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An approach to *Bordetella avium* as a universal poultry live vaccine platform for the expression of foreign antigens

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An Approach to *Bordetella avium* as a Universal Poultry Live Vaccine Platform for the Expression of Foreign Antigens

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Abstract

The Shenandoah Valley of Virginia is a major poultry growing region in the United States, leading to a strong interest in controlling poultry zoonotic pathogens in order to prevent economic losses and transmission to humans. *Bordetella avium*, a Gram negative bacterium, is the causative agent of the upper respiratory illness, bordetellosis, in turkeys. An existing bordetellosis vaccine decreases severity of the disease but does not limit infection or transmission. An improved vaccine for bordetellosis is needed for better efficacy. Our research involves developing *B. avium* as a universal poultry live vaccine platform that will protect against bordetellosis and also serve to express foreign antigens. *B. avium* is hypothesized to have the ability to stably express foreign antigens, such as those from important poultry pathogens as *Campylobacter jejuni*, *Clostridium perfringens*, and Hemorrhagic enteritis virus (HEV), and produce a strong immune response to multiple pathogens following intranasal vaccination. The gene encoding Baa1, an autotransporter involved in host colonization in *B. avium* but shown to be non-essential, was selected as the location for heterologous genes, to produce an attenuated *B. avium* strain and express the foreign antigens at the cell surface. The potential vaccine delivery plasmid design consisted of the plasmid pBBR1-MCS, a broad host range plasmid, into which the Baa1 autotransporter was inserted with the foreign antigen of interest replacing the passenger domain of the autotransporter gene. Antigens from *C. jejuni*, such as a flagellin protein and an amino acid binding protein, known to elicit immune responses in poultry have been cloned into the passenger domain of the autotransporter gene to form the plasmid construct in the *E. coli* plasmid pCR2.1. After moving the construct into pBBR1-MCS, the expression will be tested in *B. avium*. Success of these efforts will allow this vaccine platform to be used for the delivery of any number of foreign antigens and response to pathogen threats.

Introduction

Bordetella avium is a Gram negative bacterium that is responsible as the causative agent of bordetellosis in turkeys. Bordetellosis is an upper respiratory tract disease in which the bacterium colonizes on the ciliated cells of tracheal epithelium (1). The disease primarily occurs in 2-3 week old poults and is characterized by mucus accumulation in the trachea and nares, depression, weight loss, and sneezing and coughing called "snicking" (1). The Malformation of tracheal rings can lead to tracheal collapse and suffocation (1). Other species of *Bordetella*, including *B. pertussis* and *B. bronchiseptica*, show preferential binding to ciliated tracheal epithelial cells, contributing to signs of bordetellosis in their respective hosts (1).

Morbidity in young turkeys is high due to secondary infections by *Escherichia coli*. This causes several million dollars in losses to the poultry industry each year (2). The Shenandoah Valley of Virginia is a major poultry region in the USA, leading to a strong interest in controlling zoonotic pathogens in poultry, including *B. avium*, to prevent economic losses and transmission to humans. Current treatments do not effectively manage bordetellosis (2). An existing bordetellosis vaccine in very low usage in the field, as reported by veterinarians in the region, decreases severity of the disease; however, it does not limit infection or spread. Thus, a better vaccine for bordetellosis is needed. The current vaccine, however, demonstrates that live attenuated *B. avium* vaccines can be used in highly susceptible poultry populations without serious harm. The delivery of heterologous antigens from bacteria important to human health could easily be administered through intranasal/ocular vaccination or in drinking water of young poults (3).

Using bioinformatics and literature searching, genes for two outer membrane proteins from *B. avium*, HagB and Baa1, were explored as possible locations for the insertion of heterologous

genes from other poultry pathogens, both to produce an attenuated *B. avium* strain and express foreign antigens at the cell surface. Unknown issues were raised in previous research about the lack of understanding of the secretion mechanism of HagB, a hemagglutinin (4), and led to the focus on Baa1, an autotransporter involved in host colonization (5).

Live vaccines use naturally occurring bacterial transport systems to deliver antigens to the surface. Autotransporters are an example of effective delivery systems and allow for protein stability and ease of detection of the antigen in the live vaccine (6). An autotransporter is a protein using a secretion system consisting of an N-terminal signal sequence to target proteins to the Sec apparatus, a passenger domain exposed to the bacterial surface (7), and a conserved C-terminal pore-forming domain through which the N-terminal passenger domain is exported (8). The N-terminal passenger domain is translocated out of the membrane into the extracellular environment and can be cleaved to be released from the cell surface (7) (Figure 1). Baa1 specifically acts as a type V secretion system. Autotransporters are able to perform virulence related functions; their virulence factor proteins make them ideal candidates for subunit vaccines, using the antigen to stimulate the immune system, due to their surface localization and high antigenicity (5).

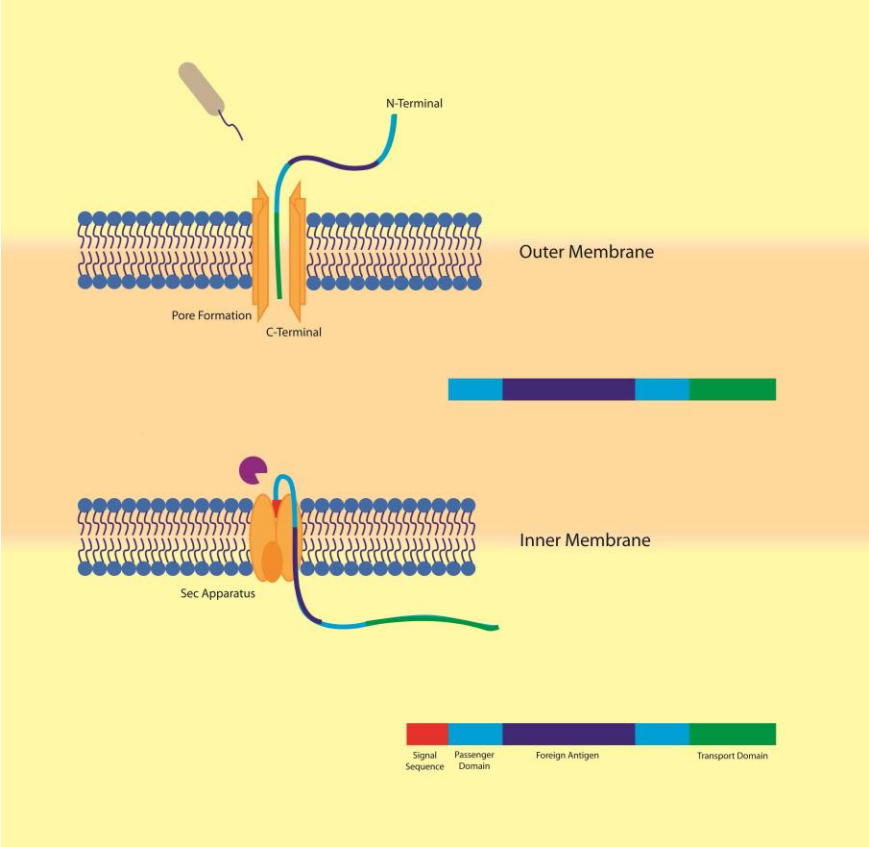

Figure 1. Model of the translocation of the modified Baa1 autotransporter through the cell membrane. The signal sequence (red) targets the protein to the Sec apparatus within the inner membrane. The signal sequence is cleaved and the passenger domain (light blue) moves through the C-terminal pore formation (green) to the extracellular environment. Foreign antigens of interest (blue) are depicted within the passenger domain.

This research involved developing *B. avium* as a universal poultry live vaccine platform for the expression of foreign antigens. It is hypothesized that *B. avium* can stably express foreign antigens, such as those from important poultry pathogens as *Campylobacter jejuni*, *Clostridium perfringens*, and Hemorrhagic enteritis virus (HEV) (Table 1), to produce a strong immune response to multiple pathogens following intranasal vaccination. *B. avium* was chosen as the live vaccine platform due to its inability to infect humans, proven antigenicity, and limited toxicity to susceptible avian species (2, 9). *Campylobacter jejuni* is one of the leading causes of food-borne illnesses in humans, specifically cambylobacter gastroenteritis, in which contaminated poultry is

the primary source (10). CjaA, a substrate binding protein component of the ABC transport system, and FlaA, a flagellin protein, are two possible proteins of interest to be expressed. Some of the proteins secreted through *C. jejuni* flagella are found to be associated with virulence (11). The flagella proteins are highly conserved between *Campylobacter* species and the proteins are highly immunogenic in poultry, validating their potential use in a vaccine (10). CjaA also encodes a highly immunogenic protein conserved among *Campylobacter* species, which is necessary to be an effective vaccine (12). Immunization of chickens with a recombinant *Salmonella* vaccine strain expressing the *C. jejuni cjaA* gene has proven to be a successful candidate for vaccine delivery (12, 13). Poultry are a key reservoir for food borne infections, establishing the need for an effective vaccine.

Table 1. Zoonotic diseases of poultry to which a new or improved vaccine is needed.

Bacteria or Virus	**Susceptible poultry species**		**Problem(s) with available vaccine(s)**	**Promising antigen candidate(s) for use in subunit vaccines**
	Chickens	**Turkeys**		
Avian flu	X	X	Viral propagation in tissue culture; re-assortment	H5 (14-16)
Avian pneumovirus (APV)	X	X	Viral propagation in tissue culture	Small non-glycosylated hydrophobic protein (SH) (17-20)
Chlamydophila psittaci		X	None available	Major OMPs (19, 20)
Hemorrhagic enteritis virus (HEV)		X	Viral propagation in spleen or tissue culture; immunosuppressive	Hexon (w/ 100 kDa protein), Fiber knob (21-23)
Avian hepatitis E virus	X		None available	Orf2 capsid (24-26)
Campylobacter jejuni	X		None available	CjaA, Flagellin gene *fsp*A1 (10-13)
Clostridium perfringens	X	X	None available	Alpha-toxin, NeTb (27, 28)

Methods

Primer Design

A bioinformatics analysis of the potential delivery plasmid design consisted of the *E. coli* plasmid pCR 2.1 with the Baa1 autotransporter and the foreign antigen of interest inserted into the passenger domain of the autotransporter. A promoter region of 250bp upstream of the Baa1 autotransporter was cloned into the plasmid construct in order to test the expression in a plasmid before moving the vaccine construct into the chromosome. Antigens known to elicit immune responses in poultry from *C. jejuni*, *C. perfringens*, and HEV, specifically *flaA* and *cjaA* of *C. jejuni*, were cloned into the passenger domain of the autotransporter gene. Alanine and histidine linkers were engineered into the construct as shown in Figure 2. Antibodies to a portion of the passenger domain are available against a predicted antigenic epitope, antibodies to the histidine epitope are commercially available, and antibodies to *cjaA* and *flaA* are available through collaborators. The design of the hypothetical plasmid construct is illustrated by the plasmid in Figure 3.

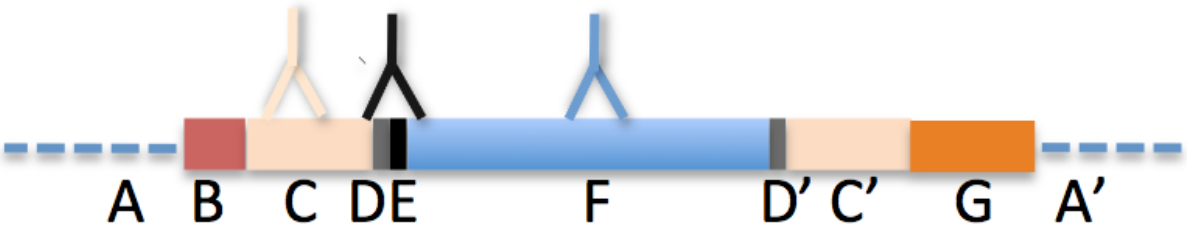

Figure 2. Schematic of chimeric delivery plasmid. A,A'-cloning vector; B-signal sequence; C,C'-Baa1 passenger; D,D'-alanine linkers; E-histidine linker; F-foreign epitope DNA; G-portion of transfer domain of Baa1. Regions B/C and C'/G provide DNA for homologous recombination.

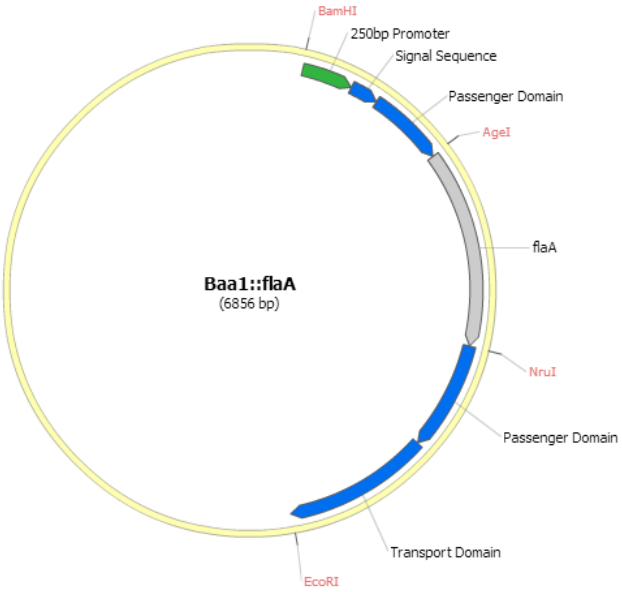

Figure 3. Schematic of modified pCR 2.1 plasmid with the Baa1 autotransporter and the foreign antigen of interest.

Polymerase Chain Reaction

Three regions of the plasmid construct were amplified by Polymerase Chain Reaction (PCR). Region 1 consisted of the promoter region, signal sequence, and part of the passenger domain. Region 2 consisted of the foreign antigen of interest, *flaA* or *cjaA*. Region 3 consisted of part of the passenger domain and the transport domain. The regions were amplified using the primers listed in Tables 2 and 3 for *flaA* and *cjaA*, respectively. The final volume of the PCR mixture for regions 1 and 3 was 50µl, and it contained dH_2O, 10X KOD buffer, 0.2mM dNTPs, 1 mM $MgSO_4$, 200ng 197N *B. avium* DNA, 0.3µM forward primer, 0.3µM reverse primer, and 1 U/µl KOD Hot Start Polymerase. Samples were run with the following program: 2 minutes at 94°C, 30 cycles of 15 seconds at 94°C, 30 seconds at 67°C, and 10 seconds at 68°C, and 2 minutes at 68°C. The final volume of the PCR mixture for region 2 was 25 µl, and it contained dH_2O, 250ng M1 *C. jejuni* DNA, 0.4 µM forward primer, 0.4 µM reverse primer, and 1X Promega Master Mix. Samples were run with the following program: 2 minutes at 95°C, 30 cycles of 15

seconds at 95°C, 1 minute at 50°C, and 1 minute at 68°C, and 5 minutes at 68°C. The PCR product length was verified with gel electrophoresis. DNA samples were purified using Zymo Clean and Concentrator kit.

Table 2. Primer design for *flaA* construct.

Region 1	Forward	GCGGGATCCGGAAAAATCCCAGCTCCCGG
	Reverse	CGGACCGGTGCCGCCACTGTTGACGGTGC
Region 2	Forward	GCGACCGGTGCGGCAGCGGCGGCACACCACCATCACCACCATTTGGTTTCAGCTATCAATGC
	Reverse	CGGTCGCGAAATGCCGCCGCTGCCGCTTGTAATAGTTTTAAAACAT
Region 3	Forward	GCGTCGCGACAGTTCGGTGCAGTTTCAAT
	Reverse	CGGGAATTCTTAAGGCCTGTCTTGAGTTC

Table 3. Primer design for *cjaA* construct.

Region 1	Forward	GCGGGATCCGGAAAAATCCCAGCTCCCGG
	Reverse	CGGACCGGTGCCGCCACTGTTGACGGTGC
Region 2	Forward	GCGACCGGTGCGGCAGCGGCGGCACACCACCATCACCACCATATGAAAAAAATACTTCTAAG
	Reverse	CGGTCGCGAAATGCCGCCGCTGCCGCAATTTTTCCACCTTCAATCA
Region 3	Forward	GCGTCGCGACAGTTCGGTGCAGTTTCAAT
	Reverse	CGGGAATTCTTAAGGCCTGTCTTGAGTTC

Restriction Enzyme Digest

The volume of the restriction enzyme mixture was 50 µl, and it contained 1 µg DNA, 1X NEB buffer, 0.5 units NEB restriction enzyme, and up to 50 µl dH_2O. The reaction was incubated at 37°C for 1 hour. The restriction enzyme digest product length for the vector and insert was verified with gel electrophoresis.

Ligation and Transformation

Ligations of the individual regions with the pCR 2.1 vector were performed using the TOPO TA Cloning Kit, with a vector to insert ratio of 1 to 4. Once the individual regions were ligated into their respective plasmid, these plasmids were ligated together using T4 DNA Ligase, with a vector to insert ratio of 1 to 3. Transformations were performed using the TOPO TA Cloning Kit.

Results

Constructs of the Baa1 autotransporter with the foreign antigen of interest were assessed through bioinformatics analysis, including Basic Local Alignment Search Tool (BLAST) analysis, to confirm that the primer design was correct and that the genes were in frame and in the correct orientation (Figure 4). The hypothetical construct sequence, created using the software Gene Construction Kit, was searched through BLAST to confirm the presence of each of the domains, including the passenger and transport domains of the autotransporter and the foreign gene of interest. Within the construct of *flaA*, the domains that appeared were a transpeptidase, passenger, autotransport, and flagellin domain. Within the construct of *cjaA*, the domains that appeared were a transpeptidase, passenger, autotransport, and periplasmic binding protein domain.

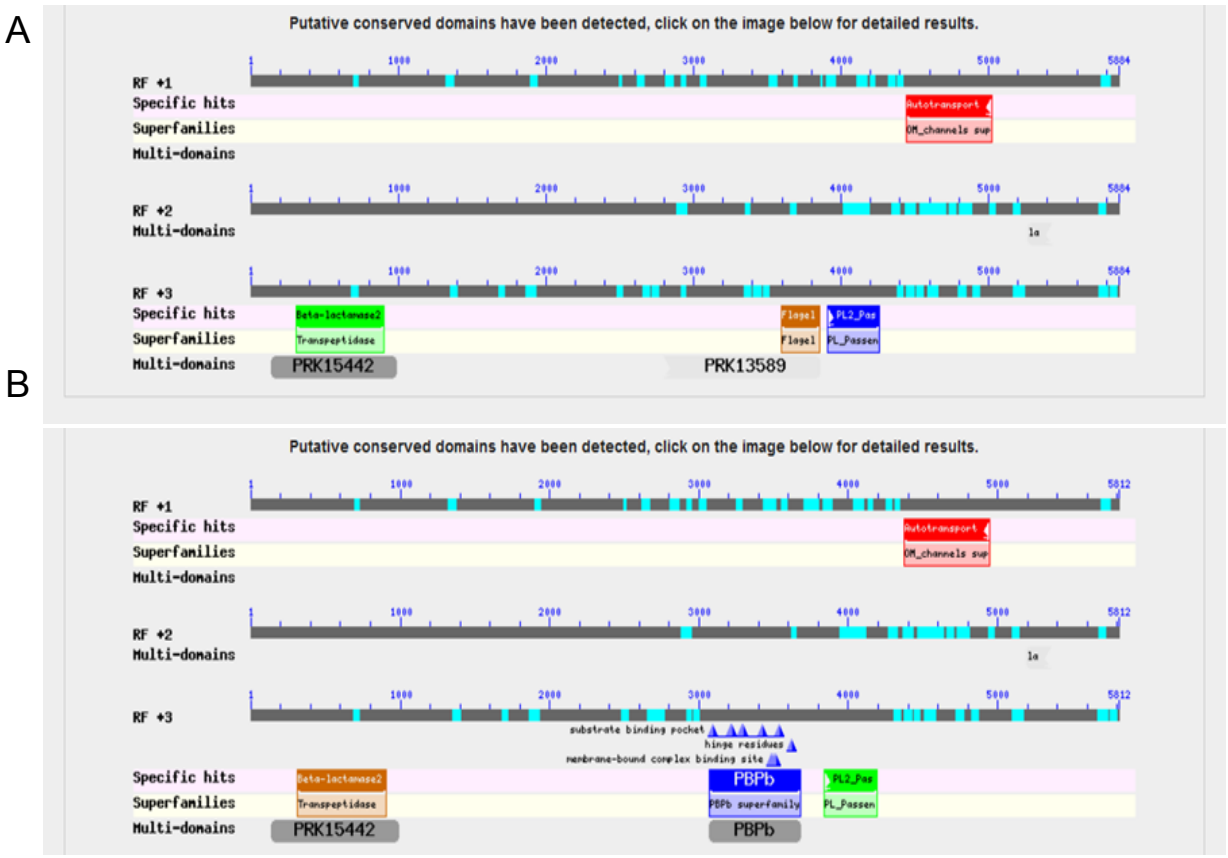

Figure 4. BLAST analysis of Baa1 constructs. BLAST results show putative domains within the searched sequence. A-FlaA; B-CjaA.

Two approaches were taken to create the plasmid construct. The first approach involved amplifying the individual regions, cutting the regions with restriction enzymes, and ligating the regions together before inserting them into the *E. coli* plasmid pCR 2.1. PCR primer sets were designed for the three regions of the construct to be amplified, the promoter to the beginning of the foreign antigen in the passenger domain, the foreign antigen of interest, and the end of the foreign antigen in the passenger domain to the end of the transport domain (Figure 3). A product was produced for each of the regions with a size of 775bp, 912bp for *flaA*, and 1243bp, respectively (Figure 5). Restriction enzyme digests were performed using AgeI and NruI to ligate

the regions together. This approach was successful only in joining two of the fragments, regions 1 and 2, rather than all three.

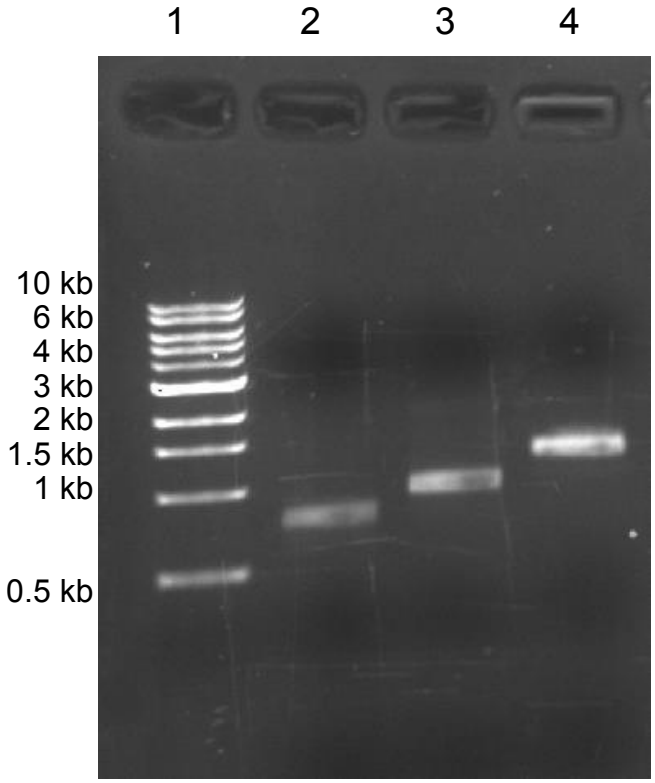

Figure 5. Agarose gel electrophoresis of PCR amplified regions of Baa1 construct. Lane 1: 1kb Ladder; Lane 2: Region 1; Lane 3: Region 2 (*flaA*); Lane 4: Region 3.

The second approach involved inserting each individual region into the *E. coli* plasmid pCR 2.1. Once each region was successfully inserted separately into pCR 2.1, a ligation was performed between two of the plasmids to join the promoter and transporter regions. The ligation product was transformed into *E. coli* and confirmed through sequencing. The promoter and transporter regions were cloned together and confirmed through restriction enzyme digest with AgeI in which one band was predicted to occur at 6kb (Figure 6). However, the clone containing the promoter region was difficult to grow. The *E. coli* grew more slowly at 37°C as well as 22°C and colonies appeared mucoid. The clone containing the promoter and transporter region

appeared to be unstable since very few colonies contained both inserts. Putative clones that may contain *flaA* and *cjaA* with the promoter and transporter regions are currently being screened using biotin labeling (Dr. Kiran Nehra).

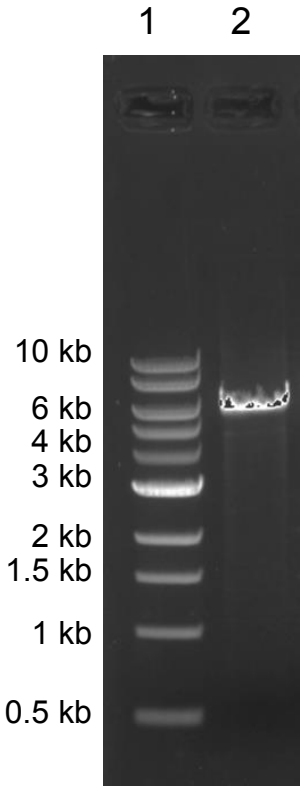

Figure 6. Agarose gel electrophoresis of restriction enzyme digest of pCR 2.1 with promoter and transporter ligated regions of the Baa1 construct. Lane 1: 1kb Ladder; Lanes 2: pCR 2.1 with promoter and transporter regions digested with AgeI.

Discussion

The development of a live-attenuated *B. avium* vaccine is necessary for controlling the emergence of zoonotic infections in poultry and improving the current vaccination methods. The vaccine will be developed to present a variety of foreign antigens derived from numerous pathogens with zoonotic potential. This method of vaccine development proves advantageous due to the low cost of vaccine production, the possible maintenance of the vaccine strain in a population of animals, the simultaneous delivery of bacterial antigens that act as immunological adjuvants, and the generation of distinctive serologic fingerprints in immunized animals (29-31). The use of a bacterial organism as a vaccine carrier over viral recombinant vaccines relies on minimal reactogenicity and maximal immunogenicity (31).

Many locations within the chromosome of *B. avium* could have been chosen for the insertion of heterologous antigens. Cell surface structures usually work well for live vaccines so the antigen can be presented at the surface. Some possible locations in *B. avium* that were considered were flagella structural genes, fimbrial structural genes, the HagB hemagglutinin, and the Baa1 autotransporter. The current strategy using the Baa1 autotransporter is based on assumptions that have not been proven. The expression, secretion, and antigenic properties of this strategy have not been shown; however, these properties can only be tested by examining the cell surface structure's potential use within the *B. avium* vaccine delivery system.

The Baa1 autotransporter system is well understood and its location for the insertion of foreign poultry antigens is likely to be successful (5). Many live bacterial vectors have been successfully used as antigen delivery systems, such as *Salmonella* spp., *Shigella* spp., *Staphylococcus* spp., and *Bacillus anthracis* (31). One successful delivery system involved using *Salmonella enterica* serovar Typhimurium, expressing the *C. jejuni* amino acid binding protein

CjaA. The delivery system significantly reduced the bacterial loads in turkeys and supported the potential use of CjaA-based vaccines for controlling *C. jejuni* in poultry (12, 13). Success of the efforts to use *B. avium* as a vaccine platform will allow for the delivery of any number of foreign antigens and respond to a number of poultry pathogen threats.

The two methods described in the results provide different approaches to construct the final plasmid of interest. The different approaches allow us to evaluate the best method for plasmid construction in order to create a successful plasmid with the foreign antigen of interest. Some difficulties were encountered during this project, such as the lack of success in PCR production of multiple fragments. Protocols have been modified to produce successful PCR products by altering the annealing temperature and polymerases. Another difficulty encountered was the negative effects of the promoter region on the host bacterium. The construct with the promoter region was difficult to grow and the construct with the promoter and transporter regions was not stable. The construct will be moved into a low copy number plasmid, such as pBBR1-MCS, since the use of low copy number vectors has improved the performance of bacterial strains as vaccine carriers (31).

Successful plasmid constructions will be transferred intact into the broad host range plasmid pBBR1-MCS. The chimeric pBBR1-MCS will be moved into *B.avium Δbaa1* by conjugation. Expression will be confirmed through western blot analysis to insure that the fusion is effectively being transcribed and translated. Cell surface localization of the foreign epitopes will be assessed through fluorescent detection by microscopy. The effect on attachment to turkey tracheal rings as well as the toxicity to ciliated tissue cultures will also be evaluated.

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