Spring 2017

Construction of a heterologous vaccine against Bordetella avium and Campylobacter jejuni utilizing the B. avium transporter, Baa1

Rachel Korba
James Madison University

Construction of a Heterologous Vaccine against *Bordetella avium* and *Campylobacter jejuni* utilizing the *B. avium* Transporter, Baa1

An Honors College Project Presented to

the Faculty of the Undergraduate

College of Integrated Science and Technology and College of Science and Mathematics

James Madison University

---

by Rachel Marie Korba

May 2017

Accepted by the faculty of the Department of Integrated Science and Technology, James Madison University, in partial fulfillment of the requirements for the Honors College.

FACULTY COMMITTEE:

Project Advisor: Louise Temple, Ph.D.,
Professor, Department of Integrated Science and Technology

Reader: Stephanie Stockwell, Ph.D.,
Associate Professor, Department of Integrated Science and Technology

Reader: Christopher Berndsen, Ph.D.,
Assistant Professor, Department of Chemistry

HONORS COLLEGE APPROVAL:

Bradley R. Newcomer, Ph.D.,
Dean, Honors College

PUBLIC PRESENTATION

This work is accepted for presentation, in part or in full, at the Integrated Science and Technology Symposium on

Friday, April 21st, 2017.

Table of Contents

List of Figures

Acknowledgements

I would like to express my gratitude to my advisor, Dr. Louise Temple for her immense amount of advice and guidance. I also acknowledge the other members of my committee, Dr. Stephanie Stockwell and Dr. Christopher Berndsen, for their aid in the writing of my thesis. Furthermore, I acknowledge Kiran Nehra from the DCR University of Science and Technology as well as previous students from the Temple lab for their contributions on this project. Finally, I would like to acknowledge the Honors College and Department of Integrated Science and Technology for the funding and facilities to complete this research project and present it at various conferences.

Abstract

A Gram negative species of bacteria, *Campylobacter jejuni*, is the leading cause of food poisoning worldwide. Humans often contract food poisoning after ingesting contaminated poultry. Detecting the presence of *C. jejuni* in poultry is difficult because it is part of the natural flora and does not cause symptomatic infection. In a related manner, *Bordetella avium* is a Gram negative species of bacteria that causes bordetellosis in poultry. This disease is similar to whooping cough caused by the related pathogen of humans, *B. pertussis*. Though the mortality rate for bordetellosis is low, it weakens the birds' immune systems, often leading to secondary infections. The aim of this project was to construct a vaccine platform capable of immunizing poultry against both pathogens—*B. avium* and *C. jejuni*—thus reducing disease in birds and humans. A heterologous construct can be made utilizing the *B. avium* autotransporter Baa1 that plays a role in host cell attachment. Autotransporters are comprised of three genetic regions: promoters to drive expression, and encoded passenger and transporter domains. The transporter is a beta barrel anchored in the outer membrane. The passenger domain is translocated through the transporter and secreted to the outer surface of the bacterial cell. An antigenic *Campylobacter* gene was cloned into the encoded passenger domain of the Baa1 autotransporter in the context of a suicide plasmid. Tri-parental mating was done to promote homologous recombination of the construct into the *B. avium* chromosome. After concluding the construct was not in the chromosome most likely due to instability, a plasmid was synthesized and the codons of the *Campylobacter* region were optimized. Tri-parental mating was completed again. All steps were verified with PCR and gel electrophoresis. A *B. avium* transconjugant containing the chimeric *cja::baa1* construct was isolated. Future work will involve gene expression and challenge studies.

Introduction

Though not frequently known as a major concern of human health, *Campylobacter jejuni* is a Gram negative bacterial species that is the leading cause of gastroenteritis worldwide (1). In 1996, *C. jejuni* was detected in stool samples at a higher rate than notorious food-borne disease agents, *Salmonella* and *Shigella* combined (1). It is also more prevalent than *E. coli* O157: H7 (1). Although there are fourteen different species of *Campylobacter*, 99% of human infections are caused by *C. jejuni*. Symptoms of gastrointestinal illness caused by *C. jejuni* include diarrhea, fever, and abdominal cramps. The spread of this disease in humans is mainly through contaminated poultry. In the USA, Europe, and Australia, 50%-70% of all *Campylobacter* infections have been attributed to consumption of chicken (1). This food-borne pathogen is of great concern, especially in the poultry industry.

Another bacterial species of concern in the poultry industry is *Bordetella avium. B avium* is a Gram negative bacterium that causes bordetellosis in turkeys. Bordetellosis is similar to whooping cough in humans, which is caused by another *Bordetella* species—*B. pertussis* (2). *B. avium* colonizes many bird species, including commercially grown and wild birds (3), but only causes disease in commercially grown turkeys (2). It is naturally avirulent in chickens, but can still colonize them (2). The mortality rate for the disease in turkeys is low, however, bordetellosis results in the weakening of many young turkeys' immune systems thus allowing opportunistic pathogens to infect the birds. Collectively, this causes several million dollars in losses to the U.S. turkey industry each year (2). Furthermore, bordetellosis also impairs the growth of turkeys, adding to the financial loss that the industry suffers (2). Since *B. avium* is found in poultry and because there is currently no effective treatment nor effective vaccine for bordetellosis, it is a

particularly pressing issue in the local economy in which the largest poultry industry in the nation is located.

Though seemingly disconnected issues, *C. jejuni* and *B. avium* are related because they are both found in poultry. Though one pathogen infects humans and one infects poultry, a vaccine can be made to immunize against both utilizing the *B. avium* cellular machinery. A new or improved vaccine against *C. jejuni* to protect human health is needed. Utilizing the colonization capabilities of *B. avium* to ciliated cells in poultry (described in more detail below), a dual vaccine platform is proposed that will combat both *B. avium* and *C. jejuni*. This duel vaccine would primarily aim to protect against *C. jejuni* infections in humans and *B. avium* in poultry. Preventing economic loss to the poultry industry would be an indirect positive outcome.

B. avium (as well as all infectious *Bordetella* species) has a preference for attaching to the ciliated cells of its host (4). *B. avium* attaches to turkey tracheal epithelial cells (2). The autotransporter, Baa1, is thought to play a role in *B. avium* attachment and virulence (5). In many Gram negative species, autotransporters make up the largest protein family (6). They are usually comprised of a C-terminal beta-barrel-shaped transporter domain anchored in the outer membrane and an N-terminal passenger domain that crosses the outer membrane through the beta barrel to be exposed on the cell surface. The autotransporter Baa 1 is considered a virulence factor with the passenger domain contributing to the virulence of the pathogen, though autotransporters may serve other purposes. Some examples autotransporter-mediated virulence mechanisms include protein/ lipid degradation, serum resistance, and host cell attachment (5). While N-terminal passenger domains are similar in structure between different bacterial species, their functions vary greatly. The C terminal beta-barrel domain is a highly conserved structure for transport across the membrane but can vary greatly in the sequence (6).

Stockwell et. al. (2011) provided a functional analysis of the first putative *B. avium* autotransporter, Baa1. The *baa1* sequence was predicted utilizing bioinformatics; a 48% similarity at the amino acid level was seen between Baa1 and the known *E.coli* tissue adhesion autotransporter, AIDA-I (7). Due to the similarity of Baa1 to AIDA-1, it was hypothesized that Baa1 is involved in *B. avium* host cell attachment. To test this hypothesis, a knockout mutant was created (i.e., Δ*baa1*). Loss of the *baa1* gene resulted in a decrease of attachment to turkey tracheal cells (8). The mean percentage of attachment of wild type cells was 5.17% whereas the mean of the knockout mutant was 0.89% (8). This significant difference in attachment signified that Baa1 plays a role in *B. avium* attachment to turkey tracheal cells (8).

The Baa1 autotransporter in *B. avium* is a good candidate for a heterologous vaccine combating *B. avium* and *C. jejuni* because the mutant still attaches to the host, but is less virulent than the wild type (5). If an antigenic Campylobacter region is substituted for the wild type passenger domain, the C. jejuni protein will be secreted to the surface of the cell via the B. avium transporter domain. This chimera will plausibly result in the recognition of both bacterial cells by the turkey host's immune system thus effectively immunizing against C. jejuni and B. avium. The method and location of host cell attachment of *B. avium* in poultry validates the suitability of a potential live vaccine.

In this work, a heterologous vaccine was built by using molecular techniques to make a construct containing a *C. jejuni* gene in the passenger domain in place of the wild type *B. avium* passenger domain. FlaA and CjaA were two *C. jejuni* antigenic proteins that were used to replace the Baa1 passenger domain. FlaA is a flagellar protein and CjaA is an outer membrane protein (9). The known antigenicity of these proteins increase the likelihood that the host turkey will recognize and produce protective antibodies for *C. jejuni.* Steps in developing this platform

included constructing the Baa1 autotransporter to contain the foreign gene of interest (i.e., CjaA or FlaA) in the passenger domain on a *B. avium* suicide plasmid, and then transferring the construct from the *E. coli* suicide plasmid into the chromosome of *B. avium* by means of tri-parental mating. Allelic exchange was then confirmed. Expression, attachment, and *in vivo* challenge studies will be done as a continuation of this work.

Materials and Methods

Construction of the baa1::cjaA and baa1::flaA Suicide Plasmids

Plasmid DNA Extraction

Qiagen QuickLyse MiniPrep kit (Valencia, CA) was used to extract plasmids containing *cjaA* and *flaA* (unpublished, Temple, L.M.). Bacterial suspensions of each *E. coli* strain containing the aforementioned plasmids were made by swabbing colonies from a plate into 2 mL of sterile water. Using materials from the kit, cells from 1.5 mL of suspension were pelleted by centrifugation then resuspended in 400 µL of ice cold lysis solution. The lysate was incubated at room temperature for 3 minutes then transferred to a column, and centrifuged for 1 minute at 13,000 rpm. Next, 400 µL QLW buffer was added and centrifuged again. The spin column was transferred to a new microcentrifuge tube and 50 µL of the provided elution buffer was added to the column and centrifuged for 1 minute to release the plasmid DNA. The preparation was analyzed by gel electrophoresis and Nanodrop.

Restriction Enzyme Digest of the B154 Backbone and PCR of the C. jejuni Inserts

A double digest of plasmid B154 (containing the promoter and transporter regions consecutively) was performed. The digest contained the following: 110 µL mini prep B154 plasmid DNA, 1.5 µL AgeI enzyme, and 12 µL NE Buffer I. The digest was incubated at 37°C overnight and confirmed by gel electrophoresis. Next, 1.5 µL of the *NruI* enzyme and 13 µL of NE Buffer 3 were added to the previous digest of *AgeI*. The digest was incubated at 37°C overnight and confirmed by gel electrophoresis.

The *cjaA* and *flaA* inserts were amplified from their respective plasmids to isolate them using Polymerase Chain Reaction (PCR). The total volume of the reaction was19 µL and consisted of 10 µL of GoTaq Green polymerase, 8 µL dH_2O, 0.5 µL forward primer, 0.5 µL

reverse primer, and a colony of the *E. coli* with the respective *C. jejuni* inserts. The samples were run at the following settings: 1 cycle of 95°C for 2 minutes, 95°C for 15 seconds, 30 cycles of 47°C for 1 minutes, 68°C for 1 minute, and 1 cycle of 68°C for 5 minutes.

DNA Ligation and Transformation

The double-digested B154 plasmid containing the promoter and transporter regions was ligated with the PCR products of *flaA* or *cjaA* using the Quick Ligation™ kit (Valencia, CA) (Figure 1). The ligation mixture for *cjaA* was: 2µL concentrated plasmid, 8µL PCR product of double-digested *cjaA*, 10µL 2X Quick Ligation Buffer, and 1µL T4 DNA Ligase. The ligation mixture for *flaA* was: 5µL concentrated plasmid, 5µL PCR product of double digested *flaA*, 10µL 2X Quick Ligation Buffer, and 1µL T4 DNA Ligase. The tubes were mixed thoroughly and centrifuged briefly before incubating at room temperature for 20-30 minutes and then stored in the freezer.

T7 Express competent *E. coli* cells (NEB, Ipswich, MA) were thawed on ice for 10 minutes. Next, 5µL of each ligation mix was added to separate tubes and put on ice for 30 minutes. The tubes were heat shocked at 42°C for exactly 10 seconds and then immediately transferred to ice for five minutes. Next, 950µL room temperature SOC was pipetted into each tube and then the tubes were placed in the 37°C shaker for 60 minutes. Meanwhile, ampicillin selection plates (100 µg/mL) were warmed in the 37°C incubator. After shaking, the tubes were mixed by flicking and inverting. Then, 250µL of the transformed cells were spread on one plate. The remaining cell/ligation mixture was spread on another

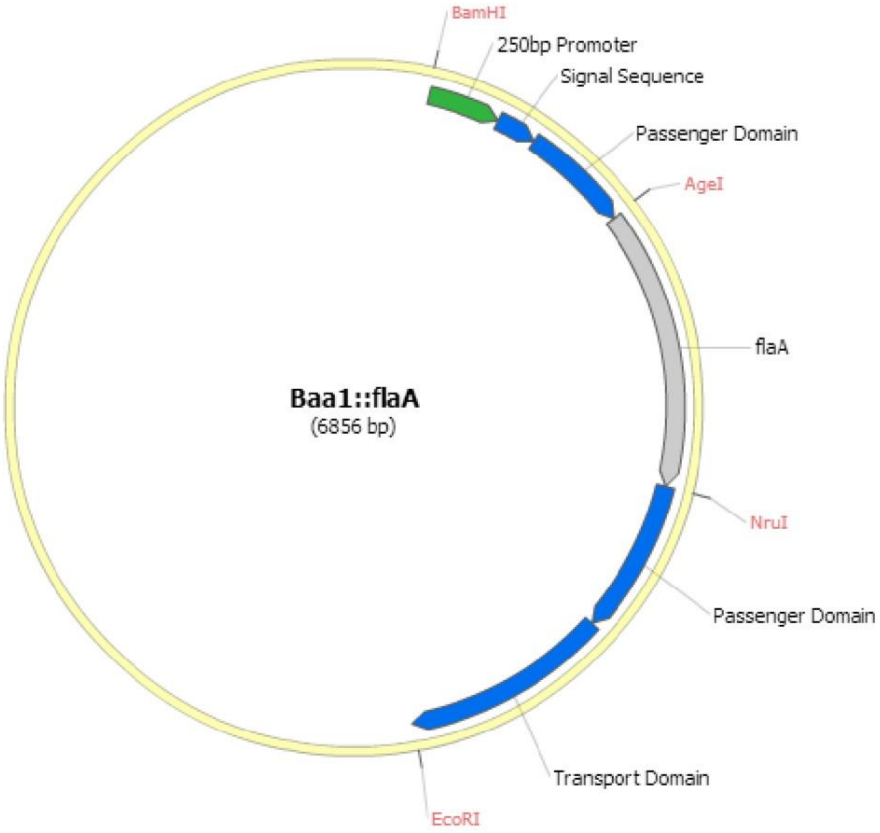

Figure 1. Plasmid Construct of Chimeric baa1 containing flaA/ cjaA in the Passenger Domain. *The flaA region was inserted into the wild type passenger domain using AgeI sites. A similar plasmid—containing the cjaA region—was also made. The flaA/ cjaA regions were inserted in the B154 backbone.*

Polymerase Chain Reaction

Putative baa1::*cjaA* and baa1::*flaA* clones were amplified using Polymerase Chain Reaction (PCR) to confirm the presence of the *flaA* and *cjaA* genes. The reaction mixture had a total volume of 19 µL and consisted of 10µL of GoTaq Green polymerase, 8 µL dH_2O (with the exception of the positive control which had 7 µL), 0.5 µL forward primer, 0.5 µL reverse primer, and a colony of the transformed *E. coli* with the respective plasmid transformed into it. The positive control contained 1µL of the respective *flaA/ cjaA* DNA from the plasmid that the inserts were originally amplified from. The samples were run at the following settings: 1 cycle of

95°C for 2 minutes, 95°C for 15 seconds, 30 cycles of 47°C for 1 minutes, 68°C for 1 minute, and 1 cycle of 68°C for 5 minutes.

Putative transconjugants in *B. avium* were amplified using PCR to confirm the presence of the *flaA* and *cjaA* genes. The reaction mixture had a total volume of 20 µL and consisted of 10 µL of KOD Master Mix (Sigma-Aldrich, St. Louis, MO), 7 µL dH_2O (with the exception of the negative control which had 8 µL), 0.5µL forward primer, 0.5µL reverse primer, and 1 µL of 40 ng/µL extracted chromosomal DNA from the putative transconjugants. The positive control contained 1µL of the plasmid DNA. The samples were run using the same thermocyles as described above. The only changes were the annealing temperatures (which were dependent upon the primers used; see results) and the temperature of extension (70°C).

Gel Electrophoresis

PCR products were observed using gel electrophoresis. One percent agarose gels were made using 0.5 g agarose, 50mL 1X TAE buffer, and one microliter of 10mg/mL ethidium bromide. A 1 kb ladder (NEB, Ipswich, MA) was used as the standard. The gels were run at 110 volts/400 amps for approximately 40 minutes. Gels were imaged on a Bio-Rad ChemiDoc MP imager.

Movement of baa1::flaA/ baa1::cjaA Construct into the B. avium Chromosome

Tri-Parental Mating

The positive *baa1::flaA* and *baa1::cjaA E. coli* clones were mated with *B. avium* 197N (10) with the help of *E. coli*/pRK2013 (11) to facilitate homologous recombination (12). A bacterial suspension of each strain was made by swabbing colonies from a plate into 3mL of LB broth. All suspensions were normalized to and optical density (OD) at 600nm of 1.0 (12). Next, 400µL of the *B. avium* suspension was mixed with 50µL of the *E. coli* (pRK2013) (11)

suspension and 50µL of the *E. coli flaA* or *cjaA* suspensions in a microcentrifuge tube. Five hundred microliters of each mating mixture was plated on a thin LB plate to provide absorption and allow for close contact. The plated mixtures were incubated at 37°C for 6-8 hours. Then, the mating mixtures were transferred to the selection plates (150µg/mL kanamycin, 30µg/mL naladixic acid, 100µg/ mL ampicillin) using cotton swabs in a single primary streak. Negative controls were mock mating mixtures, containing the recombinant *E. coli* clones, *E.coli/*pRK2013 (11), and *B. avium* 197N. The selection plates were incubated for 24 hours, after which the secondary and tertiary streaks were completed and incubated at 37°C for 48 hours. If single colonies were observed, DNA was extracted from each clone by the DNeasy kit (Quiagen, Valencia, CA). When using the synthesized plasmid clone in the tri-parental mating, only kanamycin and naladixic acid (not ampicillin) were used as selective agents.

The resulting colonies were patched on plates containing kanamycin as the selectable marker. The constant antibiotic pressure insured stability of the integrated plasmid. PCR was used to screen the putative transconjugants for the foreign *Campylobacter* region after passaging on kanamycin plates. Once verified, the kanamycin selection was removed and the putative transconjugants were passaged in LB broth to encourage a second crossover event. One colony was inoculated into 2 mL LB Broth. A dilution series of the passaged clones was made and 100 µL of the 10^{-6}, 10^{-7}, and 10^{-8} dilutions were plated to obtain individual colonies. Chromosomal DNA was extracted from each colony and the second homologous crossover was verified by PCR.

DNA Extraction of B. avium

The Qiagen DNeasy Blood and Tissue kit (Valencia, CA) was used to extract chromosomal DNA from *B. avium*. One milliliter of *B. avium* grown in broth overnight was

centrifuged for one minute at 14,000 rpm and the supernatant was removed. The pellet was resuspended in 200µL PBS. Next, 20µL proteinase K was added and incubated at 37°C for 15 minutes. Then, 200µL Buffer AL was added. After vortexing, the mixture was incubated at 56°C for 10 minutes immediately followed by the addition of 200µL of 100% ethanol. After transferring the mixture to a mini spin column, the column was centrifuged at 8,000 rpm for one minute. Next, 500µL Buffer AW2 was added and the column was centrifuged at 14,000 rpm for three minutes. The DNA was eluted with 100µL of hot distilled water and incubated at room temperature for 1 minute. The column was centrifuged for one minute at 8,000 rpm. The released and captured DNA was saved; the concentration of which was measured by Nanodrop.

Construct Synthesis and Codon Optimization

The inability to isolate stable single crossover clones of *B. avium* carrying the recombinant *flaA* or *cjaA* plasmid led to the decision to have the construct synthesized by Invitrogen (Carlsbad, CA). The *cjaA* gene was used as the foreign antigen insert between the promoter and transporter regions of *baa*1 (Figure 2) because the *cjaA* gene was shorter and therefore cheaper to have synthesized. The GC content of the synthesized *cjaA* coding region was optimized that match that of *B. avium*; *C. jejuni* has a 35% GC content whereas *B. avium* is 61% GC. The program used for this optimization was http://genomes.urv.es/OPTIMIZER/. A kanamycin resistance gene was also engineered into this plasmid as a selectable marker.

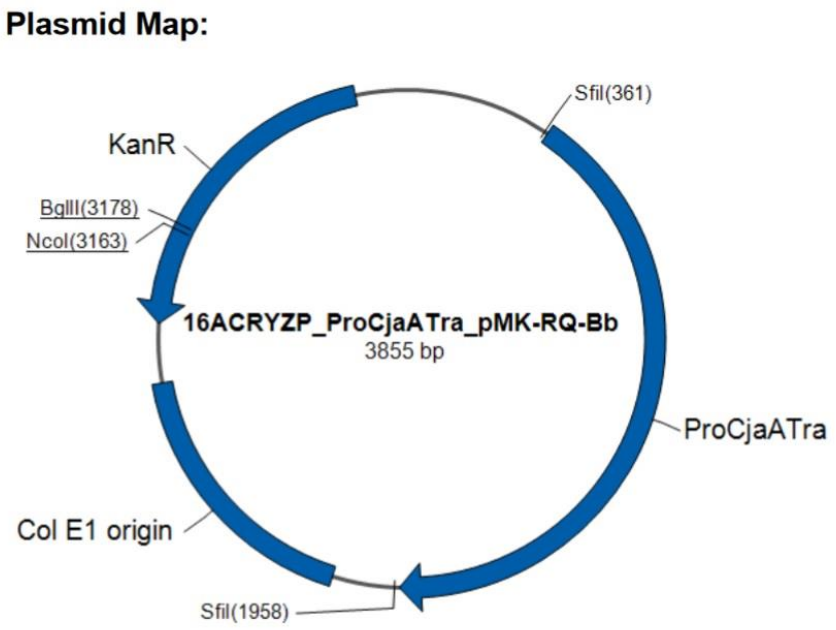

Figure 2. Map of the Synthesized Plasmid Containing the GC-Optimized cjaA Region. *The synthesized plasmid was 3855 bp long, with a 1577 bp long baa1::cjaA region.*

Primer Design

Primers were made to amplify a region of the newly synthesized construct using the computer program SnapGene (Table 1). One primer pair was selected to flank the promoter/ transporter regions and another was made to flank the *cjaA* region. They were all designed to be around 20 base pairs long, with annealing temperature close to 65°C (Table 1). Similar annealing temperatures allows for amplification using different combinations of primers. These primers were used to verify first and second homologous recombination crossover events

Table 1. Primers for the Synthesized Plasmid used in Verification of Homologous Recombination

Primer Combination	Sequence (5' to3')	Annealing Temperature (°C)	Fragment Length (bp)
ProCjaTra-F	TAGGCCTCTATGCGACGCGC	68	1519
ProCjaTra-R	AGCAGGCGGTACTCGTAAGGC		
CjaO-F	TCACCATCATCATGCGGCAGCG	69	1215
ProCjaTra-R	AGCAGGCGGTACTCGTAAGGC		

Colony Morphology Study

An investigation of the colony morphology (i.e., size) of the transconjugants was done by passaging them on different media: kanamycin/ naladixic acid, kanamycin, LB, and TSA plates.

Results

The initial goal was to produce a plasmid containing the *baa1* promoter and transporter-encoding region fused to a portion of *cjaA* or *flaA* (Figure 1). PCR was utilized to amplify the *C. jejuni* genes. The amplified PCR products were ligated with the double digested B154 (*AgeI* and *NruI*) plasmid backbone and transformed into *E. coli*. Successful ligation/transformation was verified by growth on ampicillin selection plates and colony PCR of the *cjaA* and *flaA* regions (Figure 3). Clones CjaA-10 and CjaA-13 tested positive for the recombinant plasmid (Figure 3). One clone, FlaA-14, was positive for the recombinant plasmid.

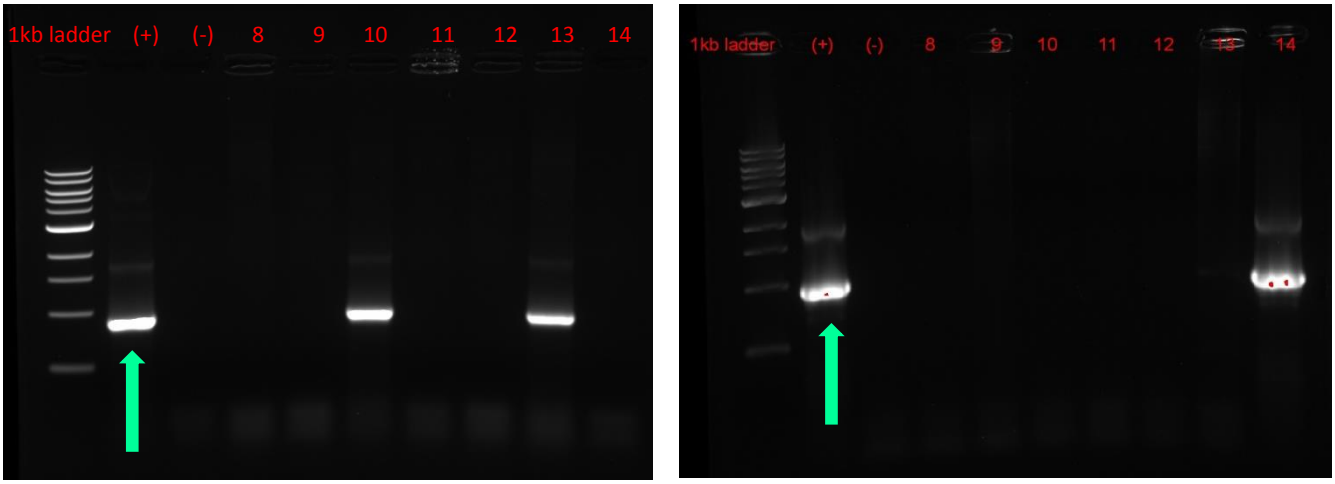

Figure 3. Confirmation of the Ligation and Tranformation of cjaA and flaA into pB154. *Gel electrophoresis showing the cjaA and flaA genes were successfully ligated in between the promoter and transporter region of Baa1 in the E. coli vector B154. The gel of samples to test the cjaA-containing clones is on the left; flaA-containing samples were assessed in the gel on the right. The arrows indicate the positive control bands of cjaA (~800 bp) and flaA (~900 bp). The sample bands (lanes 10 and 13 (left) and 14 (right)) were the correct size.*

The successfully transformed *E. coli* strains were propagated using LB plates containing ampicillin. Glycerol stocks were made for long-term storage. Next, a tri-parental mating was performed with one of the transformed *E. coli* strains for each *Campylobacter* insert: CjaA-13 and FlaA-14. Many colonies grew on the kan/amp/nal selection plates (Figure 4). Chromosomal DNA was extracted from colonies on the tri-parental mating selection plates. Using the same *flaA/ cjaA* primers used to verify the *E.coli* transformation, single crossovers events into *B. avium* 197N were assessed via PCR. Neither the *cjaA* nor the *flaA* regions were present in the putative transconjugants. Different combinations of primers, different annealing temperatures, and different DNA polymerases were used to verify the first crossover—with no success. During this PCR optimization process, only nonspecific products were observed. In other cases, no bands were observed in association with the isolated *B. avium* transconjugants (Figure S1). A purified recombinant plasmid (pB154(*cjaA*) or pB154(flaA))was used as the positive controls in all experiments and yielded visible bands at their respective locations each time.

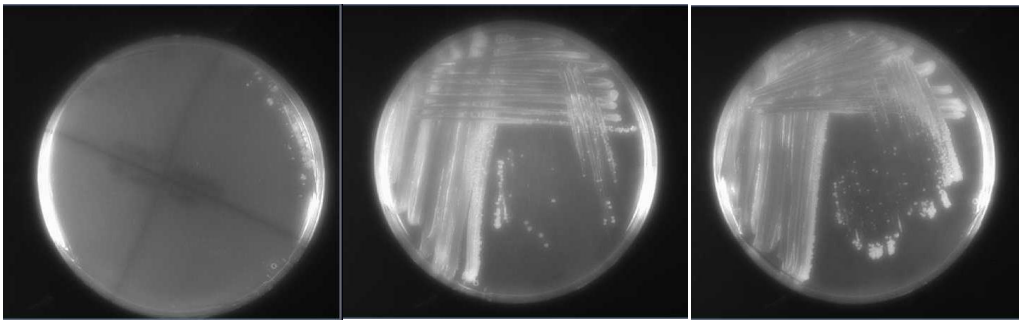

Figure 4. Tri Parental Mating Selection Plates. *Resulting growth after the tri-parental mating with the ligated plasmid, B. avium 197N, and pRK2013. The negative control plate testing the three strains individually is on the left, the cjaA mating is in the center, and the flaA mating is on the right. Note the numerous individual colonies on both the cjaA and flaA plates. It is atypical to obtain so many putative transconjugants following a mating procedure.*

PCR was done on the putative transconjugants to amplify the promoter region of the *baa1* gene. This was done to ascertain the quality of our purified chromosomal DNA. That is, if it was possible to amplify sequences found in wild type *B. avium*. The purified recombinant

plasmids were used as positive controls. All putative transconjugants showed a band at the expected size: ~250 base pairs (Figure 5).

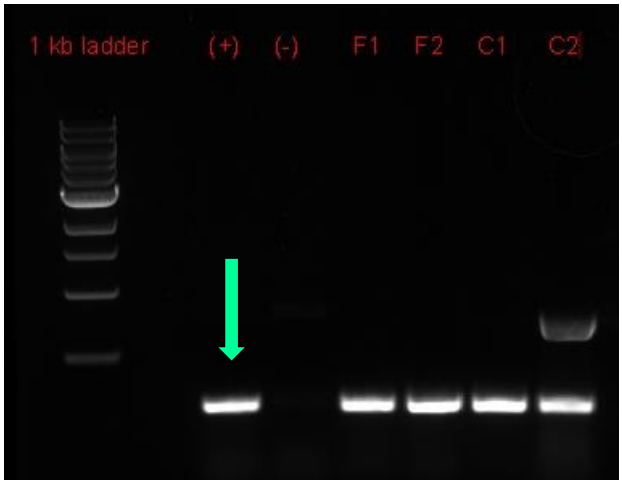

Figure 5. Verification of Amplifiable Regions in the Putative Transconjugants. *Gel electrophoresis showing that the baa1 promoter region (251 bp) of the B. avium chromosome were amplifiable in clones F1, F2, C1, and C2.*

With the results that the *cjaA* and *flaA* regions were not amplifiable (Figure S1), but other regions (such as the promoter region and the kanamycin resistance gene) (Figure S2) were, we concluded that the *Campylobacter* genes were unstable in a *B. avium* background due to incompatibility of GC content. As a result, we decided to abandon the manual cloning method in lieu of purchasing a synthesized version of the desired plasmid. In this version of the *cjaA* construct, we optimized the *cjaA* encoding fragment to better match the GC content of *B. avium* (61%). Only one plasmid was synthesized and it contained the optimized *cjaA* gene in between the promoter and transporter region of *baa1* (Figure 2) because the *cjaA* gene was shorter than *flA* and therefore cheaper to have synthesized. The purchased plasmid contained a kanamycin resistance gene as a selectable marker.

The newly synthesized plasmid was transformed into *E. coli* and recombinant clones were confirmed by PCR. Another tri-parental mating was done utilizing *E. coli* containing the synthesized plasmid, pRK2103 (11), and *B. avium* 197N. These mating plates looked more typical of tri-parental mating plates, with fewer colonies forming in the first quadrant (Figure 6). These colonies were passaged on kan plates initially. Chromosomal DNA was extracted and PCR was done utilizing the new primers that were designed (Table S1) to verify that the construct had crossed over into the chromosome during the tri-parental mating.

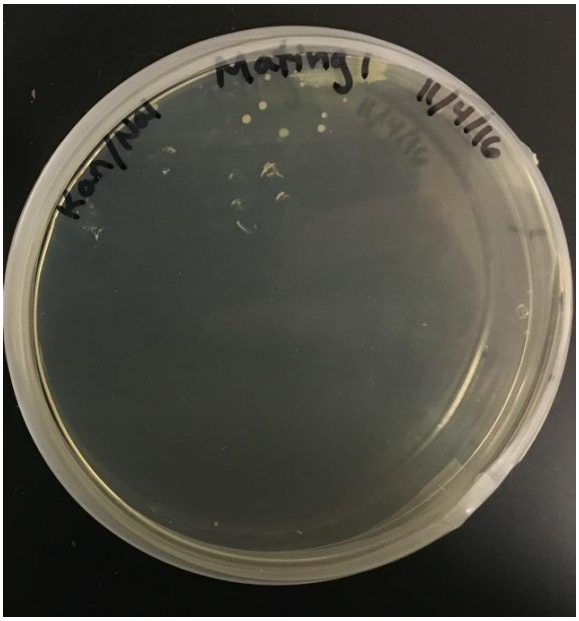

Figure 6. Tri-Parental Mating Selection Plate with Synthesized Plasmid. *This LB plate contained kanamycin and naladixic acid to select for the putative B. avium transconjugants and displays a typical number of colonies arising from a tri-parental mating.*

Different combinations of the ProCjaTra-F, ProCjaTra-R, CjaO-F, and CjaO-R primers were used to verify the first crossover event into *B. avium*; a temperature gradient was used to assess which annealing temperature yielded the most robust singular bands. The ProCjaTra-F/ProCjaTra-R primer set was a pair that gave the properly sized band on the gel (1,519 base pairs) (Figure 7). Another band around the same intensity appeared below the band of interest at

just over 1,000 base pairs. This is believed to be the amplicon of the wild type copy of *baa1* that would also appear in the transconjugants after the first crossover occurs Annealing temperatures over 60°C gave more distinct, but less intense banding patterns whereas annealing temperatures under 60°C gave less distinct but higher intensity bands. When using this primer set again, 60°C was chosen as the annealing temperature.

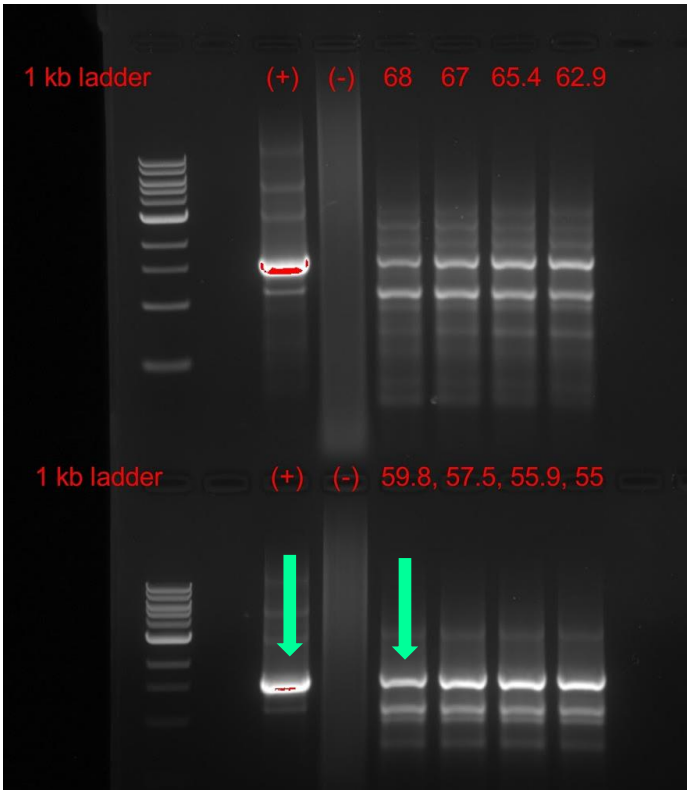

Figure 7. Verification of the First Crossover using a Temperature Gradient PCR. *Gel electrophoresis of a temperature gradient PCR reaction on the chromosome of one sample (called BPCT-1)of the putative transconjugants to verify the first crossover. The annealing temperature ranged from 55°C to 68°C (indicated above each lane). ProCjaTra-F and ProCjaTra-R primers were used. The arrow on the left is indicating the positive control band and the arrow on the right is indicating the band of interest.*

The CjaO-F/ ProCjaTra-R primer combination also resulted in the expected band size of 1,215 base pairs (Figure 8), with the addition of another smaller intensity band immediately

beneath it, just above the 1,000 base pair marker. Future reactions with this primer set used 65°C as the chosen annealing temperature.

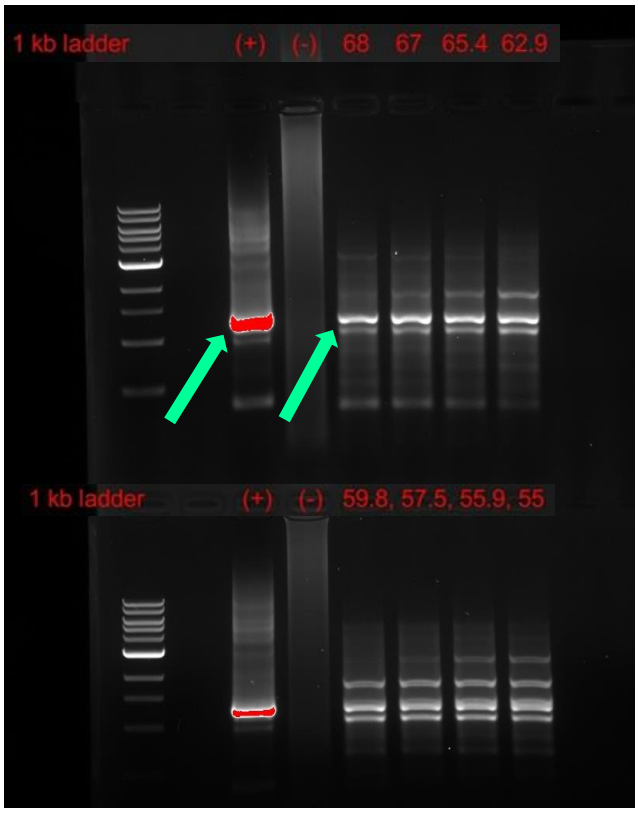

Figure 8. Verification of the First Crossover using a Temperature Gradient PCR Using CjaO-F and ProCjaTra-R Primer Combination. *Gel electrophoresis of a temperature gradient PCR reaction on the chromosome of one sample (BPCT-1)of the putative transconjugants to verify the first crossover. The annealing temperature ranged from 55°C to 68°C. CjaO-F and ProCjaTra-R primers were used. The arrow on the left is indicating the positive control band and the arrow on the right is indicating the band of interest.*

These results indicated that the first crossover event was successful, and we had recombinant *B. avium* transconjugant. Next, kanamycin was removed from the media to allow the loss of the plasmid backbone from the chromosome. Unusual colony morphologies were observed during this passaging process. Interestingly, these morphologies were not when the transconjugants were under kanamycin selection (Figure 9). When under selection, the colonies

were opaque, mucosal, and punctiform. After taking the selection away, a mixed morphology arose (Figure 9). Colonies were still opaque and mucosal, but some colonies were bigger and some remained punctiform.

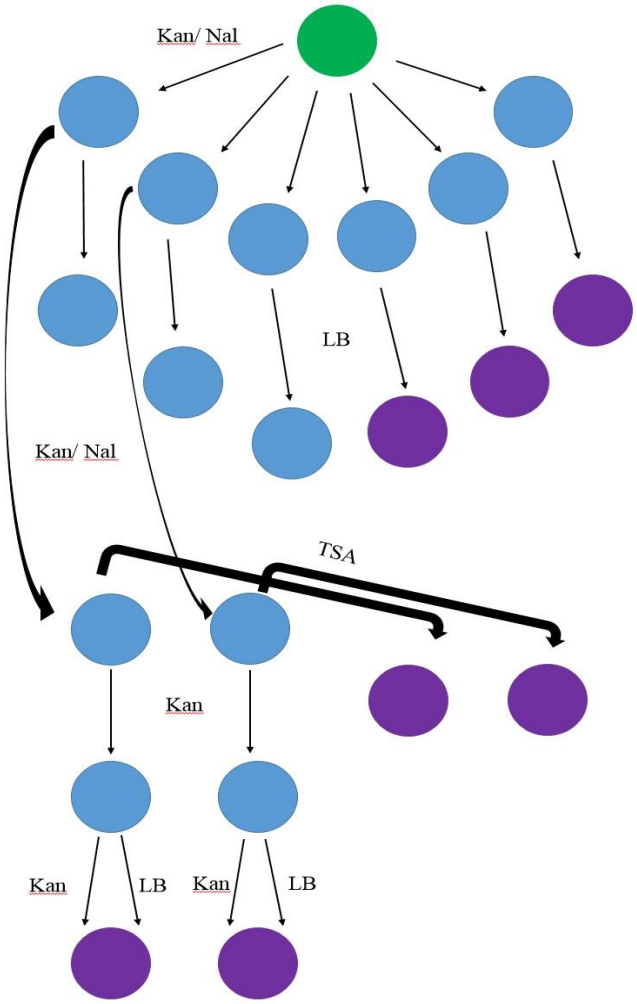

Figure 9. Flow Chart Diagram and Results of Mixed Colony morphology study. *A flow chart that indicates the passaging of colonies from one plate to another, the type of media they were transferred to, and the resulting colony sizes. The green plate indicates the selection plate that the tri-parental mating was plated on. The blue plates indicate growth of punctiform colonies. Purple plates indicate larger colonies.*

Kanamycin selection was removed and two *B. avium* clones were serially passaged in LB broth. Cultures were diluted and plated to obtain isolated colonies. Of these two separate plates containing isolated colonies, chromosomal DNA was extracted from five colonies from each plate. The CjaO-F/ ProCjaTra-R primer pair was used in a PCR reaction to validate the second crossover, and thus the completion of homologous recombination. The positive control displayed a correct band size of 1,215 base pairs (Figure 10). Other nonspecific bands were present in the positive control. All samples with the exception of sample 2-13 displayed a prominent band at the expected size—approximately 2,000 base pairs. Sample 2-13 displayed a prominent band at approximately 1,200 base pairs (Figure 10). All samples displayed multiple nonspecific bands.

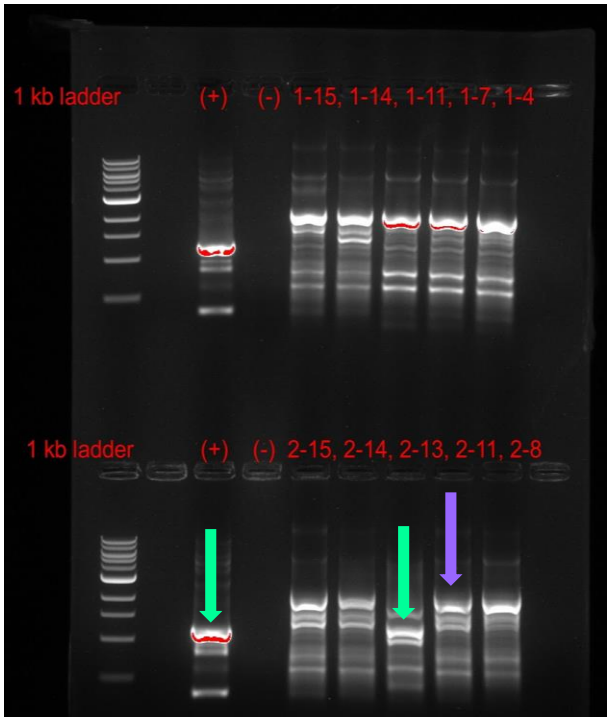

Figure 10. Verification of the Second Crossover on Ten Different Passaged B. avium Transconjugants*. PCR was done on chromosomal DNA to verify the second crossover event, resulting in the loss of the wild type copy. The annealing temperature was 65°C and CjaO-F and ProCjaTra-R primers were used. The green arrows indicate the positive control band at 1215 bp (left) and successful transconjugant (center). The purple arrow (right) indicates a nonspecific band and therefore an unsuccessful transconjugant.*

A follow-up PCR reaction was completed to reduce the nonspecific bands by using a higher annealing temperature. Both CjaO-F/ ProCjaTra-R and ProCjaTra-F/ ProCjaTra-R primers were used. The ProCjaTra-F/ ProCjaTra-R primer set used a 68°C annealing temperature whereas the CjaO-F/ ProCjaTra-R primers used a 69°C annealing temperature. Random bands were visible for 1-15 using the ProCjaTra-F/ProCjaTra-R primer set. Also, a band mirroring the positive control at 1,500 base pairs was visible in 2-13 (Figure 11). However, the intensity was weak and there were still other nonspecific bands. When using the CjaO-F/ ProCjaTra-R primers, no bands were visible in the 1-15 sample indicating the *cjaA* gene was not present in that sample's chromosome. This confirmed 1-15 was a negative transconjugant, but still showed the possibility of 2-13 being a true transconjugant because the largest and brightest band matched the positive control at approximately 1,200 base pairs.

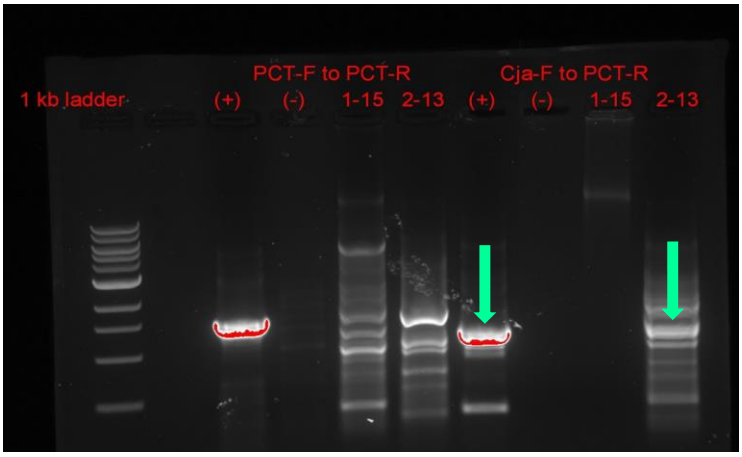

Figure 11. Verification of Recombinant B. avium clones containing the baa1::cjaA construct. *Gel electrophoresis of a PCR reaction on the samples 1-15 and 2-13 using ProCjaTra-F and ProCjaTra-R primers at 68°C and CjaO-F and ProCjaTra-R primers at 69°C. The mint green arrows indicate the positive control band at 1215 bp (left) and successful transconjugant 2-13 (right).*

Discussion

This purpose of this project was to construct a recombinant *B. avium* strain that could be used as a heterologous vaccine platform to combat the causative agent of food poisoning, *C. jejuni*, and simultaneously immunize against the causative agent of bordetellosis in turkeys, *B. avium*. This vaccine could be administered to turkeys to combat bordetellosis and reduce *Campylobacter* contamination of poultry products. Doing so would indirectly reduce human food poisoning. Making a vaccine that combined would immunize against a human pathogen and an avian pathogen would give turkey farmers an economic incentive to actively advocate for this vaccine. All parties benefit, including turkey farmers, the poultry, and the consumers. This vaccine could relieve multiple related illnesses.

Though initially expression of the construct via plasmid was the ideal, getting the construct into a plasmid that replicated in *B. avium* proved problematic. Tri-parental matings with a recombinant suicide plasmid had to be done to deliver the *baa1-cjaA* construct into the chromosome to produce a stable chimera. This was the most direct approach with the greatest likelihood for expression. To produce the recombinant suicide plasmid, molecular cloning of an antigenic *C. jejuni* gene (i.e., *cjaA*) into the passenger region of the Baa1-encoding autotransporter was the primary strategy. Since the Baa1 autotransporter was shown to be involved in tracheal cell attachment and is also antigenic, this protein was a good candidate for the heterologous vaccine platform. Apparent instability of the *C. jejuni* genes in the *B. avium* chromosome led to the synthesis of the plasmid and simultaneous codon optimization of the *cjaA* gene as a way to combat the instability. After tri-parental mating and primer design, first and second crossovers in the process of homologous recombination were verified resulting in a successful *B. avium*/ *C. jejuni* transconjugant.

Multiple attempts were made to verify that the construct crossed over into the *B. avium* chromosome. Various annealing temperatures, polymerases, and controls were used to detect the construct to no avail. However, evidence that other regions of the chromosome were amplifiable (Figure 5) led to the conclusion that this was not necessarily a matter of PCR, but rather instability of the *C. jejuni*- containing construct within the *B. avium* chromosome. *B. avium* has a GC content of 61% whereas *C. jejuni* has a GC content of approximately 30%. This difference impacts translation of proteins within these two organisms and is thought to be a main reason of the instability of the construct. Since other regions of the chromosome were amplifiable, it was reasonable to conclude that the specific *C. jejuni* regions were not maintained due to instability as a result of GC content incompatibility. In theory, synthesizing the construct would make the *cjaA* region more stable when put in the *B. avium* chromosome.

Aside from synthesizing the construct, PCR optimization played a major role in this project. For example, when verifying the first crossover using ProCjaTra-F/ProCjaTra-R (Figure 7) and CjaO-F/ ProCjaTra-R (Figure 8), unexpected nonspecific bands were present in the samples, but were not in the positive control that was the synthesized plasmid. These bands may have been showing that some primer was annealing to the wild type copy of the promoter/ transporter regions that would have still been in the chromosome after the first crossover.

In an attempt to verify that 2-13 was indeed positive for the *cjaA* region, multiple PCR reactions were completed. Nonspecific bands were present (Figure 11), but there were fewer than the first round of PCR verification for the second crossover. This gel showed bands in the expected size range though, which was convincing evidence that a stable chimera was produced, but more conclusive evidence would be preferable. It is still unclear as to why a clean PCR product could not be obtained. One possibility was that the primers were not well designed and

that they should be designed to be longer and potentially with a higher annealing temperature. Different strategies to primer design should be tested for optimization of PCR to obtain a larger product yield. Eventual sequencing of the chimera should be done as the most conclusive evidence to show the *cjaA* construct was transferred into the chromosome. Essentially, the part of the process proving to be the most difficult was not the mating itself, but rather the detection and verification of the *Campylobacter* gene in the *B. avium* chromosome.

The size difference in colonies when removing kanamycin resistance was an interesting secondary finding of this study. This morphology difference may be due in part to the expression of the *cjaA* gene in *B. avium*. A *C. jejuni* gene has never been inserted into baa1 before, so phenotypic consequences of doing so were unknown. If this insertion is unknowingly reducing the fitness of the *B. avium* cell, the second crossover is more likely to regenerate the wild type copy of the gene rather than the mutation (13). It is quite possible that the different sizes of the colonies correspond to which copy of *baa1* they contained. If the morphology was truly due to the expression of CjaA in a *B. avium* cell, future expression studies can potentially elucidate why the morphologies differed.

In this study, we continued the construction of a heterologous vaccine combatting the causative agent of food poisoning and bordetellosis, *C. jejuni* and *B. avium* respectively. Future work will involve the testing of the transconjugant 2-13 in a turkey tracheal cell adhesion assy. Previous work showed that loss of Baa1 reduced *B. avium* adhesion to the host cell, but did not affect infection rate in turkeys (5). It is important that this mutant version of Baa1 adheres to the host to elicit an immune response. A preliminary study using standard methodology (10) will be done to assess how well the *cjaA*-encoded protein is translocated to the surface of the bacterial

cell. In vivo studies facilitated by partners at the VA/MD School of Veterinary Medicine (VT) will be performed to further study the mutant. Finally, challenge studies will be performed.

Supplemental Data

Table S1. *Primers for the Synthesized Plasmid Containing the Optimized ProCjaTra Gene*

Primer Name	Sequence (5’ to 3’)	T_m (°C)
ProCjaTra-F	TAGGCCTCTATGCGACGCGC	66
ProCjaTra-R	AGCAGGCGGTACTCGTAAGGC	66.47
CjaO-F	TCACCATCATCATGCGGCAGCG	66.4
CjaO-R	ATCACCACGTCGTCGGCCTTC	66.47
NewCjaJunct1-F	TCAACAGTGGCGGCACCGGTCA	68.26
NewCjaJunct1-R	GGCCTTCACCTCGTCGCCGAAAT	68.12
NewCjaJunct2-F	CCCAGGGCGGAAAAATCGTGCTC	68.12
NewCjaO-R	GGAAGAACTGCTCCTGGCCCAGC	69.9

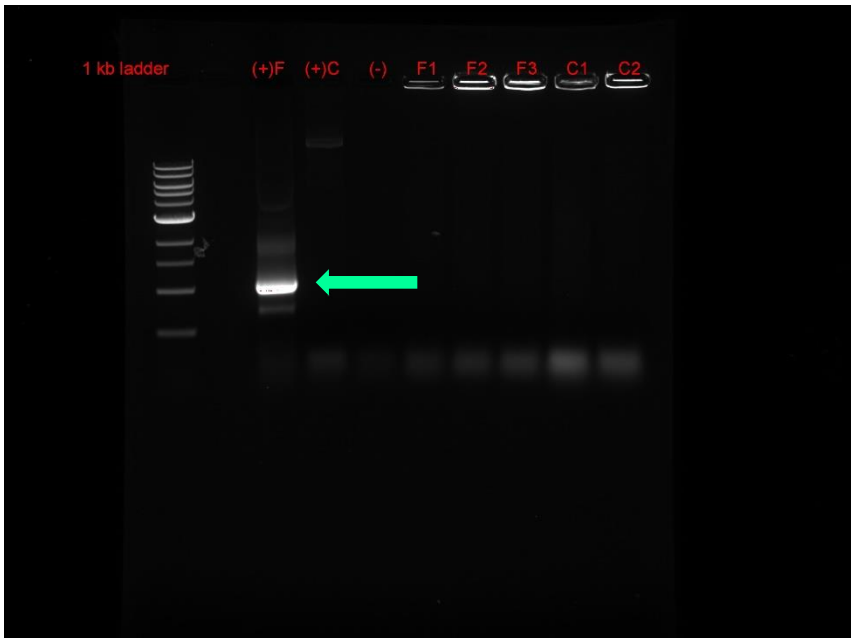

Figure S1. Campylobacter Regions Absent in B. avium Chromosome. *Gel electrophoresis image showing colony PCR of putative transconjugants. No Campylobacter bands are shown indicating instability in the B. avium chromosome. The arrow is indicating the proper band size of the flaA control at 912 base pairs.*

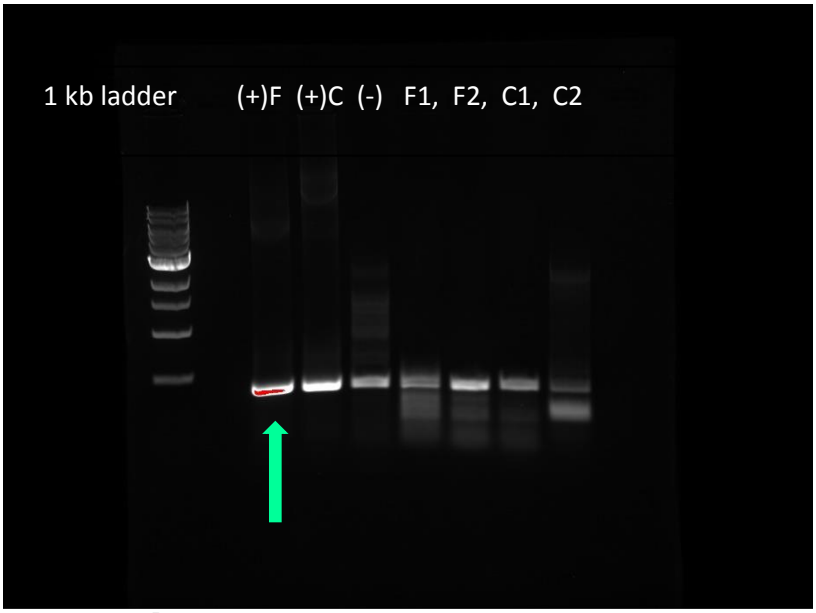

Figure S2. Amplifiable Kan^R Gene Amplifiable in Putative Transconjugants. *Though the Campylobacter gene was not present after PCR analysis, the kanamycin resistance gene was amplifiable via PCR on the chromosomes of the putative transconjugants. The arrow is indicating the positive control band of the kanamycin resistance gene at just under 500 base pairs.*

References

1. Allos BM. 2002. Campylobacter jejuni infections: update on emerging issues and trends. Food Saf 31.
2. Jackwood MW, Saif YM. 2013. Bordetellosis (turkey coryza), p. 774. *In* Calnek BW Barnes HJ, BCW et al. (ed.), . Blackwell Pub. Professional, Ames, Iowa.
3. Raffel TR, Register KB, Marks SA, Temple L. 2002. Prevalence of Bordetella avium infection in selected wild and domesticated birds in the eastern USA. J Wildl Dis 38:40.
4. Arp LH, Cheville NF. 1984. Tracheal lesions in young turkeys infected with Bordetella avium. Am J Vet Res 45:2196–2200.
5. Stockwell SB, Kuzmiak-Ngiam H, Beach NM, Miyamoto D, Fernandez R, Temple L. 2011. The autotransporter protein from Bordetella avium, Baa1, is involved in host cell attachment. Microbiol Res 167:55–60.
6. Benz I, Schmidt MA. 2011. Structures and functions of autotransporter proteins in microbial pathogens. Int J Med Microbiol 301:461–468.
7. Sherlock O, Schembri MA, Reisner A, Klemm P. 2004. Novel roles for the AIDA adhesin from diarrheagenic Escherichia coli: cell aggregation and biofilm formation. J Bacteriol 186:8058–8065.
8. Stockwell SB, Kuzmiak-Ngiam H, Beach NM, Miyamoto D, Fernandez R, Temple L. 2011. The autotransporter protein from Bordetella avium, Baa1, is involved in host cell attachment. MicrobiolRes 167:55.
9. Wenman WM, Chai J, Louie TJ, Goudreau C, Lior H, Newell DG, Pearson AD, Taylor DE. 1985. Antigenic Analysis 21:108–112.
10. Temple LM, Weiss AA, Walker KE, Barnes HJ, Christensen VL, Miyamoto DM, Shelton CB, Orndorff PE. 1998. Bordetella avium virulence measured in vivo and in vitro. Infect Immun 66:5244–5251.
11. Figurski DH, Helinski DR. 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. Proc Natl Acad Sci U S

A 76:1648–52.

12. Walker KE, Weiss AA. 1994. Characterization of the dermonecrotic toxin in members of the genus Bordetella. Infect Immun 62:3817–3828.

13. Bloor AE, Cranenburgh RM. 2006. An Efficient Method of Selectable Marker Gene Excision by Xer Recombination for Gene Replacement in Bacterial Chromosomes An Efficient Method of Selectable Marker Gene Excision by Xer Recombination for Gene Replacement in Bacterial Chromosomes. Society 72:2520–2525.