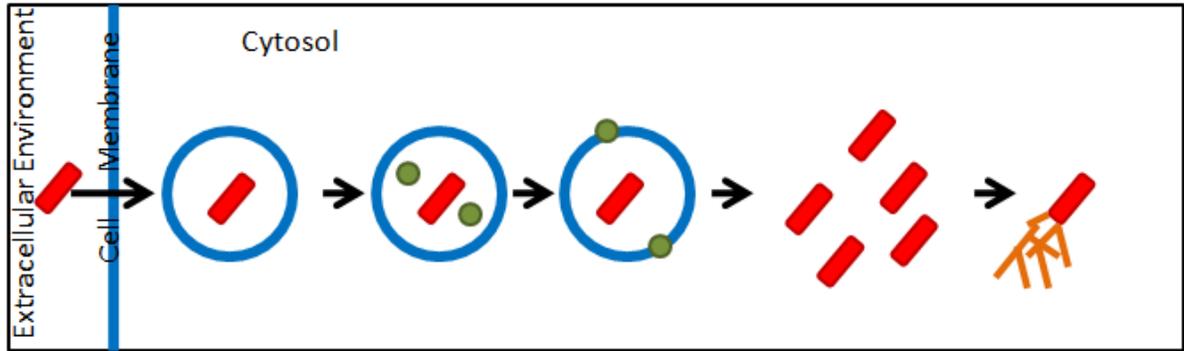
B.
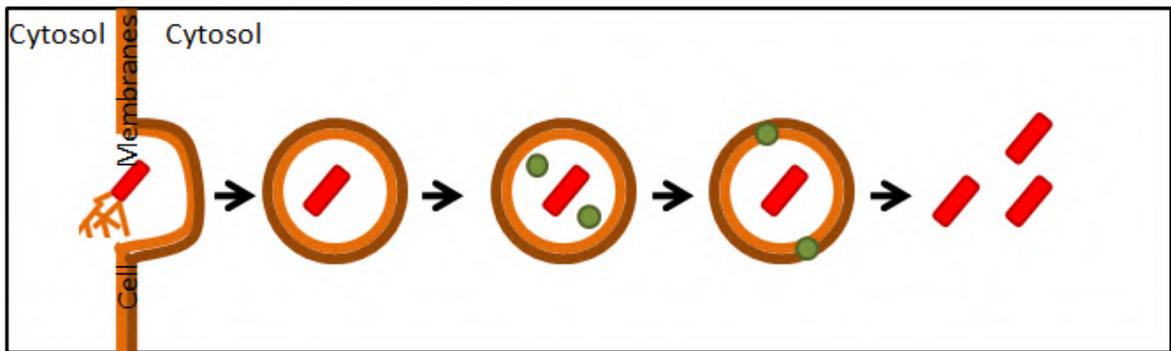
Once the *S. flexneri* is free in the cytosol, it can commence replication. In order to examine what allows some bacterial species to replicate in the cytosol while others cannot, *Escherichia coli* engineered to express an invasin protein from *Yersinia pseudotuberculosis* and to coat itself with listeriolysin O, a protein required by *Listeria* species to escape the vacuole. This allowed the normally extracellular bacteria, *E. coli*, to replicate in the cytosol (Ray, et al. 2009). This phenomenon has also been found in *Bacillus subtilis* engineered to express listeriolysin O (Monack and Theriot, 2001). However, in another study, it was found that when various bacteria were microinjected into epithelial cells, only those previously known to live and replicate in the cytosol were able to do so, while the other species including: Yersinia enterocolitica, E. coli strain 536, and B. subtilis; failed to replicate (Goetz, et al., 2001). To explain this paradox, it has been proposed that necessary changes take place while the bacteria are inside the vacuole in order to allow for cytosolic growth (Ray 2008).

While *S. flexneri* is non-motile in the environment, it polymerizes actin to gain motility within the cytosol. This actin-based motility can help facilitate the spread of the bacteria to neighboring cells (Ray 2008). The effector protein IcsA binds to tubulin oligomers to inhibit microtubule formation, while indirectly promoting polymerization of actin (Viega 2006). Actin polymerization occurs at one pole of the bacterium and provides it with propulsive force needed to move through the cytosol. Critical for actin-based motility are the bacterial outer membrane protein, VirG, and the host neural Wiskott-Aldrich syndrome protein, N-WASP. VirG and Cdc24 bind and activate N-

WASP, which in turn recruits the actin-related proteins 2 and 3, known as Arp2/3 (Yoshida 2006).

 Direction of the Research

Previous work done in our lab has identified several potentially novel requirements for the pathogenesis of *S. flexneri* (Runyen-Janecky 2002). Green fluorescent protein (*gfp*) promoter fusions were created in *S. flexneri* and activated through differential fluorescence induction. The *gfp* is promoter-less so activation of *gfp* indicates that the promoter it was fused to was activated under the growth conditions. The cells were grown either in L broth or in Henle cells and fluorescence activated sell sorting was used to sort cells based on fluorescence. Of the genes that were found to be up-regulated during intracellular growth, three are involved in metabolic processes: *uhpT*, *bioA,* and *lysA,* which encode a sugar phosphate transporter, an enzyme involved in biotin synthesis, and an enzyme involved in lysine synthesis, respectively. A *uhpT* disruption mutant was unable to utilize glucose-6-phosphate as its sole carbon source; however, it was able to exhibit normal plaque formation on epithelial cell monolayers. *bioA* was found to be up-regulated 5 fold, while *lysA* was found to be up-regulated 3 fold (Runyen-Janecky, 2002).

This upregulation in the intracellular environment was also found in a microarray study that examined *S. flexneri* growing in Hela cells. The microarray data identified *bioA* and *lysA* among genes upregulated intracellularly (Lucchini, 2005).

My research seeks to examine the necessity of *bioA* and *lysA* in *S. flexneri* pathogenesis and intracellular survival by knocking out the genes of interest and examining their virulence in Hela cells.

Biotin Synthesis and Importance

Biotin is a coenzyme in various carboxylase reactions, for example the pyruvate to oxaloacetate reaction in the gluconeogenesis pathway. The chemical structure of the biotin molecule is shown in Figure 3A. The *bioA* gene is a part of the biotin operon which encodes enzymes that catalyze the reactions responsible for producing biotin. The gene product of *bioA* is diaminoperalgonic aminotransferase (DAPA AT).   Figures 3B and 3C diagram the pathway and genes involved in the production of biotin by *S. flexneri* as well as the layout of the biotin operon.

Figure 3: Biotin Biosynthesis:

A. Chemical structure of biotin. A carboxyl group is bound to a hydrocarbon chain bound to the sulfur containing ring.

B. Synthesis pathway of biotin. The bioA gene product, diaminoperalgonic aminotransferase, catalyses the conversion of 7-KAP to 7-DAPA.

C. Organization of biotin synthesis related genes. The genes involved in biotin synthesis are located in the biotin operon.

A.

B.

Pimeloyl CoA
      bioF
7-Keto-8-aminopelargonate (7-KAP)
      bioA
7,8-Diaminopelargonate (7-DAPA)
      bioD
Dethiobiotin
      bioB
Biotin
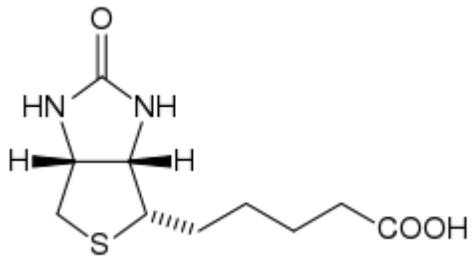
C.

In work done on *Mycobacterium tuberculosis*, also an intracellular pathogen of humans, a suicide substrate for DAPA AT was able to bind and stop the activity of the enzyme. When DAPA AT was inhibited, growth was completely inhibited on biotin-free Luria Bertani agar medium. This process was found to be reversible by adding biotin to the medium (Mann 2006). Since biotin is essential for t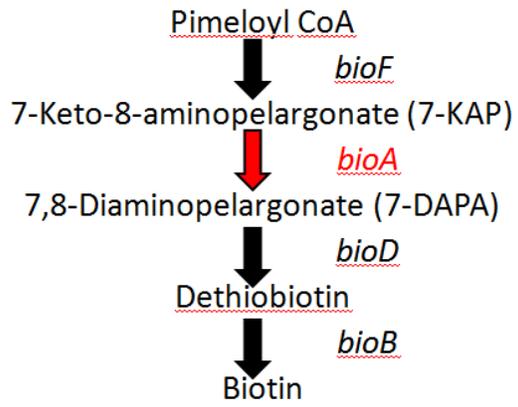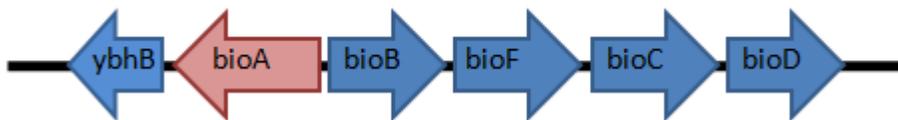he *M. tuberculosis* auxotroph to grow, it may follow that biotin is vital for the growth of *S. flexneri* as well. The biotin biosynthetic pathway has the potential to be a target for the development of antibiotics because humans do not synthesize biotin. An inhibitor of DAPA AT could be useful since this enzyme is not produced or required by humans.

Researchers in Japan performed a genetic analysis of a Bacillus subtilis strain that was unable to synthesize its own biotin. *Bacillus subtilis* Natto OK2 is used commercially in the fermentation of soybeans and required biotin for growth. The genetic defects in this strain are in the *bioW* and *bioF* genes and result in biotin auxotrophy.   The strain does not exhibit wild type growth in minimal medium unsuplemented with biotin. The growth curve exhibits a death phase after 4 hours of growth followed by a second growth phase after 6 hours. The strain reaches stationary phase at an OD_{650} of 0.1 while the wild type reaches stationary phase at an OD_{650} of 1.0 (Sasaki, 2004).

<u>Lysine Synthesis and Importance</u>

Lysine synthesis has been recently examined as a possible target for new antibiotic agents. Not only is lysine a component of proteins, but the last precursor in its biosynthetic pathway, *meso*-diaminopimelate, is also a component of the peptidoglycan cell wall. The lysine chemical structure and biosynthetic pathway is shown in Figure 4. An antibiotic that targeted lysine synthesis would not be expected to cause adverse reactions in humans because mammals do not synthesize lysine (Hutton 2003). The *lysA* gene encodes for diaminopimelate decarboxylase (DD). DD catalyzes the last step in the lysine biosynthetic pathway, and the product of that reaction is lysine (Patte 1996).

A gene involved in lysine synthesis in the fungus *Aspergillus fumigatus* has been shown to influence virulence in a mouse model of infection. *LysF* was deleted in *A. fumigates,* and the end result of the mutation was to create an *A. fumigates* lysine auxotroph. The resulting auxotroph was avirulent at low doses (Liebman, et al, 2004). This suggests a demand for the biosynthesis of lysine for virulence.

Figure 4: Lysine Biosynthesis

A. Lysine Chemical Structure. Lysine is a positively charged amino acid.

B. Lysine Biosynthetic Pathway. The substrates and genes involved in lysine synthesis. The *lysA* gene product catalyses the last reaction in the pathway, the conversion of *meso*-diaminopimelate to lysine by diaminopimelate decarboxylase.

C. Organization of lysine synthesis related genes. *lysA* is located between the *galR* gene and the *lysR* gene. The remainder of the lysine biosynthesis genes are located around the genome.

A.

B.

Aspartate semialdehyde

dapA

Dihydrodipicolinate

dapB

Tetrahydrodipicolinate

dapD

N-Succinyl-α-amino-ε-keto-pimelate

dapC

N-Succinyl diaminopimelate

dapE

LL-Diaminopimelate

dapF

Meso-diaminopimelate

lysA

Lysine

C.

galR   lysA   lysR
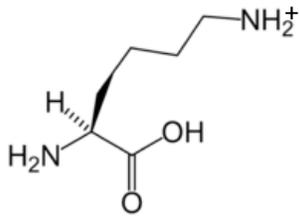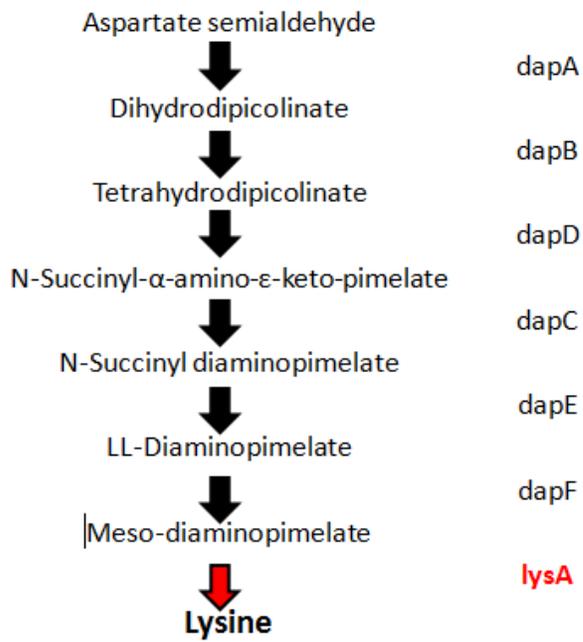
Materials and Methods

Bacterial Strains

The bacterial strains used in this research are listed in Table 1. *S. flexneri* strain 2457T serotype 2a was used as the wild type strain. Cloning was performed using $CaCl_2$ competent *E. coli* DH5α. Keio strains JW0757 and JW2806 were obtained by request from the Keio Collection (Baba, et al., 2006).

Primer List

The primers utilized in this research are listed in Table 2. Unless otherwise noted, all primers were designed by the author using Clone Manager Professional Software Suite (Sci Ed Central). Primers were ordered from Invitrogen and were resuspended at 100μM.

Polymerase Chain Reaction and Ligations

PCR and ligations were performed according to Ausubel et al, 2003. Both reactions were run in an Applied Biosystems GeneAmp thermo cycler. PCRs were performed using Taq DNA polumerase provided by New England Biolabs (Ipswich, MA) or Pfx DNA polymerase provided by Invitrogen (Carlsbad, CA).

The standard PCR mix contained 2 units of polymerase enzyme, 800μM dNTP mixture, 2μM oligonucleotide primers, buffer solution, and either template DNA or a colony picked from a plate. For reactions using Pfx, $MgSO_4$ was added at a

concentration of 1 mM. The standard reaction conditions for Taq reactions were 30 cycles of 30 seconds of 94°C for denaturation, 30 seconds of annealing, 1 minute at 72°C for each 1000bp for elongation, and 10 minutes at 72° for a final elongation. The standard reaction conditions for Pfx reaction conditions were 30 cycles of 15 seconds at 94°C for denaturation, 30 seconds of annealing, 1 minute at 68°C for each 1000bp for elongation, and 5 mutes at 68°C for a final elongation. PCR products were electrophoresed on 0.75% agarose gels.

Ligations were carried out using T4 DNA ligase and its corresponding buffer. Reactions were allowed to ligate at 16°C overnight in a thermocycler.

Table 1: Bacterial strains used in this research:

Strain	Characteristics	Reference
E. coli Strains:		
MG1655	K-12	(Blattner et al., 1997)
W3110	F⁻ IN(*rrnD-rrnE*)*1*	(Henderson et al., 1993)
DH5α	*endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1 Δ(lacZYA-argF)U169 deoR [Δ80dlac Δ(lacZ)M15]*	(Hanahan et al. , 1983)
JW0757	Keio strain, bioA-, KanR	(Baba et al. , 2006)
JW2806	Keio strain, lysA-, KanR	(Baba et al., 2006)
S. flexneri Strains:		
2457T	Wild type *S. flexneri* serotype 2a	(Wei et al., 2003)
Δ*bioA*	2457T bioA::Kan	This work
Δ*lysA*	2457T lysA::Kan	This work

Table 2: List of primers used in this research.

Primer Name	Sequence	Tm (C°)
lysAfor	5'TTCCCGTAGGCTGATGGAGTG3'	56
lysArev	5'TGGCGAACCAGACGAAAGC3'	56
lysA2for	5'TGGAGCGTTTCAGTGAGTC3'	55
lysA2rev	5'AAGGTCATACCCGCATTGG3'	55
bioAfor	5'GCGCCTGAGATTCACTCAAC3'	55
bioArev	5'TACCAGACCAGAGCCAAACC3'	55
PD4for*	5'GTCCGGTGCCCTGAATGAAC3'	56
PD4rev*	5'GGAGCGGCGATACCGTAAAG3'	56

(*) denotes primers provided by Aja Gore.

Plasmid Purification

Plasmid purification was done using a GenElute Plasmid Miniprep kit from Sigma-Aldrich (St. Louis, MO). The kit was used according to the manufacturer's directions. The isolated DNA in the eluate was stored at -20°C.

Restriction Enzyme Digests

Restriction enzyme digestions were purchased from New England Biolabs (Ipswich, MA). The digestions were incubated in water baths at the manufacturer's recommended temperature for at least 4 hours. Restriction enzyme buffers and BSA were also provided by New England Biolabs and were included as recommended for each enzyme. The digested fragments were subjected to gel electrophoresis and the band was excised and extracted using a Sigma GelElute Gel Extraction Kit. The protocol provided by Sigma was utilized and the fragments were eluted into 50μl of the provided elution solution.

Tissue Culture Maintenance

The Henle cells were cultured using modified eagle medium (MEM) purchased from Invitrogen. To prepare the MEM complete medium, 10% fetal bovine serum, 10% tryptose phosphate broth, 2mM glutamine, and non-essential amino acids were added to the stock MEM. The non-essential amino acids (Sigma Aldrich) included L-alanine at 89 μg/L, L-asparagine·H_2O at 150μg/L, L-aspartic acid at 133μg/L, L-glutamic acid at 147μg/L, glycine at 75μg/L, L-proline at 115μg/L, and L-serine at 105μg/L. The Henle

cells were split using a trypsin working solution of 80/20 mixture of versine and trypsin stock solution. The versine was made by adding 1.5 ml 0.5% phenol red to 500 ml PBS and filter sterilizing the solution. The PBS-D was made by adding 50 ml 10X PBS and 5 ml 0.1m EDTA to 450 ml sterile water. The trypsin stock solution was made by adding 0.25 ml trypsin, 0.1g Na2EDTA, and 0.3 ml 0.5% phenol red to 100 ml PBS.

P1 Transduction

P1 phage transduction was utilized to introduce knock-out mutations in the genes of interest. The phage stock and protocol were provided by Dr. Ian Molineaux's laboratory. The stock phage was amplified to a titer of 4.16×10^9 pfu/ml by growing the phage in *E. coli* MG1655 and then harvesting the phage lysate. The lystate was stored at 4°C. The phage lysate was titered by plating the lysate with *E. coli* MG1655. Keio collection strains JW0757 and JW2806 were used as the donors of the target DNA region. Strain JW0757 has the *bioA* gene replaced with a kanamycin cassette while strain JW2806 has the *lysA* gene replaced with the kanamycin cassette.

Growth Curve Protocols

Growth curves were examined in both LB medium, recipe in Appendix A, and in minimal medium both with and without supplementation with lysine or biotin. The minimal medium used was T medium (Appendix A). Biotin was supplemented at a final concentration of 100μM and lysine was supplemented at 300μM, recommended for *E. coli* auxotrophs (Davis, et al.).

The strains to be examined with the growth curve were grown overnight with shaking in test tubes at 37°C. In the morning the optical density (OD) was read using a Beckman DU 640 Spectrophotometer. The overnight cultures were then diluted to an OD_{650} of 0.02 in the indicated medium. The samples were then placed into the 37°C incubator shaker for the duration of the growth. The OD was read every hour for a 12 hour period and then once more at 24 hours. To increase the accuracy of the OD readings, once the undiluted OD_{650} of the culture reached 0.5, the remaining time point samples were diluted 1:10 in cuvettes with either T medium or L broth, depending upon the growth curve medium.

Plaque Assay Protocol

Plaque assays were performed using confluent Henle cell monolayers. The following protocols were adapted from Oaks, et al. (1985). Henle cells were grown to confluent monolayers in 6 well plates (Corning Incorporated Costar 3506). The monolayers were washed with PBS and fresh medium was added prior to infection. The *S. flexneri* strains were streaked out on TSBA containing 0.01% Congo red, 50mM NaCitrate, 100μM biotin, 300μM lysine, and 50μg/ml kanamycin (only for the kan[R] strains). Colonies that bound Congo red were used to inoculate L broth and grown in a 37°C shaking incubator until they reached mid log phase. The cell concentration was estimated by multiplying the OD_{650} at mid log by 8×10^8 cells/ml. The samples were then diluted to concentrations of 10^5 and 10^4 cells/ml.

Henle cell monolayers in 6 well plates with fresh MEM medium were inoculated with the dilutions of bacteria. The plates were centrifuged in an IEC Centra GP8 centrifuge at 1000 RPM at room temperature for 10 minutes and placed into a 37°C/5% CO_2 incubator for 45 minutes. After incubation the medium was replaced with fresh medium containing 10 μg/ml gentamycin. The mutants were assayed both with and without biotin or lysine supplementation. When added, the final concentration of lysine was 300μM and biotin was 100μM.

The plates were placed back into the 37°C/5% CO_2 incubator to allow for plaque formation. The medium was changed every 24 hours. After 48 or 72 hours, the medium was removed from the well and the remaining monolayer was washed twice with 1 ml of PBS. Each monolayer was then stained with Wright/Geimsa stain for 60 seconds, and the stain was removed by aspiration. The wells were rinsed with water and the number and size of visible plaques were recorded.

Attachment Assay Protocols

Attachment assays examine the proportion of the auxotrophs attaching to the surface of epithelial cells in comparison to wild type *S. flexneri*. These protocols were adapted from Hale and Formal, 1981. The attachment assays require Henle cell monolayers that are subconfluent, about 50% confluency. Cultures of *S. flexneri* were grown to mid-log while shaking at 37°C. The cultures were pelleted and then resuspended at a concentration of $2x10^9$ cells/ml. The resuspension volume was calculated by multiplying the OD_{650} by $8x10^8$ cells/ml.

The Henle cell sub confluent monolayers were infected with approximately 2×10^8 cells of resuspended bacteria. The plates were centrifuged at 1,000 RPM for 5 minutes and placed in the 37°C/5% CO_2 for 15 minutes. The plates were removed from the incubator and the media was removed from the wells. The wells were washed with PBS, and fresh medium containing 10 µg/ml gentamycin was added to the wells. The plates were placed back into the 37°C/5%CO2 incubator for 1.5 hours. The plates were removed from the incubator and the medium was aspirated. The wells were washed twice with 1 ml of PBS, and the monolayers were stained with Wright/Giemsa.

Invasion Assay Protocols

Invasion assays were then set up to examine the rate that the auxotrophs invaded the epithelial cells in comparison to wild type *S. flexneri*. Invasion assays were carried out with protocols similar to the attachment assays and were adapted from Hale and Formal, 1981. The difference in the protocols was the incubation and centrifugation times. The plates were centrifuged at 1,000 RPM for 10 minutes and then placed in the 37°C/5% CO_2 incubator for 30 minutes.

Complementation Protocol

After assays were completed, a plasmid carrying the *lysA* gene was constructed to complement the *lysA* auxotroph using plasmid pWKS30. pWKS30 was modified from the pSC101 plasmid (Wang and Kushner, 1991). It contains the *lacZα* gene with a multiple cloning site, and it carries ampicillin resistance. Transformants can be selected

on ampicillin and screened on X-gal and IPTG to identify those in which the *lacZ* gene has been disrupted. The vector map of pWKS30 is shown in Figure 5.

The plasmid was digested with SmaI and ligated to the HincII/HpaI-digested *lysA* fragment. T4 DNA ligase was used with its corresponding T4 DNA liagase buffer. The ligation mix was placed in a thermocycler at 16°C overnight. The ligation mix was then added to *E. coli* DH5α $CaCl_2$ competent cells (Ausubel et al. 2003). The transformed cells were plated on 25 μl/ml ampicillin + IPTG + X-gal L agar in order to allow for blue/white screening. PCR was performed using primers for2 and rev2. The products were sequenced and BLAST analyses confirmed the presence of the *lysA* gene.

Transformation of S. flexneri with pWKS30

The G Buffer method of transformation was used to produce a competent *S. flexneri* Δ*lysA* mutant and transform it through electroporation with the pWKS30 plasmid carrying *lysA* (Occhino et al. 1998). G Buffer is a solution consisting of 137mM sucrose and 1mM HEPES (pH 8). Electroporation was carried out at 200Ω, 25 mF, and 24V. The transformation mix was plated on TSBA containing 0.01% Congo red, 50mM NaCitrate, and 25μg/ml ampicillin. To confirm the presence of the plasmid carrying the *lysA* gene, plasmid purification and PCR screening using the for2 and rev2 primers was completed on eight transformants. The *lysA* gene was found in the plasmids isolated from all transformants.

Figure 5: pWKS30 Plasmid Map

Multiple Cloning Site:
-Ecl136II
-SacI
-BstXI
-SacII
-NotI
-XbaI
-BamHI
-SmaI
-XmaI
-PstI
-EcoRI
-EcoRV
-HindIII
-ClaI
-AccI
-HincII
-SalI
-XhoI
-EcoO109I
-ApaI
-PspOMI
-Acc65I
-KpnI

pWKS30
5434bps

pSC101 rep

bla

lacZ

MCS

Bpu10I
BbvCI
EcoNI
NdeI

Xcml

AhdI BsaI

ScaI
TatI
XmnI BssSI
Alw44I

BciVI

DrdI
DraIII
BsaAI

SapI
PshAI
BbsI

BseRI
AflII

BstAPI NruI

Results

Using transduction, a *S. flexneri* 2457T *lysA* deletion mutant was created. Keio donor strains were used for transducing kanamycin resistance cassettes into the genes of interest in this project. The Keio strains are a collection of *Escherichia coli* K-12 BW25113 strains that have non-essential genes removed and replaced with a kanamycin resistance cassette flanked by flippase (FLP) recognition target sites (Baba 2006). Transducing the mutation from the Keio mutants allows the creation of in-frame, single gene knock-out mutants of the *bioA* and *lysA* genes and enables screening on selective medium containing kanamycin.

The putative mutants were screened by PCR using primers specific for regions inside the kanamycin cassette and regions specific to the regions flanking the kanamycin cassette. The primers used for screening the Δ*lysA* putative mutant were lysAfor and lysArev. The primers used for screening Δ*bioA* putative mutants were bioAfor and bioArev. The results of gel electrophoresis with these putative mutants are included in Figure 6. The lanes with reactions using the internal kanamycin primers and the flanking gene primers show that the *E. coli* donors and the putative mutants contain the kanamycin cassette. The lanes with reactions using just the primers for the flanking regions show the band size of the putative mutants is the same as the band size of the *E. coli* donor and not the *S. flexneri* wild type. The primer set lysAfor and lysArev bind to sites upstream and downstream of the *lysA* gene. The primer set bioAfor and bioArev bind to sites upstream and downstream of the *bioA* gene. The size difference between the putative mutants and

the *E. coli* donor to the *S. flexneri* 2457T wild type is slight. Primers PD4for and PD4rev

bind to sites within the kanamycin cassette carried by the donor and inserted into the

Δ*lysA* and Δ*bioA* mutants. PCRs that use the PD4 primers, in either the forward or

reverse directions, should not have any binding when using 2457T as the template.

Figure 6: PCR screening results of the Δ*lysA* and Δ*bioA* mutants.


A: The gel image of the PCR reactions run the Δ*lysA* putative mutants run on a 0.75%

agarose gel. Lanes labeled as "ladder" contain 5 μl of λ DNA digested with HindIII.

B: The gel image of the PCR reactions run on the Δ*bioA* putative mutant run on a 0.75%

agarose gel. Lanes labeled as "ladder" contain 5 μl of λ DNA digested with HindIII.

A: Δ*lysA* putative mutants
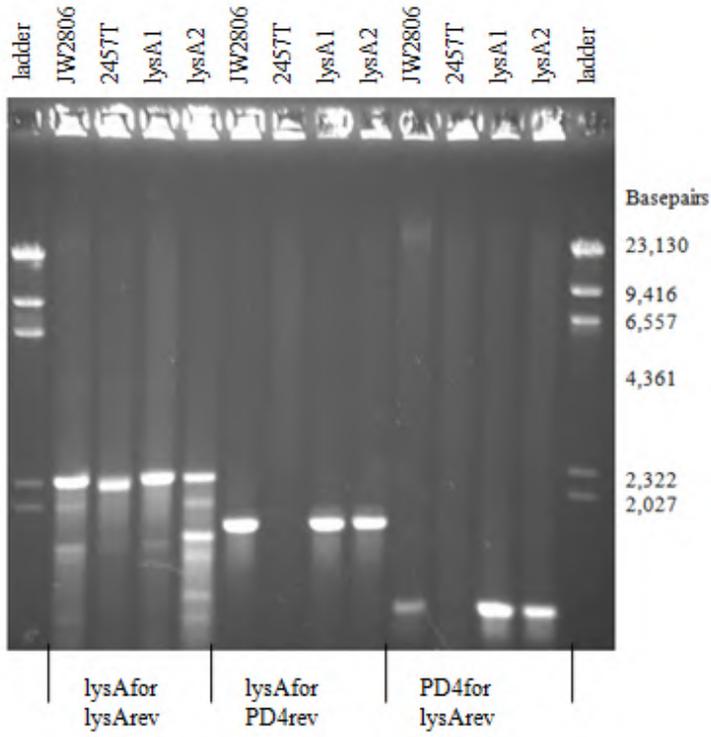
B: Δ*bioA* putative mutants
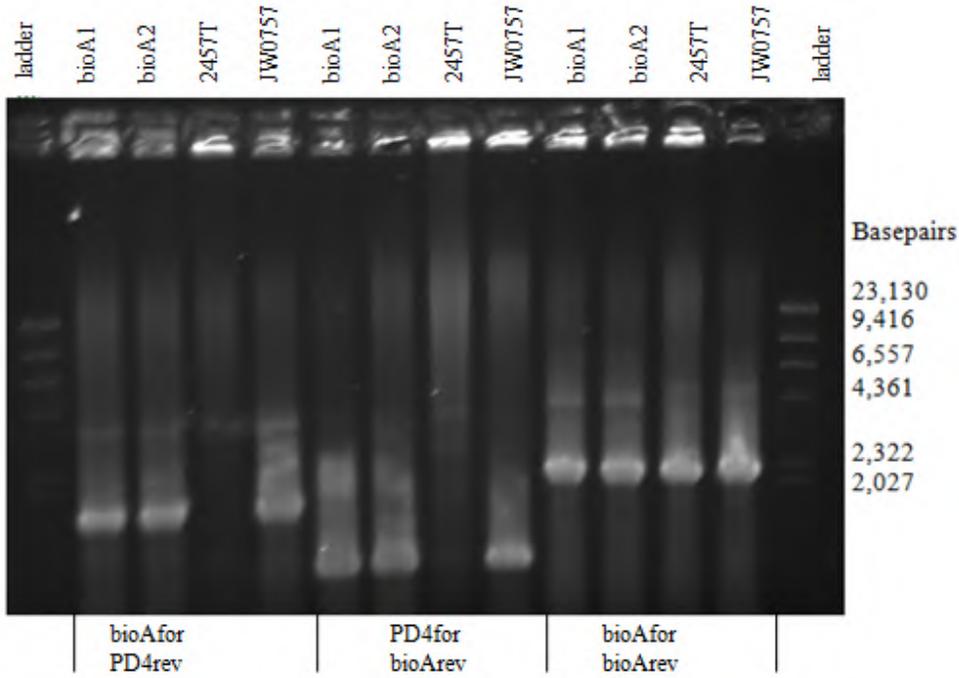
Growth Curves

Growth curves for the Δ*lysA* mutant have shown wild-type growth rates when grown in L broth (Figure 7A). However, there is a transition into stationary phase at a lower OD_{650} when the mutant is grown in minimal medium, relative to the wild type in minimal medium. When the mutant is supplemented with lysine at a concentration of 100uM in minimal medium, the transition to stationary phase occurs at a higher OD_{650} but still does not exhibit wild type behavior. The growth curve was performed again with the lysine supplementation increased to 300μM, however, all four times it was repeated the Δ*lysA* grew at a rate faster than wild type *S. flexneri*. For future work the growth curve can be performed using different minimal mediums.

Growth curves were performed as a preliminary observation of the effects of the mutations. If a growth defect was noted in the growth curves, such as a longer lag phase, the defect would need to be taken into account when the strains were grown for use in the virulence assays. When the auxotrophs were growing in a rich medium, any growth defect would have raised concerned over whether a secondary mutation had occurred. Growth was examined in minimal medium to monitor effects of the auxotrophs growing without the compound they could not produce. They were gown in the minimal medium with biotin or lysine supplemented to test whether or not wild type growth could be restored in minimal medium. The results are shown in Figures 7 and 8. Growth was more similar to wild type for both auxotrophic strains when they were supplemented.
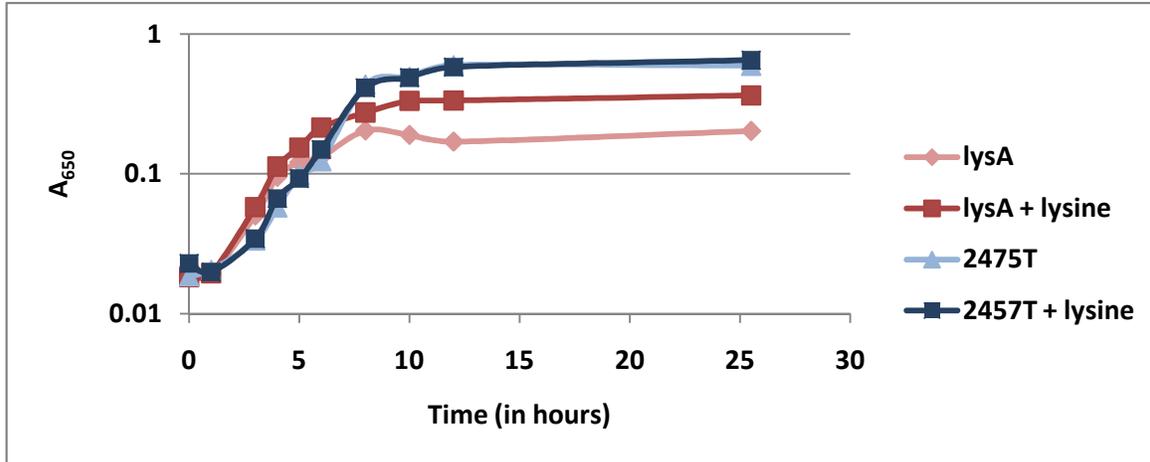
Figure 7: Δ*lysA* Growth Curves

A: The Δ*lysA* mutant grows similarly to wild type 2457T in L broth. 24 hour growth curve done in L broth. Time points were taken every hour for 12 hours and then again at 24 hours.

B: The Δ*lysA* mutant grows better in minimal medium with lysine supplemented. The strains were examined both without lysine and in the presence of lysine (100uM). Time points were taken every hour for 12 hours and then again at 24 hours.

A: L broth growth curve:
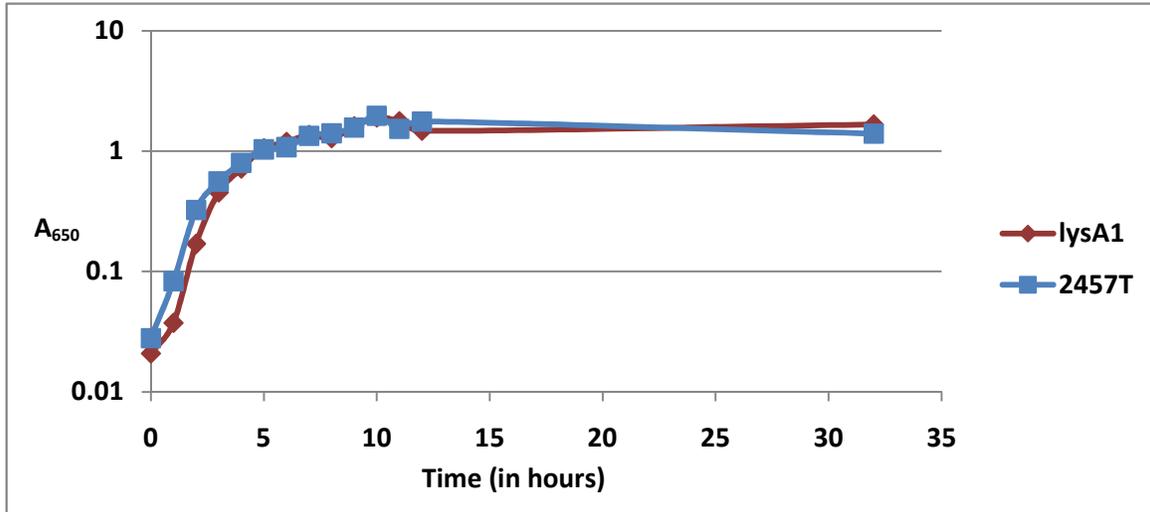
B: Minimal medium growth curve, with and without supplementation with 100μM lysine.

Transduction was also used to create a Δ*bioA* deletion mutant. Growth in L broth

for the deletion mutant is at the same rate as growth of the wild type in L broth, Figure

8A. Growth in minimal medium, T medium, is considerably different than wild type,

Figure 8B. The mutant undergoes a defect in growth at about 2 hours of growth in the

incubator. The growth curve done for the *E. coli* donor biotin auxotroph also showed a

dip in growth at about 5 hours of growth.

Figure 8: Δ*bioA* Growth Curves

A: The Δ*bioA* mutant grows similarly to wild type in L broth. Time points were taken

every hour for 12 hours and then again at 24 hours.

B: The Δ*bioA* mutants exhibit a death and recovery phase in minimal medium. JW0757

is the *E. coli* donor strain that has Δ*bioA* knocked out. At approximately 2 hours, the

Δ*bioA* mutant *S. flexneri* begins to enter a death phase, and at approximately 5 hours the

Δ*bioA E. coli* exhibits a growth defect. They both began to grow again, possibly picking

up a suppressor mutation.

A: L broth growth curves
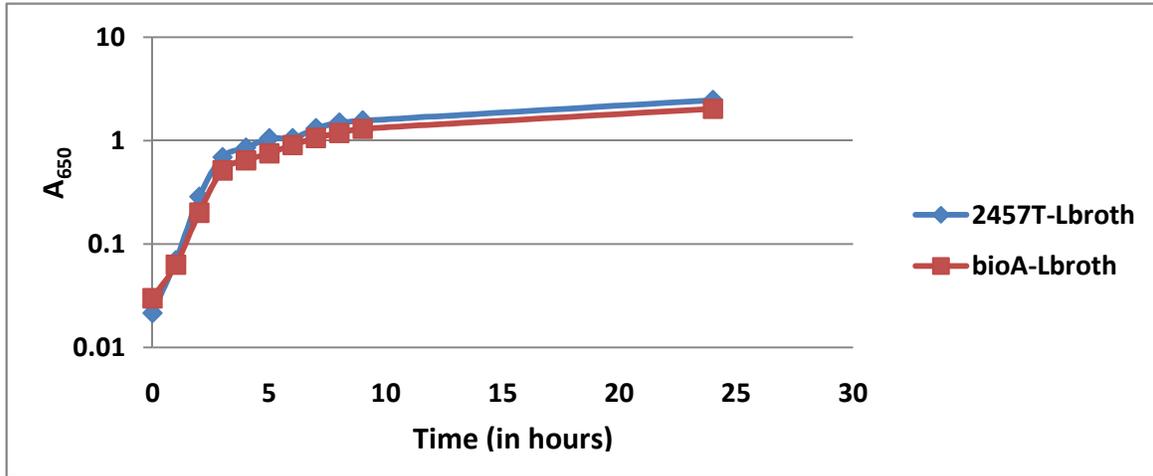
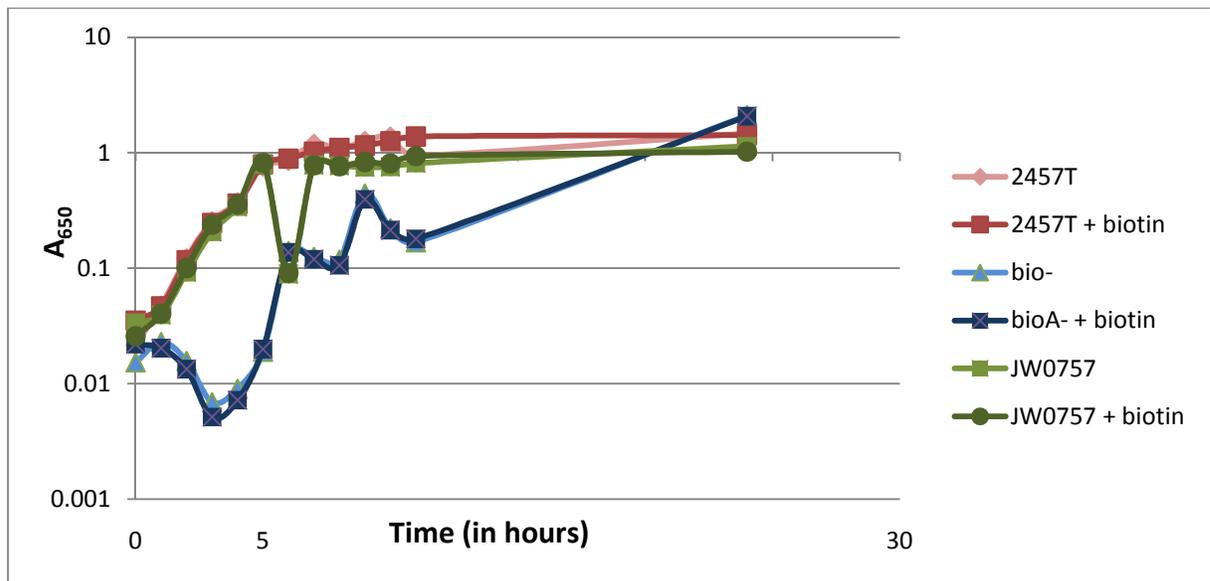B: Minimal medium growth curves, with and without supplementation of 100 μM biotin.

Shown in green is the *E. coli* donor strain, in red is *S. flexneri* wild type 2457T, and in

blue is the *S. flexneri bioA* deletion mutant.

<u>Plaque Assays</u>

Plaque assays of Henle cell monolayers were done with the ΔlysA mutant. The
monolayers were stained and examined 48 and 72 hours post-infection (Figure 9A). The
wild type was used as a control for plaque number and size, although the wild type assay
is only shown at the 48 hour time point. The monolayers infected by the wild type were
completely destroyed by 72 hours. The ΔlysA mutant formed plaques more slowly than
wild type with no plaques visible after 48 hours. At 72 hours post-infection, the
monolayers showed the formation of fuzzy plaques indicating incomplete lysis of the
epithelial cells by the ΔlysA mutant. Since the number of plaques was equivalent to the
wild type this would indicate that the ΔlysA mutants are able to attach and invade the
epithelial cells, but the mutants may not be replicate or spread intracellularly as well as
wild type.

Plaque assays of Henle cell monolayers were also done with the ΔbioA mutant
(Figure 9B). Henle cell monolayers were infected with 10^6, 10^5, and 10^4 cfu/ml of *S.
flexneri* 2457T or ΔbioA mutant. The plaque assays were done in medium both with and
without 100uM biotin supplementation. The infected monolayers were then incubated at
37C/5%CO_2 for 48 and 72 hours. The monolayers were then stained and examined 48
and 72 hours post-infection. It was found that the amount of plaque formation was
slightly lower in ΔbioA, although numerous plaques were formed after 48 hours. The
plaques formed were clear, indicating complete lysis, unlike with the *lysA* deletion
mutant. At 72 hours post-infection the monolayers displayed much larger plaques,

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although the wild type *S. flexneri* had completely destroyed the monolayer by 72 hours.

There was no noticeable difference between the size and number of plaques formed when

the infected monolayers were incubated with medium containing biotin supplementation

compared to the monolayers with medium containing no biotin. This was observed for

both Δ*bioA* and the wild type *S. flexneri*.

Figure 9: Plaque Assay Results.

A: The *S. flexneri* Δ*lysA* did not plaque as efficiently as wild type *S. flexneri* 2457T. A. The Δl*ysA* infected monolayer 48 hours post-infection. B. The Δ*lysA* infected monolayer 72 hours post infection, showing "fuzzy" plaques. C. The WT 2457T infected monolayer 48 hours post-infection, showing clear, completely lysed plaques.

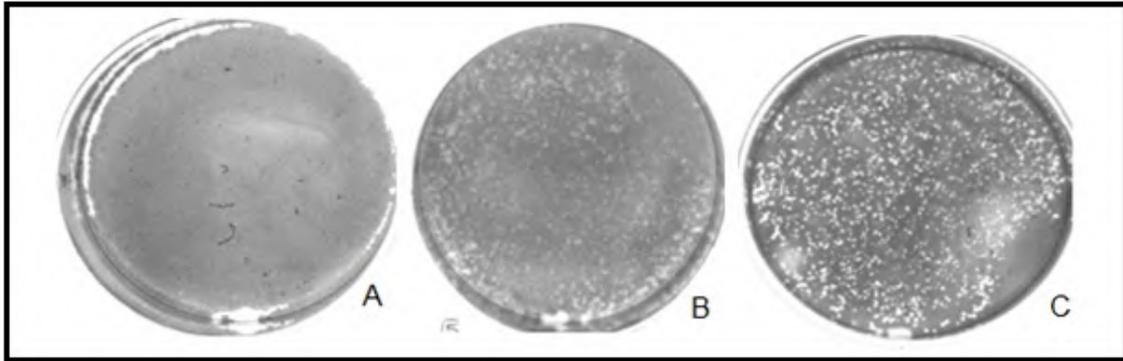B: The Δ*bioA* defective *S. flexneri* did not plaque as efficiently as wild type *S. flexneri* 2457T. A. The Δ*bioA* infected monolayer 48 hours post-infection. B. The Δ*bioA* infected monolayer 72 hours post-infection. C. WT 2457T infected monolayer 48 hours post-infection, showing clear, completely lysed plaques.

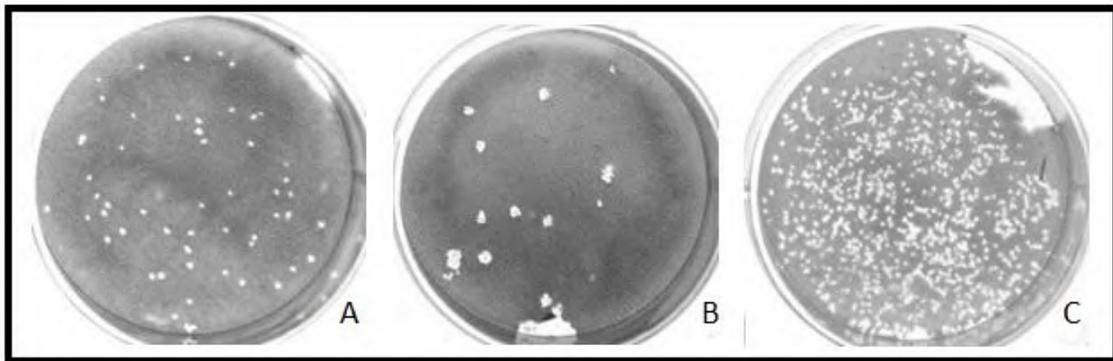A: Δ*lysA* 48 hours   Δ*lysA* 72 hours   WT 48 hours

B: Δ*bioA* 48 hours   Δ*bioA* 72 hours   WT 48 hours

<u>Invasion and Attachment Assays</u>

To examine the cause of the differences in plaquing ability, invasion and attachment assays were also performed on the $\Delta lysA$ and $\Delta bioA$ mutants. In the attachment assays, subconfluent Henle cells were examined for the presence of attached bacteria. The number of individual bacteria attached was counted for 100 Henle cells in triplicate. The $\Delta lysA$ mutant formed clumps of bacteria in both invasion and attachment assays, with clumps of up to 23 bacteria attached to Henle cells in the attachment assay. The wild type did not exhibit this phenotype in the attachment assay; individual bacteria were found to be attached to the Henle cells.

In the invasion assay, 300 subconfluent Henle cells were examined for the presence or absence of intracellular bacteria. A cell was considered to have been invaded if three or more bacteria were present. The $\Delta lysA$ mutant invaded the Henle cells at a higher rate than the wild type, 55% of epithelial cells counted were invaded by $\Delta lysA$ compared to 33% by wild type. The clumping of the $\Delta lysA$ mutant was also visible in the invasion assay, and the clumps were even larger than in the attachment assay, causing the results to be skewed. The invasion assay requires that three or more bacteria have invaded a Henle cell for that Henle cell to be considered invaded. The clumping phenotype of the biotin auxotroph conferred an advantage in that assay. The wild type was more likely than the mutant to have only one or two bacteria invading a Henle cell than the $\Delta bioA$ mutant. The clumping behavior was also visible in vitro in minimal

medium and L broth. There was more settling of bacteria in the bottom test tubes containing the *ΔlysA* mutant compared to wild type *S. flexneri*.

Invasion and attachment assays were also performed on Henle cell monolayers using the Δ*bioA* mutant and the 2457T wild type. The Δ*bioA* mutant were able to attach at a higher rate than the wild type *S. flexneri*, the wild type averages 0.6 bacteria per epithelial cell while the Δ*bioA* averaged 1.1. The invasion rates were much more similar; the Δ*bioA* mutant invaded 34% of the epithelial cells while the wild type invaded 28%. Biotin was present in the Henle medium so there were no virulence assays done of the Δ*bioA* mutant grown without biotin supplementation.

<u>Plasmid Complementation</u>

Once it was confirmed that the Δ*lysA* mutant was carrying the plasmid, through plasmid extraction and PCR, a growth curve was done using T medium, with and without supplementation of 300μM lysine. The growth curve of the complemented Δ*lysA* mutant compared to the wild type 2457T and the uncomplemented Δ*lysA* is shown in Figure 10. The plaque assay results are shown in Figure 11.

Figure 10: The growth curve of *S. flexneri* Δ*lysA*/pWKS30:*lysA* in T media, with and without the supplementation of 300μM lysine.
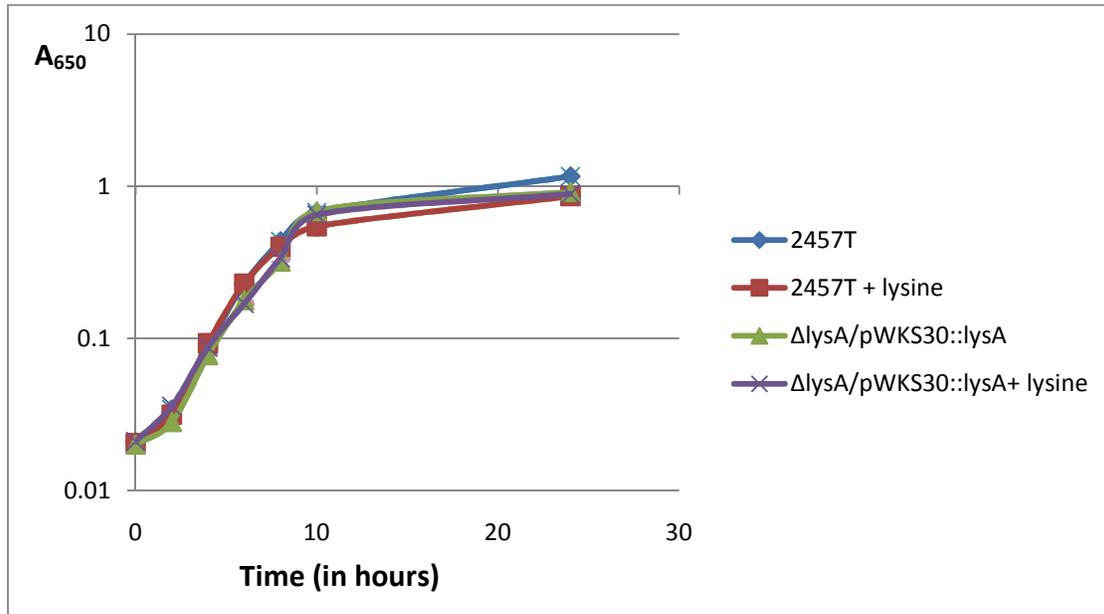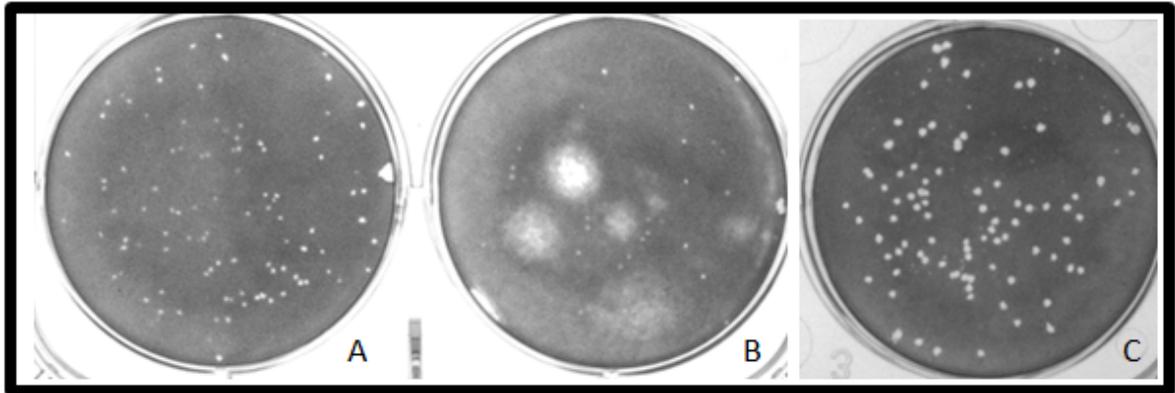
Figure 11: Plaque assay comparing the plamid complemented Δ*lysA* mutant to the wild type and the Δ*lysA* mutant. The Henle cell monolayers were stained at 48 hours post infection. A is the wild type 2457T, B is the Δ*lysA* mutant, and C is the plasmid complemented Δ*lysA* mutant.

Discussion

The auxotrophic strains grew similarly to wild type in L broth, and both strains did not grow as well as wild type in minimal medium. However, when the minimal medium was supplemented with either biotin or lysine, wild type growth rates were still not achieved by either auxotrophic strain. This was unexpected since the deficiency in these strains should be solely in lysine or biotin biosynthesis. The effect was more pronounced in the biotin auxotroph, where the addition of biotin had no noticeable effect of counteracting the growth defect observed when there was no supplementation. The lysine auxotroph supplemented with lysine did show some improvement in growth over the auxotroph that was grown unsupplemented, but the growth was not at the level the wild type displayed. This occurrence could be given further attention in additional experiments. It is possible that the levels of supplementation recommended for *E. coli* auxotrophs may not be sufficient in supplying *S. flexneri* with the nutrients it needs for a wild type growth rate.

The growth curve for *S. flexneri* missing the *bioA* gene showed a decrease in growth and subsequent recovery that was also exhibited by the *E. coli* Δ*bioA* strain used as a donor for the transduction. This growth pattern was also found in a *B. subtilis* biotin auxotroph, although it was able to regain wild-type growth through supplementation with biotin (Sasaki, 2004). The dip in growth was found to occur after the auxotroph had already begun to grow in the minimal medium. One possible explanation for this

behavior is that a toxic intermediate is being produced and the mutants could be picking up a suppressor to allow for growth.

The *lysA* deletion mutant was unable to produce clear, completely lysed plaques when it was compared to the plaquing ability of wild type *S. flexneri* 2457T. The process that the mutation is affecting remains unresolved. The attachment assay did not show a significant decrease in the ability of the mutant to attach to the surface of a Henle cell, and the invasion assay showed a higher rate of invasion than wild type. Since the defect is not in attachment or invasion of epithelial cells, but is still causing turbid plaques, it is a possibility that the inability to synthesize lysine prevents the mutant from growing intracellularly or invading neighboring epithelial cells at the wild type rate. The "fuzzy" phenotype of the plaques formed by the *lysA* deletion mutant may be an indicator of the inability of the mutant to lyse the infected epithelial cells. If the Δ*lysA* mutant was unable to utilize lysine inside the epithelial cell, this may point to the concentration of lysine in the cytoplasm being too low to support bacterial growth. The lysine that was present may be associated with its tRNA and unable to be utilized by the *S. flexneri*. In order to make conclusions, future work will need to be carried out to examine the intercellular spread of this mutant. Future work can also be done in examining additional genes in the lysine operon.

It was expected that the deletion mutants would not be able to plaque as efficiently, if at all, without supplemented nutrients. The upregulation of these genes while *S. flexneri* is growing intracellularly indicates that they may be critical for intracellular

survival, replication, or growth or that these genes may just be required for optimal growth. The defect in plaquing was not seen as dramatically in the *bioA* deletion mutant as in the *lysA* deletion mutant. Wild type plaquing was also not seen when the auxotrophs were supplemented with biotin and lysine. While it was expected that these genes would be necessary for intracellular growth because they have been found to upregulated, it is interesting to note that adding biotin and lysine to tissue culture media cannot bring back wild type plaque formation. This holds some implications for proposed amino acid pools in eukaryotic cells. If the pools of free amino acids in the cytosol are very small, the cytosolic environment would be more difficult for intracellular growth than previously thought. Further research would aim to elucidate the concentrations of amino acids and vitamin cofactors in the epithelial cells that could potentially be utilized by the *S. flexneri*.

The presence of turbid plaques in a plaque assay indicates incomplete lysis of the epithelial cells. This would indicate that not all of the infected epithelial cells are being lysed and releasing more *S. flexneri* into the extracellular environment. The inability of some of the *lysA* deficient bacteria to lyse the epithelial cells could be caused by interruptions in the normal life cycle of *S. flexneri* at various stages. Alternatively, the incomplete lysis could be due to the Δ*lysA* mutation causing a delayed lethality in the mutants. The auxotrophic intracellular bacteria may be dying because of complications of the lysine deficiency prior to inducing lysis, resulting in the characteristic fuzzy

plaques. This could also account for the lower and slower frequency of plaquing in the Δ*lysA* mutant.

The observation of finding fewer Δ*bioA* plaques could be caused by a possible defect interfering with the intracellular life cycle of the biotin auxotroph. Since the plaques are clear and completely lysed, the issue may be in the replication of the *S. flexneri* rather than in the lysing of the epithelial cells. The defect could also be affecting intracellular spread, since the invasion and attachment assays were similar to wild type. *S. flexneri* undergoes many changes in order to spread between epithelial cells after initially replicating inside the infected epithelial cells. Processes that could account for a defect in intracellular spread would include aberrant actin polymerization and problems with inducing pseudo-pinocytosis or vacuole lysis.

Complementation of the *bioA* and *lysA* deletion mutants via plasmids carrying *bioA* and *lysA* is a future direction of this work. Complementation of the deleted genes is vital to ensuring that the target genes are responsible for the phenotypes of the mutants. It is expected that once the *bioA* auxotroph is complemented with the plasmid that it will exhibit wild type growth and virulence.

Δ*lysA* has been successfully complemented with a plasmid carrying the *lysA* gene. Δ*lysA* carrying the plasmid grew at a rate comparable to wild type in minimal media, independent of supplementation with lysine. Plaque assays using the plasmid complemented Δ*lysA* showed clearly lysed plaques in the Henle monolayers. Since the

plasmid carrying *lysA* was able to restore wild type behavior to the auxotroph, this lends support to *lysA* being required for virulence.

The Δ*lysA* clumping phenotype could possibly be due to a defect in the bacterial cell walls. The last precursor for the synthesis of lysine, *meso*-DAP, is also a constituent of peptidoglycan. Conceivably, the build-up of *meso*-DAP could be affecting the cell membranes, causing the *ΔlysA* bacteria to clump together. In order to test, this future research could involve engineering deletion mutants in lysine synthesis genes that precede the production of *meso*-DAP. If those mutants clumped the defect may be caused by the loss of lysine biosynthesis rather than a build-up of *meso*-DAP.

In order to determine if the build-up of *meso*-DAP or the absence of lysine is causing the clumping phenotype of the Δ*lysA* mutant, work can be done on deleting additional lysine biosynthesis genes and examining the consequences of those deletions. If it is the loss of lysine biosynthesis that causes the phenotype, deleting any gene in the pathway should result in the reduced pathogenicity in the virulence assays. If the build-up of *meso*-DAP causes the phenotype, deleting any genes prior to the synthesis of *meso*-DAP should allow for the wild type pathogenicity of the mutant.

Appendix A

Luria Broth

NaCl   5g

Tryptone   10g

Yeast extract   5g

dH$_2$O   1000ml

Add all components to 1000ml dH$_2$O. Use stir bar and stir plate to homogenize the solution. Aliquot 300 ml into glass bottles and autoclave. To make L agar, prior to autoclaving add 5 g of agar to 300ml L broth.

T Medium

NaCl   5.8g

KCl   3.7g

CaCl$_2$ 0.113g

MgCl$_2$-6H$_2$O   0.1g

NH$_4$Cl   1.1g

KH$_2$PO$_4$ 0.272g

Na$_2$SO$_4$ 0.142g

Tris   12.1g

dH$_2$0   1000ml

Dissolve all components in 1000ml dH$_2$O. While stirring pH the medium to 7.4 using highly concentrated HCl. Aliquot 300 ml into glass bottles and autoclave. Add 4.8 g agar to 100 ml T medium prior to autoclaving in order to make T agar. After autoclaving add 0.4% and 2 μg/ml nicotinic acid to the medium.

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Vita

Laura Ann Coughlin is the middle child of John and Carol Coughlin. She grew up in Scottsdale, Arizona with her two brothers, Scott and Mark and her family's many and varied pets. In high school she spent her summers at Northern Arizona University, participating in the Four Corners Upward Bound Math and Science program. In addition to being exposed to graduate school life she made many life-long friends. She attended the University of Arizona majoring in microbiology with a minor in chemistry. She took full advantage of growing up in the great state of Arizona and hiked the Grand Canyon, Havasupai Canyon, Sabino Canyon, and trails in the Superstition Mountains. In her free time she enjoys painting, kayaking, and taking her dog Binky to the dog park. After graduation she plans on traveling and applying to PhD programs around the world.

Permanent address:

22419 N. Los Caballos Dr.
Scottsdale, AZ 85255

This thesis was typed by the author.