CLONING A VIRULENCE GENE FROM *BORDETELLA AVIUM*

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ABSTRACT

Bordetella avium (*B. avium)* causes bordetellosis, a disease similar to whooping cough, in turkeys and other birds. *B. avium* has proteins on its outer membrane called fimbriae which attach to the cilia in the turkey's trachea. The aim of this project was to isolate the fimbrial genes in order to synthesize fimbrial proteins and to eventually create a component vaccine. Polymerase chain reaction was performed on four different *B. avium* genes expected to code for fimbriae: *fim*A, *fim*1, *fim*2, and *fim*3. Three genes were successfully amplified, and were subsequently ligated into the cloning vector, pET151/D- TOPO. The ligated plasmids were then transformed into *Escherichia coli (E. coli)* to allow the plasmids to replicate. The plasmids were isolated and examined, and although electrophoresis did not prove that ligation had occurred, the *E. coli* were cultivated to synthesize the fimbrial protein from the plasmid DNA. Protein gel analysis showed that these cells did not contain proteins of the expected size, which leads to the conclusion that the fimbriae genes were not properly expressed. Future research should continue attempts to synthesize the fimbriae protein because this is an essential step to developing a working component vaccine.

INTRODUCTION

Bordetellosis is a contagious upper respiratory tract infection that infects birds worldwide, including turkeys, chickens, finches, macaws, ostriches, and others [1]. It is caused by the Gram negative bacterium, *Bordetella avium* (Figure 1). This bacterium is related to *Bordetella bronchiseptica* which causes kennel cough in dogs and *Bordetella pertussis* which is responsible for whooping cough in humans.

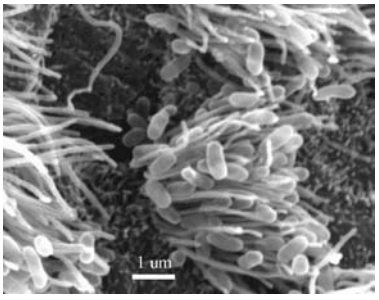


Figure 1: Scanning electron micrograph showing *B. avium* attaching to the cilia of a turkey's trachea. [2]

Bordetella avium most prominently affects commercially-grown turkeys, especially ones that are two to six weeks of age. It harms the host by colonizing the ciliated epithelium in the tracheal mucous which results in inflammation and eventually destruction of the outer tissue of the trachea [1]. Some telling signs of bordetellosis include altered vocalization, sneezing, conjunctivitis, mucous discharge from the oculonasal cavity, and weight loss. The infection itself is not lethal; rather, the bacteria impair growth of the host and incite other toxins to affect other tissues of the body. This makes the birds susceptible to secondary infection, most commonly *E. coli*, which can be lethal. While bordetellosis harms birds on a global scale, it is especially important to study its effects on turkeys. Turkeys are an integral part of the poultry market and the sick and dying birds cause the economies of the United States, Canada, and other countries to lose several million dollars annually [3].

Attempts have been made to treat or prevent bordetellosis through both antibiotics and vaccines, but with no great degree of success. *B. avium* is sensitive to some antibiotics but these do not cure the disease or even lessen its effects [4]. The only existing commercially available vaccine for *B. avium* is a live mutant strand produced by nitrosoguanide mutagenesis. However, this only reduces the effects of the disease rather than preventing it or containing the infection [5].

Vaccines, by definition, are supplements consisting of a killed or weakened pathogen or a component of that pathogen that, when dispensed, stimulate the immune system to build up antibodies with the ability to do away with that particular type of pathogen should it infect the host in question at a later date [6]. In humans, for example, administering the polio vaccine to children stimulates their immune systems to produce antibodies that, if the body should become infected with the polio virus at some point later in life, the immune system would "know" how to eradicate it. In other words, vaccines build up "memories" in the immune system of how to overcome different types of pathogens.

There are several different types of vaccines that are used to prevent infection. Inactivated vaccines make use of dead pathogens to stimulate the immune system. Live, attenuated vaccines incorporate laboratory-cultured and grown pathogens that are not virulent (weakened). Even though they are no longer virulent, the immune system will still be able to form a "memory" of that pathogen. Toxoid vaccines are made from pathogenic toxins that have been inactivated, yet, like the live, attenuated vaccine, still manage to elicit immune response. Conjugate vaccines allow immunization in immature immune systems against pathogens that often disguise themselves in other forms through attaching readily-recognizable pathogens to their outer coats. Recombinant vector vaccines supplant harmful genetic material from its own cell or capsid into that of a different weakened virus or bacteria. Finally, subunit (or component) vaccines consist of pathogenic *parts* of a particular antigen. Like the toxoid or live, attenuated vaccines, these still manage to stimulate the production of antibodies [7]. In the case of *Bordetella avium*, a subunit vaccine consisting of the bacterial protein would be the best plan of action for inoculation.

Bordetella avium has projecting from its outer membrane structures called fimbriae (sometimes referred to as pili) that help in attachment to the ciliated tracheal cells of turkeys. There is one major structural protein that makes up the fimbriae, and recent sequencing of the *B. avium* chromosome shows that there are at least eight copies of what appear to be variants of the

main structural *fim* gene. As aforementioned, it seems that the *B. avium* fimbriae are essential to attachment. Therefore, a component vaccine making use of the fimbriae seems the ideal path to take toward immunization [3].

In order to make a vaccine for *Bordetella avium*, the fimbrial proteins have to be reproduced in sizable quantities. The most efficient way to accomplish this task is to clone the stretches of DNA within *Bordetella avium*'s genetic code that encodes for these proteins. This is achieved through a process called polymerase chain reaction (PCR) used to amplify DNA (Figure 2).

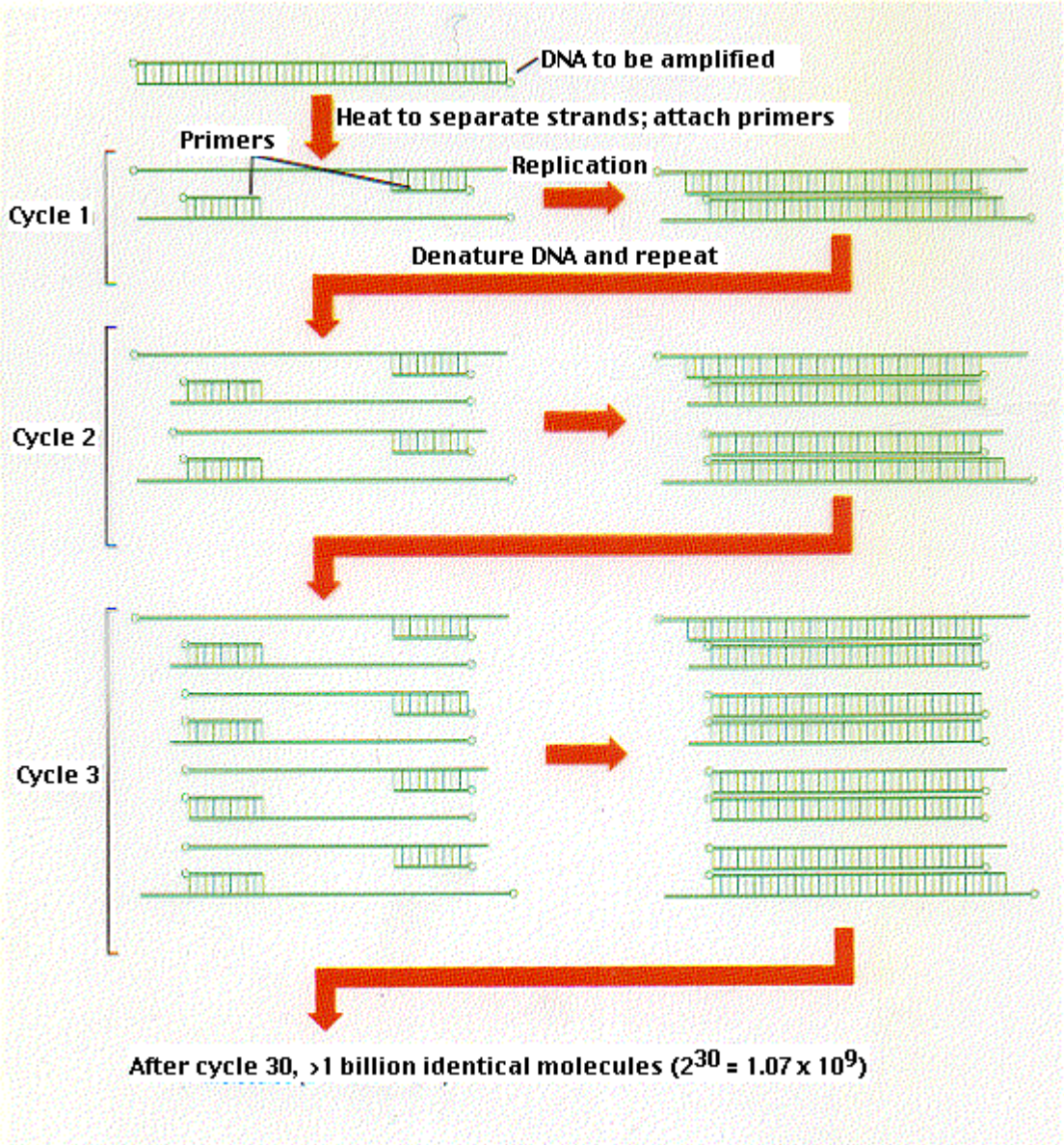


Figure 2: The cycles of PCR [8].

The first step in PCR is determining the sequence of the gene that will be replicated; more specifically, finding about 20-45 nucleotides that start the gene sequence on one strand of the double helix of DNA, and finding the last 20-45 nucleotides of the opposite strand. These short

single-stranded DNA sequences are called primers. Once the primers have been created, they are mixed with the *Thermus aquaticus* (*Taq)* DNA polymerase enzyme, the chromosomal DNA to which the gene that will be replicated belongs, and free dideoxy nucleotides. Denaturation separates the two strands of DNA, and the primers anneal to their complementary strands. The *Taq* polymerase uses the free nucleotides to copy the rest of the DNA strands. This cycle of denaturation and polymerization continue until the desired amount of DNA is replicated.

The products of the PCR are linear fragments of *Bordetella avium*'s DNA that encode for the fimbrial proteins. Such DNA is unstable in bacteria. However, if the DNA is connected to a plasmid, a circular ring of double-stranded DNA, the gene would be replicated along with the bacterial cell. This experiment used a cloning vector, or an incomplete plasmid that would be completed by the fimbriae gene code. The cloning vector used, pET151/D-TOPO, also has special features, engineered in the plasmid by molecular biologists (Invitrogen) to optimize the use for cloning (Figure 3).

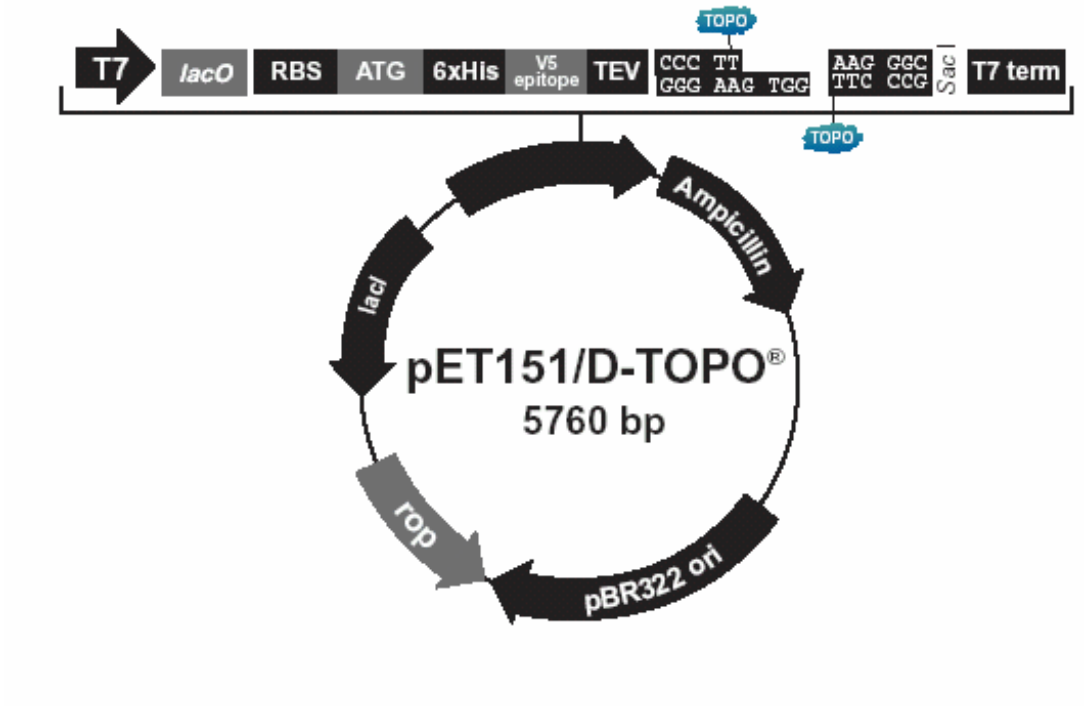


Figure 1: The plasmid pET151/D-TOPO used in the cloning vector (Invitrogen).

One beneficial feature installed in our plasmid by Invitrogen is the presence of the enzyme topoisomerase at the plasmid's cloning site. Normally, a PCR product is attached to a plasmid during the process of ligation, in which the enzyme DNA ligase forms phosphodiester bonds between the sticky ends, or unbonded single strands of DNA, of the plasmid and PCR product. The plasmid and PCR product are then connected in a double-stranded DNA circle. However, the plasmid used in this experiment was already bonded with the enzyme topoisomerase, which connected the plasmid and PCR product using phosphodiester bonds. (Figure 4).

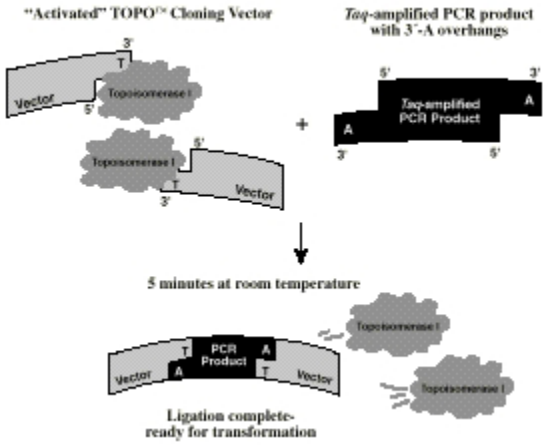


Figure 4. PCR product is ligated into a vector via topoisimerase [9].

Another beneficial characteristic of this plasmid is its T7 promoter, which allows for transcription of the gene that encodes for the fimbrial protein within the plasmid (Figure 5). A repressor protein binds to the *lacO* site and restricts RNA transcription. Transcription is promoted when the plasmid is presented with a homologue of the monosaccharide lactose. The repressor protein binds to the lactose, releasing its grasp on the *lacO* site, and allowing for the T7 RNA polymerase to transcribe. This process is called induction, and it allows for production of large amounts of the cloned gene to be transcribed into RNA and subsequently translated into protein.

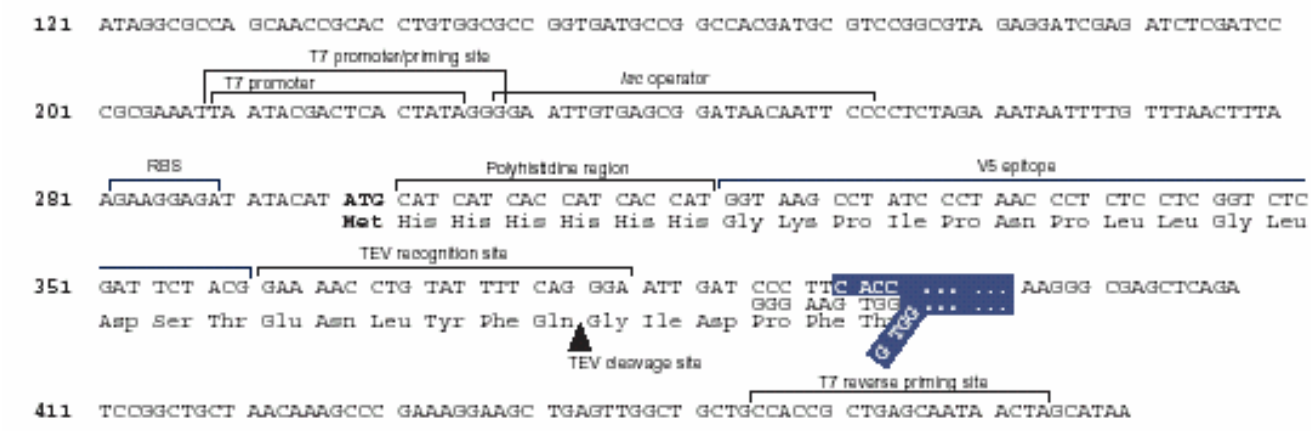


Figure 5: The base pair sequence of the pET151/D-TOPO plasmid (Invitrogen).

This plasmid also has another characteristic that would be useful in purifying the protein of the cloned gene. Near the cloning site, the plasmid has a histidine tag, a successive strand of six codons that encode for the amino acid histidine. When put into a column with nickel-nitriotrilacetic acid (Ni-NTA), a compound with a nickel ion attached, any histidine amino acids will bind to the nickel ion and thus attach to the immobilized NTA. Since histidine tags do not naturally occur in plasmids, the only proteins attached to the column will be the ones made by these pET151/D-TOPO plasmids. To elute the protein, imidazole is added and will compete with the protein for the nickel ion.

METHODS

Polymerase Chain Reaction

Primers were designed using the DNA calculator on http://www.sigma-genosys.com/order_DNA Calc.asp (Table 1).

Primer Name	Forward Sequence	Reverse Sequence
Fim A	5'-CAC CGC GGC AGA CGG CAC GAT CAC TAT TC-3'	5'-CCT GTT ACG CCT CAG GGG TAC ATG ACA G-3:
Fim 2	5'-CAC CGC CGA CGG GCT GAT CAC CAT C-3'	5'-GGG GCC ACG TCA GGG ATA AAC G-3'
Fim 3	5'-CAC CGC GGC AGA CGG CAC GAT CAC TAT TC-3"	5'-CCT GTT ACG CCT CAG GGG TAC ATG ACA G-3'

Table 1: Sequences of the primers used in the PCR.

The primers all had concentrations of 10 picomoles per reaction mixture.

To create the PCR, the reagents were first kept on ice. Then, 10.5µl of sterile water were added to a tube. Next, 5.0µl of GC melt and 5.0 µl of buffer were added. Then, 1.0µl of the template (*B. avium* 197N), 2.0 µl of mixed forward and reverse primers, 1.0 µl of dNTPs (dideoxynucleotides), and 0.5 µl of DNA polymerase (Clontech GC cDNA Advantage Polymerase) were combined with the existing mixture. After the reagents were all mixed, the samples were placed into the PCR thermocycler at 94°C for 3 minutes and 30 cycles of 94° (15 seconds), 58°C (15 seconds), and 68°C (30 seconds). Finally, the thermocycler was set at 68°C for 4 minutes and then at 4°C until the samples were retrieved. This process was repeated for each set of *fim* gene primers.

After the samples were retrieved, an analysis of 5 µl of the sample on a 1.8% agarose gel was completed. The gel was made by adding 0.6 g of agarose to 30 mL of TAE with ethidium bromide (buffer) in a 100 mL beaker with a stir bar. The TAE contained 40 mM Tris-acetate and 2 mM Na_2EDTA, and had a pH of 8.5. The beaker with the solution and the stir bar were placed on a heating plate and the solution was heated until it turned clear. Meanwhile, the gel caster was prepared by placing the gel holders and the comb into their appropriate slots. When the agarose solution was ready, the contents of the beaker (except the stir bar) were poured into the gel former. When the gel hardened, the comb was removed and buffer was added until it covered the surface of the gel. The wells were numbered starting with the well closest to the individual loading the gel. Five µl of the sample was mixed with 3 µl of loading dye and all 8 µl of the solution were placed into the first well. The loading dye was made up of 0.25% bromophenol blue and 30% glycerol in 10 µl Tris-HCl, and had a pH of 8. In the second well, 10 µl of 1kb ladder (standard) was loaded, and the gel was run for 30 minutes at 80 volts.

Ligation

After the PCR products were obtained, ligation was performed; that is, the replicated *fim* gene was combined with the cloning plasmid, known as a vector. Two µl of each PCR product was pipetted into a mixture containing 1 µl of the vector pET-151D/TOPO, 1 µl salt solution, and 2 µl of sterile water. These combined mixtures were then put on ice for 15 minutes while the PCR product combined with the plasmid.

Transformation

Next the ligated plasmids were moved from the test tube into an *E. coli* strain, the Top 10 strain (Invitrogen) in order to cultivate and replicate the plasmids, in a process called transformation. Three µl of each plasmid mixture was added to 30 µl of *E. coli* cells. Positive and negative controls were also prepared; the positive control consisted of 1µl of a previously prepared plasmid and 30 µl of *E. coli,* while the negative control was contained only 30 µl of *E. coli.* These samples were then placed on ice for 30 minutes. After being chilled, they were moved to a 42°C water bath for 30 seconds and then back to ice for another 30 seconds. A 250 µl SOC medium of Tryptone, yeast extract, NaCl, and $MgCl_2$ was then mixed into the tubes. After this the tubes of bacteria were placed in a 37°C rotating incubator for an hour. After sterilizing a glass spreader with alcohol, 50µl of each sample was spread onto separate plates of Luria-Bertani agar with 100µg/ml ampicillin. On another set of LB-Amp plates, 200µl of each sample was spread. A plate with 200µl of the negative control and plate with 50µl of the positive control were also prepared.

Miniprep (Qiagen Spin-Prep Kit) and Restriction Digest

After the bacteria were incubated, single colonies were selected and inoculated into 2 ml Brain Heart Infusion (BHI) broth with 100 µg/ml ampicillin**.** After allowing the bacteria to grow for several hours, we collected and suspended it in another solution. Microtubes containing 1.5ml of each *E. coli* plasmid sample were centrifuged, and the supernatant was removed, leaving a bacterial pellet. Each sample was combined with 250µl of buffer P1 and then vortexed. Next, 250µl of the alkaline solution P2 was then added to each bacterial suspension, disrupting the bacterial cell walls, and the suspensions were inverted three times; the suspensions became cloudy and viscous. Next, 325µl of acidic N3 solution was added to each bacteria sample. Three inversions of the samples yielded even cloudier and more viscous solutions. The tubes were then placed into the microfuge at top speed for 10 minutes. This process broke the bacterial cell walls and precipitated proteins and chromosomal DNA while retaining the plasmid in the supernatant.

Next, the supernatant was pipetted out of the microtubes, leaving the white precipitate behind, and each was added to individual spin columns in separate 2ml microtubes. These were then spun for one minute in a microcentrifuge, after which the remaining effluents in the 2ml catch tube were discarded. The plasmid DNA captured in the white matrix was then washed by adding 750µl of solution PE to the columns, which were centrifuged once again. The effluent was discarded, and the microtubes were microcentrifuged once again to remove all the traces of the effluent-ethanol inside.

The columns were then transferred to 1.5ml microfuge tubes and 50µl of sterile water was added to each. The water was allowed to sit in the microfuge tubes for ten minutes, when the tubes were centrifuged at full speed for one minute. After discarding the columns, leaving the plasmid DNA in solution in the microfuge tubes, the restriction enzymes were prepared. Restriction enzyme digests cut the plasmid at certain points, depending on which enzyme is used. Restriction enzymes are selected on the basis that they cut the vector at only one point, and the gene being cloned at one point. The circle will then be cut into two linear pieces. The restriction enzyme *BclI* was selected because it cuts the *fim*A, *fim*2, *fim*3 genes and the pET151/D-TOPO vector genes at only one location each; thus the *BclI* restriction enzyme can be used for all samples. Sixteen µl of each plasmid preparation, 2µl of buffer, and 2µl of the restriction enzyme *BclI* were pipetted into clean microtubes. These were centrifuged for a few seconds to collect all the liquid at the bottom, and then incubated at 50°C.

In order to analyze the restriction enzyme digests of the plasmids next a 1.2% agarose gel (TAE with ethidium bromide and agarose) was prepared as described above, but using 0.36g agarose, and 30ml of buffer. After injecting the DNA kbp ladder and the samples into the wells, the electrophoresis gel was run for 45 minutes at 80 V.

Protein Expression

The construct was transformed into the BL21 strain of *E. coli* as previously described. A single colony was then added to a 2 mL Brain Heart Infusion (BHI) broth, a bacteria medium (Fisher Science), which was inoculated with 100 µg/mL ampicillin. This was grown at 37°C with shaking until the OD_{600} (optical density at 600 nanometers) was between 0.5 and 0.8. At this point, the bacterial growth is exponential.

Isophenylthiogalactoside (IPTG), an inducer for promoting gene expression, was added to one of the cultures for a final concentration of 1 mM. A duplicate was left with no IPTG. These were grown again at 37°C with shaking for four hours. The cultures were centrifuged for two minutes at 13000 X G in order to harvest the cells. The media was poured off to leave behind a pellet of cells, which were then resuspended in a lysis buffer (50 mM potassium phosphate, pH 7.8; 400 mM NaCl; 100 mM KCl; 10% glycerol; 0.5% Triton X-100; 10 mM imidazole) by pipetting up and down. This was then frozen using liquid nitrogen and left to thaw for two minutes, in order to break open the cell and release the cytoplasmic contents. 8 µL of the crude ligase were added to 2 µL of a dye, glycerol bromophenol blue, and the resulting samples were run in a gel. The gel was a 14% Sodium dodecylsulfate- polyacrylamide gel (SDS-PAGE) electrophoresis. All gels and reagents, unless indicated otherwise, were from Invitrogen. The samples were put into wells in the gel and the gel was run at 150 V for 30 minutes. The gel was then stained using a Daiichi silver stain II kit from Owl Separation Systems.

RESULTS

The first process run was PCR. This process served to amplify a given fragment of DNA. In this case, four of the *B. avium fim* genes, were amplified. After performing the PCR, the PCR

products were run on an agarose electrophoresis gel to determine their approximate lengths (Table 2).

Table 2: Length of *fim* gene as compared to the length of the PCR products.

Sample	Expected Length	Actual Length
Fim A	543 bp	~500 bp
Fim 1	549 bp	~500 bp
Fim 2	579 bp	~500 bp
Fim 3	565 bp	~500 bp

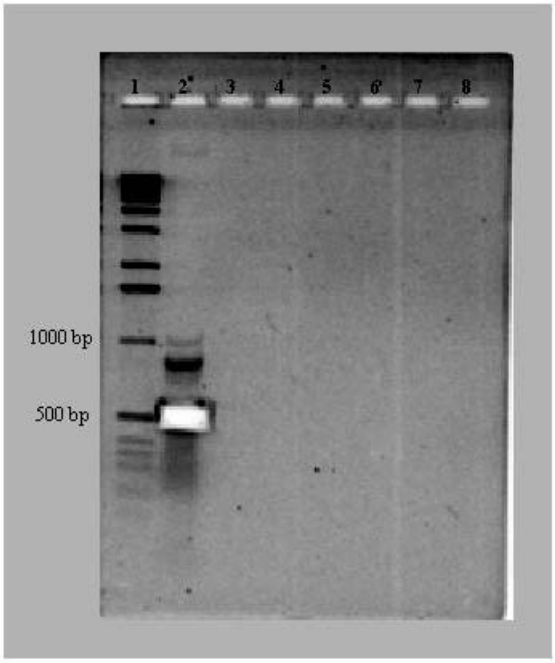
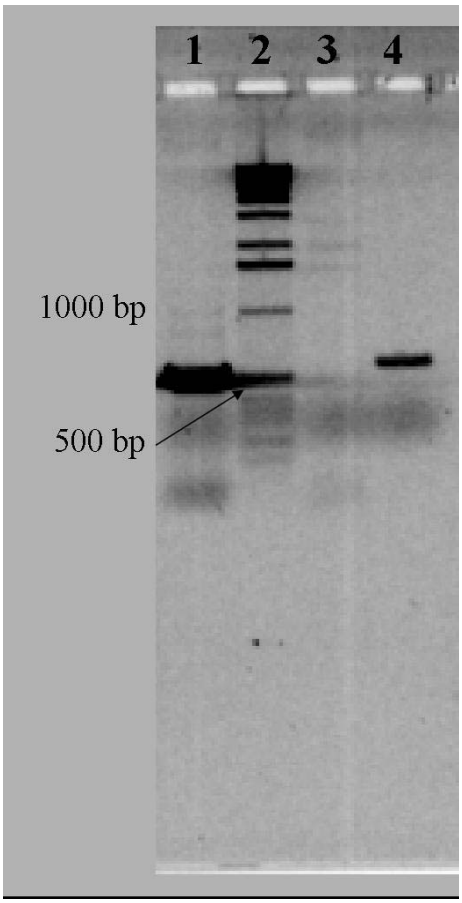


Figure 6. Agarose gel after PCR of the *fim* genes. Lane 1 contains a 1 kb DNA ladder, and Lane 2 contains Fim 2 PCR products at 500 bp.

The first agarose electrophoresis gel shows multiple bands of PCR product (Figure 6) Bands of DNA appeared at approximately the 1000 bp mark, 900 bp mark, and 500 bp mark. In order to isolate the correct PCR product, the 500 bp band was extracted, since that was the expected size. The extracted DNA fragment was then run again on a new gel to ensure that it was the correct PCR product (Figure 7).



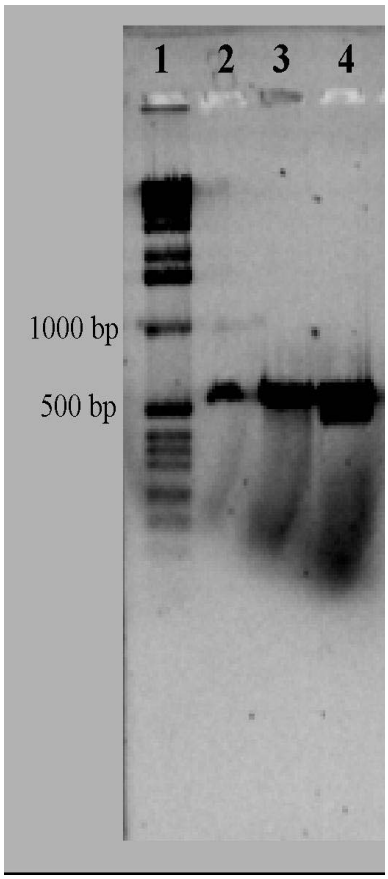


Figure 7 (left) Agarose gel. Lane 1 contains *fim*A PCR product, lane 2 contains the 1 kb DNA ladder, lane 3 contains *fim*1, and lane 4 contains *fim*2 extraction products.

Figure 8 (right) Agarose gel. Lane 1 contains the 1 kb DNA ladder while lanes 2, 3, and 4 contain *fim*3 PCR products.

The next agarose gel from the PCR showed mixed results. Lane 1 shows a band of *fim*A sample at about 500 bps, and lane 3 shows no Fim 1 PCR products. The extraction from the first gel was placed in lane 4, and the DNA was now isolated to those strands at approximately 500 bps rather than the array from the first gel.

A third agarose gel was run to insure that the *fim*3 PCR products were purely the expected gene (Figure 8). Lanes 2, 3, and 4 show bands that are a little more than 500 bps, as well as smears from bromophenol blue loading dye. This indicates that the PCR process effectively amplified the Fim 3 DNA sequence.

After the polymerase chain reaction was completed, the PCR products were ligated into competent E. coli cells. The cloning vector, pET151/D-TOPO, that was used carried resistance to the antibiotic ampicillin. After ligation, the bacteria were inoculated onto LB-Amp agar plates, which contain ampicillin. Only the bacteria cells that successfully acquired the new plasmid would be able to grow on the agar plates, since the rest would not be resistant to the antibiotic, and would be destroyed. What resulted were single colonies of bacteria (Table 3).

Table 3: Number of E. coli Colonies After Transformation

Sample	Number of Colonies
Positive Control	50
Negative Control	0
Fim A	7
Fim 2	30
Fim 3	20

In order to determine whether the restriction enzymes cut the cloning vector in the correct places, another agarose electrophoresis gel was run to determine the resulting bp lengths (Figure 9).

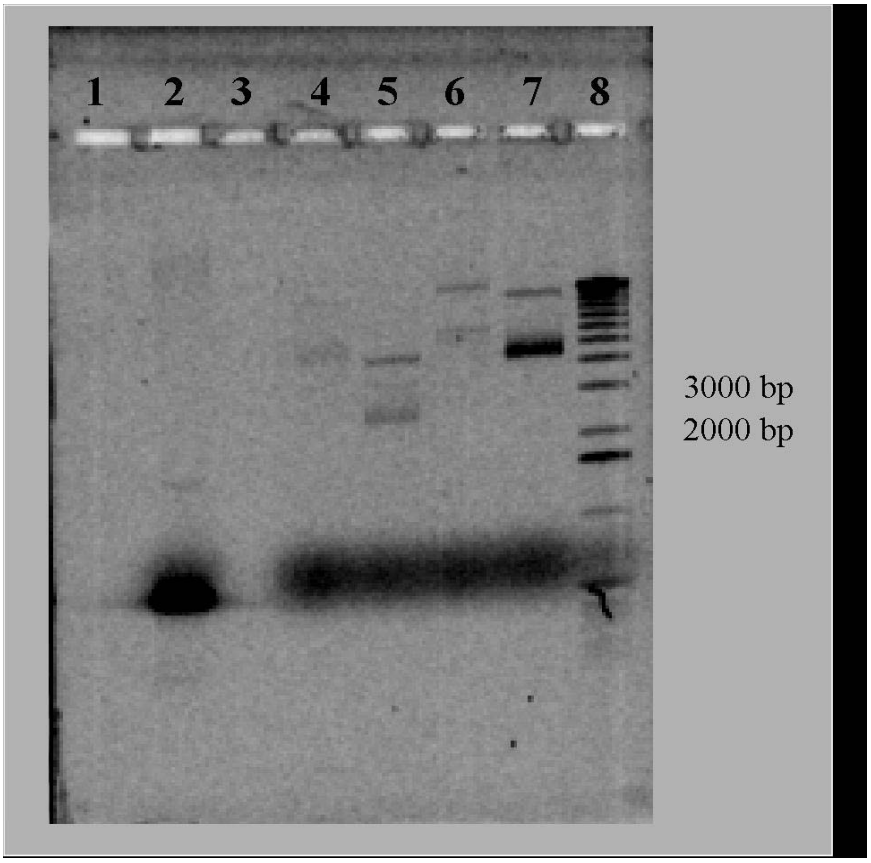


Figure 9 Agarose gel. *fim*2 test digestions were run in lanes 2, 4, 5, 6, and 7, and a 1 kb DNA ladder is run in lane 8.

This gel shows the bp lengths of *fim*2-pET151/D-TOPO plasmid after being exposed to the *BClI* restriction enzyme. Bands are sporadic, appearing at about 5000 and 1500 bps in lane 2, 3500 bps in lane 4, 3500 and 2500 bps in lane 5, and 5500 and 4500 bps in lanes 7 and 8. The smear at the bottom is the bromophenol blue and glycerol loading dye.

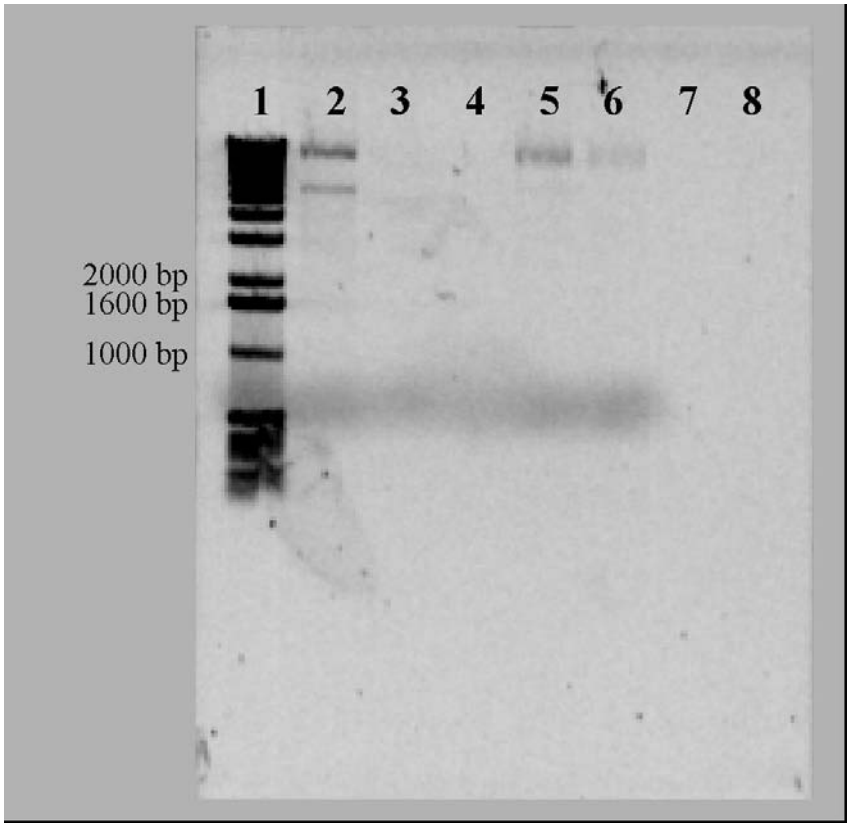


Figure 10 Agarose gel. Lane 1 contains the 1 kb DNA ladder, lane 2 contains digested *fim*A-pET151/D-TOPO, and lanes 3, 4, 5, and 6 contain 4 samples of *fim*3-pET151/D-TOPO.

The second agarose gel of the restriction enzyme digest contains the *fim*A- and *fim*3-pET151/D-TOPO plasmids after being exposed to the *BClI* restriction enzyme. Lane 2 contains 5500 and 4500 bp bands, and lanes 5 and 6 contain 5500 bp bands (Figure 10).

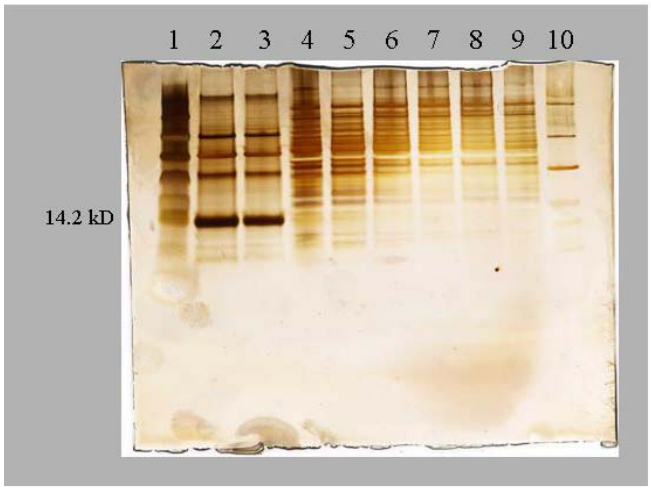


Figure 11 Polyacrylamide gel. Lane 1 contains a silver stained standard, lanes 2 and 3 contain fimbrial proteins taken from the membrane of *B. avium* cells, lane 4 contains *fim*3 non-induced proteins, lane 5 contains *fim*2 non-induced proteins, lane 6 contains *fim*A non-induced proteins, lane 7 contains *fim*2 induced proteins, lane 8 contains *fim*A induced proteins, lane 9 contains *fim*3 induced proteins, and lane 10 contains another silver-stained standard.

Even though the restriction enzyme digest agarose gels did not show the expected bp bands, gene expression and protein analysis were performed (Figure 11). In lanes 4-9, strong bands have not appeared by the 14.2 kD mark that was supplied by the actual fimbrial protein in lanes 2 and 3. However, bands are very faint on the standard in lane 10, so it was possible that staining was not completed correctly. Thus, another protein polyacrylamide gel was run (Figure 12).

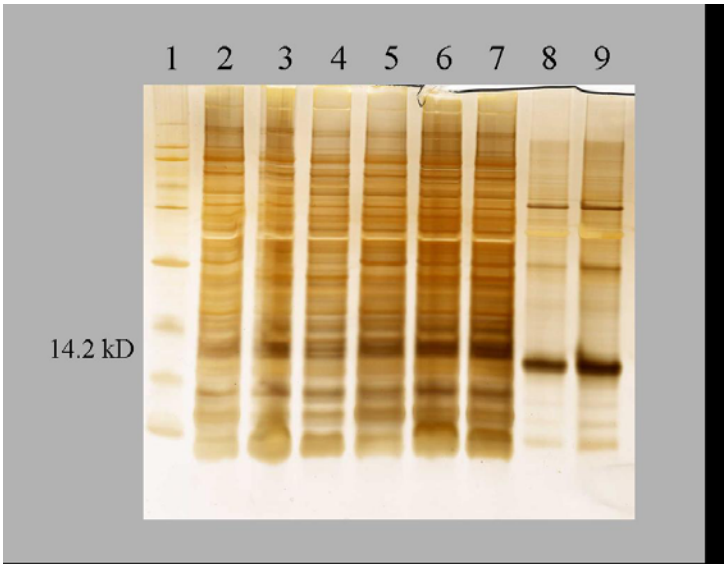


Figure 12 Polyacrylamide gel. Lane 1 contains a silver stained standard, lane 2 contains *fim*3 induced protein, lane 3 contains *fim*A induced protein, lane 4 contains *fim*2 induced protein, lane 5 contains *fim*A non-induced protein, lane 6 contains *fim*2 non-induced protein, lane 7 contains *fim*3 non-induced protein, and lanes 8 and 9 contain fimbrial protein extracted from *B. avium* cells.

The second polyacrylamide gel shows bands at the expected 14.2 kD length for both induced (lanes 2, 3, and 4) and noninduced (lanes 5, 6, and 7) samples (Figure 12). Lane 1 is the silver-stained sample, and lanes 8 and 9 contain fimbrial proteins extracted from the outer membrane of *B. avium* cells. In addition, calculations have been done using data from the specific enzymes and cloning vector in order to estimate the weights of the various fimbrial proteins (Table 4).

Table 4: Weights of Fimbrial Proteins

Sample	Expected Weight	Actual Weight
Fim A	18,292 D	Undetected
Fim 2	19,104 D	Undetected
Fim 3	18,806 D	Undetected

DISCUSSION

Analysis

PCR, as discussed before, is a very useful tool in amplifying DNA. Agarose electrophoresis is a necessary check to see if the segments of DNA produced are the length expected from sequencing of the gene. There is a chance that the cycles of varying temperature did not allow time for the primers to anneal or the double helix DNA to denature, in which case the full chromosomal DNA would remain intact without being duplicated. There is also a chance that a primer mutated and attached to a different set of codons elsewhere on the chromosomal DNA. In this case, DNA strands of lesser or greater bps than expected would appear on the gel. However, PCR in this case was successful; each *fim* gene was expected to be 543-579 bps, and all of the bands of *fim* gene (saving *fim*1) showed bands at or slightly above the 500 bp mark, which means that they contain 500-600 bps. This is correct, so it can be assumed that the PCR was performed correctly and amplified the targeted segment of DNA.

The ampicillin-resistant gene on the pET151/D-TOPO vector can help in determining which bacteria contain the vector. *E. coli* that underwent transformation were placed onto Luria-Bertani plates containing 100 μg/ml ampicillin. If the plasmids were contained in the *E. coli*, the bacteria would have survived and formed ampicillin-resistant single colonies. If the plasmid was not contained in the *E. coli,* it would have died. The negative control contained no bacteria with ampicillin resistance, and thus no colonies survived. The positive control contained all *E. coli* that were ampicillin-resistant, so the bacteria thrived and formed colonies. The plates with the *fim* genes contained some colonies; since the positive and negative controls gave the expected results, it can be inferred that these colonies were ampicillin-resistant and thus contained the plasmid with the ampicillin-resistance gene.

The plasmids were then extracted from the *E. coli* that contained them in order to determine if the plasmids did indeed contain the *fim* genes. After the restriction enzyme digest was performed, an agarose gel was run to determine if the *BClI* restriction enzyme had cut the plasmids in the correct places (Table 5).

Table 5: Lengths of Test Digestion Products

Fim gene	Expected length I	Expected length II	Actual length I	Actual length II
A	4987 bp	1316 bp	Uncut	Uncut
2	5398 bp	941 bp	Uncut	Uncut
3	5384 bp	941 bp	Uncut	Uncut

The agarose gels showed scattered results. In general, they all contained bands at the 5500-6000 bp mark, which is the length of the full, uncut DNA. In some cases, a second band appeared about 1000 bps lower. These were not shorter DNA, but rather supercoiled DNA, while the higher ones were the nicked DNA. Nicked DNA results when the restriction enzyme used only cuts the plasmid DNA once, not twice. This results in a strand of linear DNA, which travels through the gel extremely slowly. The lower bands were probably not cut by the restriction enzyme at all, thus leaving the plasmid DNA in its circular shape. This would travel through the gel faster than the nicked DNA, but still not yield the same results as properly cut plasmids. These results clearly indicate that our restriction enzymes must have been ineffective, since they show only two versions of a complete vector, rather than two shorter pieces.

Although the test digestions did not work in this experiment, protein gel analysis was pursued because the restriction enzymes used were believed to be ineffective. Before the gel was run, induction was performed on half of the samples. The pET151/D-TOPO vectors came with a repressor protein attached to the *lacO* site, which would release and allow for RNA transcription if monosaccharide lactose was present. Half of the *E. coli* were exposed to monosaccharide lactose, forcing transcription and assumedly protein translation and expression. The other half of the samples were not exposed to lactose, and were thus non-induced. Concurrently, fimbrial proteins were taken from *B. avium*, to act as a standard and to be compared with the *B. avium* protein spectrum. It would be expected that the induced proteins would appear in their respective lanes as a strong band at the 14.2 kD mark, and the non-induced proteins would leave a weak band if any. However, all *E. coli* samples had weak bands at the 14.2 kD mark, which indicates that there was no difference in expression of proteins of that weight in the induced and non-induced samples. This signifies that either the repressor protein was not removed in the induced protein samples, or that the *fim* genes were not present.

Reasons Why the Fimbrial Proteins Were Not Expressed

Since there was no confirmation that cloning had fully succeeded, a component vaccine could not be created. However, this does not mean that there is no hope for a bordetellosis vaccine in the future. There could be technical reasons why the cloning did not succeed and the proteins were not expressed. Perhaps the ligation conditions should have been different. Also, the sample of *BClI* used in the restriction enzyme digest could be tested on other plasmids to confirm that it is simply an ineffective enzyme. If that is the case, then another restriction enzyme should be used to validate the ligation of the *fim* gene into the pET151/D-TOPO plasmid. If that restriction enzyme succeeds in cutting the vector into two shorter pieces, then the error occurred post-ligation; if it still does not succeed in cutting the vector in two places, the error occurred pre-ligation.

Pre-ligation error might have occurred because of faults in the cloning vector. The sample of pET151/D-TOPO vectors that have been used in this experiment have not been successful in cloning other samples of genes. There might be an error in the topoisomerase's function to form phosphodiester bonds between the gene sample and the vector.

Post-ligation error might have occurred simply because not enough colonies were used in transformation. Despite the presence of ampicillin in the agar plates used, some *E. coli* colonies without resistance, that is, without the pET151/D-TOPO plasmid, might have survived. This might be because a frame shift occurred; that is, a nucleotide was added or deleted in transcription, and a different set of amino acids was coded for since the codons started and stopped in different places.

Conclusion

There is enough evidence to show that the error might have been technical rather than because of the *fim* genes to justify encouraging a repeat of this process in the future. Different restriction enzymes, cloning vectors, LB-Amp agar plates, and amount of colonies should be tried in various experiments. A different restriction enzyme would help determine if the gene was correctly inserted into the vector. A different cloning vector, which must be cut with a restriction enzyme rather than the given topoisomerase, might give researchers more control over the ligation process although consume more time. A different set of agar plates might ensure that the only remaining colonies contained the ampicillin-resistant gene. A different, i.e. greater, number of colonies would make the chance of choosing ones with a frame shift smaller. There is great hope in the future for success of the replication of fimbrial proteins and the creation of a component vaccine.

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Figure B: A token of gratitude to all those who helped the project.

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