Transposon-mediated mutagenesis in *Bordetella avium* and cloning and Western immunoblot analysis of antigens

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PREFACE

This thesis consists of a general introduction, a review of the literature, two separate papers (Sections I and II), a general summary, literature cited, and acknowledgements. The master's candidate, Randy Dean Leyh, is the senior author and principal investigator for each of the papers.

GENERAL INTRODUCTION

Bordetella avium causes bordetellosis, an economically important disease of turkeys. Flocks with bordetellosis usually exhibit mild upper respiratory disease and decreased weight gain. Morbidity can be high but mortality is generally quite low, unless bordetellosis is complicated by secondary invaders. *Escherichia coli* is the most common pathogen seen in these cases, and when it is involved mortality may be high. A vaccine consisting of a temperature-sensitive mutant is available to producers to help control bordetellosis, but it does not provide adequate protection. Other means of control, such as improved management of the environment, have been effective but may be expensive and difficult to implement. *Bordetella avium* possesses several virulence factors that may be involved in disease production. These factors are not well understood but it appears that *B. avium* must be able to colonize the upper respiratory mucosa and elaborate some substance(s) that affects the epithelium and possibly other tissues.

Genetic techniques have been used extensively by researchers to examine bacterial virulence factors. Specific mutations caused by transposons allow a comparison between virulence of the wild-type strain and mutants that vary only in one aspect from the original strain. The use of recombinant techniques allows the isolation and cloning of portions of the bacterial genome that may code for important molecules involved in pathogenesis. Once isolated, a gene product can be studied, and its role in virulence may be determined. These techniques may prove

to be invaluable in producing new and better vaccine strains. In the following study, genetic manipulation of *B. avium* was examined. Various transposon-insertion mutants were generated to be used in future research into the pathogenesis of bordetellosis. A genomic library of *B. avium* was generated in *E. coli*, and the recombinants were screened for antigen production.

LITERATURE REVIEW

Filion et al. first isolated a small gram-negative bacterium from young turkey poults exhibiting respiratory disease.[31] Later, Simmons et al. isolated a gram-negative rod from laboratory poults with acute respiratory disease.[104] This bacterium was used to experimentally infect other poults. The organism was initially classified as _Alcaligenes faecalis_.[101] Based on morphological, physiological, nutritional, genetic, and serological studies, this bacterium was recently placed in the genus _Bordetella_, and the name _B. avium_ was proposed.[56]

Bordetella avium is a small, motile, nonfermentative, gram-negative rod that causes rhinotracheitis, or bordetellosis, in turkey poults.[101,104] Bordetellosis is characterized clinically by oculonasal discharge, sneezing, dyspnea, tracheal collapse, and decreased weight gain.[40,95] Uncomplicated bordetellosis in turkeys is generally characterized by high morbidity and low mortality. The mild upper respiratory disease caused by _B. avium_ is frequently complicated by other infectious agents, of which _Escherichia coli_ is the most common.[46,95] In these complicated cases of infection death rates may reach significant levels.

Gross pathologic changes of bordetellosis include mild to moderately severe rhinitis, excessive tracheal mucus and exudate,[40,95] distortion of tracheal rings, and tracheal collapse.[4,5] _Bordetella avium_ colonizes the ciliated respiratory epithelium, associating with

the basal area of cilia.[5] Colonization of ciliated epithelium leads to progressive loss of ciliated cells and discharge of mucus from goblet cells.[4,5] The tracheal mucosa becomes distorted and thrown into folds, and mucous glands become dilated, cystic, and depleted of mucus. Ultrastructurally, membrane-bound crystals are common in the cytoplasm of ciliated and nonciliated epithelial cells, and are composed of numerous parallel, electron dense filaments with a periodicity of 7 nm.[5] Moderate numbers of heterophils, lymphocytes, and macrophages infiltrate the deep mucosa followed by the appearance of widely spaced lymphoid nodules and the eventual appearance of plasma cells.[4]

Bordetella avium appears to play a role in upper respiratory disease in broiler chickens. Experimentally-induced infections with B. avium resulted in variable responses from broilers.[11,75] In a study by Montgomery et al., seven out of eight isolates colonized the trachea, but only two isolates produced mild clinical signs of respiratory disease.[75] Prevalence of B. avium in North Carolina commercial broiler flocks was examined during the winter months, and 62% of tested flocks were infected.[12] Examination of broiler flocks with respiratory disease resulted in isolation of B. avium 75% of the time. Only 29% of the flocks without respiratory disease gave positive isolates. Though the role of B. avium as a primary pathogen in broiler respiratory disease is questionable, the prevalence of this bacterium may indicate possible involvement in the overall respiratory disease complex of broilers. Bordetella avium does not appear to produce respiratory disease in leghorn chickens.[75]

Bacterial Adhesion

Bacterial pathogens contain many virulence factors that allow them to infect a host and cause disease. These virulence factors may allow a bacterium to invade the host while evading the host's immune system. These factors can vary greatly between different species of bacteria. The initial event in colonization or invasion by many bacteria is adherence to epithelial cells of the host.[83] Bacteria may have specific surface molecules that allow attachment to various receptors on animal cells.

Fimbrial adhesion

Adhesins can be in the form of fimbriae (pili) in gram-negative organisms. Fimbriae are filaments composed of protein subunits which radiate from the bacterial surface.[15,23] They are 2-7 nm in diameter and up to 2-3 um in length. The most common type of fimbriae seen on *E. coli* is the type 1 or D-mannose sensitive fimbriae.[23,33] This type of fimbriae adheres to many types of erythrocytes and animal cells.[23,33,96] *Escherichia coli* type 1 fimbriae are composed of a single type of protein subunit, with a molecular weight of 17,500.[96] Type 1 fimbriae are found on other gram-negative organisms. *Salmonella typhimurium* strain LT2 expresses type 1 fimbriae that hemagglutinate horse and chicken erythrocytes.[57] The protein subunit of *S. typhimurium* fimbriae has a molecular weight of 21,000, and is chemically different from the *E. coli* fimbrial subunit. Type 1 fimbriae that mediate adhesion to

eucaryotic cells are also observed in other species of Salmonella,[26] as well as in Shigella flexneri,[25] Klebsiella sp.,[18,24] and Enterobacter sp.[32] Enterobacterial type 1 fimbriae preferentially bind structures found in short oligomannose chains of N-glycosyl-linked glycoproteins.[32] Although these fimbriae are all classified type 1, different genera bind to different carbohydrate moieties on the glycoprotein receptors. Also, differences exist in the genes encoding type 1 fimbriae.[18] Phenotypic expression of type 1 fimbriae requires multiple gene products. Differences in the number and molecular weights of the fimbrial accessory proteins are seen between genera.

The majority of pyelonephritogenic E. coli isolates exhibit D-mannose resistant adherence to uroepithelial cells.[109] Adherence is mediated by P-fimbriae which are immunologically but not morphologically indistinguishable from type 1 fimbriae. The receptor for P-fimbriae is a digalactoside moiety of glycosphingolipids located on P blood group human erythrocytes. Escherichia coli type 1 and P-fimbriae are encoded by genes located on the chromosome.[18,47]

Enterotoxigenic E. coli strains exhibit several types of mannose-resistant fimbriae. In contrast to type 1 fimbriae, these fimbriae adhere to intestinal epithelium of a limited number of animal species and agglutinate only a few species of erythocytes.[23,33] The majority of these fimbriae in animals are classified as K88, K99, 987P, or F41 antigens.[33] Each type of fimbriae is composed of a polymer of protein subunits. The subunits have variable molecular weights and amino acid compositions, but each fimbrial type contains only one kind of subunit.

The K88 and K99 adhesins are encoded by genes contained on conjugative plasmids.[84,106] Genes for the F41 and 987P fimbriae are located on the chromosome.[22,76] The small intestinal brush border receptors for K88 and 987P antigens appear to be glycoproteins.[21,108] A glycosphingolipid found on horse erythrocytes may serve as the receptor for K99 fimbriae.[105]

Other gram-negative bacteria utilize fimbriae for adhesion to erythrocytes and target cells. Fimbriae have been observed with rough colony types of Moraxella bovis.[97] Calves could be infected with M. bovis grown in the fimbriated phase but not with M. bovis grown in the non-fimbriated phase.[53] Attachment of Neisseria gonorrhoea to human erythrocytes and epithelial cells is also mediated by fimbriae.[17,86]

Non-fimbrial adhesion

Bacterial attachment to target cells can also be mediated by non-fimbrial surface structures. Lipid or lipoprotein surface components of Mycoplasma pneumoniae appeared to be involved in adhesion because of their ability to block hemadsorption-inhibiting antibodies.[107] Treatment of erythrocytes and tracheal epithelial cells with neuraminidase prevented bacterial adhesion; therefore, it was concluded that neuraminic acid was a component of the receptor for the bacterial adhesin. Mycoplasma gallisepticum was shown to bind to glycophorin, a sialoglycoprotein component of erythrocyte membranes.[35] Adhesion was inhibited by neuraminidase treatment of erythrocytes.

Bordetella adhesion

Bordetella species preferentially attach to ciliated epithelium of the upper respiratory tract of man and animals.[68,113] This attachment is considered to be an important virulence factor in disease caused by these organisms. Several mechanisms are involved in adhesion to erythrocytes and host epithelium.

Fimbriae are ubiquitous among B. bronchiseptica species.[61] The fimbriae consist of variable amounts of three serologically related protein subunits with molecular weights of 21,000, 22,000, and 24,000. Attachment of B. bronchiseptica to the cilia of swine nasal epithelial cells was mediated by a fimbrial-like appendage visible with electron microscopy.[120] Isolates were also able to attach to and agglutinate horse and dog erythrocytes.[10] The bacterial ligand appears to be proteinacious and heat labile.[10,85,120] The presence of a non-fibrial hemagglutinin was indicated when removal of pili increased hemagglutination.[10] A non-fimbriated isolate was also capable of agglutinating dog erythrocytes. Plotkin and Bemis indicated at least two mechanisms are involved in adherence of B. bronchiseptica to hamster lung fibroblasts in vitro.[85] One mechanism involved N-acetylglucosamine as an essential component of the fibroblast binding site. The other mechanism was facilitated by several divalent cations. In contrast, adherence of B. bronchiseptica to swine nasal epithelial cells in vitro could be blocked by sialic acid-containing glycoconjugates. This indicates that these compounds might function as receptors on the host cell surface.[50] Also, B. bronchiseptica has a strong affinity for

mucin, which might aid in attachment of this bacterium to the swine nasal cavity.

Several protein antigens of *B. pertussis* are thought to be involved in adhesion of the organism to the ciliated mucosa of the upper respiratory tract in man. Two agglutinogens of serotypes 2 and 6, are fimbriae.[20,121] The fimbriae of serotypes 2 and 6 consist of single protein subunits with molecular weights of 22,000 and 21,500, respectively. The two subunit types have functionally conserved regions and share NH_2-terminal sequence homology in 15 of 21 amino acids.[20] Serotype 2 and 6 fimbrial subunits have antigenic regions which are similar to the *B. bronchiseptica* fimbrial subunits; however, intact *B. pertussis* serotype 2 fimbriae and *B. bronchiseptica* fimbriae are only weakly cross-reactive serologically.[61] *Bordetella pertussis* fimbriae do not possess any hemagglutinating activity.[20,121]

Bordetella pertussis possesses two chemically, morphologically, and immunologically distinct non-fimbrial protein hemagglutinins, the filamentous hemagglutinin (FHA) and pertussis toxin.[2,98] The FHA appears as filaments 2.5 to 3.0 nm in diameter and 45 nm in length with a molecular weight of 220,000.[7,49] Pertussis toxin is a 117,000-molecular-weight protein made up of spherical molecules approximately 6 nm in diameter.[2,110] Both FHA and pertussis toxin are secreted into growth medium *in vitro*, and can bind to bacteria and to respiratory epithelial cilia.[1,112] Tuomanen and Weiss found that both pertussis toxin and FHA were required for effective adherence to human ciliated respiratory-epithelial cells *in vitro*.[112] They suggest that pertussis

toxin and FHA act in concert to promote attachment to human ciliated respiratory-epithelial cells by serving as bifunctional molecules bridging receptors on bacterial and ciliary surfaces. Treatment of Staphylococcus aureus, Streptococcus pneumoniae, and Haemophilus influenzae isolates, or the respiratory epithelial cilia with exogenous B. pertussis hemagglutinin resulted in increased adherence of these respiratory pathogens; therefore, the secreted adhesins may not only be involved in adherence of B. pertussis, but may function as a means of attachment for typically non-adherent secondary invaders.[111] Urisu et al. noted the involvement of FHA in in vitro adhesion.[114] They observed that FHA-positive isolates of B. pertussis without fimbriae exhibited high adherence to human WiDr cells, whereas FHA-negative isolates containing serotype 2 fimbriae adhered poorly. Antibody to FHA inhibited adherence of B. pertussis but antibody to pertussis toxin did not. This made the toxin's involvement in adhesion unclear. A transposon-induced mutant deficient in the production of FHA was nearly as virulent for infant mice as the wild-type.[117] It was suggested that even though FHA may not be involved the mouse-model, it still may be important in human pertussis. The exact role of these adhesins in disease is unknown.

Bordetella avium exhibits a similar ability to attach to the ciliated respiratory epithelial cells.[68] The bacterium associates with epithelial cell cilia.[5] Knob-like structures on the surface of the bacterium are visible by electron microscopy and appear to associate with the cilia. Material that is similar in appearance is also seen on

cilia within bacterial colonies on the tracheal mucosa. These structures may function in bacterial adhesion. A hemagglutinin is present in pathogenic isolates of *B. avium*;[90,102] however, this hemagglutinin appears not to be associated with fimbriae because purified fimbriae from *B. avium* do not hemagglutinate guinea pig erythrocytes.[51] Jackwood et al. found 100% correlation between hemagglutination of guinea pig erythrocytes and virulence.[52] Two transposon-induced hemagglutination-negative mutants of *B. avium* exhibited reduced adherance to the turkey trachea.[6] Reversion of one mutant to hemagglutination-positive status was accompanied by the return of much of its capacity to adhere.

Fimbriae have been observed on virulent *B. avium* isolates.[51,102] The fimbriae were composed of a single protein subunit with a molecular weight of 13,100.[51] Indirect immunofluorescence using a fimbriae-specific polyclonal antiserum indicated that isolated fimbriae did adhere to the surface of the mucosal lining of turkey tracheal organ cultures. The fimbriae-specific antiserum inhibited *in vitro* adherence of *B. avium* to respiratory epithelium. It was concluded that fimbriae were involved in *in vitro* attachment to the tracheal mucosal surface.

Bordetella Toxins

Many virulent bacteria, once established in a host, produce or elaborate various toxins involved directly with the pathogenesis of disease. All virulent strains of *B. pertussis* produce three distinct toxins in vitro: heat-labile or dermonecrotic toxin (HLT), lipopolysaccharide (LPS) or endotoxin, and pertussis toxin.[116] Other toxins may also be involved in diseases caused by members of the genus *Bordetella*.

Heat-labile toxin

Bordet and Gengou noted that supernatant fluid from *B. pertussis* cultures was lethal when given intraperitoneally or intravenously to guinea pigs, rabbits, and mice.[14] When administered subcutaneously this material was dermonecrotic. Partially purified toxin, when given intravenously to mice at sublethal doses, caused a marked splenic atrophy or lienotoxicity.[48] These toxic properties were lost during heating at 56 C for 10 min and were attributed to HLT.[48,62,80]

Bordetella pertussis, *B. bronchiseptica*, and *B. parapertussis* produce HLT with similar biological and toxic properties.[16,29] *Bordetella pertussis* HLT antitoxin is capable of neutralizing toxins from all three species.[28] *Bordetella pertussis* HLT is a 102,000-molecular-weight protein and is composed of two polypeptides with molecular weights of 24,000 and 30,000.[80] The majority of HLT is located in the cytoplasm, but some appears to be exposed on the surface

of the bacterium.[62] In contrast, HLT isolated from B. bronchiseptica had a molecular weight of 190,000 and contained two polypeptide chains with molecular weights of 75,000 and 118,000.[58]

The role of HLT in pathogenesis of whooping cough is unclear.[62,77,116] Nakase and Endoh noted that purified HLT induced vasoconstriction which resulted in ischemia and anoxia and lead to necrosis of the affected tissue.[80] They also observed that HLT inhibits an activity of Na^+-K^+ ATPase. This inhibition might cause constriction of the vascular smooth muscles, and might be involved in dermonecrosis and splenic atrophy. HLT activity appears to be directly correlated to the ability of B. bronchiseptica to produce nasal turbinate and lung lesions in neonatal swine.[92] Incubation with HLT-containing extracts resulted in damage to swine nasal tissue in vitro.[58,79] A crude cell-free sonicated extract of B. bronchiseptica with a high level of HLT produced turbinate atrophy in piglets when administered intranasally.[41]

Lipopolysaccharide

Bordetella pertussis possesses LPS with biological properties characteristic of those found in other gram-negative bacteria.[8,116] When endotoxin from several B. pertussis strains was analyzed on hydroxylapatite columns, two LPS peaks (LPS-I and LPS-II) were observed.[59] These two lipopolysaccharides were present in the bacterial cell in the ratio of 2:3, and both exhibited chemical and biological properties of true endotoxins. Under similar experimental conditions, endotoxin preparations from E. coli, S. typhimurium, and S. flexneri

were eluted as single peaks. Two different polysaccharides (PS-I and PS-II) and two lipids (lipid A and lipid X) were isolated from B. pertussis endotoxin.[60] It was suggested that LPS-I and LPS-II contain PS-I and PS-II, respectively, in association with lipid A.[59] The position of lipid X in B. pertussis endotoxin complex was not determined.

Lipid A represents the toxic portion of LPS in enterobacterial organisms.[65] The biological properties of the chemically-isolated lipid components of B. pertussis LPS were very different, with lipid X retaining all biological endotoxic properties, and lipid A exhibiting reduced pyrogenicity and no toxicity and Shwartzman reactivity.[8] Lipid A did retain its antiviral activity, and its adjuvant power was higher than that of intact endotoxin.

Pertussis toxin

Probably the major toxin of B. pertussis involved in pathogenesis of whooping cough is pertussis toxin.[44,91] Though the exact role of pertussis toxin in whooping cough has not been shown, a non-toxin-producing mutant was severely impaired in the ability to cause pertussis in infant mice.[117] Pertussis toxin is also known as pertussigen, leukocytosis-promoting factor or lymphocytosis-promoting factor (LPF), histamine-sensitizing factor, LPF-hemagglutinin, and islet-activating protein.[91,116] Pertussis toxin is a 117,000-molecular-weight globular protein that can be dissociated into five dissimilar subunits.[110] Tamura et al.[110] have proposed an A-B structure for pertussis toxin

which is similar to several other bacterial toxins.[34] The A (active) protomer contains the subunit S-1 (with a molecular weight of 28,000) and the B (binding) oligomer contains four subunits S-2 (23,000), S-3 (22,000), S-4 (11,700), and S-5 (9,300) in a molar ratio of 1:1:2:1.[110] The S-1 subunit is the biologically active portion of pertussis toxin.[9,55,110] The S-1 subunit is not active with intact cells, and has to be associated with the B oligomer to become accessible to its site of action on the inner surface of the membrane of intact cells.[55] To become active, it appears the S-1 subunit must be released from the pertussis toxin complex. This activation may be accomplished by a cell membrane-associated enzyme.

Pertussis toxin causes increased production of cellular cAMP in mammalian cells by mediating transfer of the ADP-ribose moiety of NAD to the inhibitory (N_i) component of the membrane adenylate cyclase complex.[55] Inhibition of adenylate cyclase is prevented by ADP-ribosylation of the N_i subunit. The A protomer of pertussis toxin mediates ADP-ribosylation.[55,64,110] The change in cellular levels of cAMP may be responsible for many of the biological effects of _B. pertussis_.[42] Pertussis toxin causes excess intracellular cAMP accumulation by stimulatory agents and prevents inhibition of intracellular cAMP accumulation by inhibitory agents. The affected cells are impaired in their ability to respond to normal homeostatic regulation.

Pertussis toxin may also mediate effects independent of the adenylate cyclase system.[44,91] While possessing no ADP-ribosylation

activity, the B oligomer is able to elicit insulin-like effects on glucose transport in fat cells and cause mitogenic effects on T lymphocytes.[78] Pertussis toxin inhibits _in vitro_ natural killer (NK) cell cytotoxicity for tumor target cells.[43,44] This inhibition occurs without any change in the cellular cAMP levels. Release of arachidonic acid and granular enzymes from neutrophils is inhibited by pertussis toxin.[13] This inhibition of neutrophil function occurs by mechanisms that do not involve cAMP.

Bordetella pertussis, _B. parapertussis_, and _B. bronchiseptica_ have many common antigens, but pertussis toxin is only produced by _B. pertussis_.[94] The pertussis toxin is encoded by an operon containing five closely linked genes (one for each subunit) under the control of one promotor.[63] The pertussis toxin gene was used as a probe to examine the chromosomal DNA of several strains of _B. parapertussis_ and _B. bronchiseptica_.[66] The gene was found in all strains examined but there was no evidence of expression. The pertussis toxin genes of _B. parapertussis_ and _B. bronchiseptica_ were 98.5% and 96% homologous, respectively, to the corresponding gene in _B. pertussis_.[3] Mutations common to _B. parapertussis_ and _B. bronchiseptica_ in the polycistonic promotor region were thought to affect the transcription of this gene. When placed under the control of an _E. coli_ promoter, the S-1 subunits from both species where expressed. The subunits exhibited the same ADP-ribosylating activity as the S-1 subunit of _B. pertussis_.

Extracytoplasmic adenylate cyclase

Other toxins of *Bordetella* may have roles as virulence factors. Virulent *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* isolates produce extracytoplasmic adenylate cyclase which is released into the culture medium during exponential growth.[27] The adenylate cyclase produced by *B. pertussis* is cell-associated, but the enzyme of the other two species appears to be loosely associated with the cells. A later study indicated that the majority of adenylate cyclase activity is located in the outer membrane and is exposed to the surface.[82] Molecular weights of adenylate cyclase enzymes from the three species range from 68,000 to 70,000.[81,82] All three adenylate cyclase molecules share two epitopes but other antigenic differences are present. Antiserum prepared against *B. bronchiseptica* adenylate cyclase protected mice against challenge with *B. bronchiseptica* but not *B. pertussis*. Antisera prepared against *B. pertussis* adenylate cyclase toxin protected mice against *B. pertussis* challenge.

Hewlett et al. suggest that the adenylate cyclase toxin conforms to the A-B subunit model, with the A subunit consisting of two components, an adenylate cyclase enzymatic site and a regulatory site.[45] Calmodulin can react with the regulatory site and stimulate the activity of the toxin 1000-fold.[45,118] The B subunit would contain the binding site allowing adenylate cyclase to enter the cell and catalyze the production of cAMP from endogenous ATP.[45]

Extracts of adenylate cyclase toxin have profound effects on mammalian cells. Internalization of *B. pertussis* adenylate cyclase by

neutrophils and macrophages caused large increases in intracellular cAMP.[19] This accumulation of cAMP suppresses phagocyte functions including superoxide production, chemotaxis, and bacterial killing. Incubation of NK cells with extracytoplasmic adenylate cyclase nearly eliminated their cytotoxic activity.[43,45]

The adenylate cyclase toxin appears to be an important virulence factor in diseases caused by Bordetella species. A Tn5-insertion mutant of B. pertussis with no adenylate cyclase activity exhibited a large drop in virulence when compared to the virulent parent strain.[117] Bordetella bronchiseptica isolates that did not possess adenylate cyclase were not virulent.[81] A monoclonal antibody specific for this protein toxin passively protected mice against rhinitis and pneumonia.[74]

Tracheal cytotoxin

Goldman et al. examined HLT, adenylate cyclase, and pertussis toxin, and found these toxins created no cytopathological effects on hamster tracheal ring organ cultures.[37] A tracheal cytotoxin (TCT) was isolated which caused epithelial cytopathic changes comparable to that seen during B. pertussis infection. When exposed to this partially purified toxin, the ciliary activity was inhibited, with eventual extrusion of the ciliated epithelial cells. Synthesis of tracheal epithelial cell DNA was also inhibited. The TCT from culture supernatants of B. pertussis strains is composed of alanine, glutamic acid, glycine, cysteine, diaminopimelic acid, muramic acid, and glucosamine, and has an approximate molecular weight of 1800.[36,37] The

TCT is chemically indistinguishable from the major fragment of soluble peptidoglycan which is released from growing *B. pertussis* cultures.[93]

Bordetella avium toxins

Compared with other *Bordetella* species, much less is known about the toxins of *B. avium*. Virulent *B. avium* isolates produced cytotoxicity of ciliated epithelial cells in tracheal organ cultures from one-day-old turkeys.[39] A correlation was observed between the ability of *B. avium* to cause *in vitro* cytotoxicity of ciliated cells and *in vivo* pathogenicity in turkey poults.[38] The ciliated epithelial cells appeared to be the primary target of this toxic effect, with no degenerative changes seen in the lamina propria. Marshall et al. suggested that the toxic component may be an integral part of the bacterial cell because cytolysis appeared to require close contact between the bacterium and the tracheal epithelium.[67] The possible involvement of LPS in this disease was considered. In contrast, Van Alstine and Arp using tracheal organ cultures from five-week-old poults, found that the addition of live *B. avium* resulted in no cytotoxicity to the epithelial cells.[115] Cytopathologic change in turkey tracheal epithelial cells *in vivo* is a progressive event requiring several days of exposure to *B. avium* with minimal evidence of progenitor cell hyperplasia.[5] The appearance of membrane-bound cytoplasmic crystals in the respiratory epithelium of turkeys infected with *B. avium* may indicate a cytotoxic activity that interferes with the normal assembly of protein subunits in the cytoplasm. This toxin may have a role in

delayed replacement of lost epithelial cells and the protracted nature of the clinical disease.

Intraperitoneal administration of filtered sonicates of virulent *B. avium* isolates were lethal for poults and mice.[88] This toxic activity was inactivated by heating at 56 C for 30 min. Inactivation with formalin and proteolytic enzymes suggested that this toxin was a protein. This heat-labile toxin (HLT), when given intradermally, produced hemorrhagic lesions in turkeys and guinea pigs.[89] *Bordetella avium* HLT from several isolates appeared to be antigenically similar; however, these HLT were different antigenically from *B. bronchiseptica* HLT. Filtered sonicates containing *B. avium* HLT did not cause any metabolic or morphologic changes in the epithelium of turkey tracheal organ cultures.[89,115] When repeatedly administered intranasally to turkey poults, the toxin-containing sonicate did not produce any clinical signs or gross lesions.[87]

Several *B. avium* strains produce a histamine-sensitizing factor in mice and turkey poults.[103] The histamine-sensitizing factor is similar to that produced by *B. pertussis* but it is not as potent.

A heat-stable toxin (HST) is excreted by or is loosely associated with the *B. avium* cell surface.[99] This toxin was not inactivated by heat (85 C for 30 min) but was inactivated by trypsin. When given intraperitoneally the toxin was lethal to mice, but no observable changes were produced in turkey poults and Japanese quail chicks. Rabbit antiserum prepared against HST neutralized the toxin, but convalescent turkey antiserum did not. Thymus weights were decreased in

poults given HST by intraperitoneal injection.[119] The thymus medullary area was significantly less in the HST treated birds.

A number of alterations in the immune system occur in turkeys with bordetellosis. Because B. avium is non-invasive, it has been suggested that a toxin is released which affects lymphocytes in various ways.[70,72,73,119] Bordetella avium infected poults exhibited histopathologic changes in and decreased size of the thymus.[100] Decreased T lymphocyte mitogenic response to concanavalin A was noticed in turkeys infected with B. avium. The humoral immune system appeared to be functioning normally in infected birds. Infected poults exhibited enhanced phytohemagglutinin-delayed hypersensitivity response,[72] increased graft-vs-host response of splenic lymphocytes,[73] enhanced in vitro blood leukocyte migration,[70] and no difference in immediate hypersensitivity response to bovine serum albumin.[69] Serum levels of corticosterone were increased in B. avium infected poults.[71] It was suggested that this increase might also affect on T lymphocyte function.[100]

Apparently, a number of adhesins and toxins are associated with B. avium. These adhesins and toxins might play an important role in infection caused by this agent and in the corresponding immune response of turkey poults. The work in this thesis was directed towards facilitating the study of these virulence factors. Genetic engineering techniques were adapted for use in the study of this agent.

SECTION I. TRANSPOSON-MEDIATED MUTAGENESIS IN *BORDETELLA AVIUM*

This manuscript has been accepted for publication by the American Journal of Veterinary Research.

SUMMARY

Transposon-mediated mutagenesis was used to introduce specific mutations in the chromosome of *Bordetella avium*. The transposon Tn*5* was transferred by conjugation to *B. avium* from *Escherichia coli* strain SM10 by the suicide plasmid vector pSUP1011. A defined minimal medium for growth of *B. avium* was developed for scoring transposon-containing mutants for specific nutritional requirements. Approximately 1% of all mutants tested were in some form, auxotrophic and these organisms were grouped into 13 classes. Also, two classes of hemagglutination-negative mutants were isolated. Stability of Tn*5* insertion was examined, and frequencies of reversion less than 10^{-7} were observed, indicating stable mutations.

INTRODUCTION

Bordetella avium causes bordetellosis in turkeys.[29,33] Clinically, this disease is characterized by oculonasal discharge, sneezing, dyspnea, tracheal collapse, and decreased weight gain.[16,29] Ability to adhere to the mucous membranes of the upper respiratory tract is an essential step for initiation of infection caused by organisms in the genus Bordetella.[38] A filamentous hemagglutinin isolated from B. pertussis is involved in bacterial adhesion to epithelial and tracheal ciliated cells.[37,39] In one study, the presence of a heat-labile hemagglutinin in several B. avium strains was shown not to be correlated with pathogenicity;[32] however, in a more recent study, Jackwood et al. found 100% association between ability to hemagglutinate guinea pig red blood cells and virulence.[20]

Bacterial mutants have been used extensively for biochemical and pathogenesis studies.[18,37,42] Transposon-mediated mutagenesis has been used to create nutritional and virulence factor mutants in many species of bacteria.[1,23,26,31,41] Transposon insertion normally results in a single interruption of the genome.[6] The antibiotic-resistance determinant contained on the transposon allows easy selection for insertion events and further genetic manipulation of mutations.[6,23]

Little is known about the biochemical and genetic basis of pathogenicity in B. avium. The purpose of the study reported here was to isolate and partially characterize transposon-induced nutritional and hemagglutination-negative mutants.

MATERIALS AND METHODS

Bacterial Strains

Bordetella avium strains 75 and 838 were isolated in Iowa from turkeys with bordetellosis.[2,4] A spontaneous streptomycin-resistant mutant, 838-S, was derived from 838. Virulent *B. avium* strains NCD, 4671, and W were obtained from D. G. Simmons, North Carolina State University, Raleigh, North Carolina.[32] *Bordetella avium*-like strains 031, 101, and 023 were received from Y. M. Saif, Ohio Agricultural Research and Development Center, Wooster, Ohio. *Escherichia coli* strain J5(RP4) contains the 34 Md conjugative plasmid RP4 which encodes for ampicillin, kanamycin, and tetracycline resistances.[12] *Escherichia coli* strain MV12(R388) carries the conjugative plasmid R388 containing resistances for trimethoprim and sulfonamides.[11] Strains J5 and MV12 were obtained from Madelon Halula, Virginia Commonwealth University, Norfolk, Virginia. *Escherichia coli* strain SM10(pSUP1011) containing the mobilizable vector plasmid pSUP1011 that carries the kanamycin-resistant transposon Tn*5* and a chloramphenicol resistance determinant, was obtained from A. G. Atherly, Iowa State University, Ames, Iowa.[6,35] The nalidixic acid-resistant *E. coli* strain 711 was received from Thomas Casey, National Animal Disease Center, Ames, Iowa.

All bacteria were maintained at -70 C in 20% glycerin/80% brain heart infusion (BHI, Difco Laboratories, Detroit, MI) broth mixture. All isolates were purified by picking individual colonies and re-

streaking them before any examination. The identity of all cultures was verified by use of standard biochemical tests. Antibiotic susceptibilities were determined according to the method of Bauer et al.[5]

Media

Brain heart infusion broth or BHI agar containing 1.5% agar (Bacto agar, Difco Laboratories, Detroit, MI) were used for routine cultures unless otherwise indicated. Both media were supplemented with thymine (20 ug/ml) and adenine, guanine, cytosine, and uracil (5 ug of each/ml). Streptomycin (500 ug/ml), kanamycin (100 ug/ml), tetracycline (25 ug/ml), trimethoprim (25 ug/ml), nalidixic acid (20 ug/ml), ampicillin (100 ug/ml), and chloramphenicol (100 ug/ml) were added to BHI agar as required. Tryptose blood agar (Difco Laboratories, Detroit, MI) containing 5% bovine blood was used for growth of hemagglutination-negative mutants.

Growth of _B. avium_ strains 75 and 838 was compared on the following minimal salts media: M9,[27] M63,[27] complete defined synthetic,[28] and modified Stainer-Scholte.[24] Nutritional components were prepared as concentrated stock solutions and maintained at 4 C. Nutrients were filter sterilized (0.2 um, Gelmon Sciences Inc., Ann Arbor, MI) and added to agar that had been autoclaved with the remaining water and cooled to 48 C. All minimal defined media were solidified with 1.5% agar (Bacto agar noble, Difco Laboratories, Detroit, MI). Streptomycin

(100 ug/ml) and kanamycin (50 ug/ml) were added to minimal defined agar as needed. All nutrients and antibiotics were obtained commercially (Sigma Chemical Co., St. Louis, MO).

Conjugation

The transposon Tn*5* was transferred by conjugation to *B. avium* by the *E. coli* mobilizable vector plasmid pSUP1011.[35] These conjugations and all others between *E. coli* strains and *B. avium* strains were performed on BHI agar.[9] Mixtures of bacteria in a 1:2, donor:recipient ratio, were allowed to mate for various times without antibiotics. The cells were harvested and dilutions were streaked on appropriate antibiotic-containing media. *Escherichia coli* donors were counter-selected against by addition of streptomycin to the medium. Kanamycin or nalidixic acid was used to counter-select against *B. avium* donors. Conjugation frequencies were expressed as the number of transconjugants per donor cell at the end of mating.

Classification of Auxotrophs

Strain-75 isolates containing Tn*5* inserts from six separate SM10(pSUP1011) matings were velveteen-replicated from BHI agar to minimal defined agar. Colonies that did not grow on minimal agar were further characterized for specific auxotrophic requirements by a system of pooled nutrients.[13] All auxotrophs were transferred to chloramphenicol-containing medium to test for loss of vector plasmid

pSUP1011.

Reversion Analysis of Auxotrophs

Stability of Tn5 insertion in representative isolates from each class of auxotrophs was determined by reversion tests on antibiotic-free media. Auxotrophs were grown to a concentration of approximately 10^9 colony-forming units (CFU) per ml in BHI broth, washed once in minimal medium broth, concentrated 50 to 100-fold, and streaked on minimal medium agar without antibiotics in 0.2-ml aliquots. Viable cell counts of concentrated bacteria were determined by colony counts of serial dilutions on BHI agar. After 72 hours, revertants were velveteen-replicated to minimal medium agar containing kanamycin to determine whether reversion to prototrophy was related to loss of kanamycin resistance.

Hemagglutination

Guinea pig red blood cells were collected via cardiac puncture and mixed with sodium citrate to a final concentration of 0.5%. Red blood cells were washed once in phosphate-buffered saline solution (PBSS, 0.0132 M phosphate, 0.15 M NaCl, pH 7.3) and suspended to a concentration of 0.9% (PCV in PBSS). *Bordetella avium*, Tn5 insertion mutants were grown on BHI or blood agar at 35 C for 24 hours and washed from the agar surface with PBSS and diluted to approximately 5 x 10^{10} CFU/ml in PBSS. Two-fold dilutions of bacteria were prepared in 50 ul

PBSS in U-bottomed microtiter plates (Dynatek Laboratories, Alexandria, VA). An equal volume of red blood cells was added to each well and the plates were incubated at 25 C for 1 hour and were observed for hemagglutination. Titers were recorded as the inverse of the highest dilution yielding complete hemagglutination.

DNA Isolation

Plasmid DNA was isolated,[22] and electrophoresis was carried out in 0.7% agarose (Bethesda Research Laboratories, Gaithersburg, MD) in Tris acetate buffer (40mM Tris [pH 7.9], 2mM Na_2 EDTA, 19 mM acetic acid) at 5 V/cm for 2 1/2 hours. Gels were stained with ethidium bromide (1 ug/ml). Plasmid DNA from *E. coli* strain SM10 was isolated by an alkaline lysis procedure,[8] and further purified by cesium chloride gradient.[25] Chromosomal DNA was isolated from *B. avium* strains, as previously described.[19]

Restriction Endonuclease Digest and Southern Hybridization

Chromosomal DNA from several *B. avium* strains was digested to completion with *Eco*RI, using conditions recommended by the supplier of the enzyme (Bethesda Research Laboratories, Gaithersburg, MD). Agarose gel electrophoresis of the digested DNA was carried out in Tris acetate buffer at 1 V/cm for 20 hours. The gel was depurinated in 0.25M HCl for 15 minutes, placed in Tris-borate buffer (89mM Tris [pH 7.8], 2mM Na_2 EDTA, 89mM boric acid) for 30 min, and transferred to a nylon membrane

(Hybond N, Amersham Corp., Arlington Heights, IL) by the method of Southern.[36] The pSUP1011 DNA was labeled with photoactivatable biotin (Clontech Laboratories, Inc., Palo Alto, CA) and used as a probe for hybridization.[14] Hybridization and color determination by use of a streptavidin-alkaline phosphatase conjugate were carried out, as described by the supplier (Bethesda Research Laboratories, Gaithersburg, MD).

RESULTS

Defined Minimal Medium

Tests were conducted to determine basal nutritional requirements of B. avium strains 75 and 838. Of all defined media examined, complete defined synthetic medium supported best growth of both strains and was used exclusively in all tests. Addition of L-cystine, L-methionine, niacin, and Ca pantothenate was required for growth of colonies within 30 hours. Also, growth of both strains was stimulated by inclusion of L-glutamic acid and L-aspartic acid. Contents of the minimal medium are listed in Table 1. Growth of 17 representative strains of B. avium was examined on this minimal medium. All organisms grew except one strain.

Conjugation

Broad host range plasmids R388 and RP4 were transferred to virulent strains of B. avium (Table 2). Plasmid DNA isolated from transconjugants and donor strains had identical electrophoretic mobility (Figure 1). In addition, B. avium isolates containing RP4 were resistant to the unselected plasmid-mediated antibiotic determinants. These results indicated that the entire conjugative plasmid had been transferred.

Transconjugants from the matings between B. avium strain 75 and E. coli MV12(R388) were isolated after a 30-minute mating (Table 2).

Table 1. Composition of *Bordetella avium* minimal medium

Nutrient	Concentration (g/L)
Salts	
$K_2HPO_4 \cdot 3\ H_2O$	7.0
KH_2PO_4	2.0
Na_3 citrate $\cdot$ 2 H_2O	0.4
$MgSO_4 \cdot 7\ H_2O$	0.05
$(NH_4)_2SO_4$	1.0
Dextrose	5.0
Vitamins	
Niacin	0.0012
Ca pantothenate	0.00025
Amino acids	
L-glutamic acid	0.1
L-aspartic acid	0.09
L-cystine	0.02
L-methionine	0.01

Table 2. Conjugation frequencies in matings between *Escherichia coli* strains and *Bordetella avium* strains

Recipient	Donor	Mating time (min)	Selection for transfer of[b]	Frequency[a]
75	MV-12(R388)	30	Tp	6.2×10^{-4}
4671	75(R388)	90	Tp	4.5×10^{-1}
W	MV-12(R388)	90	Tp	1.0×10^{-2}
W	75(R388)	90	Tp	$4.8\text{-}5.3 \times 10^{-2}$
711	75(R388)	90	Tp	4.1×10^{0}
75	J5(RP4)	30	Te	0
75	J5(RP4)	60	Te	1.1×10^{-6}
75	J5(RP4)	90	Te	$0.7\text{-}2.0 \times 10^{-5}$
75	J5(RP4)	120	Te	$1.6\text{-}5.7 \times 10^{-4}$
75	J5(RP4)	240	Te	6.4×10^{-4}
711	75(RP4)	90	Km	1.2×10^{0}
838-S	75(RP4)	90	Te	0
NCD	75(RP4)	90	Te	4.0×10^{-6}
75	SM10(pSUP1011)	60	Km	1.7×10^{-4}
75	SM10(pSUP1011)	90	Km	$0.4\text{-}2.6 \times 10^{-4}$
75	SM10(pSUP1011)	120	Km	$1.8\text{-}4.2 \times 10^{-5}$
75	SM10(pSUP1011)	240	Km	4.3×10^{-5}
838-S	SM10(pSUP1011)	90	Km	$0.9\text{-}2.2 \times 10^{-5}$
NCD	SM10(pSUP1011)	90	Km	1.3×10^{-5}
711	W	90	Km	1.9×10^{-3}

[a]Transconjugants/donor cell at end of mating.

[b]Tp = trimethoprim, Te = tetracycline, Km = kanamycin.

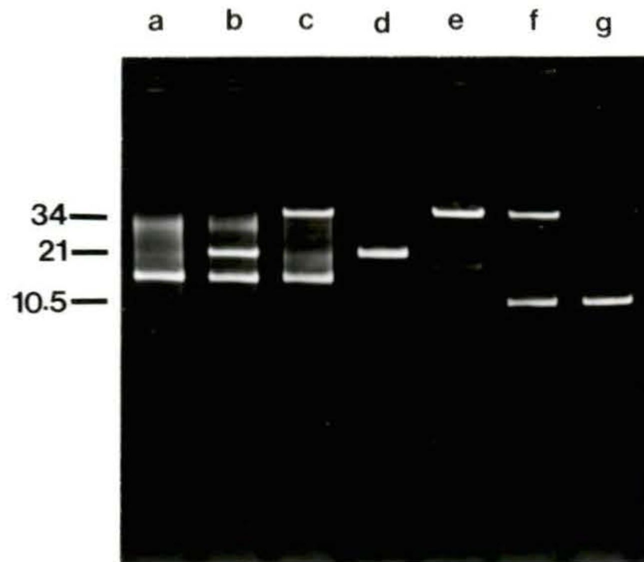

Figure 1. Agarose gel electrophoresis of plasmid DNA from *Escherichia coli* and *Bordetella avium* strains. A - *B. avium* 75; B - *B. avium* 75(R388); C - *B. avium* 75(RP4); D - *E. coli* MV12(R388); E - *E. coli* J5(RP4); F - *B. avium* NCD(RP4); G - *B. avium* NCD. Molecular weight standards are given in megadaltons.

Conjugation between strains 75 and *E. coli* J5(RP4) proceeded for 60 minutes before any transconjugants were observed, and as mating times increased beyond 60 minutes, frequency of conjugation also increased (Table 2). Various results were noticed in matings between other *B. avium* recipient strains and J5(RP4). Matings with 838-S produced no transconjugants; however, matings involving strain NCD as recipient had frequencies of conjugation similar to those found with strain 75 (Table 2).

Strain 75(R388) was mated with *B. avium* strains W and 4671. Conjugal transfer of R388 was observed in all cases, with frequencies of conjugation slightly higher than those obtained from matings with *E. coli* donors (Table 2). *Bordetella avium* strains 75(R388) and 75(RP4) were able to redonate their plasmids back to *E. coli* strain 711. The frequencies of transfer of these matings were much higher than those of the corresponding matings that used *E. coli* strains as donors and *B. avium* strains as recipients (Table 2).

During conjugation between *E. coli* MV-12(R388) and *B. avium* strain W, kanamycin resistance was acquired by MV-12(R388). Results of plasmid analysis indicated transfer and stable maintenance of an approximately 35 megadalton plasmid from strain W to strain MV-12(R388). The plasmid had high frequency of transfer and encoded for kanamycin-neomycin and sulfonamide resistance in *E. coli* strain 711 (Table 2).

Transposition of Tn5

Bordetella avium strains 75, 838-S, and NCD were mated with E. coli strain SM10(pSUP1011), and kanamycin-resistant organisms were isolated (Table 2). Results of electrophoresis of plasmid DNA isolated from representative recipients showed that the vector pSUP1011 was not maintained autonomously and therefore, transposition had occurred (Figure 2). These isolates were examined for loss of pSUP1011-mediated chloramphenicol resistance. Most isolates were sensitive to chloramphenicol; however, 1.2% remained resistant, indicating maintenance of the chloramphenicol-resistance gene from pSUP1011 in these recipients. Plasmid DNA with the electrophoretic mobility of pSUP1011 was not isolated from these resistant strains and no further determinations were done (Figure 2).

In matings between strains SM10(pSUP1011) and 75, 90-minute mating times yielded optimal numbers of transpositions. Longer mating times resulted in lower frequencies of conjugation; therefore, all further matings were carried out for 90 minutes (Table 2).

Characterization of Auxotrophs

Approximately 1% of 6,105 Tn5-containing mutants were auxotrophs. These isolates were divided into 13 groups on the basis of nutritional requirements (Table 3). Each class was represented by various frequencies. Uracil-, histidine-, and adenine-requiring mutants accounted for 46% of the total (Table 3). Chromosomal DNA isolated from

Table 3. Bordetella avium auxotrophs

Type	No.	%
Uracil	11	19
Histidine	10	17
Adenine	6	10
Phenylalanine-tyrosine	5	8
Threonine	5	8
Tyrosine	5	8
Aromatic[a]	4	7
Isoleucine-valine	4	7
Tryptophan	3	5
Valine	2	3
Adenine-thiamine	2	3
Glycine	1	2
Lysine	1	2
Total	59	100

[a]Requires phenylalanine, tyrosine, and tryptophan.

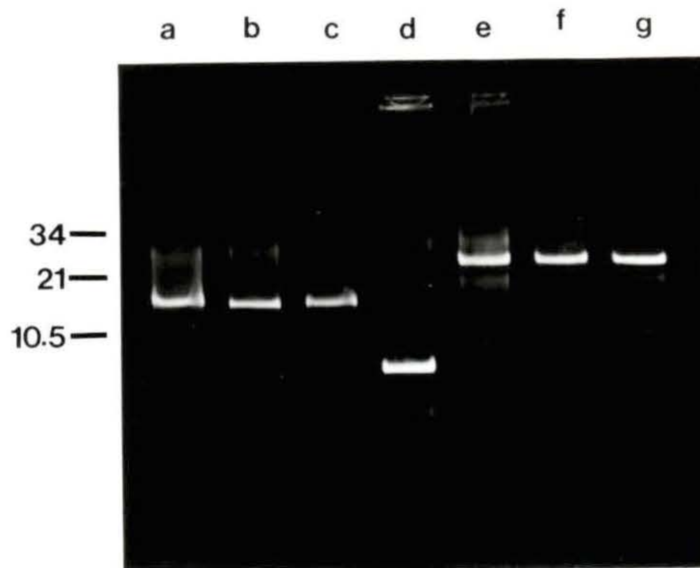

Figure 2. Agarose gel electrophoresis of plasmid DNA from Escherichia coli and Bordetella avium strains. A - B. avium 75; B - B. avium 75 with chromosomal insert of Tn5; C - B. avium 75 with chromosomal insert of Tn5, chloramphenicol resistant; D - E. coli SM10(pSUP1011); E - B. avium 838 with chromosomal insert of Tn5, chloramphenicol resistant; F - B. avium 838 with chromosomal insert of Tn5; G - B. avium 838. Molecular weight standards are given in megadaltons.

single representative mutants in these three classes was probed with pSUP1011 DNA. Each mutant contained a single insert of Tn5 in various sizes of restriction fragments between 9 and 30 kilobases.

Stability of Tn5 insertions was determined by examining reversion to prototrophy. Rates of 2 X 10^{-7} to less than 10^{-10} revertants per CFU were determined. All revertants examined had loss of kanamycin resistance except one adenine-requiring mutant.

Hemagglutination

Strains 75 and 838-S Tn5 insertion mutants were examined for loss of hemagglutination. Of 2,806 strain 75 isolates tested, only 3 had loss of hemagglutinating ability (Table 4). No hemagglutination negative mutants of strain 838-S were isolated from 2,085 colonies tested.

Strains 75-1 and 75-2 showed no hemagglutination when grown on BHI agar, but when grown on blood agar prior to testing, they regained the ability to agglutinate guinea pig red blood cells (Table 4). The third mutant, 75-3, exhibited no hemagglutination when grown on either medium (Table 4). All auxotrophs showed normal hemagglutination, except for 6 uracil-requiring mutants that exhibited reduced hemagglutination (Table 4). *Bordetella avium*-like strains 031, 101, and 023 showed no hemagglutination (Table 4).

Growth of strain 75-3 was inhibited on blood agar compared to that of the wild-type strain 75. When 10^{10} mutants were spread onto a blood

Table 4. Hemagglutination titers of various wild-type and mutant *Bordetella avium* strains

Strains	Medium[a]	Hemagglutination titer[b]
75	BHI	16 to 32
75-1	BHI	< 2
75-1	BA	32
75-2	BHI	< 2
75-2	BA	16
75-3	BHI	< 2
75-3	BA	< 2
75-3 revertant	BHI	32
838-S	BHI	16 to 32
101	BHI	< 2
031	BHI	< 2
023	BHI	< 2
75-uracil auxotroph	BHI	2 to 4

[a] BHI = brain heart infusion agar, BA = blood agar.

[b] Titers are expressed as the inverse of the highest dilution yielding complete hemagglutination.

agar plate, revertants could be observed as fast-growing colonies on a lawn of much slower-growing mutants with a reversion rate of 6 x 10^{-8} revertants per CFU. Examination of several revertants revealed ability to hemagglutinate and loss of kanamycin resistance (Table 4).

Chromosomal DNA from 75-1, 75-2, 75-3, 75-3 revertant, and a representative uracil-requiring mutant with reduced hemagglutination was probed with pSUP1011 DNA. Single Tn*5* insertions in 75-1, 75-2, 75-3, and the uracil-requiring mutant were found in *Eco*RI fragments of various sizes between 9.5 and 30 kilobases. The revertant of strain 75-3 had no hybridization with pSUP1011 DNA.

DISCUSSION

Establishing a means of gene transfer is an important step in genetic characterization of an organism. Conjugative plasmids RP4 of the P incompatibility group and R388 of the W incompatibility group mediated self-transfer into several pathogenic *B. avium* strains. Replication of broad host range plasmids has been reported in *B. pertussis* and *B. bronchiseptica*.[17,40] In our study, the conjugative plasmids appeared to transfer into and replicate normally in *B. avium*. Self-transfer of R388 from *E. coli* donors to *B. avium* recipients occurred at a rate up to 2500-fold greater than that of RP4, and no RP4-containing transconjugants were obtained in the mating with *B. avium* strain 838-S as recipient (Table 2). Higher transfer frequencies of RP4 were expected, and this might indicate the presence of restriction barriers in *B. avium* strains for RP4. Stable maintenance of recipient strain plasmids in the presence of conjugative plasmids indicated they were in different incompatibility groups.

In a study by Simmons et al., the plasmid in *B. avium* strain W was found not to carry any virulence factors.[34] In our study, the plasmid was shown to encode self-transfer. The plasmid co-existed with R388 in *E. coli* strain MV-12, which indicated it was not in the W incompatibility group. This plasmid and an R plasmid isolated from *B. bronchiseptica* were of similar size and both encoded sulfonamide resistance.[17] Broad-host range plasmids are commonly found in gram-negative bacteria and it is not unusual to find them in virulent *B.*

avium strains.

Development of a nutritionally defined medium for growth of *B. avium* was essential for isolation and characterization of *Tn5*-induced auxotrophs. Growth of most of the *B. avium* isolates on minimal medium indicates similar nutritional requirements for most strains. This similarity was not observed with *B. pertussis* in which major strain differences exist.[24] The specific requirement of several phase-I and phase-IV *B. pertussis* strains for L-cystine and niacin, as well as other nutrients, was comparable to that for *B. avium* isolates.

Transposon-mediated mutagenesis in *B. avium* resulted in 13 auxotrophic classes and 2 types of hemagglutination mutants. This indicates the ability of *Tn5* to transpose to many sites in the chromosome. Southern analysis with pSUP1011 DNA as a probe resulted in hybridization with single *Eco*RI fragments of chromosomal DNA from each mutant strain examined. Single bands of hybridization indicated single insertions in the chromosome because *Eco*RI does not cleave *Tn5*.[21] Approximately one-half of all auxotrophs isolated could be grouped into 3 classes. This apparent non-random insertion is characteristic of *Tn5*.[7] Reversion rates indicated *Tn5* insertion was very stable. Reversion of mutation was linked to loss of kanamycin resistance, and therefore loss of *Tn5*, in all cases except one. This observation may result from separate transposition of the IS*50* element from *Tn5*, producing a mutation not linked to kanamycin resistance.[1,6]

The chloramphenicol resistance gene on the vector pSUP1011 is not contained on a transposon and is stably maintained by the plasmid.[10,35]

When all B. avium Tn5 insertion mutants were examined, 1.2% remained resistant to chloramphenicol. This indicates that all or part of pSUP1011 remained in the recipient. Retention of pSUP1011 DNA in the recipient strain has not been observed with this vector system in other bacteria.[1,35] Plasmid profiles of representative chloramphenicol-resistant transconjugants indicated that pSUP1011 was not replicating autonomously in the cell (Figure 2); therefore, all or part of the plasmid probably integrated into the chromosome.

Isolation of 3 hemagglutination mutants from 4900 strain 75 and 838 transposon mutants examined indicates hemagglutination is controlled by a small area of the genome or it is in a region into which Tn5 does not frequently insert. However, each of these 3 mutants was created by an insertion in a different EcoRI fragment, suggesting a fairly large area of the chromosome is involved in hemagglutination. The first class of hemagglutination-negative isolates required some component of blood agar, possibly in blood or serum, that is not in BHI agar. The second type of hemagglutination mutant was stable even when grown on blood agar.

Among all auxotrophs examined for loss of hemagglutination, about one-half of the uracil auxotrophs had reduced activity. In many bacteria, the pyrimidine synthesis genes are spread throughout the genome.[3,30] Insertion of Tn5 into one of these genes might have had some polar effect on a nearby hemagglutinin gene, or mutation in the uracil gene might affect transcriptional initiation of the hemagglutination gene through a regulatory circuit.[15]

Transposon-induced filamentous hemagglutinin-negative mutants have been isolated in *B. pertussis*.[41] These mutants have been used for studying the filamentous hemagglutinin and its effect on adherence to human cells.[37,41] Hemagglutination-negative mutants of *B. avium* should prove to be of value in the study of the hemagglutinin and its effect on adhesion of the bacterium to tracheal epithelium of the turkey.

Availability of a bank of specific transposon-induced auxotrophs might allow further investigation into the virulence mechanisms of *B. avium*. Stability of the mutants isolated in our study should permit examination of the effect of mutation on pathogenesis by experimental infection of turkeys.

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SECTION II. CLONING AND WESTERN IMMUNOBLOT ANALYSIS OF ANTIGENS OF *BORDETELLA AVIUM*

SUMMARY

A *Bordetella avium* genomic library was prepared in *Escherichia coli* strain JM109 using the plasmid cloning vector pUC19. Approximately 13,000 recombinant colonies were screened for antigen production by a colony immunoblot procedure. Hyperimmune rabbit antiserum was used as primary antibody. A total of 134 immunoreactive *E. coli* isolates were selected for plasmid and Western blot analysis. The recombinant plasmids contained *B. avium* DNA inserts with an average size of 7-10 kilobases (kb) and ranging from 1.5 kb to greater than 30 kb. Of the 134 immunoreactive colonies examined by Western blots, 120 exhibited one or more antigen bands. The antigens were grouped into more than 30 groups based on electrophoretic mobility. Antigens with molecular weights of less than 6,000 to greater than 80,000 were represented in these groups. Seven size groups contained the majority (76) of the recombinants examined with molecular weights of less than 6,000 to approximately 55,000. The results indicate that *B. avium* DNA is expressed in *E. coli* JM109 and the antigens produced are relatively stable.

INTRODUCTION

Bordetella avium is the cause of bordetellosis in turkeys.[20,24] Clinically, this disease is characterized by oculonasal discharge, sneezing, dyspnea, tracheal collapse, and decreased weight gain.[8,20] A predominant lesion of bordetellosis is a progressive loss of ciliated respiratory epithelium and goblet cells with replacement by nonciliated cells.[1,2]

Bordetella avium attaches to ciliated epithelium of the respiratory tract of turkeys.[2,16] The bacterium associates with the basal area of the cilia.[2] Raised, knob-like structures on the surface of *B. avium* appear to be associated with the cilia, and may function in bacterial adhesion. Virulent strains of *B. avium* possess a hemagglutinin.[12] Two transposon-induced hemagglutination-negative mutants were less adherent to tracheal mucosa compared to the parent strain; however, reversion of one of the mutants to hemagglutination-positive status resulted in the partial return of adherence.[3] *Bordetella avium* isolates have fimbriae that are polymers of a single protein subunit with a molecular weight of 13,100.[11] The fimbriae may be involved in *in vivo* attachment to the tracheal mucosal surface but do not appear to be involved with hemagglutination.

Two toxins have been isolated from cultures of *B. avium*. A heat-labile toxin is lethal for turkey poults and mice when given intraperitoneally.[18] Intradermal injection of this toxin causes dermonecrosis in turkeys and guinea pigs.[19] A heat-stable toxin is

lethal to mice but not to turkeys;[22] however, decreased thymus weights have been observed in turkeys given this toxin.[27] Both toxins are considered to be proteins.[18,22] Several virulent *B. avium* isolates also produce a histamine-sensitizing factor for turkeys and mice.[23] The histamine-sensitizing factor may be similar to that produced by *B. pertussis* but it is not as potent. The role of these toxins in pathogenesis of bordetellosis is not understood.

The role of the above factors in bordetellosis and the turkey's immune response to these and other antigens is unknown. Cloning of DNA and expression of recombinant proteins from pathogenic bacteria in *Escherichia coli* allows the potential production of individual proteins in large quantities. Availability of large amounts of proteins aids in the study of the immune response of the host to these molecules. Also, the function, structure, and genetic organization of bacterial components such as fimbriae, toxins, and outer membrane proteins might be examined. In the present study, we generated a *B. avium* genomic library in *E. coli* with the plasmid vector pUC19. The resulting recombinants were screened for antigen expression and grouped according to size of antigen produced.

MATERIALS AND METHODS

Bacterial Strains and Media

Bordetella avium strains 75 and 838 were isolated in Iowa from turkeys with bordetellosis.[1,5] *Bordetella avium* strain NCD was obtained from D. G. Simmons, North Carolina State University, Raleigh, North Carolina. *Escherichia coli* strains 71-18(pUC19) and JM109 were received from John Mayfield and Greg Mahairas, respectively, Iowa State University, Ames, Iowa.[26]

Bacteria were maintained at -70 C in 20% glycerin/80% brain heart infusion (Difco Laboratories, Detroit, MI) broth mixture. All isolates were initially purified by picking individual colonies and re-streaking. The identity of all cultures was verified by use of standard biochemical tests. *Escherichia coli* and *B. avium* strains were grown at 37 C on LB medium containing 1.5% agar.[7] Ampicillin (100 ug/ml) was added to LB medium as required. Minimal medium was used for growth of *B. avium* strains for bacterin production (as previously described in Section I).

DNA Isolation

Plasmid DNA from *E. coli* strain 71-18(pUC19) was isolated by an alkaline lysis procedure,[6] and further purified by cesium chloride gradient.[14] Chromosomal DNA was extracted from *B. avium* strain 75 as previously described.[10]

Construction of Recombinant Library

Restriction endonuclease enzymes were used according to the instructions of the manufacturer (Bethesda Research Laboratories, Gaithersburg, MD). Chromosomal DNA was partially digested with the restriction endonuclease Sau3A I and approximately 200 ug of digested DNA was layered on a 10-40% sucrose gradient and centrifuged at 24,000 rpm (SW-27 rotor, Beckman Instruments, Inc., Palo Alto, CA) for 21 hr.[14] Fractions (0.5 ml) were collected and 5 ul samples from each were electrophoresed in a 0.4% agarose gel. Fractions containing 7-10 kilobases (kb) of DNA were diluted in 1 volume of Tris-EDTA buffer (TE, 10 mM Tris [pH 8.0], 1 mM Na_2EDTA), yeast tRNA (100 ug/fraction) was added as a carrier, and the DNA was precipitated at -20 C in 2 volumes of 95% ethanol. DNA from each fraction was resuspended in 10 ul TE buffer, and used in ligation reactions. The vector DNA was digested to completion with BamHI, and dephosphorylated with calf intestinal alkaline phosphatase, following conditions of the supplier (Boehringer Mannheim Biochemicals, Indianapolis, IN). Ligation of chromosomal and vector DNA with T-4 DNA ligase was according to the procedure of Maniatis et al.[14] Approximately 200 ng of ligated DNA was transformed into competent *E. coli* strain JM109 cells using a high efficiency procedure.[9] Transformed cells were spread on LB agar containing ampicillin and incubated overnight.

Screening of Recombinant Library

Bordetella avium strains 75, 838, and NCD were grown on minimal medium agar at 37 C for 40 hr. The cells were resuspended in phosphate-buffered saline solution (PBSS, 0.0132 M phosphate, 0.15 M NaCl, pH 7.3) to an OD of 1.0 at 620 nm, and inactivated with formaldehyde. An aluminum hydroxide suspension (Mann Research Laboratories, Inc., New York, NY) was added to whole inactivated cells to a concentration of 15%, and the bacterin was injected subcutaneously to hyperimmunize two rabbits. Anti-B. avium serum was absorbed with E. coli JM109(pUC19), diluted 1:400, and used to screen the recombinant colonies. Transformed colonies were transferred to nitrocellulose membranes (BA-85, 45 um, Schleicher and Schuell, Inc., Keene, NH) and lysed.[17] Blocking of membranes using bovine serum albumin, incubation with anti-B. avium serum and secondary antibody (biotinylated goat anti-rabbit IgG diluted 1:2000), and color determination using steptavidin-alkaline phosphatase conjugate (1:4000), were carried out as descibed by the supplier (Bethesda Research Laboratories, Gaithersburg, MD).

Sodium Dodecyl Sulfate-Polyacrylamide Electrophoresis (SDS-PAGE) and Western Blot Analysis

Immunopositive E. coli were grown for 18 hrs on LB agar, washed from the plates with PBSS, pelleted by centrifugation, and resuspended in PBSS. The concentration of each isolate was standardized to an OD of 1.0 at 600 nm, and 0.5 ml of each suspension was pelleted by

centrifugation. The pellet was resuspended in 40 ul of sample buffer (62.5 mM Tris [pH 6.8], 3% sodium dodecyl sulfate, 1% dithiothreitol, 0.01% bromophenol blue, 20% glycerol) and heated for 5 min at 95 C. The proteins were separated by discontinuous SDS-PAGE using a 4% stacking gel, and a 6-20% gradient separating gel.[13] Electrophoresis was carried out at a constant current of 25 ma/gel through the stacking gel, and 35 ma/gel in the separating gel, until the tracking dye reached the end of the gel.

Immediately following SDS-PAGE, the proteins were electrophoretically transferred to nitrocellulose sheets (BA-85, 45 um, Schleicher and Schuell, Inc., Keene, NH), by a modification of the procedure of Towbin et al.[25] Proteins were transferred in a transblot cell (Bio-Rad Laboratories, Richmond, CA) at a constant current of 75 ma/gel for 18 hrs using transfer buffer (25 mM sodium phosphate [pH 7.5]). The nitrocellulose sheets were blocked in TTMS buffer (10 mM Tris [pH 7.4], 150 mM NaCl, 0.05% Tween 20, 5% nonfat dry milk [Carnation Co., Los Angeles, CA]) for 1 hr at 25 C. After blocking, the sheets were incubated for 2 hr at 25 C with rabbit antiserum diluted 1:100 in TTMS buffer. Following three 10 min washes in TTMS buffer, the nitrocellulose sheets were incubated for 1 hr at 25 C with biotinylated goat anti-rabbit IgG antibody diluted 1:1000. The washing step was repeated, and the sheets were added to streptavidin-alkaline phosphatase conjugate, diluted 1:4000 in TTMS buffer without non-fat dry milk. Incubation and color determination were carried out as described for the colony immunoblot procedure.

RESULTS

Cloning and Screening of Antigen Producing Colonies

Approximately 13,000 ampicillin resistant, recombinant *E. coli* colonies were screened for *B. avium* antigen production using the colony immunoblot procedure. Immunoreactive colonies were purified by re-streaking, and were re-examined by colony immunoblot for antigen production. A total of 134 isolates were immunoreactive on this second screen. Isolation and restriction endonuclease analysis of the plasmids from the 134 immunoreactive colonies indicated the presence of *B. avium* chromosomal inserts. The recombinant plasmids contained DNA inserts with an average size of 7-10 kb and ranging from 1.5 kb to over 30 kb.

Western Blot Analysis

Of 134 immunoreactive isolates examined by Western blot analysis, 120 exhibited one or more antigen bands that reacted with rabbit anti-*B. avium* serum. The antigen bands appeared quite sharp, with a lack of smaller molecular weight immunoreactive degradation products. The isolates were placed into approximately 30 groups based on electrophoretic mobility of the antigens expressed. Antigens with apparent molecular weights less than 6,000 to greater than 80,000 were represented. Seven size groups contained 76 of the total number of recombinants examined, with molecular weights of these antigens from less than 6,000 to approximately 55,000.

DISCUSSION

A *B. avium* genomic library was prepared in *E. coli* strain JM109. In our study, it appears that *B. avium* DNA is expressed in *E. coli*. The plasmid pUC19 contains a functional beta-galactosidase promoter that can be used by *E. coli* for transciption of the foreign DNA insert.[26] The expression product would be a fusion protein of part beta-galactosidase and part foreign protein. In this case, however, *E. coli* strain JM109 contains the *lac* I^q mutation which causes overproduction of the *lac* repressor and subsequent repression of the beta-galactosidase promoter.[4,26] Though this repression is probably not complete, it suggests that the *B. avium* promoters are being used by *E. coli* for transcription. The lack of degradation products on Western blots indicates the antigens produced by *E. coli* are relatively stable.

Bordetella avium antigens produced by *E. coli* recombinants were grouped into over 30 size groups, however, there may not be over 30 different antigens represented. Different portions of the same gene may be cloned, and the resulting proteins might contain the same antigenic determinant, but have different molecular weights. Further examination of the recombinants might be useful in determining the groups of antigens produced. It is likely, however, that many different antigens are represented in this group of recombinants. Two groups of recombinant *E. coli* isolates expressed antigens with molecular weights in the range of the fimbrial antigen. It is possible that some of these antigens may be important in the immune response of the turkey to *B.*

avium. Also, some of the recombinant antigens might represent one or more of the toxins.

Cloning and expression of DNA from *B. avium* represents an important technique that can be used in the study of this organism. Many virulence factors of bacteria have been examined using this technique.[4,10,17,21] Several potentially important virulence factors of *B. avium* have been demonstrated, but their role in pathogenesis is unknown. The availability of a set of antigen-producing recombinants may aid in the future evaluation of these potential virulence factors and the interaction of *B. avium* with the immune defense mechanisms of its host.

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SUMMARY AND CONCLUSIONS

Conjugation in *Bordetella avium* was described. Two broad host range plasmids, RP4 and R388, were transferred by conjugation from *Escherichia coli* to several *B. avium* strains. The plasmids appeared to transfer into and replicate normally in *B. avium*. The recipient strains were able to donate these plasmids to other strains of *B. avium* and *E. coli*. This indicates that there was no major modification of function of the plasmids in *B. avium*. An approximately 35-megadalton plasmid found naturally in *B. avium* strain W appeared to mediate self-transfer to *E. coli*. Kanamycin-neomycin and sulfonamide resistance was also transferred with this plasmid.

A defined minimal medium was developed for growth of *B. avium*. Similar nutritional requirements were noted for most strains examined. The defined minimal medium was used for scoring transposon-derived mutants for specific nutritional requirements. The transposon Tn*5* was transferred by conjugation to *B. avium* using the suicide plasmid vector pSUP1011. Approximately 1% of all Tn*5*-insertion mutants were auxotrophs. These auxotrophs were placed into 13 classes based on their nutritional requirements. Two classes of transposon-derived hemagglutination-negative mutants were isolated. One type of uracil-requiring mutant exhibited reduced hemagglutinating ability. The Tn*5* insertions were relatively stable, with frequencies of reversion less than 10^{-7}. All mutants examined by Southern hybridization had one copy of Tn*5* in their chromosomes.

A *B. avium* genomic library was prepared in *E. coli* strain JM109 using the plasmid cloning vector pUC19. The recombinant *E. coli* isolates were screened by a colony immunoblot procedure for antigen expression, using rabbit hyperimmune antiserum prepared against whole *B. avium* cells. Immunoreactive colonies were selected and examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western immunoblot procedure. Of 134 isolates examined, 120 exhibited one or more antigen bands. These antigens were grouped into 30 groups based on electrophoretic mobility. Molecular weights of the antigens expressed ranged in size from less than 6,000 to greater than 80,000. The recombinant antigens appeared to be relatively stable in *E. coli*.

Availability of specific transposon-generated mutants and a group of recombinant antigens should aid future research into the virulence mechanisms of *B. avium*. Comparison of wild-type *B. avium* strains and transposon-generated mutants deficient in hemagglutination may be used to help determine the role of the hemagglutinin in adhesion. Turkey challenge studies using auxotrophic mutants could be used to evaluate changes in virulence of *B. avium* which may be associated with auxotrophy. Recombinant antigens isolated in our study can be evaluated with Western immunoblot procedure using convalescent *B. avium* turkey antiserum. This procedure might allow identification of antigens which are immunologically recognized by turkey poults. These antigens might be important in the immune response of the turkey to *B. avium*. Antiserum prepared against the recombinant antigens could be used to determine if any offer passive protection to turkey poults when

challenged with virulent *B. avium* isolates. The recombinant antigens can be used to study specific virulence factors of *B. avium* such as fimbriae and toxins. Availability of recombinant proteins should aid in the study of the function, structure, and genetic organization of these virulence factors.

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