To the Graduate Council:

I am submitting herewith a dissertation written by Eugene Hugh Burns, Jr. entitled "Involvement of Fimbriae in Attachment by *Bordetella bronchiseptica*." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Microbiology.

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INVOLVEMENT OF FIMBRIAE IN ATTACHMENT BY *BORDETELLA BRONCHISEPTICA*

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Eugene Hugh Burns, Jr.

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ABSTRACT

Bordetella bronchiseptica is a Gram negative respiratory tract pathogen which infects a wide variety of animal species including dogs, pigs, and guinea pigs. Infection and colonization of ciliated respiratory epithelial cells by *B. bronchiseptica* has been associated with atrophic rhinitis in pigs and acute tracheobronchitis (kennel cough) in dogs. The first step in colonization is attachment of bacteria to the host cells. *B. bronchiseptica*, like the human pathogen, *Bordetella pertussis*, produces several proteins which could act as adhesins, including filamentous hemagglutinin (FHA), pertactin, and fimbriae. It is believed that attachment by *B. pertussis* is mediated primarily through the action of FHA in concert with pertactin and pertussis toxin. *B. bronchiseptica*, however, in order to infect a wider range of animal species, may utilize different adherence mechanisms than *B. pertussis*. Despite experiments suggesting that fimbriae are not necessary for attachment by *B. pertussis*, there has been data suggesting that fimbriae may play a role in adherence by *B. bronchiseptica*. Therefore, experiments were undertaken to further explore the role of fimbriae in attachment by *B. bronchiseptica.*

Two approaches were used to examine the involvement of fimbriae in attachment. The first approach involved determination of relative amounts of fimbrial proteins expressed on the surface of *B. bronchiseptica* cells and comparison of these results with attachment ability. A monoclonal antibody specific for non-denatured serotype 2 fimbrial filaments was developed and characterized in this laboratory. This antibody was used in a pre-adsorption ELISA to measure serotype 2 fimbrial expression levels on *B. bronchiseptica* cells. Thirty-six strains of *B. bronchiseptica* from pigs, dogs, guinea pigs, and four other species were tested using this assay. Strains originally isolated from pigs exhibited significantly more reactivity with monoclonal antibody CF8 than those from guinea pigs. Strains originally isolated from dogs, however, were highly variable in

reactivity suggesting that dogs are capable of being infected with a wider variety of *B. bronchiseptica* strains than pigs or guinea pigs. Levels of reactivity in this assay were compared with attachment ability in an *in vitro* attachment assay using Vero cells. No correlation was seen between attachment ability and reactivity with monoclonal antibody CF8. However, a correlation was observed between attachment ability and expression of a 24 kD fimbrial subunit protein which reacts in Western blots with antibody specific for serotype 2 fimbriae, the same fimbrial serotype as recognized by CF8.

The second approach to exploring the role of fimbriae in attachment involved cloning a gene affecting expression of fimbriae and examining attachment ability of strains carrying this cloned gene. A genomic library was constructed from *B. bronchiseptica* strain 110H and screened with oligonucleotide probes derived from each end of the *B. pertussis fim2* gene. A cosmid clone reacting with both of these probes and designated pGB710 was introduced into *B. bronchiseptica* strains 17640 and R-5. Expression of serotype 2 fimbriae, as measured by ELISA with monoclonal antibody CF8 and examination of fimbrial protein profile on SDS-PAGE, increased in each of these strains after introduction of pGB710. Attachment ability of strain R-5 also doubled when cosmid pGB710 was present. This increase in attachment ability could be inhibited by pre-incubation of the bacteria with monoclonal antibody against serotype 2 fimbriae but not with antibodies against FHA or pertactin. In addition, recombinational inactivation of the gene reactive with the serotype 2 fimbrial oligonucleotide probes prevented the increase in attachment in strain R-5. These data suggest that serotype 2 fimbriae are involved in attachment by *B. bronchiseptica.*

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LIST OF ABBREVIATIONS

BG	Bordet Gengou Medium
dATP	Deoxyadenosine Triphosphate
dCTP	Deoxycytidine Triphosphate
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic Acid
dUTP	Deoxyuridine Triphosphate
EDTA	Disodium Ethylenediaminetetraacetic Acid
ELISA	Enzyme Linked Immunosorbent Assay
ET	Enzyme Electromorphotype
FHA	Filamentous Hemagglutinin
HRP	Horseradish Peroxidase
kb	Kilobases
kD	KiloDaltons
kV	Kilovolts
LB	Luria Bertani Medium
O.D.	Optical Density
PBS	Phosphate Buffered Saline
REGW	Ryan Einot Gabriel Welsh Multiple F Test
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SSC	Sodium Chloride, Sodium Citrate Buffer
TAE	Tris Acetate EDTA Electrophoresis Buffer
TBS	Tris Buffered Saline
TdT	Terminal Deoxynucleotidyl Transferase

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

Introduction

Bordetella bronchiseptica is a Gram negative respiratory pathogen of animals. It has been associated with atrophic rhinitis in swine (87, 209, 286) and acute tracheobronchitis (kennel cough) in dogs (33, 39, 98, 100) as well as respiratory infections in a wide variety of other animals (99, 101, 311). Development of disease first requires colonization of the ciliated respiratory epithelium by the bacteria. Attachment leading to colonization is mediated by the combined effects of several adhesins also produced by the closely related species, *Bordetella pertussis*. These adhesins include filamentous hemagglutinin (FHA) and pertactin which was initially identified in *B. bronchiseptica* (49, 194, 231, 289, 332). *Bordetella* species also produce fimbriae. In other bacteria such as *Escherichia coli* and *Neisseria,* fimbriae commonly function as adhesins (90, 278, 364). However, a number of studies with *B. pertussis* have indicated that fimbriae are not involved in attachment by this species (234, 270, 278). Due to the close evolutionary relationship and high degree of physiological and genetic similarity between *B. pertussis* and *B. bronchiseptica*, it is often assumed that these two organisms have identical pathogenic traits (122, 169, 181).

There are, however, differences between *B. bronchiseptica* and *B. pertussis* in terms of host preference. *B. pertussis* infects only humans while *B. bronchiseptica* is commonly isolated from various animals (71, 100, 101, 209, 311). Since *B. bronchiseptica* infects a broader range of hosts than *B. pertussis*, it may require or utilize different adhesins for attachment. Despite studies discounting the role of fimbriae in *B. pertussis* attachment, involvement of fimbriae in attachment by *B. bronchiseptica* cannot

be ruled out. Dugal reported that thin filaments presumed to be fimbriae could be seen by electron microscopy radiating from *B. bronchiseptica* cells and touching the cilia (90). Antibodies against fimbriae inhibited attachment in *in vitro* attachment assays (127) and prevented colonization of the lungs of experimentally infected mice (278). These data suggest that fimbriae may play a role in attachment by *B. bronchiseptica.* This dissertation describes studies designed to explore the relationship between fimbriae and attachment by *B. bronchiseptica.*

Background

The Genus *Bordetella*

The genus *Bordetella* consists of four species of Gram-negative, respiratory tract pathogens of humans and animals. *Bordetella pertussis*, the etiologic agent of whooping cough, infects only humans (71). *Bordetella parapertussis* infects primarily humans but the organism has also been isolated from sheep (84). *B. parapertussis* infection can cause a mild respiratory disease similar to that caused by *B. pertussis*, but infections usually remain asymptomatic (191). *Bordetella avium* is primarily a pathogen of birds, causing a condition known as coryza in turkeys (15, 173, 260). *Bordetella bronchiseptica* has been associated with atrophic rhinitis in swine (87, 209, 287) and acute tracheobronchitis in dogs (33, 38, 98, 100). *B. bronchiseptica* can also infect a wide variety of other animals including mice, guinea pigs, cats, and horses (99, 101, 311). A fifth and sixth species of *Bordetella* have recently been proposed. These species, tentatively designated *Bordetella hinzii* (80) and *Bordetella holmesii* (356a) were isolated from humans and most closely resemble *B. avium.* These species have not been extensively characterized and references to characteristics of all *Bordetella* species in this dissertation do not include these two recently discovered species.

B. pertussis was first isolated by Bordet and Gengou in 1906 on a potato extract agar which is still used today for isolation of *B. pertussis* (46). It was originally

designated *Haemophilus pertussis* (122, 258). In 1910, *B. bronchiseptica* was isolated from dogs by Ferry and named *Bacillus bronchicanis* (98). It was subsequently determined to be the same organism previously isolated from other animals and the name was changed to *Bacterium bronchisepticus* (260). *B. parapertussis* was first described by Eldering and Kendrick in 1938 and named *Bacillus parapertussis* (91). In 1952, Moreno-López proposed the genus *Bordetella* in honor of Jules Bordet and the organisms now known as *B. pertussis, B. parapertussis,* and *B. bronchiseptica* were placed in this genus (235). A *B. bronchiseptica* -like organism, now designated *B. avium,* was isolated from turkeys and first described by Hinz *et al.* (148) in 1978.

Bordetella are Gram negative coccobacilli, ranging in size from 0.2 to 0.5 μm in diameter and 0.5 to 2.0 μm in length, which are readily isolated from the respiratory tract of infected individuals (260). When grown in laboratory cultures, these organisms exist singly or occasionally in pairs. Short chains are sometimes, but not commonly, seen in cultures in the avirulent phase (260). These bacteria do not require any nutrients from blood for growth but *B. pertussis* requires the presence of blood, charcoal, or other components such as albumin or anion exchange resins in the medium to reduce the inhibitory effects of short chain unsaturated fatty acids, colloidal sulfur, or secondary metabolites usually found in agar (36, 187, 259, 262, 264). In a chemically defined medium, such as Stainer-Scholte broth, no absorbents are needed (260, 314). Aoyama *et al.* (9)compared growth of *B. pertussis* on cyclodextrin solid medium with growth on BG medium and found that *B. pertussis* grows well on this medium. Cyclodextrin has been reported to be a good growth stimulant for *Bordetella* (9, 161, 162). The other three species can be grown on nutrient agar without the need for these additives and Brucella agar is commonly used for growth of *B. bronchiseptica* (258).

All *Bordetella* are somewhat slow growing. *B. pertussis* produces the smallest colonies and is the slowest growing of the *Bordetella* species. Mature colonies of *B. pertussis* are present after three to six days of growth on Bordet-Gengou (BG) agar at

37°C (260). *B. bronchiseptica* is the fastest growing species of *Bordetella* and produces the largest colonies. On Brucella agar, colonies of *B. bronchiseptica* are visible after one day of incubation but do not reach a mature size until the next day. On BG agar, mature colonies of *B. bronchiseptica, B. parapertussis,* and *B. avium* develop within two to three days of incubation. *B. parapertussis* colonies are surrounded by a zone of diffuse brown pigment on peptone agar due to the action of tyrosinase on the tyrosine in the medium (260). This discoloration of peptone agar can be used to differentiate *B. parapertussis* from the other *Bordetella* species.

All species of *Bordetella* can exhibit at least two distinct colony types on BG medium (97, 167, 182, 189, 196, 222, 226, 302). Leslie and Gardner first described four phases in 1931, which they designated Phase I to Phase IV (196). Phase I *Bordetella*, now commonly referred to as virulent phase, produce colonies that are small (≤ 1 mm), domed, and hemolytic (depending on strain). Cells in the avirulent phase, also called phase IV, produce much larger, flat, non-hemolytic colonies. Avirulent phase colonies also grow more rapidly than those in the virulent phase (254). When *Bordetella* switch from virulent phase to avirulent phase, they stop producing certain virulence factors among which are filamentous hemagglutinin (FHA), fimbriae, pertactin, adenylate cyclase toxin, and dermonecrotic toxin (78, 129, 296, 297, 351). Each of these factors will be described in more detail in later sections of this chapter. The loss of these virulence factors due to phase change has been correlated with a similar reduction in pathogenicity (165, 255, 256, 349).

Switching between phases can occur by two different mechanisms termed phase variation and modulation. Both phase variation and modulation occur naturally in all species of *Bordetella*. Phase variation is caused by a mutation, usually a deletion, in the *Bordetella* virulence gene, *bvgS* (226, 229, 318, 319). Phase variation, therefore, can cause a change in phenotype from the virulent to the avirulent phase but the frequency of reversion back to virulent phase is small (227, 318, 319). Modulation is the name given to

a similar but reversible change in phenotype. In the presence of certain environmental factors, *Bordetella* will switch from virulent phase to avirulent phase (188, 222, 223, 298). Melton and Weiss reported that the addition of sulfate ions to the growth medium would induce phase change in *B. pertussis* (222). Ishikawa and Isayama induced modulation in *B. bronchiseptica* by the addition of crystal violet to the medium (165). Jackwood reported that similar conditions could cause modulation in *B. avium* (167). It has also been found that the presence of nicotinic acid or growth at low temperature (25°C) will induce modulation (188, 222, 223). Modulation is controlled by a transmembrane environmental sensor protein, BvgS, and is reversed when the modulating agent is removed (188). Regulation of virulence factors, phase variation, and modulation will be discussed later in this chapter.

All species of *Bordetella* are strictly aerobic and non-fermentative (258, 260). Amino acids, principally glutamate, and other organic acids are used as primary energy sources rather than sugars. *B. pertussis* and *B. parapertussis* deaminate proline, alanine, aspartic acid, serine, and glycine. *B. bronchiseptica* can utilize all of the common amino acids except arginine, lysine, and histidine (260). *B. avium* can utilize only glutamic acid, aspartic acid, proline, succinate, and pyruvate as energy sources (173). Members of the genus *Bordetella* are also inactive in many standard biochemical tests. They do not liquefy gelatin, hydrolyze starch or casein, produce indole, or produce H_2S (258, 260). *B. bronchiseptica*, *B. avium*, *B. parapertussis* and most, but not all, strains of *B. pertussis* are catalase positive (260). The nitrate, urease, and oxidase tests can be used to differentiate between species. All species except *B. parapertussis* are oxidase positive. Strains of *B. bronchiseptica* are nitrate test positive; the other species are generally negative (258, 260). *B. avium* can produce nitrate only when the medium is supplemented with NAD and serum (260). *B. pertussis* and *B. bronchiseptica* strains are urease test positive with reduction of urea occurring rapidly, usually within four hours in *B. bronchiseptica* (228,

258, 260). *B. parapertussis* and *B. avium* are negative in the urease test (260). In the avirulent phase, *B. bronchiseptica* produces peritrichous flagella and therefore, can exhibit motility (260) *B. avium* produces peritrichous flagella and exhibits motility in the virulent phase (167, 173). The other two species do not produce flagella in either phase (258).

Bordetella species are closely related genetically. Kloos *et al.* (181) performed DNA-DNA reassociation reactions between *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* and found a high degree of homology between these three species. In addition, DNA from *B. bronchiseptica* was used to transform and complement tryptophan auxotrophs of *B. pertussis* (181). Johnson and Sneath also undertook an experiment to determine relationships between *Bordetella* species (169). They report a cophenetic correlation of 0.96 between species in the genus *Bordetella* (169). Some have suggested that due to this close relationship, the genus *Bordetella* should consist of a single species (122, 181). Analysis of the pertussis toxin gene sequences (212, 245) and enzyme electromorphotype data of Musser *et al.* (238) allowed Altschul to construct evolutionary trees for the genus *Bordetella* (4). These trees suggest that the species are divergent enough to warrant separate species status and that *B. pertussis* is actually more closely related to *B. parapertussis* than to *B. bronchiseptica* as was suggested by Kloos *et al* (4, 181). In addition, Khattak *et al.* (175) have found species specific differences between macrorestriction digest patterns obtained by pulsed field gel electrophoresis.

Despite this close genetic relationship between species of *Bordetella*, there is still controversy over differentiation of the genus *Bordetella* from *Alcaligenes*, especially differentiation between *B. bronchiseptica* and *Alcaligenes faecalis* or *Alcaligenes denitrificans*. These organisms are similar both physiologically and genetically. *Bordetella* species have a mol% G + C content of DNA ranging between 66 and 70% (36, 173, 260). The mol% G + C content of DNA of *A. denitrificans* also falls in this range

(260). Johnson and Sneath found a close genetic relationship between *B. bronchiseptica* and *A. faecalis* by DNA reassociation experiments (169). Kersters and De Ley placed *B. bronchiseptica* strains in a tight cluster close to *A. denitrificans* with a phenetic correlation of 0.90 (172).

Diseases Associated With *Bordetella*

Diseases associated with *Bordetella* occur most frequently in young animals or children. *B. pertussis* is the etiologic agent of the disease whooping cough in children and *B. parapertussis* can cause a milder whooping cough-like disease. Infection with *B. parapertussis* is widespread but disease occurs in only 3–4% of those infected (191). Whooping cough is characterized by paroxysmal coughing with episodes followed by a distinctive whooping sound resulting from the forceful inhalation of air through the constricted glottis. Frequently, the paroxysms will be followed by vomiting. The paroxysmal stage usually lasts from one to four weeks but paroxysms can still be experienced for as long as six months even after patients are free of *Bordetella* (71).

Before immunization became standard practice in the United States, 73.5% of children experienced clinically recognizable pertussis by the time they reached the age of seventeen (160). Gordon *et al.*. (124) report that 95% of all individuals were infected with *B. pertussis*, with some cases being asymptomatic, at some time in their lives. *B. pertussis* infection in humans has been controlled in most of the developed world through the use of vaccines. However, the World Health Organization estimates that 600,000 deaths a year still occur due to whooping cough (71). Until recently, in this country, most infants have been vaccinated with killed whole cell *B. pertussis* mixed with an adjuvant and administered in combination with diphtheria and tetanus toxoid (DPT). Despite vaccination, the CDC reports that 1,200 to 4,000 cases of pertussis are diagnosed yearly in the United States resulting in five to ten deaths (61, 62, 63, 64, 71).

There have been reports of adverse reactions and side effects associated with the currently used whole cell vaccine. These adverse reactions include swelling and redness at the site of inoculation, fever, uncontrollable crying, seizures, neurological disorders, and sudden infant death syndrome (23, 24, 25, 27, 71, 74). Studies have been undertaken to determine if these events are caused by the DTP vaccination or are merely temporally related. Barkin and Pinchichero report that 54% of children vaccinated with the whole cell DTP vaccine experienced fever and 82% of the children had behavioral changes including irritability (27). Only 12% of the control group exhibited fever and 15% reported irritability (27). In 1979, Cody *et al.* (23, 24, 25, 74) undertook a study involving over 16,000 children immunized with whole cell DTP vaccine or the diphtheria tetanus component alone. They found significantly higher rates of redness, swelling, and pain at the site of inoculation in patients immunized with the DTP vaccine. They also observed fever, drowsiness, vomiting, anorexia, and persistent uncontrollable crying for greater than 3 hours in a significant number of patients who received the DTP vaccine (23, 24, 25, 74).

There have also been reports of more severe adverse reactions to the whole cell pertussis vaccine including seizures, neurological disorders, and death (71). Cody *et al.* (23, 24, 25, 74) found an occurrence of seizures in one out of every 1,750 vaccinations given as part of a controlled study of adverse reactions to the DTP vaccine. Ellenberg *et al.* (92) studied 54,000 children and found that 616 of these had seizures before the age of one year. Therefore, they predicted that 2 out of 10,000 children will have a seizure within 2 days of DTP vaccination by coincidence alone. The study actually found 1.5 children per 10,000 had seizures within 2 days of immunization indicating that the DTP vaccine may not cause seizures (149).

The Institute of Medicine recently reviewed the data obtained from several studies of adverse reactions to the DTP vaccine and determined that the data indicate a possible causal relationship between the whole cell pertussis vaccine and acute encephalopathy

and neurological disorders with a rate of 10.5 cases per million immunizations (81). However, the data are insufficient to indicate that vaccination causes permanent neurological damage (81). Several studies have explored the relationship between vaccination and sudden infant death syndrome (26, 40, 150, 171, 326, 328, 340). Although some of these studies show a significant increase in sudden infant death after immunization (26, 40, 328), the evidence is not conclusive (71).

Due to concern about the safety and possible side effects of the current whole cell pertussis vaccine, some countries have discontinued vaccination. In Sweden, vaccination was discontinued in 1979. Before this time, whooping cough was rare and infections that did occur were described as mild. Between 1980 and 1985, 36,729 cases of pertussis occurred in Sweden with 2,282 of these requiring hospitalization (71). The Japanese have taken a different approach to minimize the risks associated with the whole cell vaccine. Japan is currently using an acellular vaccine and does not vaccinate until the age of two years (71).

The FDA has licensed two acellular pertussis vaccines for use in the United States. In December of 1991, the acellular vaccine produced by Takeda Chemicals and used in Japan was licensed for use in the United States mixed with diphtheria and tetanus components produced by Lederle Laboratories (59, 75). This vaccine consists of FHA, pertussis toxin, pertactin, and agglutinogens (75). In August of 1992, a second acellular vaccine with similar components prepared by Connaught Laboratories was licensed for use in the U. S. (60, 75). These vaccines can only be used as the fourth and fifth dose in the immunization series for children over 15 months of age because of lack of efficacy data for children vaccinated at an earlier age since the vaccine is only used in Japan after the child reaches 2 years of age (7, 59, 60, 75). The Advisory Committee on Immunization Practices currently recommends the use of the whole cell vaccine for the first three immunizations at the age of 2, 4, and 6 months and the acellular vaccine for the

booster doses at 15 months and 4–6 years. The whole cell vaccine is still considered acceptable for these later doses (7).

B. avium is the causative agent of turkey coryza and respiratory infections in other birds. The organism has been isolated from chickens, ducks, geese, and sparrows as well as turkeys (173, 260). Symptoms of the disease include oculonasal discharge, sneezing, dypsnea, tracheal collapse, and loss of weight. The disease causes a progressive loss of ciliated cells in the trachea which leads to an accumulation of mucus and exudate in the trachea (173). Turkey coryza is highly contagious and infected birds become predisposed to other secondary bacterial infections (167, 288). Vaccination against *B. avium* using a live vaccine can cause severe adverse reactions as are reported for the pertussis vaccine (309).

B. bronchiseptica infects several species of animals including pigs, dogs, and guinea pigs (100, 209, 238). Infection by *B. bronchiseptica* has been associated with atrophic rhinitis in swine but toxigenic strains of *Pasteurella multocida* are more commonly isolated from affected pigs (66). Disease can be produced in experimentally infected pigs with *B. bronchiseptica* alone (122, 281, 286) but pigs infected with both *B. bronchiseptica* and *P. multocida* exhibit more severe symptoms than those infected with only *B. bronchiseptica* (21, 66, 73, 139, 209, 272). Animals suffering from atrophic rhinitis exhibit turbinate atrophy which sometimes results in a permanent distortion of the snout (122).

There have been several studies undertaken to determine if the presence of *B. bronchiseptica* or *P. multocida* is causing the turbinate atrophy. Rutter and Rojas experimentally infected gnotobiotic pigs with *B. bronchiseptica* and *P. multocida* (287). This study suggested that the most severe turbinate atrophy occurs when *P. multocida* is present (287). Roop *et al.* (281) observed atrophic rhinitis in experimentally infected neonatal swine and concluded that the dermonecrotic toxin produced by *B. bronchiseptica* is required for turbinate atrophy. Similar results have been obtained by

Nakai *et al* (240). *P. multocida* also produces a dermonecrotic toxin and the combined effects of these two toxins could lead to more severe disease. Experimental evidence indicates that the presence of both *B. bronchiseptica* and *P. multocida* in pigs exhibiting atrophic rhinitis is not coincidental. Backstrom and others have suggested that infection by *B. bronchiseptica* may predispose animals to infection by *P. multocida* (21, 34).

Infected animals do not gain weight as quickly as non-infected animals (122). Sows infected with *B. bronchiseptica* may have an increased frequency of stillbirth and piglets that do survive often grow at a much slower rate than normal (308). *B. bronchiseptica* has been found present in as many as 80 to 90% of farms tested (21, 87, 115, 290) and 25 to 50% of the world's swine are estimated to be infected (323). Because of this high prevalence of *B. bronchiseptica* infection among pigs, pork producers are losing approximately $200 million a year due to reduced market price for swine with facial deformities, reduced weight of animals which have been infected, veterinary fees, medicated feed, and other expenses associated with raising pigs with atrophic rhinitis (243).

B. bronchiseptica also causes acute tracheobronchitis (kennel cough) in dogs (100). Dogs with kennel cough have a dry, harsh, hacking cough usually followed by retching or vomiting (33, 122). The infection rate is higher in young animals and many animals which become infected remain asymptomatic (33). Bordetellosis can occur alone but is usually associated with other diseases such as distemper, parainfluenzae, adenovirus infections, or infections with *Mycoplasma* (33, 39). In fact, *B. bronchiseptica* is isolated in more than 75% of cases of distemper and was originally believed to be the cause of distemper (33, 98, 99, 219). There is an intranasal vaccine consisting of live, attenuated *B. bronchiseptica* which can be given to animals prior to the disappearance of maternal antibodies, before boarding in a kennel, or during an outbreak of kennel cough (306, 327). The efficacy of parenteral vaccination or direct intranasal vaccination with the live vaccine is controversial (218, 327).

B. bronchiseptica infection is also common in several other animals including guinea pigs, rabbits, cats, and horses (99, 100, 122, 238, 311). Isolation of *B. bronchiseptica* from humans is rare with most cases involving immunocompromised hosts exposed to infected animals (362). Between 1910 and 1980 there were only six reported cases of *B. bronchiseptica* infection of humans (52, 66, 100, 114, 219, 361). Since that time, there have been several reports of *B. bronchiseptica* infections in AIDS patients (5, 86, 244, 266) and other immunosuppressed individuals (29, 47).

Typical transmission of *Bordetella* is from animal to animal. Animals usually become infected through exposure to others previously infected (21). Aerosolization of the bacterium by coughing is common. There is no known reservoir of *Bordetella* in the environment, and there is only short term survival on fomites (36). Porter *et al.* inoculated six strains of *B. bronchiseptica* into phosphate buffered saline and into natural fresh and salt water samples (263). They found that *B. bronchiseptica* could grow in each of these environments and suggest that water may be a natural reservoir for *B. bronchiseptica*. They also report that *B. avium* but not *B. pertussis* or *B. parapertussis* can grow in natural fresh and salt water samples (263). *B. bronchiseptica*, however, has not been isolated from a natural water source.

Virulence Factors*

Infection and colonization by *Bordetella* species are mediated by several proteins. Among these are filamentous hemagglutinin (FHA), pertactin, pertussis toxin, and possibly fimbriae (50, 350, 289). *Bordetella*, in addition to attachment factors, also produce several toxins. Among these are an extracellular adenylate cyclase toxin, dermonecrotic toxin, tracheal cytotoxin, and osteotoxin (28, 111, 113, 121). *B. pertussis*

* Adapted from:
Bemis, David A., and Eugene H. Burns, Jr. 1993. *Bordetella.* in C. L. Gyles and C. O. Theon (ed.). *Pathogenesis of Bacterial Infections in Animals*, second edition. Iowa State University Press, Ames, Iowa. p. 201-215.Used by permission.

also produces pertussis toxin, which exhibits ADP-ribosyltransferase activity and plays a role in attachment of *B. pertussis* to ciliated cells (246). *Bordetella* virulence factors, except tracheal cytotoxin, are under control of the Bvg regulatory system, formerly called Vir (78, 129, 296, 297, 298, 299, 351). This system is similar to two component regulatory systems present in other bacteria (13). Due to this regulation, virulence factors are not always expressed by *Bordetella* but are only present when cells are in virulent phase. Table 1 lists the major virulence factors of *Bordetella* species and indicates their cellular location and function.

Toxins

Pertussis Toxin

Pertussis toxin is a 105 kD toxin secreted by *B. pertussis* but not produced by any of the other *Bordetella* species (246). Pertussis toxin, due to the large number of effects which it has on the host, has been known by several other names, including lymphocytosis promoting factor, histamine sensitizing factor, and islet activating protein (246). The toxin induces lymphocytosis, enhances vascular permeability, and promotes anaphylaxis in laboratory animals (230). Pertussis toxin is also a mitogen and an effective adjuvant as well as causing release of fatty acid from fat cells, interfering with chemotactic migration, and causing a modification in the growth pattern of Chinese hamster ovary (CHO) cells in culture (145, 236, 237, 246).

Pertussis toxin functions through ADP ribosylating a G_i protein normally involved in receptor mediated inhibition of adenylate cyclase (157, 170, 230, 334). This modification of the G protein prevents the adenylate cyclase from being turned off leading to an overproduction of cAMP second messenger in the cell. Pertussis toxin is also known to bind to other G proteins involved in the transfer of signals from receptors to phospholipase C (20, 45, 48, 339, 348) and to transducin which is involved in

Table 1: Major virulence factors of *Bordetella* species[1]

Virulence Factor	Species	Size	Location	Function
Pertussis toxin	*B. pertussis* only	105 kD	extracellular	attachment, ADP-ribosylation
FHA	all except *B. avium*[2]	150-220 kD	extracellular	attachment
Pertactin	all	68-70 kD	outer membrane	attachment
Fimbriae	all	14.4-24 kD	outer membrane	attachment?
Adenylate cyclase toxin	all except *B. avium*	around 216 kD	extracellular	inhibition of phagocytosis
Dermonecrotic toxin	all	155-190 kD	intracellular	impairment of osteogenesis
Tracheal cytotoxin	all	0.921 kD	extracellular	destruction of ciliated cells
Osteotoxin	all	80 kD	intracellular	toxicity to osteoblasts

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[2] *B. avium* has hemagglutinating activity which correlates with virulence

translocation of signals from the light receptor rhodopsin to a cGMP phosphodiesterase (211, 338). Each of these proteins to which pertussis toxin has been shown to bind are involved in signal transduction in the host cell. Uncoupling of these proteins could have a wide variety of effects on cells since signal transduction mechanisms and second messengers are used for several different functions in cells (246). *B. pertussis* mutants deficient in pertussis toxin do not colonize animals as well as parent strains producing the toxin (350).

Pertussis toxin consists of five subunit proteins designated S1 through S5 (246). These proteins are arranged into two parts in the native toxin. Part A consists of the S1 subunit and is the catalytically active portion of the toxin. Part B consists of two dimers held together by the S5 subunit. One dimer consists of S2 and S4; the other dimer contains S3 and S4 (325). The B portion of the toxin is involved in binding to cells and translocation of the A subunit across the cell membrane (246, 301).

Genes encoding the pertussis toxin subunits were cloned and sequenced by Nicosia and Locht (203, 246). The five pertussis toxin genes exist in a single 3.2 kb operon and have been designated *ptxABDEC* (43, 246). These genes encode subunits S1, S2, S4, S5, and S3, respectively. The gene encoding S4, *ptxD*, overlaps the genes for S2 and S5 (246). Each gene has its own signal sequence indicating that the pertussis toxin proteins may each be transported independently across the inner membrane and assembled in the periplasmic space or during transport across the outer membrane (246). The operon contains a promoter before the first gene, *ptxA*, and before the *ptxD* gene (203, 205, 246). Nicosia has proposed that the pertussis toxin genes are transcribed as a single polycistronic message (246). Nicosia *et al.* (245) have expressed these genes under a lambda promoter in *E. coli*. Monack *et al.* (230) introduced cloned *ptx* genes into *B. parapertussis*. They found that the genes were expressed but pertussis toxin was not secreted (230). Runeberg-Nymen *et al.* (285) have expressed the pertussis toxin S1 subunit in *Bacillus subtilis*.

Arico *et al.* (12) probed Southern blots of chromosomal DNA from *B. bronchiseptica*, *B. parapertussis*, *B. avium*, and *Alcaligenes* with probes for the *ptx* genes. They found that *B. bronchiseptica* and *B. parapertussis* reacted with the probes but *B. avium* and *Alcaligenes* did not react (12). Since *B. bronchiseptica* and *B. parapertussis* do not produce pertussis toxin, Arico *et al.* (12) cloned and sequenced the *ptx* genes from these species to determine if these genes are capable of being expressed. They found that 50% of the differences between these genes and the *ptx* genes from *B. pertussis* are in the promoter region (12). Two of these changes cause down regulation of expression in *E. coli*. Most of the changes in the structural portion of the genes are conservative and would not be predicted to effect function of the protein. To determine if *B. bronchiseptica* and *B. parapertussis* were producing *ptx* subunit proteins which may not be being assembled or transported out of the cell correctly, Arico *et al.* (12) used enzyme linked immunosorbent assays (ELISA). Northern blots were also performed to determine if the *ptx* genes were being transcribed in these species. Both the ELISA and Northern blots indicated that there is no expression from the *ptx genes* in either *B. bronchiseptica* or *B. parapertussis* (12).

Weiss *et al.* (354) used Tn5*lac* transposon mutagenesis to identify 8 genes necessary for secretion of pertussis toxin. These genes were cloned and used to direct insertion of a gentamicin cassette into the chromosomal copy of each gene to verify that disruption of the gene would prevent secretion of pertussis toxin. These genes have been designated *ptlA* through *ptlH* for pertussis toxin liberation (354). The *ptl* gene cluster is located immediately downstream of the genes for pertussis toxin. Most of the *ptl* genes are preceded by a signal sequence indicating that they are transported to the periplasmic space or the outer membrane. These genes exhibit similarity with the *virB* genes of *Agrobacterium tumefaciens* which are involved in transport of T-DNA across the bacterial outer membrane and ultimately into a plant cell (354).

Johnson and Burns placed the *ptl* genes in an expression vector designed to produce maltose binding protein fusions (168). These fusion proteins were used as

antigens to produce antibodies against the Ptl proteins which could be used in Western blots to identify the proteins encoded by each of the *ptl* genes. Using this method, proteins PtlE, PtlF, and PtlG were identified. PtlE is a 30 kD inner membrane protein. The *ptlE* gene has 32% identity with the *virB8* gene of *A. tumefaciens* (354). The protein product of this gene is also localized to the inner membrane. PtlF is an inner membrane protein which may be associated with some outer membrane components as is postulated for the VirB9 protein of *A. tumefaciens* with which *ptlF* exhibits 27% gene sequence identity (168, 354). PtlG is a 38 kD protein believed to be located in the inner membrane but tightly associated with outer membrane proteins like VirB10 of *A. tumefaciens*. *ptlG* has no signal sequence. The proteins encoded by the other *ptl* genes have not been identified (168). *B. bronchiseptica*, *B. parapertussis*, and *B. avium* hybridize with probes for the *ptl* genes but do not react with antibodies against any of the Ptl proteins (168). Cloned pertussis toxin genes can be expressed in these species but the toxin is not secreted suggesting that these species are not producing functional Ptl proteins (168, 230).

Antibodies against pertussis toxin, whether naturally acquired or passively transferred, are protective against infection by *B. pertussis* (237, 291). Pertussis toxin is a component of several acellular vaccine preparations including the Takeda vaccine which is used in Japan and has been approved for use in the United States for the fourth and fifth immunization (75). The presence of pertussis toxin in the whole cell vaccine is believed to be partially responsible for the adverse reactions associated with vaccination, especially the local redness, swelling, and fever (71).

Adenylate Cyclase Toxin/Hemolysin

Adenylate cyclase toxin, which has also been called cyclolysin, is a bifunctional protein exhibiting both adenylate cyclase and hemolysin activity (28). It is produced by all species of *Bordetella* except *B. avium*. The native form of the protein is approximately

216 kD and is secreted from the bacterial cell (118, 146). Extracellular adenylate cyclase toxin was first isolated from culture supernatant of *B. pertussis* by Hewlett and Wolf as a 70 kD protein (144). This molecule did not exhibit adenylate cyclase activity (144). Ladant *et al.* (189) have isolated 50 kD, 45 kD, and 43 kD forms which also lack activity. Weiss *et al.* (355) and Hanski and Farfel (138) have found that the adenylate cyclase activity is associated with a 216 kD protein contained in crude extracts of *B. pertussis.* This protein has been shown to react with antibodies produced against the smaller forms (216, 279) and has been purified by Hewlett *et al.* (146) and Bellalou *et al.* (32). The smaller fragments ranging from 43 to 70 kD are believed to be degradation products formed during purification procedures (116, 117, 118).

The adenylate cyclase toxin possesses both a cAMP generation domain and a hemolysin domain which are separately regulated. Rogel *et al.* (280) observed that cAMP generation by the adenylate cyclase toxin required the presence of calcium but hemolysis occurred even without calcium. Raptis *et al.* (267) treated adenylate cyclase toxin with trypsin and chymotrypsin and found that the adenylate cyclase activity could be separated from the hemolysin activity. The amino terminal fragment of the toxin possesses the adenylate cyclase activity while the hemolysin function is in the carboxyl end of the molecule (267).

After release from the bacterial cell, adenylate cyclase toxin binds to and enters the host cell. Binding and facilitating entry into the cell is the main role for the hemolysin portion of the adenylate cyclase toxin molecule (28). Gordon *et al.* (125) reported that inhibitors of receptor mediated endocytosis, including cytochalasin D and pre-treatment of cells with trypsin, did not prevent entry of the *B. pertussis* adenylate cyclase toxin into Chinese Hamster Ovary (CHO) cells in culture. These treatments did, however, prevent entry of the *Bacillus anthracis* adenylate cyclase toxin into the cells indicating that these two toxins enter cells by different mechanisms (125). *B. pertussis* adenylate cyclase toxin

was later found to enter cells through interaction with glycolipids instead of glycoproteins as are recognized by *Bacillus anthracis* adenylate cyclase toxin (118).

Entry into the target cell occurs through formation of a pore. The carboxy terminal portion of the molecule is homologous to the *E. coli* α-hemolysin and the *Pasteurella haemolytica* leukotoxin (118, 320). These molecules are both known to act through the formation of pores in the cell membrane (320). All three of these proteins are part of the RTX family of toxins (28, 320). RTX toxins exhibit repeated amino acid sequences in certain regions of the protein. *Bordetella* adenylate cyclase toxin contains fifteen repeats of a thirteen amino acid sequence and five repeats of a nine amino acid sequence in the C-terminal hemolysin portion of the molecule (116). The repeat regions are involved in calcium binding and target cell binding as well as secretion from the cell (44, 207, 303). Sebo *et al.* (303) have found that the repeat sequences from the *B. pertussis* adenylate cyclase toxin can be recognized and used as secretion signals by the *E. coli* α-hemolysin accessory proteins.

After entry into the cell, mediated by the hemolysin, the amino terminal adenylate cyclase domain begins to catalyze the production of cAMP (116). This portion of the molecule is highly similar to mammalian adenylate cyclase and to *B. anthracis* adenylate cyclase (117). It has a calmodulin binding domain and cAMP production is activated by calmodulin (116). This increase in intracellular cAMP levels causes a decrease in normal anti-bacterial functions of both neutrophils and macrophages. Alveolar macrophages isolated from rabbits infected with *B. bronchiseptica* do not produce superoxide in response to stimuli (76). Confer *et al.* (76) observed the same inhibition of response in human alveolar macrophages exposed to *B. pertussis* adenylate cyclase toxin. Masure *et al.* (215) compared a mutant strain of *B. pertussis* which was unable to produce adenylate cyclase toxin with wild type *B. pertussis* cells and found that both strains were able to be internalized by macrophages but the strain producing adenylate cyclase toxin survived longer intracellularly due to inhibition of superoxide production and phagosome

lysosome fusion. Neutrophils also lose their ability to kill *Staphylococcus aureus* or *Coccidiodes immitis* when exposed to adenylate cyclase toxin due to inhibition of phagocytosis and the oxidative burst (76, 110, 252). Thus, through the action of this toxin, fewer *Bordetella* become internalized by cells of the immune system and those that do become internalized have an increased chance for survival (76, 110, 215, 252).

Goodwin *et al.* (123) and Khelef *et al.* (176) examined mutants of *B. pertussis* and suggested that adenylate cyclase toxin is required for initial growth of the bacteria in the lungs of experimentally infected mice. Lungs of mice infected with mutant strains of *B. pertussis* did not become colonized and the bacteria were cleared from the lungs within ten days (123, 176). When mice were infected with an adenylate cyclase toxin producing strain of *B. pertussis*, the lungs were colonized leading to a persistent infection lasting more than 40 days (123). These data combined with the observation that antibodies against adenylate cyclase toxin are protective, suggest that adenylate cyclase toxin is necessary for initial colonization by *Bordetella* (130, 131).

Adenylate cyclase toxin genes have been cloned, sequenced, and expressed in *E. coli* by Glaser *et al.* (117) and Brownlie *et al.* (53). The operon consists of five genes, four of which exhibit similarity to the *E. coli* α-hemolysin genes (116, 117). The structural gene has been designated *cyaA* and encodes the entire bifunctional protein. The *cyaBDE* genes encode proteins involved in secretion of the toxin (116, 117). *cyaE* is the only gene in the operon which does not exhibit similarity with the *E. coli* α-hemolysin genes (116). The *Bordetella* adenylate cyclase toxin must be post-translationally modified to be functional (28, 41). This modification is carried out by the product of the *cyaC* gene which exhibits significant similarity to the *hlyC* gene which encodes the protein responsible for this modification of the *E. coli* α-hemolysin (28, 41, 53, 117). The *hlyC* modification in *E. coli* is the transfer of a fatty acid residue from a charged acyl carrier protein to HlyA (41). There is speculation that the modification by CyaC may be the same since CyaC can activate the HlyA protein from *E. coli* (41).

Dermonecrotic Toxin

Dermonecrotic toxin is produced by all four species of *Bordetella* (54, 96, 111, 345). It was initially discovered in *B. pertussis* by Bordet and Gengou (46). The dermonecrotic toxin was purified from *B. pertussis* by Horiguchi *et al.* (153) and others (186, 242, 249, 367) as a single chain polypeptide with a molecular mass of 145 kD. The toxin has also been purified from the other *Bordetella* species and found to have a similar molecular mass: 155 kD in *B. avium*, and 190 kD in *B. bronchiseptica* and *B. parapertussis* (95, 111, 186, 367). Smaller, approximately 75 kD forms have been reported in both *B. pertussis* and *B. bronchiseptica* but are believed to be proteolytic degradation products (111). The toxin is intracellular and is released upon lysis of the bacteria (82, 367). Dermonecrotic toxin has also been called heat labile toxin since it can be inactivated by heating to 56°C (95, 367).

Dermonecrotic toxin produces non-ulcerating necrotic lesions when injected intradermally into guinea pigs and is lethal for mice when injected intraperitoneally (95, 367). Nakase *et al.* (241) have reported that dermonecrotic toxin from *B. pertussis* inhibits the sodium-potassium ATPase *in vitro*. Endoh *et al.* (93, 94) observed that dermonecrotic toxin caused contraction of smooth muscle in peripheral blood vessels and the resulting vasoconstriction increased perfusion pressure in lungs of guinea pigs. Splenic atrophy observed in mice injected intravenously with dermonecrotic toxin may be due to vasoconstriction of arterioles in the spleen (93). Horiguchi *et al.* (151) observed significant suppression of antibody response to sheep red blood cells and *E. coli* LPS by mice injected with dermonecrotic toxin. This suppression of antibody production was a direct result of failure of lymphocytes to proliferate and differentiate in the spleen since dermonecrotic toxin had no effect on antibody production by T or B cells *in vitro* (93, 151). Nagano *et al.* (239) isolated strains of *B. bronchiseptica* which were unable to produce dermonecrotic toxin and found that infection of guinea pigs with these strains

would not cause splenic atrophy and was protective against challenges with dermonecrotic toxin producing strains suggesting the possible use of dermonecrotic toxin mutants as vaccine strains.

Dermonecrotic toxin does not affect ciliary activity but does cause damage to swine nasal tissue (94, 111, 186, 239, 281). Extracts of *B. bronchiseptica* containing dermonecrotic toxin can produce turbinate atrophy similar to that observed in atrophic rhinitis when applied intranasally to piglets (135, 281). Kume *et al.* (186) observed damage to swine nasal turbinate tissue when incubated with *B. bronchiseptica* extracts *in vitro*. Atrophic rhinitis-like conditions have also been induced experimentally in rats, rabbits, and mice by extracts containing dermonecrotic toxin (177, 208, 295). Roop *et al.* (281) demonstrated a correlation between the level of dermonecrotic toxin production and the severity of turbinate atrophy in neonatal piglets infected with *B. bronchiseptica.*

The dermonecrotic toxin of *Bordetella* species is not functionally the same as that of *Pasteurella multocida* which is responsible for the most destructive and permanent form of atrophic rhinitis. *P. multocida* dermonecrotic toxin increases activity of osteoclasts, an activity not observed with the *Bordetella* dermonecrotic toxin (178). *Bordetella* dermonecrotic toxin impairs osteogenesis by causing damage to osteoblasts and osteoprogenitor cells (154). Horiguchi *et al.* (152, 154) found that cultured osteoprogenitor cells exposed to purified dermonecrotic toxin become irregular in shape with small blebs on their surfaces. The cells remained viable and continued to proliferate but at a reduced growth rate (154). These cells exhibited greatly reduced ALP activity and type I collagen levels, both of which would normally become elevated during differentiation to osteoblasts (154).

Horiguchi *et al.* (152) also found that dermonecrotic toxin stimulated DNA synthesis in osteoblasts but instead of inducing proliferation, it resulted in the production of polynucleated cells (152). Down regulation of DNA synthesis has been associated with normal differentiation of osteoblasts (152). Stimulation of DNA synthesis may prevent

this normal differentiation process. Thus, turbinate atrophy caused by *Bordetella* is due to inhibition of osteogenesis, not to osteoclastic resorption, as in *P. multocida* infection (152).

The *B. pertussis* dermonecrotic toxin gene has been cloned and sequenced by Walker and Weiss (341). The open reading frame encodes a 1,451 amino acid protein which would have a molecular mass of 159 kD. No sequences similar to *E. coli* promoter regions were found (341). However, several other *Bordetella* promoters do not resemble promoters from *E. coli* or each other, including the promoter for the *aroA* gene (213), the pertactin gene (68), and the fimbrial genes (357). The gene also does not contain the heptamer binding sequence for BvgA (283) or the 20 base pair repeats required for expression of pertussis toxin and adenylate cyclase toxin (158, 341). Dermonecrotic toxin is, however, known to be *bvg* regulated. Walker *et al.* (341) probed chromosomal DNA from other *Bordetella* species with a probe specific for the dermonecrotic toxin gene from *B. pertussis*. This probe reacted with each *Bordetella* species except *B. avium* (341). Antibodies against *B. pertussis* dermonecrotic toxin also did not cross-react with dermonecrotic toxin from *B. avium* (341). The *B. avium* dermonecrotic toxin also exhibits functional differences from the dermonecrotic toxins of the other *Bordetella* species. Mice injected subcutaneously with *B. avium* dermonecrotic toxin develop dermonecrotic lesions in 13 hours (341). Lesions develop more rapidly, usually in 3 hours, with dermonecrotic toxin from each of the other species (341). These data suggest that *B. avium* dermonecrotic toxin is genetically, antigenically, and functionally different from that of the other *Bordetella* species.

Antibodies against *B. pertussis* dermonecrotic toxin do not cross-react with *P. multocida* dermonecrotic toxin (341) and the dermonecrotic toxin gene from *B. pertussis* does not exhibit homology with the *P. multocida* dermonecrotic toxin gene (341). However, both the *B. pertussis* and *P. multocida* dermonecrotic toxin genes exhibit homology with the CNF1 and CNF2 genes from *E. coli* but not in the same regions (341).

The cytotoxic necrotizing factor (CNF) of *E. coli* causes tissue culture cells to become multinucleated and causes necrosis on rabbit skin (250). CNF1 affects the Ras related GTP binding proteins of the Rho family which regulate assembly of focal adhesion and stress fibers in eukaryotic cells (103, 250). CNF2 mediates actin polymerization through modification of Rho proteins. The nature of this modification of G proteins is not known but it is not ADP-ribosylation or phosphorylation (250). CNF2 also causes an increase in DNA synthesis in quiescent cells. These effects (stimulation of DNA synthesis, multinucleation of cells, necrosis on animal skin) have all been observed with both the *P. multocida* and *B. bronchiseptica* dermonecrotic toxins (95, 152, 154, 178, 250). These similarities in function combined with the observed structural similarities would suggest that the dermonecrotic toxin of *Bordetella* may be related to the CNF proteins of *E. coli*. Other bacterial toxins, including Staphylococcal epidermal cell differentiation inhibitor EDIN and the botulinum C3 toxin, have also been found to be members of this CNF-like dermonecrosis inducing toxin family (156, 163, 321, 341).

Osteotoxin

The osteotoxin was originally identified in *B. avium* by Gentry-Weeks (113). Western blots using polyclonal antibodies against the osteotoxin showed a band of similar size in all *Bordetella* species. The osteotoxin exists as a dimer of two 41 kD subunit proteins containing one molecule of pyridoxal 5' -phosphate each (113). Gentry-Weeks *et al.* (113) showed that extracts of osteotoxin from *B. avium* cause MC3T3-E1 osteogenic cells in tissue culture to change from a normal fibroblastic morphology to a spherical morphology. The cells also detached from the plate and produced clear blebs at the cell surface. These cells were determined to be dead by Trypan blue exclusion experiments. Similar effects were observed on fetal bovine trabecular cells, osteosarcoma cells, and embryonic bovine tracheal cells (113). In *Bordetella* infections, there is progressive

degeneration and deterioration in bone-forming osteoblast cells. This damage can be attributed to the effects of dermonecrotic toxin and osteotoxin (113, 152, 154).

The gene encoding osteotoxin from *B. avium* has been cloned, sequenced, and expressed in *E. coli* by Gentry-Weeks (113). This gene exhibits a 38% identity and 60% similarity with the genes for pyridoxal 5' -phosphate dependent β-cystathionases from *E. coli*, *Salmonella typhimurium*, and rat liver. The osteotoxin gene has been designated *metC* in accordance with the corresponding genes from *E. coli* and *S. typhimurium* (113). The enzyme β-cystathionase is involved in converting L-cystathionine to L-homocysteine, ammonia, and pyruvic acid (104, 105). The purified osteotoxin from *B. avium* has been found to catalyze this reaction in *in vitro* tests (113). The osteotoxin gene does not contain the Met box regulatory sequence found in β-cystathionase genes from other organisms (113). The Met box is involved in repression of β-cystathionase production in the presence of excess methionine (31, 356). The lack of a Met box in the *Bordetella* osteotoxin gene indicates that the gene is expressed constitutively and at a high level (113).

Osteotoxin can be isolated from culture supernatant of *B. avium* and therefore, it may be secreted from the cells (113). However, the osteotoxin gene does not contain a signal sequence which is usually necessary for secretion from the cell. The gene also does not encode the amino acid sequence or carboxyl terminal secondary structures necessary for secretion by alternate pathways. Therefore, it is possible that the osteotoxin remains inside the cell and is released upon cell death and lysis (113). Due to the potentially high density of bacteria in the trachea of infected animals, a significant amount of β-cystathionase may be released into the environment at the site of infection (113).

Tracheal Cytotoxin

Tracheal cytotoxin is a soluble monomer of peptidoglycan released from actively growing cells of all *Bordetella* species (111, 121, 77, 120). Goldman and Cookson

determined the chemical composition to be N-acetylglucosaminyl- 1, 6-anhydro N-acetylmuramylalanyl- γ- glutamyldiaminopimelyl-alanine produced by cleavage of intact peptidoglycan, not by release of precursors (120, 282). Rosenthal *et al.* (282) have reported that greater than 95% of the soluble peptidoglycan fragments released from *B. pertussis* are tracheal cytotoxin indicating a specific cleavage and release rather than a generalized breakdown of cell wall components due to a turnover process. The molecule has a molecular weight of 921 Daltons (79, 123, 142) and the tracheal cytotoxin of *Bordetella* is identical to a peptidoglycan monomer released by *Neisseria gonorrheae* which is responsible for destruction of ciliated cells in the Fallopian tubes (106).

Muramyl peptides have several biological activities including adjuvanticity, arthritogenicity, and somnogenicity (1, 70, 185). The pertussis vaccine is known to serve as a good adjuvant; Goldman and Cookson propose that part of this effect is due to tracheal cytotoxin (120). In addition, Goldman *et al.* (120) report that the tracheal cytotoxin is identical to the human sleep promoting factor FSu. Drowsiness is also reported as a common side effect of the whole cell pertussis vaccine (74). Antibodies against tracheal cytotoxin are difficult to produce. There are two reasons for this rarity of antibodies. First, the tracheal cytotoxin is identical to the human sleep promoting factor FSu, and similar molecules may exist in animals (120). Second, since tracheal cytotoxin is a component of bacterial cell walls, animals may become tolerant due to the presence of normal flora.

Goldman *et al.* (119, 121) reported that in the presence of the *Bordetella* tracheal cytotoxin, ciliary activity was decreased in hamster tracheal ring cultures and ciliated cells lost their normal elongated shape becoming rounded with a constriction of their apical end. In addition, mitochondria became swollen and exhibited decreased amounts of cristae (121). Intercellular junctions also deteriorated leading to extrusion of the cells (79). This loss of ciliated cells caused accumulation of mucus, bacteria, and foreign particles which may lead to coughing and increased susceptibility to secondary infections

(119, 120). Besides causing loss of ciliated cells, Cookson *et al.* (79) observed that the tracheal cytotoxin inhibited DNA synthesis in epithelial cells. Therefore, division and differentiation of the basal cell population was delayed, preventing replacement of the ciliated cells extruded due to the action of the tracheal cytotoxin (79). Treatment of tracheal rings in culture with *B. pertussis* tracheal cytotoxin led to pathological changes similar to those seen in pertussis (79)

Tracheal cytotoxin competed with serotonin for binding sites on hamster tracheal epithelial cells. Tracheal cytotoxin binds to this site with low affinity and it is not known if this is the only tracheal cytotoxin binding site present. The mechanism of action of tracheal cytotoxin is believed to be through production of IL-1α by respiratory epithelial cells (141, 142, 143). Muramyl peptides stimulate IL-1 production by monocytes and the sleep promoting effects of muramyl peptides are caused by increases in IL-1(141, 142, 143). Heiss *et al.* (142) observed that hamster tracheal epithelial cells in culture will produce IL-1 when exposed to tracheal cytotoxin from *B. pertussis*. She also reported that the effects of tracheal cytotoxin can be reproduced by IL-1 alone (142). IL-1 induces production of nitric oxide and the effects of tracheal cytotoxin may actually be caused by this nitric oxide production (141, 143). Nitric oxide synthase inhibitors block the effects of tracheal cytotoxin (141).

In addition to being toxic to ciliated respiratory epithelial cells, tracheal cytotoxin is toxic for neutrophils when present at a concentration of 10^{-6} M (85). At low concentrations, Cundell *et al.* (85) report that tracheal cytotoxin did not inhibit chemotaxis or chemiluminescence by neutrophils. It also does not induce IL-1 production by neutrophils or activate complement (85). However, high concentrations of tracheal cytotoxin are produced by *B. pertussis* in culture (up to 10^{-6} M) and it is possible that even higher concentrations of tracheal cytotoxin may be present in the microenvironment around respiratory epithelial cells which would indicate that killing of neutrophils may be occurring *in vivo* (79, 85).

Adhesins

Filamentous Hemagglutinin (FHA)

Filamentous hemagglutinin is a high molecular weight protein which has been demonstrated in all *Bordetella* species except *B. avium* (164, 248). FHA has been purified by Arai (10), Skelton (310), and Selmer (305) by different methods including affinity chromotography with monoclonal antibodies and filtration through butyl Sepharose 4B. Purified FHA from *B. pertussis* produces several bands on SDS-polyacrylamide gels ranging in size from 58,000 to 220,000 Daltons (8, 164, 206). The smaller forms are believed to be proteolytic degradation products since antibodies which recognize these forms also bind to the larger forms (305, 164).

B. bronchiseptica produces a hemagglutinin with a similar range of sizes on SDS-PAGE. Ohgitani *et al.* (248) purified a 150 kD protein from *B. bronchiseptica* which they report to be the intact FHA; larger forms are believed to be precursor molecules since the structural gene for FHA encodes a 367 kD protein which is posttranslationally modified to the mature form, which is 220 kD in *B. pertussis* (88). *B. bronchiseptica* mutants deficient only in the production of this 150 kD protein lose the ability to agglutinate bovine red blood cells (248). Agglutination of bovine red blood cells has been associated with virulence of *B. bronchiseptica* for pigs. Arp *et al.* (15) have reported that mutants of B. *avium.* which have lost hemagglutinating activity adhere less well to tracheal epithelial cells. This evidence suggests that *B. avium* may produce FHA or a similar protein which has not been detectable using *B. pertussis* antibodies. The presence of this hemagglutinating ability in *B. avium* has been associated with virulence (15).

FHA is a filamentous structure originally believed to be fimbrial in nature (8, 10). Filaments have a length between 40 and 100 nm and a diameter of approximately 2 nm (10, 248). FHA is secreted from the cell but remains associated with the outer membrane

and is a major attachment factor of *Bordetella* (289, 346). Tuomanen *et al.* (332) studied attachment of *B. pertussis* FHA mutants to human ciliated respiratory epithelial cells. Leininger *et al.* (194) examined the ability of epithelial cells, CHO cells, and HeLa cells to bind to purified FHA. They found that FHA could promote binding of each of these cell types and mutant *B. pertussis* cells deficient in the production of FHA could not attach as well as wild type cells (194, 332). Kimura *et al.* (179) also observed that mutant *B. pertussis* strains deficient in FHA could not colonize the tracheas of mice but could still colonize the lungs.

Since FHA is not membrane bound, it is possible that it may serve as a mediator of attachment for other bacteria present in the respiratory tract. Tuomanen has shown that *Streptococcus pneumoniae, Haemophilus influenzae,* and *Staphylococcus aureus* can adhere to cilia when FHA is present (289, 330). She has termed this phenomenon "piracy of adhesins" (330). Both *Pasteurella multocida* and *B. bronchiseptica* are usually isolated from pigs with atrophic rhinitis and research has shown that colonization by *B. bronchiseptica* enhances the likelihood of the animal becoming infected with *P. multocida* (21, 37, 68). This aid in colonization by *P. multocida* may be due to the use of FHA as an adhesin by the *Pasteurella.*

The protein contains three domains involved in adhesion of *Bordetella* to eukaryotic cells: the arg-gly-asp (RGD) sequence (194), a carbohydrate binding site (271), and a heparin binding site (224). The RGD sequence is found on the extracellular matrix proteins fibronectin, laminin, and collagen and is recognized by integrin receptors (257, 261, 265). Leininger *et al.* (194) report that the RGD sequence of FHA binds to the integrin CR3 on macrophages. Binding to CR3 promotes phagocytosis by the macrophage (194). Since *Bordetella* can inhibit phagosome-lysosome fusion in neutrophils and the respiratory burst in macrophages, *Bordetella* may survive intracellularly (76, 110, 215, 252). Therefore, promotion of phagocytosis by FHA may create a reservoir of internalized *Bordetella.*

The RGD sequence, however, is not involved in binding of *Bordetella* to ciliated cells. Such attachment is mediated by the carbohydrate binding domain (271). Pre-incubation of *Bordetella* with galactose or carbohydrates containing galactose in a β1-4 linkage can competitively inhibit binding to ciliated cells (269). Tuomanen *et al.* (331) used thin layer chromatography to demonstrate binding of FHA to lactose containing glycolipids. Binding to macrophages can occur through the carbohydrate binding domain as well but such binding does not induce internalization (269).

Menozzi *et al.* (224, 225) report the presence of a heparin binding site on FHA. Heparin and heparin sulfate are common components of the extracellular matrix and heparin sulfate proteoglycans are present on the surface of most animal cells (363). Other organisms, including *Helicobacter pylori*, *Streptococcus mutans*, and *Staphylococcus aureus*, bind to sulfated polysaccharide receptors (16, 72, 201). Menozzi *et al.* (225) suggest that *Bordetella* bind first to heparin sulfate proteoglycans with low affinity and then this binding helps bring the cells into contact with other high affinity receptors on the cell surface.

FHA was previously known as fimbrial hemagglutinin (8, 10). Although now known not to be fimbrial, FHA is closely related to fimbriae. FHA is encoded by a five gene operon consisting of *fhaB, A, C, D, E* (204, 319). Locht and Willems have observed significant homology between three of these genes and fimbrial genes and have renamed these genes as fimbrial genes (204, 359, 360). *fhaA*, now known as *fimC*, is involved in regulation of synthesis and export and the protein it encodes has 26.5% amino acid identity with *Escherichia coli* PapC which is an anchor protein for *E. coli* pyelonephritis associated pili (pap, 204, 359, 360). FhaD, now known as FimB, exhibits 33.2% amino acid identity with *E. coli* PapD, which is a chaperonin protein and *fhaE*, now called *fimD*, is homologous to the *Bordetella* fimbrial genes, *fim2, fim3,* and *fimX* (204, 359, 360). *fimD* is believed to encode a minor fimbrial subunit (204, 358, 359). Mutations in *fimC* or *fimD* prevent expression of both FHA and fimbriae by *Bordetella* (204, 359, 360). The

presence of these fimbrial genes within the FHA operon and the linkage between a minor fimbrial subunit gene and expression of FHA suggests a close relationship between FHA and fimbriae. The other two genes in this operon do not show significant homology with fimbrial genes. *fhaB* is the structural gene for FHA and *fhaC* is involved in export (88, 268, 269, 318, 359). The FHA genes have been cloned and sequenced and *fhaB* has been expressed in *E. coli* under the *lac* promoter and using a two cistron system (51, 132, 133, 204, 360).

FHA is highly immunogenic and the presence of antibodies against FHA is protective against respiratory challenge in mouse models (179). Guzman *et al.* (134) immunized mice orally with an *E. coli* strain expressing FHA from *B. pertussis*. This form of immunization induced production of both systemic IgG and mucosal IgA antibodies against FHA (134). Antibodies against FHA have been reported by Sato *et al.* (292) to prevent the attachment of *B. pertussis* to HeLa and Vero cells in culture. Kimura *et al.* (179) demonstrated that antibodies against FHA are protective in adult mice against aerosol infection with *B. pertussis*. Previous investigators reported that passive immunization with antibodies against FHA does not protect mice from challenge with *B. pertussis* (236, 247).

Pertactin

Pertactin is an outer membrane protein involved in adhesion by all *Bordetella* species (50, 183, 197, 231). The 68 kD protein was first identified in *B. bronchiseptica* as a protective antigen for pigs (231). Similar proteins have since been found in the other species of *Bordetella* (50, 197). Pertactin from *B. pertussis* exhibits a relative molecular mass of 69 kD on SDS-PAGE, while that from *B. parapertussis* is 70 kD (50, 197). Brennan *et al.* (49) reported that the protein pertactin was recognized by the U. S. reference factor 3. Reference Factor 3, however, also contains antibodies against serotype 6 fimbriae; so, agglutinogen 3 is not purely pertactin or fimbriae(275).

Gotto *et al.* (128) determined by mass spectrometry that pertactin actually exists as a 60.28 kD protein which appears to be larger on SDS-PAGE due to its high proline content. Charles *et al.* (68) determined that the protein is produced as a 93 kD precursor which is posttranslationally modified to the mature form. The precursor protein possesses two RGD sequences but the carboxyl-terminal RGD sequence is not present in the mature protein (195). The RGD sequence is involved in binding of several mammalian proteins to cells including fibronectin, vitronectin, fibrinogen, and von Willebrand factor (257, 261, 265). As previously indicated, the RGD sequence is also important in adhesion by FHA (194). Leininger *et al.* (195) reported that pertactin was involved in binding of *B. pertussis* to CHO cells and that this interaction is at the RGD site. The pertactin protein also contains an NAD binding site (67, 197). This site is homologous to the NAD binding site of fragment A of diphtheria toxin (67, 197).The significance of the NAD binding site in attachment is not known since NAD binding by pertactin has not been observed and pertactin from *B. parapertussis* does not contain this site but exhibits no difference in function (197).

Pertactin was shown to be a protective antigen in mice and pigs (50, 183, 275). Brennan *et al.* (50) passively transfered antibodies against pertactin to protect mice from respiratory challenge with *B. pertussis*. Kobisch and Novotny compared the abilities of wild type and pertactin negative mutants of *B. bronchiseptica* to protect piglets when used as a vaccine (183). They found that mutants unable to produce pertactin did not induce production of protective antibodies (183). Monoclonal antibodies specific for pertactin from *B. bronchiseptica* prevented development of atrophic rhinitis in experimentally infected piglets (183).

The gene for pertactin has been cloned from *B. pertussis* and *B. parapertussis* and designated *prn* (67, 68, 197). The *B. pertussis* pertactin gene has a much higher G+C content (70.8%) than other *Bordetella* virulence genes (67). The pertussis toxin gene has a G+C content of 62.2%; the serotype 2 fimbrial gene has a G+C content of 61.5%, and

the adenylate cyclase toxin gene has a G+C content of 66.6% (67). The G+C content of the *prn* gene is similar to that of "housekeeping" genes in *Bordetella* such as the *aroA* gene (71.3%, 67). These data suggest that the *prn* gene evolved differently from the other *Bordetella bvg* regulated genes (67). The promoter of this gene does not resemble *E. coli* promoters but does show similarity with the pertussis toxin promoter (67, 68). The pertactin gene from *B. parapertussis* has 93% homology with the *prn* gene of *B. pertussis* (197). Li and Charles have expressed the *prn* gene of *B. pertussis* and *B. parapertussis* respectively, in *E. coli* on expression vectors but not under its own promoter (68, 197). The *B. pertussis* gene has also been expressed in a baculovirus insect cell expression system (69).

Fimbriae

Fimbriae are produced by all the *Bordetella* species but their role in attachment is still uncertain (83, 166, 193, 365, 366). Figure 1 shows an electron micrograph of fimbriae on the surface of *B. bronchiseptica*. Fimbriae of *B. pertussis* have been classified into two serotypes based on agglutination with standard typing sera (18). In the Eldering serotyping scheme, agglutinogens 2 and 6 correspond to fimbriae. However, agglutinogen 3, which contains pertactin and lipooligosaccharide, also contains serotype 6 fimbriae (275). The Preston serotyping scheme designates Eldering serotype 6 fimbriae as serotype 3 (49). Therefore, these fimbriae are often referred to as serotype 3/6. The U. S. reference sera, however, cannot be used for typing *B. bronchiseptica* since the reference factors have been absorbed with *B. bronchiseptica* strains (18, 200). Other antibodies which react identically to the reference sera do cross react with *B. bronchiseptica* and *B. avium* strains indicating that at least two fimbrial types exist in these species (200). Due to the activity of these antibodies, references to serotypes in *B. bronchiseptica* are becoming common.

In *B. pertussis*, fimbriae are composed of subunits of 22.5 kD for serotype 2 and 22 kD for serotype 3/6 (83, 233, 365, 366). In *B. bronchiseptica*, Lee *et al.* (193) have

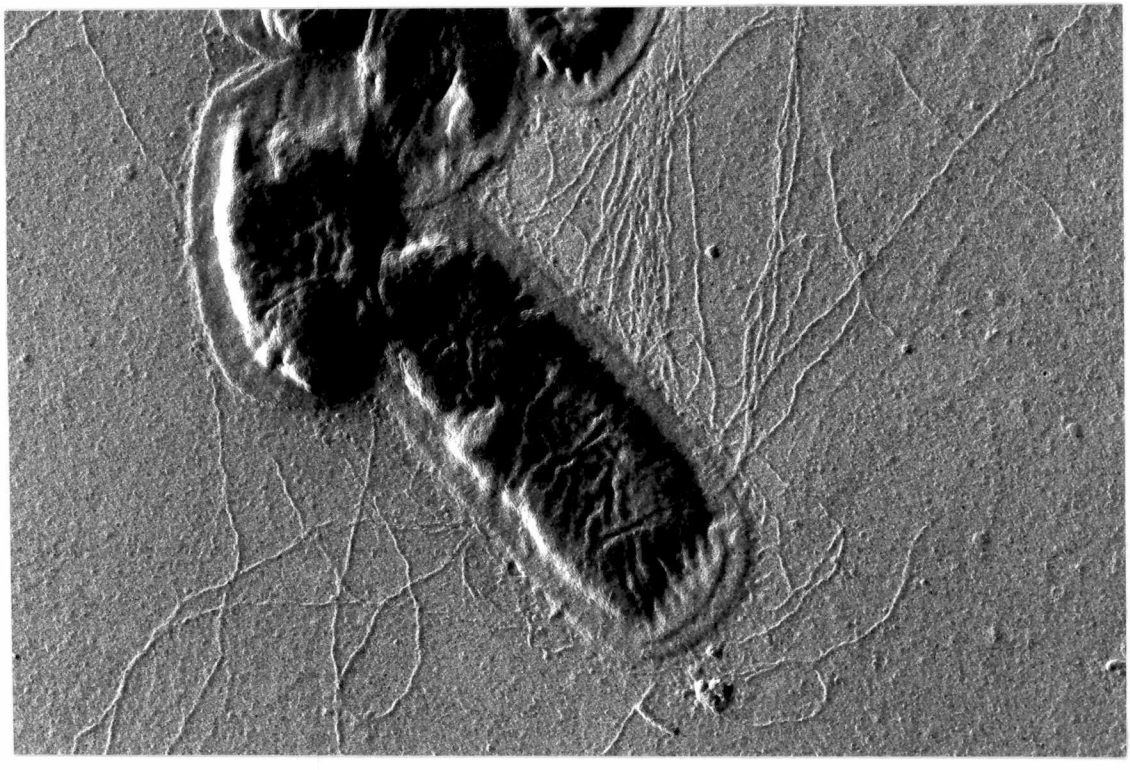

Figure 1: Fimbriae of *Bordetella bronchiseptica*.* *B. bronchiseptica* cells were shadow-casted and examined at a magnification of 34,000x.

* Source:
Bemis, David A., and Eugene H. Burns, Jr. 1993. *Bordetella.* in C. L. Gyles and C. O. Theon (ed.). *Pathogenesis of Bacterial Infections in Animals*, second edition. Iowa State University Press, Ames, Iowa. p. 201-215.Used by permission.

identified three proteins of molecular weights 21.5, 22, and 24 kD as being fimbrial subunits and in *B. avium*, three proteins of 14.4, 17, and 24 kD have been identified as fimbrial proteins (166). It is not known if these three proteins each represent a distinct fimbrial type or if they are subunits of one type of fimbriae or degredation products.

Fimbriae can be detached from the cell surface by mechanical shearing and using preparations of fimbriae enriched in this manner, Steven *et al.* (315) observed that fimbrial filaments formed paracrystalline bundles. Optical diffraction analysis showed the structure of *B. pertussis* fimbriae to be a helical repeat of fimbrial monomers (315). The fimbrial filaments are 4 nm in diameter and variable in length (17). Monoclonal antibody labeling of fimbriae has shown that cells can possess both serotype 2 and serotype 3/6 fimbriae (17, 107, 200). Pearce *et al.* (251) and Robinson *et al.* (278) have denatured fimbriae using guanidium hydrochloride. Fimbriae denatured in this manner do not reassociate into filaments spontaneously. These data suggest that accessory proteins are necessary for the proper folding and assembly of the fimbrial filaments (251, 278).

Fimbriae are involved in attachment of many bacteria to host tissue. However, the role of fimbriae in the pathogenesis of bordetelloses is not certain. Fimbriae negative *B. pertussis* mutants adhered to cilia and to macrophages as well as wild type strains (270). These mutants also colonized the lungs of experimentally infected mice (234). Monoclonal antibodies against agglutinogen 2, serotype 2 fimbriae, inhibited attachment of *B. pertussis* to Vero cells (127). Fimbriae have also been implicated in attachment by *B. bronchiseptica* (90, 234, 358). The role of fimbriae in attachment by *Bordetella* will be discussed in more detail later in this chapter.

Despite the uncertainty as to whether fimbriae mediate attachment, fimbriae have been identified as protective antigens in mice (276, 277, 365). Robinson *et al.* (276) immunized mice with purified fimbriae of one serotype and then challenged intranasally with *B. pertussis* strains of the same and different serotypes. They found that strains possessing fimbriae of a particular serotype could protect against lung colonization by

strains of that serotype but did not provide cross-protection against other serotypes (276). Such specificity is interesting since fimbriae are similar physiologically and genetically and some antibodies against serotype 3/6 fimbriae cross-react with serotype 2 fimbriae (200). Agglutinogens, including fimbriae, are important protective antigens in many of the current component and acellular pertussis vaccines including the Takeda vaccine in use in Japan and the United States (75). There is also a vaccine using *B. bronchiseptica* fimbrial subunits to induce protection against *B. pertussis* infections in humans (324).

Seven fimbrial genes have been identified in *B. pertussis* and are designated *fim2, fim3, fimX*, and *fimABCD* (202, 204, 253, 358, 359, 360). Each is present as a single copy gene in *B. pertussis* (233, 358, 359). The structural genes for the major fimbrial subunits are *fim2* and*fim3*. (202, 232). An additional gene with sequence similarity to the major fimbrial subunit genes (*fim2* and *fim3*) has been identified and designated *fimX* (253). *fimX* is not expressed in *B. pertussis* but Savelkoul has identified a 22 kD protein expressed from the *fimX* gene in *B. bronchiseptica* (294). In *B. bronchiseptica* and *B. parapertussis*, Mooi *et al.* (233) identified five to six *SalI* fragments of genomic DNA which hybridize with an oligonucleotide probe specific for the 5' end of *fim2* indicating that the fimbrial structural genes may be present in multiple copies in these species. Interestingly, this probe did not hybridize with genomic DNA from *B. avium* despite the fact that antibodies against serotype 2 fimbriae do react on Western blots (233). It is not known if each copy of the fimbrial genes is expressed or if there are several silent genes in these species. Other silent genes have been identified including the gene for pertussis toxin in *B. bronchiseptica* and *B. parapertussis* and the gene for flagella in *B. pertussis* (3, 12). One copy of each of the *B. bronchiseptica fim2, fim3*, and *fimX* genes has been cloned and sequenced by Savelkoul (294).

Walker *et al.* (342) expressed the cloned *B. pertussis* serotype 2 fimbrial subunit gene (*fim2*) in *B. bronchiseptica* and *B. parapertussis* under its own promoters and under *E. coli* promoters. These recombinant fimbrial subunits were assembled into filaments and

expressed on the cell surface as determined by ELISA and immuno-electron microscopy (342). The *B. pertussis* serotype 2 fimbrial subunit gene has also been expressed in *E. coli* under the λ P_L and P_R promoters (343).

The other fimbrial genes, *fimABCD*, are present as part of the FHA operon in *B. pertussis*, *B. bronchiseptica*, and *B. parapertussis*. Probes for these genes do not hybridize with DNA from *B. avium* (204, 360). These genes have been cloned and sequenced by Locht *et al.* (204) and Willems *et al.* (360). *fimA* is located immediately downstream from *fhaB*, the structural gene for FHA and exhibits significant homology with *fim2*, *fim3*, and *fimX* (204, 360). This gene has been cloned and sequenced and is similar in sequence to the major fimbrial subunit genes *fim2* and *fim3* with a deletion of the first 160 bases (204, 360). This gene is not expressed in any *Bordetella* species and is believed to be a pseudogene (204, 360).

Downstream from *fimA* is a putative BvgA binding site followed by the *fimB* gene (204, 360). BvgA is a regulator of several virulence related proteins in *Bordetella.* The *fimB* gene encodes a 24 kD protein which has significant amino acid identity with *E. coli* PapD (204, 360). PapD is a chaperonin protein involved in transport of pap pilin subunits (fimbrial subunits) through the periplasmic space (159). It is believed, therefore, that FimB serves the same purpose in *Bordetella* (204, 360). Strains with mutation in the *fimB* gene do not produce serotype 2 or serotype 3 fimbrial filaments as occurs in other species when fimbrial chaperonins are mutated (359).

Immediately downstream of the *fimB* gene is the *fimC* gene (204, 360). *fimC* encodes a 92 kD protein with similarity to *E. coli* outer membrane proteins involved in export of fimbrial subunits. It has 33.2% amino acid identity with the *E. coli* protein PapC (204, 360). PapC is involved in translocation of *E. coli* pap pili (fimbriae) subunits across the outer membrane (159). *Bordetella* mutants in which *fimC* has been inactivated by insertion of Tn5 did not produce FHA, serotype 2, or serotype 3 fimbriae (358). Mutation of the next gene in the operon, *fimD*, had the same effect (358). *fimD* encodes a 40 kD

protein which may be a minor fimbrial adhesin protein (358). This gene exhibits significant similarity with the *MrkD* gene of *Klebsiella pneumoniae* which encodes a fimbrial adhesin protein that binds to type V collagen (155, 204, 359, 360). Studies are currently underway to determine if FimD will also bind to collagen (358). The *fimD* gene of *B. bronchiseptica* differs from that of *B. pertussis* by only one base which would not lead to any changes in the amino acid sequence of the protein (204, 358, 360). The *fimD* gene of *B. parapertussis* differs by 34 bases which would predictably result in atein with 20 different amino acids (358). Probes for *fimD* did not hybridize with chromosomal DNA from *B. avium* (358). Mutations in *fimB* prevented expression of *fimD* which is necessary for production of FHA and fimbriae (358). Therefore, *fimB, fimC,* or *fimD* mutants do not produce fimbrial filaments.

Other Factors

Bordetella produce several other compounds that could be considered virulence factors. One of these factors is lipopolysaccharide (LPS). *Bordetella* LPS has biological activities similar to LPS from *Enterobacteriaciae*; it is lethal for galactosamine-sensitized mice, pyrogenic, mitogenic, and causes macrophage activation, and induction of tumor necrosis factor production (6, 347). There have been reports that LPS composition in *Bordetella* may be different in avirulent and virulent phase (57), and that monoclonal antibodies against lipooligosaccharide A of *B. pertussis* reduce colonization of the respiratory tract of mice experimentally infected with *B. pertussis* (6).

Bordetella also produce several outer membrane proteins which could prove to play a role in virulence. One of these is a 40 kD porin protein which has a gene sequence similar to the GroEL proteins found in other species (55, 137, 174, 184). There is also a suggestion that these porin proteins undergo qualitative changes between phases (55, 137, 174). The recently described hydroxamate siderophore, Alcaligin (originally called

Bordetellin), and the binding proteins for lactoferrin and transferrin may have a number of effects on virulence which have not been identified (1a).

Regulation

Regulation of *Bordetella* virulence factors occurs through the Bvg (*Bordetella* virulence gene) regulatory system, formerly called vir. This two component regulatory system, similar to two component systems observed in other bacteria (13), regulates expression of all the virulence factors described above except tracheal cytotoxin which is produced constitutively. The other virulence factors are produced only in the virulent phase (78, 129, 296, 297, 351). Switching between the two phases occurs spontaneously in all *Bordetella* species (3, 112). Miller *et al.* (227)report that avirulent phase variants can be isolated from a *Bordetella* culture at a rate of 10^{-3} to 10^{-6}. Mice can be inoculated with up to 10^{7} avirulent phase *B. pertussis* without being killed, whereas, inoculation with only 200 virulent phase *B. pertussis* is lethal (349). The avirulent phase cells also grow at a faster rate, produce larger colonies than virulent phase cells (254, 255), and produce several proteins not produced by virulent phase cells (3, 102). These proteins include the flagella of *B. bronchiseptica* (3).

Switching between virulent and avirulent phase occurs by two mechanisms which have been designated phase variation and modulation. Phase variation occurs as a result of a mutation in the *bvgS* gene (217, 229, 318). Monack *et al.* (229) report that phase variation in *B. bronchiseptica* can occur through the deletion of 50 to 500 base pairs of the *bvgS* gene. They found that this mechanism of phase variation occurred in six out of the fifteen avirulent phase variants they examined (229). The same phenomenon has been observed in other strains of *B. bronchiseptica* by Arico *et al.* (14). Phase variation through deletion is not reversible (229). Phase variation by deletion has not been observed in *B. pertussis*.

The mechanism of phase variation observed in *B. pertussis* and each of the other *Bordetella* species is a frameshift mutation in the *bvgS* gene (318, 226). Stibitz *et al.* (318) observed that avirulent phase strains of *B. pertussis* have a string of seven guanines in the *bvgS* gene sequence; virulent phase strains only have six guanines in this site. This frameshift mutation causes a change from virulent to avirulent phase (227, 318). The mechanism of insertion of the guanine residue at this specific site is not known. This form of phase variation is reversible at a low frequency (227, 318).

The other mechanism of phase switching is called modulation and occurs in response to environmental changes (188, 222, 223, 298). Melton *et al.* (222, 223) have characterized some of the compounds which can act as modulators in *Bordetella*. She found that organic acids must have a benzene or pyridine ring and a carboxyl group to serve as modulators (223). Nicotinic acid is one such modulator (222, 223). The presence of inorganic ions such as sulfate and chlorate can also cause modulation (188, 298). Modulation can also occur in response to growth at low temperature (28°C, 188). In the presence of these modulating conditions, *Bordetella* switch from virulent phase to avirulent phase (188). When the modulator is no longer present, *Bordetella* revert back to virulent phase (188). Melton *et al.* (223) found that modulation could be inhibited by the addition of chlorine ions to the medium. The importance of modulation in pathogenesis has not been determined. However, there are reports that *Bordetella* switch to avirulent phase when internalized by macrophages or after invading HeLa cells under laboratory conditions (214). These data suggest that the virulent phase may be necessary for initial infection and colonization but the avirulent phase may be responsible for persistence of intracellular *Bordetella.*

Modulation occurs through the action of the Bvg regulatory system. Knapp *et al.* (182) identified two genes involved in regulation of modulation by TnPhoA insertion and designated these genes *vir* and *mod.* These genes were cloned and sequenced by Arico *et al* (13). They reported that there were actually three genes involved which they

designated *bvgABC* (13). Scarlato *et al.* (297) later reported that a sequencing error had been made by Arico *et al.* (13) introducing an extra C in position 1583; the corrected sequence indicates that there are only two genes present which are now referred to as *bvgA* and *bvgS*. These genes encode a 23 kD cytoplasmic protein (BvgA) and a 135 kD inner membrane spanning protein with domains in both the periplasmic space and the cytoplasm (316).

The *bvgAS* genes are located 427 base pairs upstream of the *fhaB* gene and are transcribed in the opposite direction of *fhaB* (297). Scarlato *et al.* (297) found four promoters upstream of the *bvgA* gene. The promoters P_1 and P_3 are bvg regulated (283, 297). These promoters, therefore, are only active in virulent phase cells (283, 297). The P_2 promoter is constitutively active (297). Therefore, BvgA and BvgS are produced in all *Bordetella* but are produced at higher levels in virulent phase cells (283, 297). Promoter P_4 regulates the production of an antisense RNA whose function is unknown (297). This promoter is bvg regulated indicating that it is active only in virulent phase cells (297).

The *bvgAS* genes exhibit 95.5% DNA sequence identity between *Bordetella* species (298). Scarlato *et al.* (298) found that there are only three amino acid differences between the BvgS proteins of *B. bronchiseptica* and *B. pertussis* and there are three different amino acid changes between *B. pertussis* and *B. parapertussis* indicating that the *bvg* genes and proteins are highly conserved among *Bordetella* species. They also observed that these proteins are functionally identical and interchangeable between *Bordetella* species (298). Utsumi *et al.* (336, 337) recently discovered two *E. coli* genes with significant similarity to the *bvgAS* genes of *B. pertussis*. These genes were designated *evgA* and *evgS* and found to down regulate expression of the OmpC outer membrane protein of *E. coli* in response to the same modulators as *Bordetella* (336, 337).

The BvgS protein contains two domains, a periplasmic environmental sensor and a cytoplasmic histidine kinase (335). Under non-modulating conditions, the BvgS protein, which exists as a dimer in the inner membrane, phosphorylates BvgA (335, 344, 223).

The phosphorylated form of BvgA binds to and activates the P_1, P_2, and P_4 promoters of *bvgAS* resulting in increased expression of BvgAS and the antisense RNA (283, 284, 297, 335). Phosphorylated BvgA also binds to the promoters for certain other bvg regulated genes including *fhaB* and *fimB* (283, 296, 299, 357, 360) causing an increase in expression of FHA and FimB whose expression has been shown to up regulate transcription from fimbrial subunit genes (360).

In addition, the production of phosphorylated BvgA causes an increase in expression of a 23 kD cytoplasmic protein identified by Huh and Weiss and designated Act (158). Huh and Weiss used Southwestern blotting to discover that the Act protein binds to the promoters for pertussis toxin and adenylate cyclase toxin (158). A genetic locus affecting expression of pertussis toxin and adenylate cyclase toxin has been identified by Carbonetti *et al.* (58) and they speculate that the gene product of this locus, which may be Act, may be a histone-like protein which would influence DNA topology. Such proteins have been found in *Salmonella* and *E. coli* and have regulatory effects on several genes in these organisms (89, 147, 217). In support of this theory, Scarlato *et al.* (300) found that changes in DNA topology do effect transcription from the pertussis toxin genes.

The presence of phosphorylated BvgA also increases expression of a 34 kD cytoplasmic DNA binding protein (30). This protein binds to the promoters of the Bvg repressed genes, which are still commonly referred to as vrg's (vir-repressed genes) even though the designation *vir* is no longer used for the*bvg* regulatory system. Binding by this protein prevents the transcription of the vrg's (30). The function of most vrg's is not known but Finn *et al.* (102) made mutations in several vrg's which prevented their expression in the avirulent phase. They found that strains of *B. pertussis* with a mutation in vrg-6 were unable to colonize the lungs of mice indicating that some vrg's may be necessary for virulence (102). Akerley *et al.* (3) have shown that expression of flagella by *B. bronchiseptica* occurs only in the avirulent phase and that mutation of the *bvgS* gene to

an inactive form does cause constitutive expression of flagella. Therefore, flagella appear to be bvg-repressed in *B. bronchiseptica* (3).

Stibitz *et al.* (316) identified a putative BvgA binding site in the *fhaB* and *bvgAS* promoters. *fhaB* contains a 14 base pair inverted repeat containing the sequences TTTCCTA and TTTCTTA (316). The *bvgAS* promoters contain a 14 base pair direct repeat containing the sequences TTTCCTA and TTTGGTA (316). These sites were protected by BvgA in DNAse I protection assays (316). Beattie *et al.* (30) have identified a consensus binding sequence for the repressor protein involved in preventing expression of vrg's in the virulent phase. This consensus sequence occurs in the coding region of the vrg's and no upstream regions are necessary for bvg regulation (30). The binding site for Act has not been identified. An additional transcriptional regulator has been identified by Bannan *et al.* (22). This protein exhibits 32.9% identity with the *E. coli* transcriptional regulator FNR and has been designated Btr (*Bordetella* transcriptional regulator, 22). The function of Btr has not been determined.

Under modulating conditions, BvgA is not phosphorylated (223, 335, 344), additional transcriptional enhancement of the *bvgAS* genes does not occur (283, 284, 297, 335), and the Act protein (158), Bvg-regulated virulence factors (296, 299, 357, 360), and the repressor are not produced (30). The vir repressed genes (vrg's), such as flagella in *B. bronchiseptica*, do become activated (3). Regulation of virulence factors is diagrammed in Figure 2.

Fimbriae and Attachment

Fimbriae are involved in attachment in many other species and there is evidence for their involvement in attachment by *B. bronchiseptica* and *B. avium* (90, 364). As with most *Bordetella* virulence factors, the majority of studies performed have used *B. pertussis*. Research has indicated that in *B. pertussis*, fimbriae may be unnecessary for attachment (234, 270, 278). Mooi *et al.* (234) produced *B. pertussis* fimbrial mutants by

Figure 2: Bvg regulation in *Bordetella.* BvgS is an environmental sensor which, in the absence of modulators, phosphorylates BvgA. BvgA enhances transcription from the *BvgAS* operon, the *fhaB* gene, the repressor gene, *fimB*, *act*, and possibly the dermonecrotic toxin gene leading to increased expression of FHA, BvgA, BvgS, and dermonecrotic toxin. Production of the repressor protein prevents trancription from the Bvg repressed genes (vrg's). The presence of FimB enhances transcription of the fimbrial subunit genes. Act enhances transcription and expression from the pertcatin gene, the adenylate cyclase toxin gene, and possibly the dermonecrotic toxin gene. Under modualting conditions, BvgA is not phosphorylated, virulence factors regulated by BvgA or Act are not produced, and the vrg's are expressed. Tracheal cytotoxin is produced in both phases.

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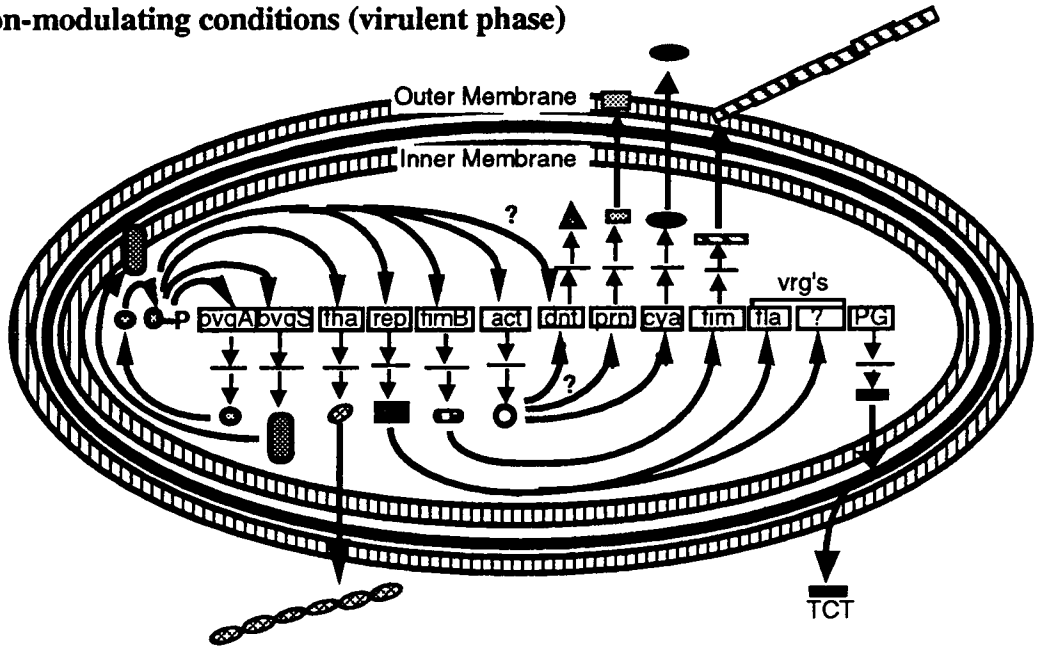
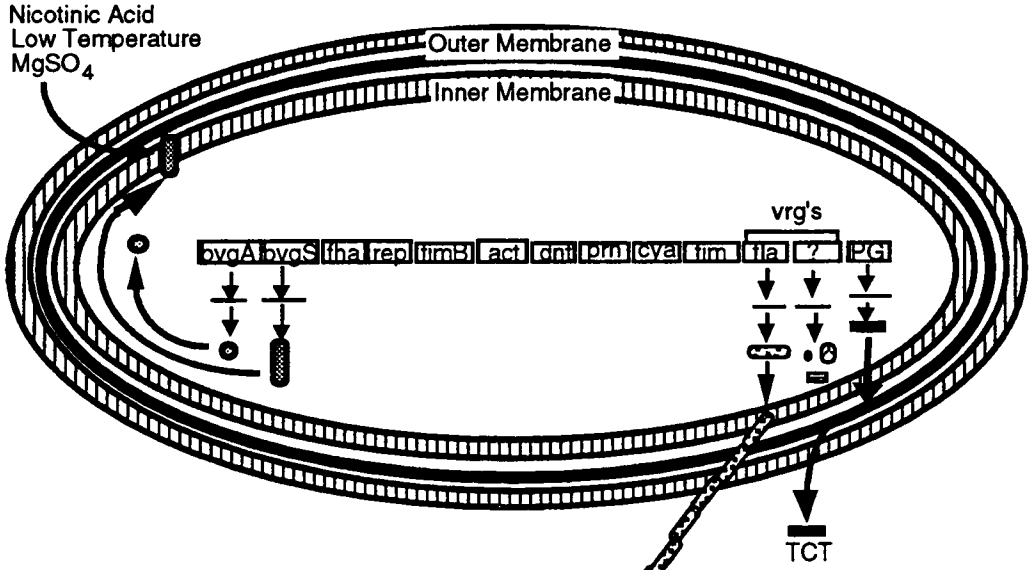
* Adapted from:
Bemis, David A., and Eugene H. Burns, Jr. 1993. *Bordetella.* in C. L. Gyles and C. O. Theon (ed.). *Pathogenesis of Bacterial Infections in Animals*, second edition. Iowa State University Press, Ames, Iowa. p. 201-215.Used by permission.

Non-modulating conditions (virulent phase)



Modulating Conditions (avirulent phase)



bvgA	**transcriptional activator**	**cya**	**adenylate cyclase toxin**
bvgS	**environmental sensor**	**fimB**	**fimbrial regulatory gene**
fha	**filamentous hemagglutinin**	**fim**	**fimbriae subunits**
act	**activator**	**fla**	**flagella**
dnt	**dermonecrotic toxin**	**vrg's**	**vir repressed genes**
prn	**pertactin**	**PG**	**peptidoglycan synthesis genes**
rep	**repressor**	**TCT**	**tracheal cytotoxin**

insertion of a kanamycin cassette into the *fim2* and *fim3* genes. By this method, they produced a *B. pertussis* strain unable to express either serotype of fimbriae. This strain, B52, when introduced into mice, colonized the nasopharynx, trachea, and lungs, as well as the native fimbriated strain BP536 (234). They did, however, notice a difference in the ability of the afimbriated strain to persist in the trachea of colonized mice (234). This difference was less than that seen with FHA mutants suggesting that FHA is more important in maintaining infection in the mouse model than fimbriae (234).

Other researchers have also constructed *B. pertussis* fimbrial mutants. Relman *et al.* (270) report that *B. pertussis* mutants deficient in the production of any one adhesin, FHA, pertussis toxin, or fimbriae, do not show decreased ability to adhere to macrophages. These data support the model that natural attachment by *Bordetella* is mediated through the action of several adhesins. Mutating just one of these primary adhesins does not cause a decrease in attachment ability since both FHA and pertussis toxin alone are sufficient for binding to animal cells (270). However, Relman's data suggest that the presence of fimbriae alone cannot mediate efficient attachment to macrophages (270).

Robinson *et al.* (278) constructed transposon insertion mutants of *B. pertussis* and studied their ability to attach to Vero cells. Vero cells are African green monkey kidney cells maintained in tissue culture. Mutants unable to produce FHA were not able to adhere to Vero cells. However, those *B. pertussis* mutants deficient only in the production of fimbriae could adhere well (278). They also found that pre-incubation of Vero cells with fimbriae did not inhibit attachment by *B. pertussis* (278).

There is still some controversy over the role of fimbriae in attachment by *B. bronchiseptica*. *B. bronchiseptica* adherence may be similar to that of *B. pertussis* with FHA playing the most prominent role with some help from other adhesins such as pertactin. There have, however, been some reports implicating fimbriae in attachment by *B. bronchiseptica*. Yokomizo *et al.* (364) reported that fimbriae are involved in attach-

ment of *B. bronchiseptica* to swine nasal epithelial cells. Dugal *at al.* examined attachment of *B. bronchiseptica* to ciliated swine tracheal epithelial cells in an outgrowth culture (90). In transmission electron micrographs of the cells, they observed thin filaments radiating from the bacteria that appeared to be in contact with the cilia. These filaments were believed to be fimbriae (90).

Additional evidence of the involvement of fimbriae in attachment of *Bordetella* is present in reports of experiments designed to inhibit attachment with antibodies. Gorringe *et al.* (127) report that monoclonal antibodies against agglutinogen 2, serotype 2 fimbriae, were able to inhibit attachment of *B. pertussis* to Vero cells. Robinson *et al.* (278) report that polyclonal antibodies against serotype 2 fimbriae were able to inhibit *B. pertussis* attachment to Vero cells and to protect mice from colonization of the lung. They suggest that antibodies to fimbriae can inhibit attachment despite the fact that afimbriated mutants still attach well because of physical blocking of other adhesins by the presence of the antibodies (278). There have also been reports that afimbriated mutants which attach well in *in vitro* attachment assays are less able to persist in the trachea of animals than *Bordetella* with fimbriae (234), suggesting that fimbriae may play a greater role in attachment in natural infection than is seen under laboratory conditions.

Analysis of the fimbrial subunit genes of *Bordetella* also suggests that fimbriae may have a role in attachment. Willems *et al.* (358) have proposed that *fimD* encodes a minor fimbrial subunit which can function as an adhesin. This gene has similarity with minor fimbrial subunit genes from *E. coli* and *Klebsiella pneumoniae* which have been shown to be involved in attachment (155, 358). A 72 bp deletion was introduced into the *fimD* gene so that its effect on attachment could be examined. However, the mutant strain did not produce any fimbriae on the surface or fimbrial subunit proteins as determined by ELISA (358). In other species, mutation of fimbrial adhesins which are only a minor component of the fimbrial filament do not prevent polymerization of the filament or expression of other subunits (364). Mutations in chaperonin genes do result in

degradation of fimbrial subunits in the periplasmic space before polymerization into filaments (234, 358). The lack of fimbrial filaments or subunits in the *fimD* mutant suggest that FimD may actually be a chaperonin or accessory protein rather than an adhesin (358). The ability of FimD to bind to cells has not been investigated.

In summary, the role of fimbriae in attachment by *Bordetella* is still uncertain. There are data suggesting that fimbriae are not necessary for attachment in *B. pertussis* but may work in concert with other adhesins (234, 270, 278). Since fimbriae function as adhesins in other species, it is likely that they function as adhesins in *Bordetella* as well. Initial experiments indicate that fimbriae play a role in attachment by *B. bronchiseptica* (90, 127, 364). Therefore, further studies on fimbriae of *Bordetella* and from species other than *B. pertussis* are necessary.

Specific Aims

This dissertation describes experiments which examine the involvement of fimbriae in attachment by *B. bronchiseptica*. Two approaches were used to explore this relationship. First, a survey of expression of fimbriae by various strains of *B. bronchiseptica* was performed so that fimbrial expression level could be compared to attachment ability. Second, a fimbrial related gene from *B. bronchiseptica* was cloned and its effect on attachment was examined. The specific aims of these experiments were:

1. To determine the expression level of *Bordetella pertussis* serotype 2 cross-reactive fimbriae in strains of *Bordetella bronchiseptica* isolated from different animal hosts and in different enzyme electromorphotypes (Chapter 2).
2. To determine if there is a correlation between production of particular fimbrial subunit proteins and reactivity with antibodies against serotype 2 fimbriae or attachment ability (Chapters 2 and 3).
3. To compare attachment ability of strains of *Bordetella bronchiseptica* in an *in vitro* attachment assay (Chapter 3).

4. To clone a fimbrial related gene from *Bordetella bronchiseptica* 110H and introduce it into *Bordetella bronchiseptica* strains which produce little or no serotype 2 fimbriae (Chapter 4).
5. To examine the effect of a cloned *Bordetella bronchiseptica* fimbrial-related gene on attachment by strains of *Bordetella bronchiseptica* which do not naturally attach well in an *in vitro* attachment assay (Chapter 5).
6. To examine the effect of antibodies against fimbriae and known adhesins of *Bordetella pertussis* (FHA and pertactin) on attachment by *Bordetella bronchiseptica* (Chapter 5).

CHAPTER TWO

SEROTYPE 2 FIMBRIAE EXPRESSION LEVELS IN *BORDETELLA BRONCHISEPTICA* *

Introduction

Bordetella bronchiseptica has been associated with atrophic rhinitis in pigs and acute tracheobronchitis (kennel cough) in dogs (38, 39, 286). *B. bronchiseptica* is also commonly isolated from the respiratory tracts of several other animals, including rabbits and guinea pigs (238). Musser *et al.* (238) used multilocus enzyme electrophoresis to compare strains isolated from different animal species. They found that strains isolated from pigs were predominately of one electromorphotype (ET), which was designated ET1. Strains isolated from guinea pigs were in ET16, and most strains in ET6 were isolates from dogs (238). These data indicated that there are measurable differences between strains isolated from different species. ET groups, defined by Musser *et al.* (238), were based on the electrophoretic mobility of 15 metabolic enzymes. Studies have not been undertaken to determine if virulence factors or adhesins might also differ between strains isolated from different host species.

B. bronchiseptica produces most of the virulence factors identified in *B. pertussis*, including filamentous hemagglutinin (164), a 68 kD protein similar to pertactin (49, 50), adenylate cyclase toxin (32), and fimbriae (193). As in *B. pertussis*, attachment is believed to be mediated by the combined effect of more than one of these proteins. Fimbriae are involved in attachment in several other bacterial species and may play a role

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* Adapted from:
Burns, Eugene H., Jr., John M. Norman, Monty D. Hatcher, and David A. Bemis. 1993. Fimbriae and Determination of Host Species Specificity of *Bordetella bronchiseptica*. J Clin Microbiol **31**: 1838-1844. Used by permission.

in attachment by *B. bronchiseptica* (90, 278, 364). Three proteins have been identified as fimbrial subunit proteins in *B. bronchiseptica* (193). If fimbriae are involved in attachment, then differences in these three fimbrial proteins may contribute to the differences in host specificity observed by Musser *et al* (238).

This chapter describes the production of a monoclonal antibody, designated CF8, which exhibits specificity for non-denatured *B. bronchiseptica* fimbriae. This antibody exhibits a pattern of reactivity similar to monoclonal antibody BPF2, which is specific for *B. pertussis* serotype 2 fimbriae (200). Preparations enriched in fimbrial proteins were obtained from *B. bronchiseptica* strains in each of the three most common electromorphotypes identified by Musser *et al.* (238), ET1, ET6, and ET16 . There were large differences in the amount of the 24 kD fimbrial subunit protein produced by each strain. Since this protein was identified by Western blot as being antigenically related to *B. pertussis* serotype 2 fimbrial subunits, an enzyme linked immunosorbent assay (ELISA) using monoclonal antibody CF8 was developed to quantify these differences. This assay indicated that there are significant differences in the amounts of CF8-reactive fimbriae between strains. These differences did not correlate with enzyme electromorphotype but did correspond to the host species from which the strain was isolated suggesting that the amount of serotype 2 cross-reactive fimbriae produced by strains of *B. bronchiseptica* may be involved in determination of host specificity.

Materials and Methods

Bacterial Strains, Media, and Growth Conditions

Bordetella bronchiseptica strains used have been described previously (37, 238) except strain R-5 which is a guinea pig lung isolate received from W. Shek, Charles River Laboratories. *Bordetella* were grown on Brucella agar (Difco, Detroit, MI) or Bordet Gengou (BG) agar prepared from BG base (BBL, Cockeysville, MD) with 15% horse

blood obtained from donor horses at the University of Tennessee College of Veterinary Medicine. All strains were grown for 48 hours at 37°C. Only colonies exhibiting virulent phase phenotype (37) were used except for strain 110NH which is an avirulent phase variant of strain 110H. Virulent phase colonies are small (≤ 1 mm), domed colonies which, depending on strain, may exhibit hemolysis.

Enrichment for Fimbrial Proteins

Bordetella from two day cultures grown on Brucella agar were scraped off plates and suspended in phosphate buffered saline (PBS) to form a 10% (w/v) solution. The suspension was homogenized five times for thirty seconds each time using the highest speed setting on an Osterizer blender (Oster Corporation, Milwaukee, WI), with two minute intervals of cooling on ice between each homogenization. The homogenate was centrifuged at 13,180 x g for 20 minutes. The supernatant fractions were successively centrifuged in this manner until no visible pellet was obtained. Proteins were precipitated by adding ammonium sulfate to a final concentration of 17.5% and leaving overnight at 4°C. This concentration of ammonium sulfate was determined in preliminary experiments with strain 110H to yield optimum precipitation of fimbrial subunit proteins with the least amount of other proteins, particularly flagella. Precipitated proteins were recovered by centrifugation at 19,160 x g for approximately one hour. The pellet was suspended in 3 ml of PBS and stored at 4°C.

Fimbrial Protein Profiles

Fimbrial protein enrichments were subjected to sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) by the method of Laemmli (190) using a 5% stacking gel and a 10% separating gel. Proteins were added to sample treatment buffer containing 2% β mercaptoethanol, heated in a boiling water bath for 5 minutes,

and electrophoresed at 20 mA per gel. Gels were fixed in methanol - acetic acid and stained with Coomassie brilliant blue R250.

After separation by SDS-PAGE, fimbrial proteins were electrophoretically transferred to nitrocellulose as described by Towbin (329). A strip from the nitrocellulose sheet was stained with India ink after transfer (136). Nitrocellulose strips containing transferred proteins were blocked with Tris buffered saline (TBS; 20 mM Tris, 500 mM NaCl, pH 7.5) containing 3% gelatin for one hour at 37°C. All washing steps employed TBS containing 0.05% Tween 20. Strips were incubated overnight at room temperature with the primary antibody in TBS containing 1% gelatin, followed by incubation for 3 hours at room temperature with a 1:2000 dilution of goat anti-mouse IgG horseradish peroxidase conjugate (BioRad, Richmond, CA). Color detection was accomplished by hydrogen peroxide and 4-chloro-1-naphthol. The development was stopped by washing with water. For the Western blots described here, monoclonal antibody BPA5 was used as the primary antibody. BPA5 is specific for a *B. pertussis* serotype 2 fimbrial subunit. BPA5 was kindly provided by Michael J. Brennan (Center for Biologics Evaluation and Research, FDA, Bethesda, MD).

Production and Characterization of Monoclonal Antibody CF8

Production and characterization of monoclonal antibody CF8 by immunogold electron microscopy was carried out by Mr. Monty Hatcher and Mr. John Norman. These experiments are briefly described here in order to show the origin and specificity of the antibody used in later work. Hybridomas were established using the Taggert Hybridoma Technology^{R} (Hyclone Laboratories, Logan, UT) following immunization of RBF/dn mice intraperitoneally with proteins isolated from fimbrial enrichments by SDS-PAGE. SDS polyacrylamide gels included pre-stained molecular weight markers (Amersham, Arlington Heights, IL). The gels were cut at each marker and the pieces generated were injected separately into mice. Hybridoma supernatant fluids were screened by Western

blot analysis. Monoclonal antibody CF8 was derived from a mouse immunized with the region of gel between the top two markers, from 97.4 to 200 kD. Ascites fluid containing monoclonal antibodies was produced in Balb/c mice. Cell debris and immiscible lipids were removed by centrifugation at 19,000 x g for one hour at 4°C. Ascites fluid was stored at -20°C. Immunoglobulin isotypes were determined by indirect ELISA with specific anti-mouse immunoglobulin reagents contained in the Mouse Monoclonal Sub-Isotyping Kit (Hyclone).

Western blots were performed as described above using fimbrial enrichments from strain 110H and purified *B. pertussis* filamentous hemagglutinin (FHA) as the antigen. Purified FHA was kindly provided by J. L. Cowell (FDA, Center for Biologics Evaluation and Research, Bethesda, MD). Monoclonal antibody F1, specific for *B. pertussis* FHA, was kindly provided by L. I. Irons (Centre for Applied Microbiology and Research, Porton Down, Salisbury, UK).

For immunogold electron microscopy, bacterial cells were labeled by floating bacteria coated grids on drops of monoclonal antibody for 30 minutes followed by gold conjugated goat anti-mouse IgG (5 nm, Janssen Life Sciences Products, Beerse, Belgium) for thirty minutes. Initial blocking, all washes and antibody dilutions were with PBS containing 2% bovine serum albumin. Cells were negatively stained with potassium phosphotungstic acid and examined by transmission electron microscopy as previously described (35).

For dot blot immunoassays, bacteria from 48 hour Brucella agar cultures were suspended in PBS and inactivated by adding formalin to a final concentration of 0.25% and holding overnight at 37°C. This suspension was diluted in PBS to an optical density of 1.2 at 465 nm. Five microliters of the suspension was spotted on nitrocellulose strips which had been pre-moistened with TBS. Enzyme based detection of the reactivity of these spots with monoclonal antibodies was performed as described for Western blot assays. A 1:3 dilution of CF8 culture supernatant was used. In addition to CF8, reactivity

with monoclonal antibody BPF2 was also determined. BPF2 is specific for non-denatured *B. pertussis* serotype 2 fimbriae (199) and was kindly provided by Michael J. Brennan, (FDA, Center for Biologics Evaluation and Research, Bethesda, MD). Bacterial suspensions prepared in the same manner as for the dot blots were used in microagglutination assays. Equal volumes of bacterial suspension were added to each well, plates were sealed, shaken to mix, incubated at 37°C for 2 hours and at 4°C for 18 - 24 hours. Agglutination was determined visually. Agglutination assays and some of the dot blots were performed by Dr. David Bemis.

Enzyme Immunoassays

Indirect ELISA

Each *B. bronchiseptica* strain to be tested was grown for 2 days on Brucella agar plates. Colonies exhibiting virulent phase morphology were picked and suspended in carbonate buffer (sodium carbonate 1.59g/l, sodium bicarbonate 2.93 g/l, pH 9.6) to an optical density of 0.4 at 595 nm. Immulon 4 plates (Dynatech Laboratories, Chantilly, VA) were coated with this suspension overnight. The plates were then blocked by incubation for one hour at 37°C in wash buffer (10 mM Tris, 0.9% NaCl, 0.05% Tween 20, pH 7.4). Two-fold serial dilutions, starting at 1:100, of ascites fluid containing monoclonal antibody CF8 were made in wash buffer. 100µl of each dilution was added to each well of the ELISA plate. The plates were incubated one hour at 37°C, washed, and goat anti-mouse IgG horseradish peroxidase conjugate (BioRad) was added as the secondary antibody. After incubation for one hour at 37°C, the plates were washed and developed using the ELISA HRP substrate kit (BioRad). Plates were read at 405 nm. Titer was determined as the point at which the absorbance was 0.1 above background.

Antibody Capture Assay

A 1:400 dilution of heat inactivated ascites fluid containing monoclonal antibody CF8 was made in carbonate buffer and 100µl of this solution was added to each well of an Immulon 2 microtiter plate (Dynatech Laboratories). After incubation overnight at room temperature, the plate was washed three times in wash buffer and 100µl of a 3% solution of bovine serum albumin in wash buffer was added to each well for blocking. The plate was incubated for one hour at room temperature and then washed three times in wash buffer. *B. bronchiseptica* strains to be tested were grown for 2 days on Brucella agar at 37°C. Colonies exhibiting virulent phase phenotype were picked and suspended to an $O.D._{595}$ of 0.6 in wash buffer. 100 µl of 2 fold serial dilutions of this suspension was added to each well of the microtiter plate which had been coated with monoclonal antibody CF8. The plate was incubated for one hour at 37°C and washed three times with wash buffer.

After washing, 100µl of a 1:100 dilution of rabbit anti-110H whole cell serum was added to each well and incubated for one hour at 37°C. Plates were washed three times with wash buffer and 100 µl of a 1:100 dilution of goat anti-rabbit IgG horseradish peroxidase conjugate (BioRad) was added to each well. After incubation for one hour at 37°C and washing three times with wash buffer, the plates were developed using the ELISA HRP substrate kit (BioRad). Plates were read at 405 nm. Titer was determined as the point at which the absorbance was 0.1 above background.

Pre-Adsorption ELISA

B. bronchiseptica strain 110H was grown for 2 days on Brucella agar plates. Colonies exhibiting virulent phase morphology were picked and suspended in carbonate buffer to an optical density of 0.4 at 595 nm. Immulon 4 plates (Dynatech Laboratories) were coated with this suspension overnight. *B. bronchiseptica* strains to be tested were

grown 2 days on Bordet Gengou agar. Colonies exhibiting virulent phase phenotype were picked and suspended in wash buffer to an optical density of 0.6 at 595 nm. Two-fold serial dilutions of ascites fluid containing monoclonal antibody CF8 were made in the bacterial suspensions and incubated at 37°C for one hour.

Bacteria were removed by centrifugation in a microfuge for 15 minutes and 100µl of the supernatant was added to the wells of the microtiter plates that had been coated with 110H and blocked by incubation for 30 minutes in wash buffer. The plates were incubated one hour at 37°C, washed, and goat anti-mouse IgG horseradish peroxidase conjugate (BioRad) was added as the secondary antibody. After incubation for one hour at 37°C, the plates were washed and developed using the ELISA HRP substrate kit (BioRad). Plates were read at 405 nm. Titer was determined as the point at which the absorbance was 0.1 above background. Each test was repeated three times.

Statistical Analysis

Statistical analysis was performed using the SAS system on the VAX cluster at the University of Tennessee Computing Center. Analysis of variance was performed using the general linear models procedure, Bonferoni t tests, and the Ryan-Einot-Gabriel-Welsch multiple F test procedure.

Results

Monoclonal Antibody Production and Characterization

A fimbrial protein enrichment was prepared from *B. bronchiseptica* strain 110H by homogenization and ammonium sulfate precipitation. SDS-polyacrylamide gel electrophoresis of this sample was performed and regions (described in Materials and Methods) cut from this gel were injected into mice for monoclonal antibody production. After formation of hybridomas, a monoclonal antibody of isotype IgG_1, designated CF8,

was identified which exhibited specificity for *B. bronchiseptica* fimbriae. Fimbrial specificity was determined by immunogold electron microscopy. Monoclonal antibody CF8 bound to some but not all of the fimbrial filaments on *B. bronchiseptica* cells (figure 3), indicating that strains of *B. bronchiseptica* may produce more than one type of fimbriae. Strains 110H, 17640, and R-5 were each subjected to immunogold labeling with CF8. It was difficult to quantify binding, but there appeared to be fewer antibody labeled filaments on strains 17640 and R-5 than on strain 110H.

Two antigenically distinct fimbrial types have been identified in *B. pertussis*. Monoclonal antibodies specific for each of these fimbrial serotypes are cross-reactive with *B. bronchiseptica* fimbriae (199). To determine if monoclonal antibody CF8 reacted with an epitope similar to one of these *B. pertussis* fimbrial serotypes, the reactivity of CF8 was compared with that of antibodies to *B. pertussis* fimbriae. Monoclonal antibody CF8 was used in dot blot immunoassays and agglutination assays with 40 strains of *B. bronchiseptica* (table 2) and three strains of *B. pertussis*. Monoclonal antibody CF8 reacted by dot blot with *B. pertussis* strain Tohama I (serotype 1.2.3.4) but not with strain 114 (serotype 1.3.6). CF8 also agglutinated *B. pertussis* strain 460 (serotype 1.2.3.4.6). The pattern of reactivity for CF8 with both *B. pertussis* and *B. bronchiseptica* strains was identical to that of monoclonal antibody BPF2, which is specific for non-denatured *B. pertussis* serotype 2 fimbriae, indicating that CF8 recognizes a *B. pertussis* serotype 2 cross-reactive epitope.

To determine if the epitope recognized by CF8 is present on denatured fimbrial subunits, Western blots were performed on fimbrial protein enrichments from *B. bronchiseptica* strain 110H (figure 4). When the fimbrial protein enrichment was boiled for one minute before electrophoresis, CF8 reacted with high molecular weight proteins producing a laddering effect. When the sample boiling time was increased to five minutes, no reactivity with CF8 was observed with high molecular weight proteins or with 21 - 24 kD fimbrial subunit proteins. Therefore, monoclonal antibody CF8

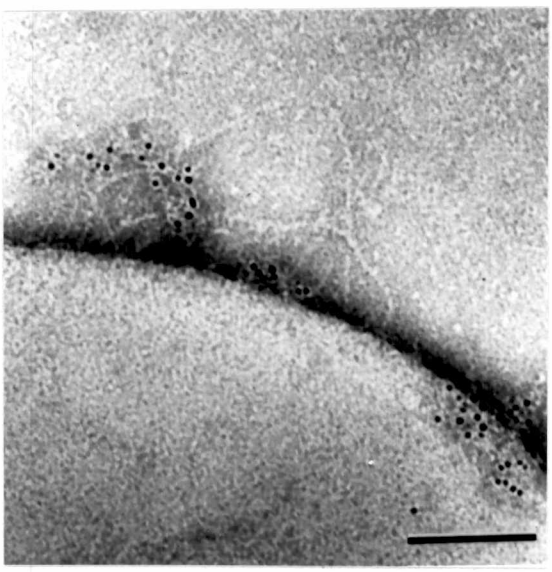

Figure 3: Binding of CF8 to fimbriae.* *B. bronchiseptica* strain 110H was immunolabeled with monoclonal antibody CF8 and anti-globulin gold conjugate and negatively stained with potassium phosphotungstate. Magnification x 165,000. Bar, 100 nm.

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* Source:
Burns, Eugene H., Jr., John M. Norman, Monty D. Hatcher, and David A. Bemis. 1993. Fimbriae and Determination of Host Species Specificity of *Bordetella bronchiseptica*. J Clin Microbiol **31**: 1838-1844. Used by permission.

Table 2: Reactivity of *B. bronchiseptica* with CF8 and BPF2[1]

	Agglutination[2]		Dot Blot[3]	
Strain	CF8	BPF2	CF8	BPF2
110H	+	+	+	+
B133	+	+	+	+
2-9 NADL	+	+	+	+
1120-A83-013	+	+	+	+
82-1711	+	+	+	+
ATR6	-	-	+	+
p2458	-	-	-	-
2	-	-	-	-
402	-	-	+	+
2535	-	-	+	+
S-55	-	-	-	-
22067	-	-	-	-
138 AnHus	-	-	-	-
H-475	-	-	-	-
D-1	-	-	-	-
Columbus	-	-	-	-
BB Ithaca	-	-	-	-
17640	-	-	-	-
482-74	-	-	-	-
695-74	-	-	-	-
SS-232-75	-	-	+	+
214	+	+	+	+
899L	-	-	-	-
87	-	-	-	-
K704	-	-	-	-
CDC-47	-	-	-	-
NYS #4	-	-	-	-
19141	-	-	-	-
WFA5Y	-	-	-	-
R-5	-	-	-	-
Rat1	+	+	+	+
SHGP1	-	-	-	-
VT115	-	-	-	-
Horse	-	-	-	-
RAT	-	-	-	-
VPIGP1	-	-	-	-
VPIEQ1	-	-	-	-
VPIFE1	-	-	-	-
85-2979	-	-	-	-
UT Dog	+	+	+	+

[1] Source:
Burns, Eugene H., Jr., John M. Norman, Monty D. Hatcher, and David A. Bemis. 1993. Fimbriae and Determination of Host Species Specificity of *Bordetella bronchiseptica*. J Clin Microbiol **31**: 1838-1844. Used by permission.

[2] + = complete agglutination (100%) determined visually

[3] + = any visible precipitate

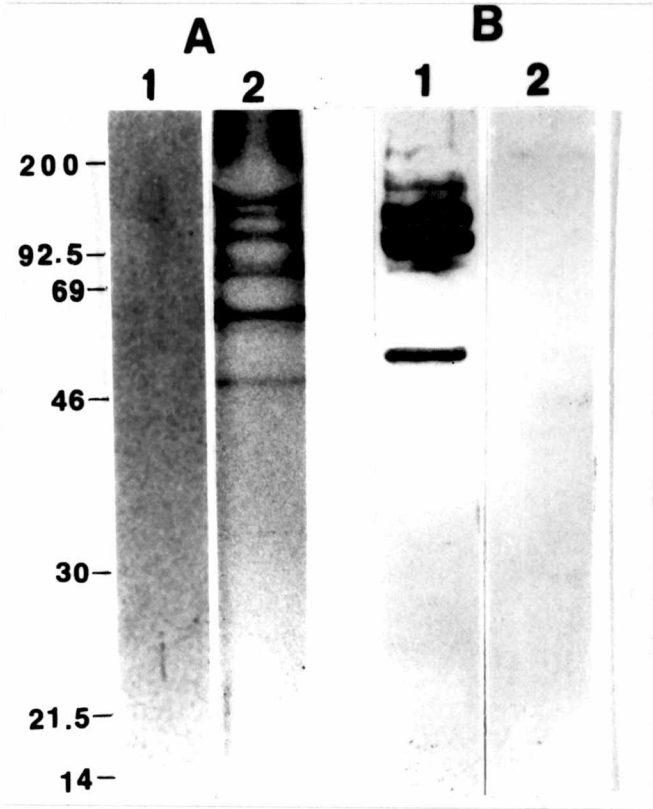

Figure 4: Western blots with CF8 and F1.* Antibody CF8 (specific for serotype 2 fimbriae) was used in panel A and F1 (specific for FHA) was used in panel B. lane 1) purified *B. pertussis* FHA, lane 2) fimbrial protein enrichment from strain 110H. Protein samples were boiled for one minute before running on the gel.

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* Source:
Burns, Eugene H., Jr., John M. Norman, Monty D. Hatcher, and David A. Bemis. 1993. Fimbriae and Determination of Host Species Specificity of *Bordetella bronchiseptica*. J Clin Microbiol **31**: 1838-1844. Used by permission.

recognizes an epitope present on non-denatured or oligomeric fimbrial units which is not present on fully denatured or monomeric fimbrial subunits obtained by treatment of fimbrial enrichments with SDS, β-mercaptoethanol, and boiling for five minutes. A similar laddering pattern has been seen with monoclonal antibody BPF2 (199), which recognizes non-denatured *B. pertussis* serotype 2 fimbriae.

Since filamentous hemagglutinin (FHA) from *B. pertussis* also appears as a series of high molecular weight bands on SDS polyacrylamide gel electrophoresis and the fimbrial protein enrichments used as antigens for the production of monoclonal antibody CF8 were not pure fimbrial preparations and could have contained FHA, it was necessary to determine if the bands being recognized by CF8 were non-denatured fimbriae or were FHA. Western blots using monoclonal antibody F1, which is specific for *B. pertussis* FHA and cross-reacts with FHA from *B. bronchiseptica* (164), were performed on purified FHA and on fimbrial protein enrichments (figure 4). Monoclonal antibody F1 did not react with the fimbrial protein enrichment and monoclonal antibody CF8 did not react with the purified FHA.

Fimbrial Protein Profiles

It has previously been reported that strain 110H possesses multiple fimbrial subunit proteins visible on polyacrylamide gels in denaturing conditions (193). It has not been determined if each of these fimbrial subunit proteins represent different fimbrial types. Since monoclonal antibody CF8 reacted differently with strains 110H, 17640, and R-5, and since reactivity with CF8 has been associated with serotype 2 fimbriae, we wanted to determine if variations in the amount of any of these major fimbrial subunit proteins corresponded to reactivity with monoclonal antibody CF8. Strains which reacted poorly with monoclonal antibody CF8 should have less of the fimbrial subunit proteins associated with serotype 2 fimbriae.

Fimbrial protein profiles were determined for eight strains of *B. bronchiseptica* (figure 5). Three predominant proteins with relative molecular masses of 21 kD, 22 kD, and 24 kD were observed. These proteins appeared to be identical in molecular mass to those previously identified as fimbrial subunit proteins (193). The heavy band at approximately 45 kD is believed to be flagella. Since the cells were grown on Brucella agar in a confluent layer in order to obtain large numbers of bacteria, it was impossible to determine if all cells used in the fimbrial protein enrichment procedure were in virulent phase. Due to the frequency of phase variation in *B. bronchiseptica*, some of the cells may have been in avirulent phase and therefore, producing flagella. This band is commonly seen in our fimbrial protein enrichments.

Strain 110H produced large amounts of each of the fimbrial proteins; strain R-5 produced much less of the 24 kD protein than it did of the other two proteins. Strain 17640 produced only small amounts of both the 24 kD and the 22 kD proteins. These strains consistently produced the same fimbrial protein profile on repeated preparations. There was slight variability in the fimbrial protein profile of strain 110H. Strain 110H typically exhibits a protein profile with large amounts of the lower and upper bands but very little of the middle band. However, in some preparations, this strain shows equal amounts of all three bands despite using identical conditions for growth and fimbrial protein enrichment. This strain always exhibits one of these two patterns.

To determine which, if any, of these fimbrial subunit proteins is associated with serotype 2 cross-reactivity, Western blots were performed using monoclonal antibody BPA5, which reacts with *B. pertussis* serotype 2 fimbrial subunits. These blots indicated that the 24 kD protein is present in fimbrial protein enrichments from each of the three strains tested even though in strain 17640 it is not easily visible on Coomassie blue stained gels (figure 6). Reactivity of the 24 kD protein with this antibody indicated that this *B. bronchiseptica* protein is antigenically related to the *B. pertussis* serotype 2 fimbrial subunit.

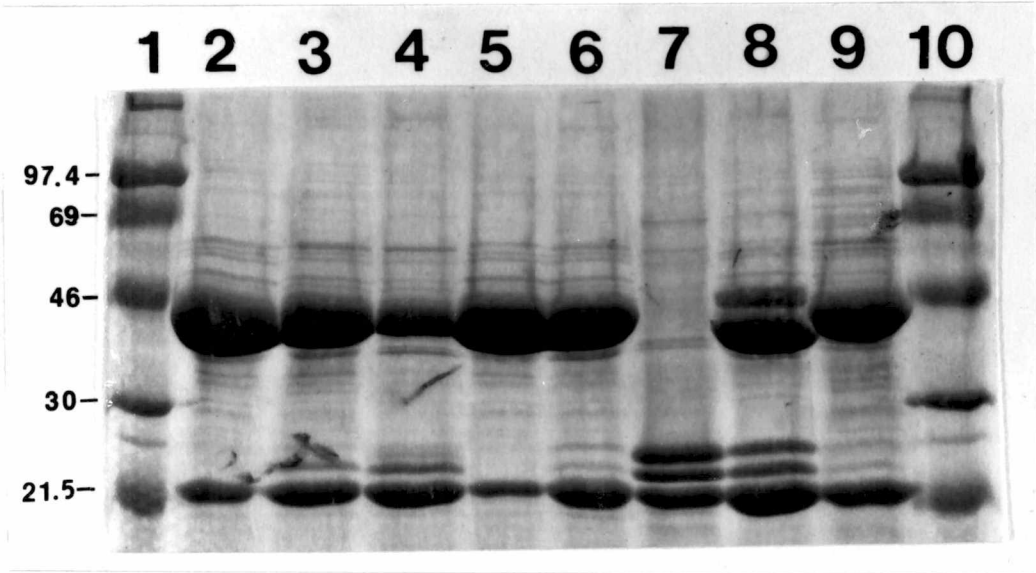

Figure 5: Fimbrial protein profiles of *B. bronchiseptica* strains. Coomassie blue stained gel of fimbrial protein enrichments from strain BB Ithaca (lane 2), 64-C-0406 (lane 3), JS 34682 (lane 4), Meisel (lane 5), Romark (lane 6), 110H (lane 7), R-5 (lane 8), 17640 (lane 9), and pre-stained markers (lanes 1 and 10).

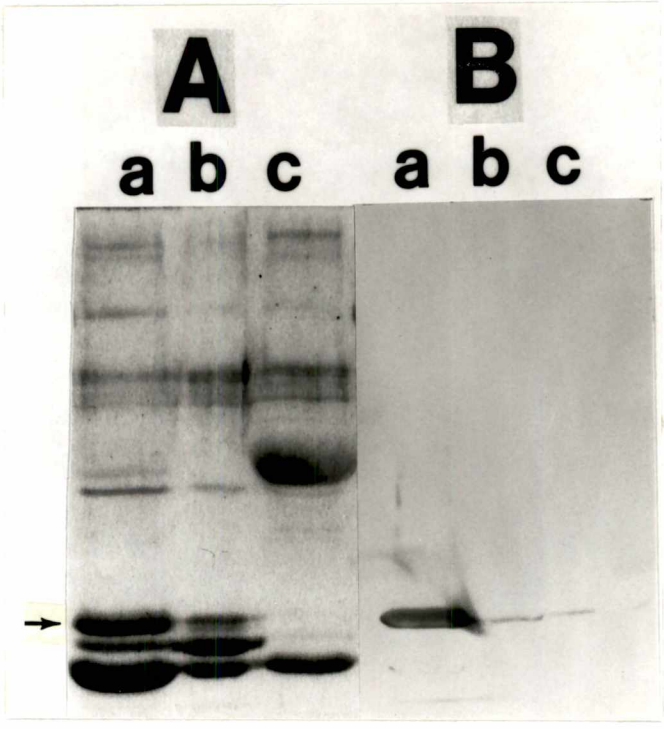

Figure 6: Western blot with BPA5.* Panel A shows the Coomassie blue stained gel and panel B shows the Western blot using monoclonal antibody BPA5. The arrow marks the 24 kD fimbrial subunit protein. *B. bronchiseptica* strains 110H (lane a), R-5 (lane b), and 17640 (lane c).

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* Source:
Burns, Eugene H., Jr., John M. Norman, Monty D. Hatcher, and David A. Bemis. 1993. Fimbriae and Determination of Host Species Specificity of *Bordetella bronchiseptica*. J Clin Microbiol **31**: 1838-1844.

Enzyme Immunoassay

Development of Assay

Fimbrial protein profiles, Western blots, and immunogold electron microscopy indicated that strains of *B. bronchiseptica* differ in the amount of CF8-reactive and *B. pertussis* serotype 2 cross-reactive fimbriae they produce. Since quantitative screening by these methods would be relatively cumbersome, we developed an enzyme immunoassay to measure indirectly the amount of this type of fimbrial antigen expressed by various *B. bronchiseptica* strains grown under identical conditions.

In development of a quantitative assay, we first tested an indirect ELISA. This assay used whole cells of *B. bronchiseptica* bound to ELISA plates as the antigen. Monoclonal antibody CF8 was used as the primary antibody. This assay was tested using strain 110H as a positive control, 110NH as a negative control, and 17640 and R-5 as test samples. Strain 110NH is an avirulent phase variant of strain 110H. It does not produce fimbriae and should not react with monoclonal antibody CF8. As shown in figure 7, strains 17640 and R-5 did not react as well as strain 110H which would indicate that these strains were expressing less serotype 2 cross-reactive proteins on their surface. These results are in agreement with the fimbrial protein profiles where it was shown that strains 17640 and R-5 do not produce as much of the 24 kD serotype 2 cross-reactive protein as strain 110H (figures 5 and 6). However, testing different strains in this assay would involve binding each strain separately to the ELISA plate. Due to differences in the surface of cells from different strains, each strain may not bind to the plate with the same affinity. Therefore, results from this assay may indicate differences in binding to the plastic rather than differences in antigen expression level.

To avoid this problem, a capture assay was developed. The capture assay involved binding of monoclonal antibody CF8 to the ELISA plates and then adding dilutions of suspensions of the strains to be tested to the wells. Strains 110H, 110NH, 17640, and R-5

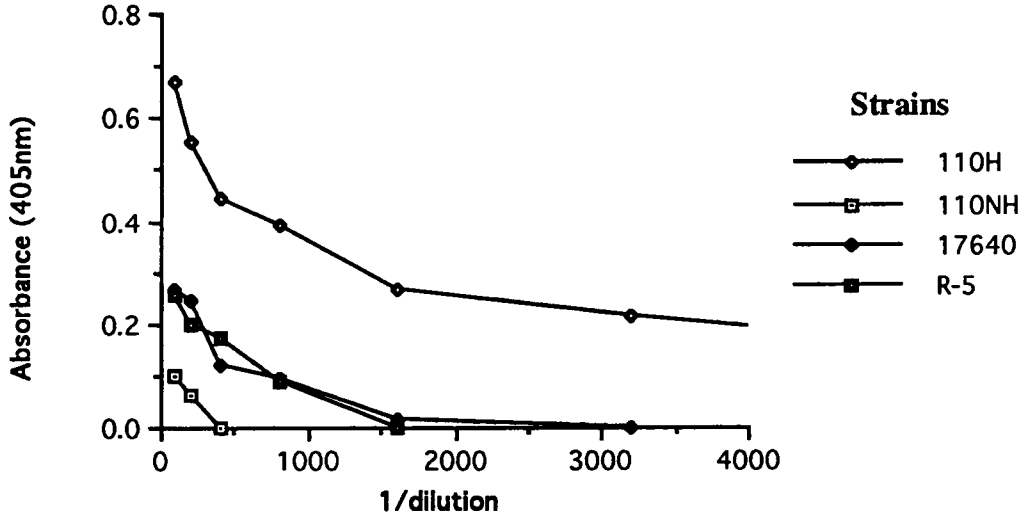


Figure 7: Indirect ELISA with monoclonal antibody CF8.

were used. Results from this trial are shown in figure 8. This assay showed results similar to that found in the indirect ELISA with 17640 and R-5 reacting at a level between 110H and 110NH. This assay, however, did not give high titers and only small differences were seen. In addition, this assay required high amounts of antibody; so, it would not be practical to use to compare large numbers of strains.

In order to use less antibody and to avoid the problems associated with binding each strain to the plate, the pre-adsorption ELISA was developed. Figure 9 diagrams the steps in this assay. This ELISA involved binding only a single strain, 110H, to the plate. A suspension of each *B. bronchiseptica* strain to be tested was incubated with monoclonal antibody CF8. The supernatant from this adsorption, which would contain any antibody not bound to the bacteria, was used as the primary antibody in a standard indirect ELISA with *B. bronchiseptica* strain 110H as the antigen. Strains expressing more immunologically reactive fimbriae would adsorb more antibody yielding a lower titer. Strains 110H, 110NH, 17640, and R-5 were used. The results are shown in figure 10 Strains 17640 and R-5 were intermediate in reactivity as compared to strains 110H and 110NH. In general, this assay eliminated the problems of low titer and high antibody usage exhibited by the capture assay and the problem of differential binding levels to the plate inherent in the indirect ELISA. Therefore, this assay was used to screen several *B. bronchiseptica* strains for expression of serotype 2 cross-reactive fimbriae.

Serotype 2 Fimbrial Antigen Expression Levels

36 strains of *B. bronchiseptica* from pigs, guinea pigs, dogs, and four other species were tested in the pre-adsorption ELISA. The results are shown in table 3. Strains isolated from pigs gave predominately low titers indicating a large percentage of the antibody was being adsorbed. Strains isolated from guinea pigs did not adsorb as much antibody as those from pigs, and strains isolated from dogs exhibited considerable variation in antibody reactivity.

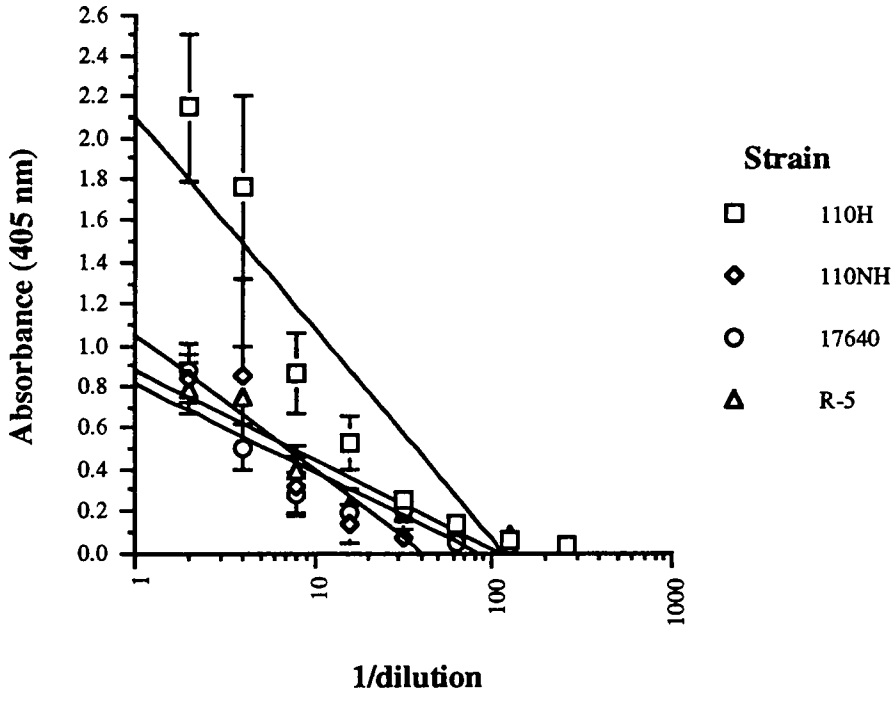


Figure 8: Capture assay. Monoclonal antibody CF8 was bound to the ELISA plate and used to capture *B. bronchiseptica* strains. Bacteria were then reacted with polyclonal rabbit anti-serum specific for *B. bronchiseptica* and goat anti-rabbit HRP conjugate was used to detect bound antibody.

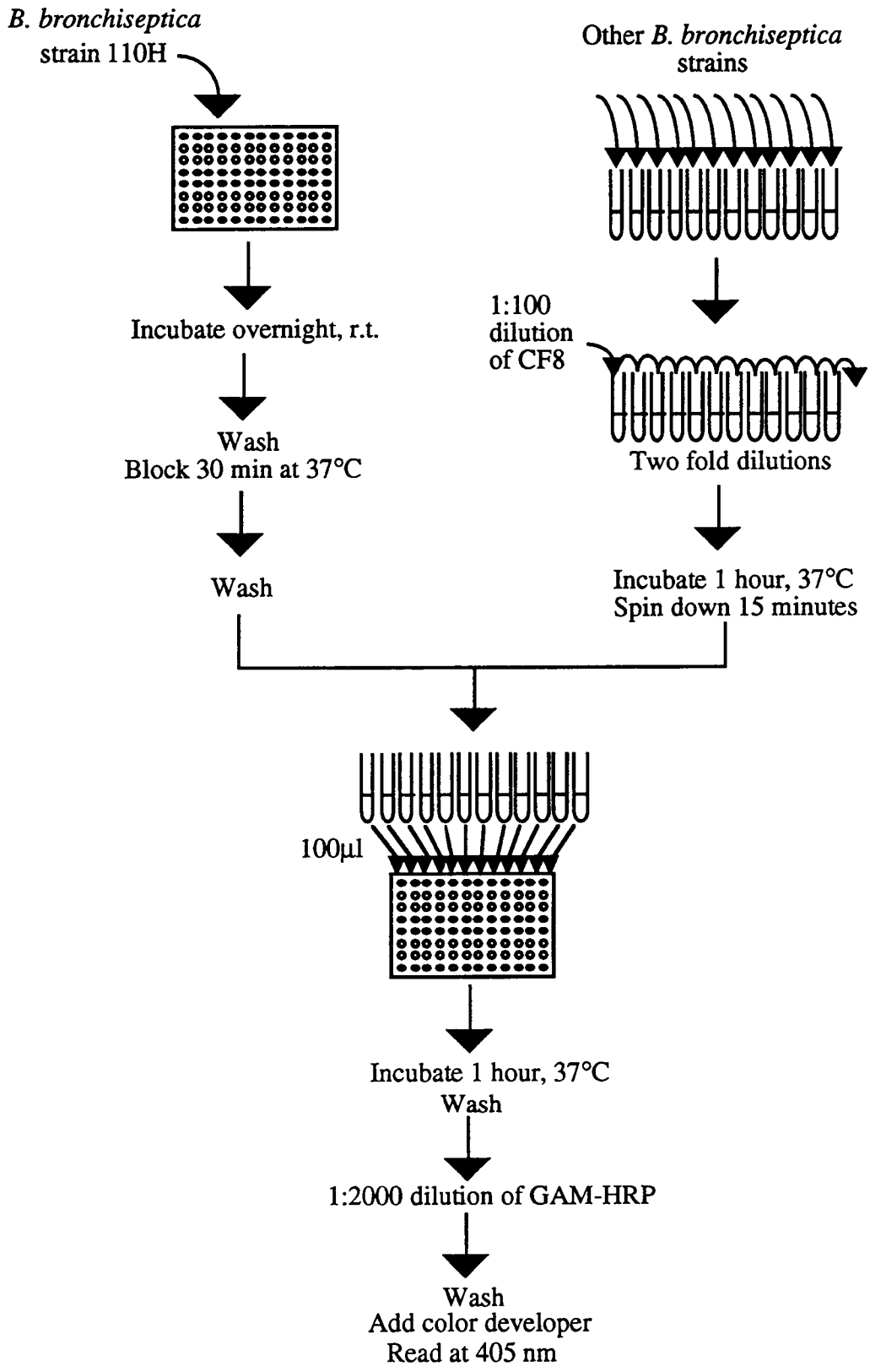


Figure 9: Pre-adsorption ELISA protocol.

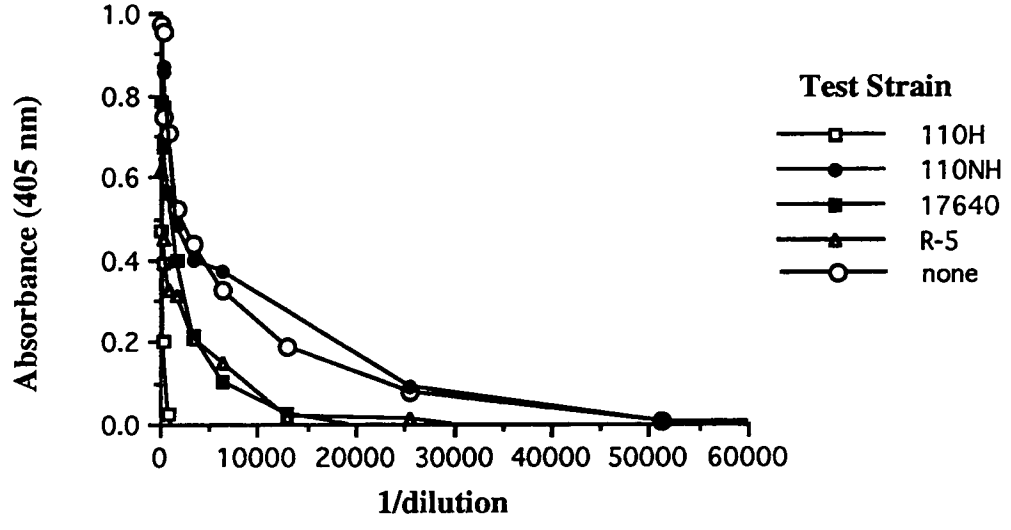


Figure 10: Pre-adsorption ELISA. Monoclonal antibody CF8 was incubated with each test strain before use in a standard indirect ELISA with strain 110H as the antigen.

Table 3: Pre-adsorption ELISA with *B. bronchiseptica* strains[1]

Strain	ET[2]	Host[3]	Titer[4]	% Reduction[5]
none	-	-	24120 ± 2973	-
110NH	1	dog	25666 ± 7025	-
110H	1	dog	1316 ± 320	94 ± 1
64-C-0406	1	dog	6896 ± 1617	71 ± 6
Congdon	1	dog	7856 ± 1375	67 ± 5
PR8053	1	dog	7056 ± 1233	70 ± 5
1120-A83-013	1	pig	1002 ± 663	95 ± 2
2-9 NADL	1	pig	1026 ± 547	95 ± 2
MBORD 303	1	pig	3184 ± 247	86± 1
MBORD 344	1	pig	1666 ± 1161	93 ± 4
82-1711	1	pig	2728 ± 2609	88 ± 10
B133	1	pig	929 ± 641	96 ± 2
MBORD 302	1	pig	5555 ± 934	76 ± 3
BTS	3	pig	4632 ± 551	80 ± 2
ATR3	ND	pig	4094 ± 386	83 ± 1
92-160c	ND	pig	1731 ± 295	92 ± 1
Phase I Tuskeegee	6	pig	4016 ± 370	83 ± 1
17640	6	dog	8296 ± 654	65 ± 2
BB Ithaca	6	dog	4000 ± 1707	83 ± 7
Meisel	6	dog	2692 ± 154	88 ± 1
JS34682	6	dog	2440 ± 528	89 ± 2
482-74	6	dog	6668 ± 1073	72 ± 4
Romark	6	dog	11836 ± 1983	50 ± 8
695-74	6	dog	7251 ± 86	69 ± 0.3
R-5	16	guinea pig	8917 ± 473	63 ± 2
VPI-GP1	16	guinea pig	8636 ± 1293	64 ± 5
SHGP-1	16	guinea pig	8903 ± 304	63 ± 1
86-2630 5T	16	guinea pig	6627 ± 926	72 ± 3
GPA	16	guinea pig	7150 ± 1375	70 ± 3
87-0599-1	16	guinea pig	5971 ± 1593	75 ± 6
V-205/211/218-1	16	guinea pig	6191 ± 2093	74 ± 8
V-205/211/218-2	16	guinea pig	7291 ± 1390	69 ± 5
V-205/211/218-3	16	guinea pig	5836 ± 1926	75 ± 7
VPI-FE1	16	cat	8447 ± 1443	64 ± 5
283494	16	rat	13170 ± 2278	45 ± 9
VPI-EQ1	16	horse	5301 ± 927	78 ± 3
ILG	16	monkey	5009 ± 388	79 ± 1

[1] Source:
Burns, Eugene H., Jr., John M. Norman, Monty D. Hatcher, and David A. Bemis. 1993. Fimbriae and Determination of Host Species Specificity of *Bordetella bronchiseptica*. J Clin Microbiol **31**: 1838-1844. Used by permission.

[2] Enzyme electromorphotype as determined by Musser *et al.* (238).

[3] Species from which strain was originally isolated.

[4] Determined as the mean reciprocal antibody dilution at the point where absorbance is 0.1 for three repetitions ± standard deviation.

[5] Calculated as difference in titer with and without antibody pre-adsorption by bacteria.

ND = Not determined

Using the general linear models analysis of variance procedure on the SAS system, the difference between these three groups was found to be significant ($P \leq 0.0002$). Bonferoni t tests and the Ryan Einot Gabriel Welsch multiple F test procedures indicated that strains from pigs and guinea pigs could be placed into separate groups in terms of reactivity with monoclonal antibody CF8. Statistically the strains isolated from dogs were placed in a group with the strains from guinea pigs (table 4). However, due to the high degree of variability in strains isolated from dogs, individual strains from dogs could be grouped with those from pigs or guinea pigs. Figure 11 illustrates the relationship between reactivity with monoclonal antibody CF8 and host species.

Reactivity with anti-fimbrial monoclonal antibody CF8 does not seem to correspond with enzyme electromorphotype (figure 12). Canine isolates in ET1 do not all exhibit the same high reactivity as porcine isolates from ET1. In addition, strains from dogs in ET1 exhibit increased variation as do canine isolates from other enzyme electromorphotypes. Porcine isolates from enzyme electromorphotypes other than ET1 are rare. Therefore, only two strains isolated from pigs outside of ET1 were tested. These strains both exhibited levels of reactivity similar to those porcine isolates in ET1. We were unable to obtain any strains isolated from guinea pigs in enzyme electromorphotypes other than ET16, but strains in ET16, isolated from animals other than guinea pigs, did not react at the same level as strains from guinea pigs. Some of these strains reacted better and some reacted worse than strains isolated from guinea pigs.

The reduction in titer after incubation with particular strains of *B. bronchiseptica*, could be due to the presence of a protease or an IgG binding protein rather than specific binding of the antibody to fimbriae. To examine this possibility, the same assay was performed using bovine serum albumin as the antigen and commercially available monoclonal anti-BSA (Sigma, St. Louis, MO) in place of CF8. If the differences in reactivity between strains is due to a protease or an IgG binding protein, then the same differences would be seen with an antibody which should not specifically recognize a

Table 4: Grouping of strains by Bonferoni T tests and REGW multiple F tests

Group[1]	Mean titer	Host
A	7314.0	Guinea Pig
A	6028.4	Dog
B	2778.9	Pig

[1] Means with the same letter are not significantly different.

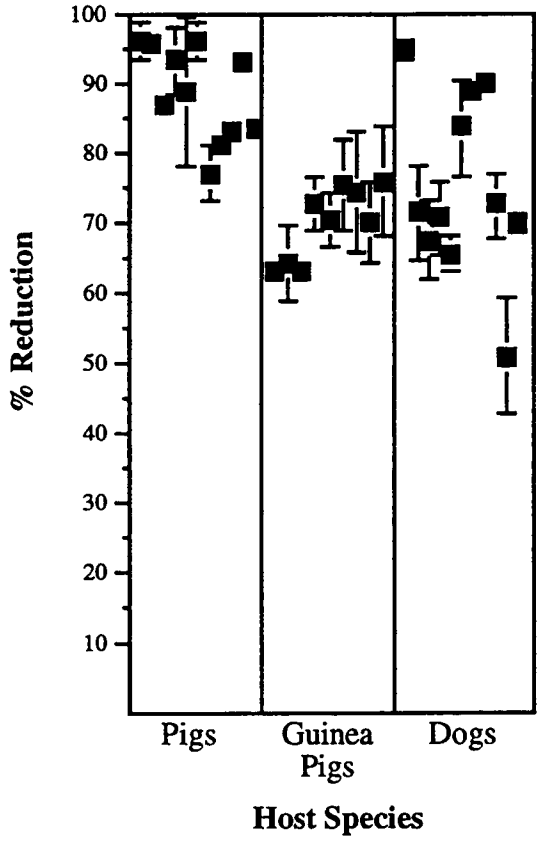


Figure 11: Relationship of CF8 reactivity to host species.

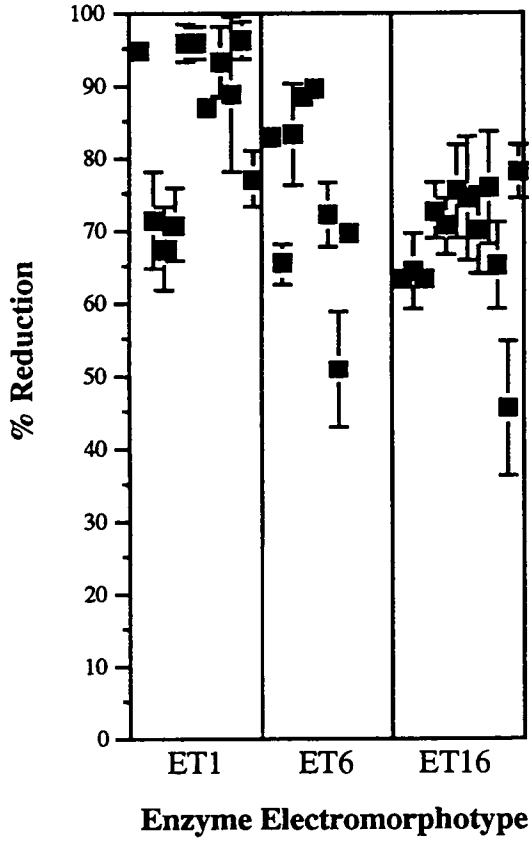


Figure 12: Relationship of CF8 reactivity to enzyme electromorphotype.

Bordetella antigen. The anti-BSA was pre-incubated with an avirulent phase strain, strain 110H, strain 17640, and strain R-5. None of these strains caused a reduction in titer, indicating that the antibody was not being bound by or proteolytically degraded by these *Bordetella* strains.

Discussion

Strains of *B. bronchiseptica* exhibited different levels of reactivity with monoclonal antibody CF8 in an enzyme immunoassay system. Monoclonal antibody CF8 is specific for *Bordetella* fimbriae. This antibody does not react with all *B. bronchiseptica* fimbriae; it reacts only with those bearing an epitope antigenically related to that found on *B. pertussis* serotype 2 fimbriae. Electron microscopic examination of *B. bronchiseptica* strains 110H, 17640, and R-5 revealed no noticeable difference in quantity, length, or diameter of fimbriae, even though these strains express different amounts of the three fimbrial subunit proteins. The 24 kD fimbrial subunit protein was identified by Western blot as being serotype 2 cross-reactive. The difference in reactivity with monoclonal antibody CF8 seen between strains may reflect differing amounts of this protein being produced or being accessible on the bacterial cell surface. This study has clearly demonstrated that a 24 kD protein is associated with the fimbrial serotype 2 determinant. It is not known at this point whether the 21 kD and 22 kD proteins represent subunits of other fimbrial serotypes or are also somehow related to serotype 2 fimbriae.

The differences in reactivity with CF8 correlate with the host species from which each strain was isolated. Strains isolated from pigs and guinea pigs each exhibited a tight range of reactivity with strains from pigs having significantly more reactivity with CF8 than those from guinea pigs. This observation suggests that there may be an increased need for the presence of serotype 2 cross-reactive fimbriae for infection of pigs by *B. bronchiseptica.* At present it is not known if strains which infect pigs exhibit high levels of serotype 2 cross-reactive fimbriae before colonization or if some unidentified

environmental factor present in the porcine respiratory tract induces antigenic switching to this type of fimbriae. Switching of fimbrial serotypes has been documented in *Neisseria* species (322). Robinson observed selection for particular fimbrial serotypes after immunization of mice with *B. pertussis*. He suggested that the recovery of fimbrial serotypes other than those present in the inoculum could be due to fimbrial serotype switching (276).

Canine isolates exhibited a much broader range of reactivity with CF8 than either strains from pigs or guinea pigs. Strains isolated from dogs included both strain 110H, which is highly reactive with CF8, and strain Romark, the least reactive strain tested from any of these three animals. This increased variability is not surprising considering that Musser *et al.* (238) found canine isolates to be more variable in enzyme electromorphotype than isolates from pigs or guinea pigs. Together, these observations indicate that dogs may be capable of being infected with a much wider range of *B. bronchiseptica* strains than other species. An unexplored possibility is that dogs may be more readily infected by strains possessing other, non-serotype 2 cross-reactive, fimbriae. Alternatively, dogs may not induce fimbrial serotype switching by *B. bronchiseptica*. Neither of these possibilities have been examined experimentally.

The Ryan Einot Gabriel Welsch multiple F test and the Bonferoni t tests each placed the canine isolates in a group with the guinea pig isolates. These tests take into account the means and variance of the titers from each group of isolates. The majority of canine isolates did have titers that fell below the pig isolates. This grouping may indicate that isolates from dogs and guinea pigs are more alike than those from dogs and pigs. Pigs may, therefore, be different from dogs in their requirement for the presence of a high level of serotype 2 fimbriae for colonization.

The observed difference in reactivity with monoclonal antibody CF8 between strains from different host species does not correlate with enzyme electromorphotype. Canine isolates in enzyme electromorphotype ET1 are variable in reactivity with CF8 just

as are canine isolates in other electromorphotypes. Porcine isolates in ET6, an enzyme electromorphotype predominately associated with infection of dogs, all exhibit levels of reactivity similar to the other strains from pigs. *B. bronchiseptica* strains from ET16, which were isolated from animals other than guinea pigs, do not fall into the narrow range of reactivity of strains from guinea pigs. This lack of correlation of reactivity with enzyme electromorphotype is interesting since Musser *et al.* (238) found definite correlation between enzyme electromorphotype and host species. This observation suggests that more than one factor may be involved in determination of host specificity. It is also interesting to speculate that if fimbriae are playing a role in determination of host specificity in *B. bronchiseptica*, then the presence or absence of particular fimbrial traits may be responsible for the restriction of *B. pertussis* to human hosts. Both *B. pertussis* and *B. bronchiseptica* produce fimbriae reactive with serotype 2 specific antibodies, but these fimbriae are not necessarily identical because they share this particular epitope.

From the data presented, four major conclusions can be reached: 1) fimbrial protein profiles differ between *B. bronchiseptica* strains, 2) strains isolated from pigs express more CF8-reactive and presumably *B. pertussis* serotype 2 cross-reactive fimbriae than strains isolated from guinea pigs, 3) *B. bronchiseptica* strains isolated from dogs vary greatly in the amount of CF8-reactive fimbriae they express, and 4) expression of CF8-reactive and *B. pertussis* serotype 2 cross-reactive fimbriae does correlate with host species but not with enzyme electromorphotype. These observations are consistent with the idea that serotype 2 fimbriae are involved in determination of host specificity of *B. bronchiseptica.*

CHAPTER THREE

ATTACHMENT OF *BORDETELLA BRONCHISEPTICA*

Introduction

The first step in colonization of the respiratory epithelium of animals by *B. bronchiseptica* is attachment to ciliated cells. In *B. pertussis*, attachment is mediated through the combined effects of several adhesins including FHA, pertactin, and pertussis toxin (50, 194, 231, 289, 332). FHA and pertactin are also believed to be involved in attachment by *B. bronchiseptica* (164, 231). Since *B. bronchiseptica* infects several animal species and *B. pertussis* only naturally infects humans (71), it is possible that the factors involved in adhesion of these two species to host cells may also be different.

In order to study adhesins of *B. bronchiseptica,* it is necessary to have an assay for attachment ability. Several attachment assays have been used by other researchers. The assay most closely paralleling natural infection is colonization of experimentally infected animals. Experimental infections of pigs, mice and other animals have been used to study colonization and onset of disease caused by *B. bronchiseptica* as well as *B. pertussis* (122, 234, 281, 286, 304). Researchers have produced atrophic rhinitis in pigs with *B. bronchiseptica* alone and in conjunction with *Pasteurella multocida* (21, 66, 73, 122, 139, 209, 272, 281, 286). Quantitative measurements of bacterial colony forming units in the trachea, bronchi, and lungs of experimentally infected animals have been used to compare the ability of various mutants to colonize animals (234).

In addition to experimental infection of animals, researchers have used tracheal ring and outgrowth cultures for attachment assays (37, 90). Such assays have the advantage of being able to be more easily worked with in the laboratory than live animals

while still using the natural host tissue for *B. bronchiseptica* infection. Dugal used tracheal ring cultures to examine the attachment of *B. bronchiseptica* (90). He observed thin filaments, presumed to be fimbriae, connecting the bacterial cells to the cilia (90). In addition to organ cultures of tracheal tissue, several cell lines have been used to study *Bordetella* attachment, among which are HeLa cells, WiDR cells, and Vero cells (127, 278, 293, 294). Robinson *et al.* (278) used Vero cells grown on cover slips to measure attachment by *B. pertussis*. Gorringe *et al.* (127) also used Vero cells to measure attachment ability of *B. pertussis*. Pre-incubation of the Vero cells with FHA, pertussis toxin, serotype 2 fimbriae, or serotype 3 fimbriae did not inhibit attachment ability (278). Pre-incubation with FHA actually increased attachment (278). Pre-incubation with FHA increases the amount of FHA present in the medium just as increased production of FHA by the bacterial cells would, causing an increase in attachment. These data support the theory that FHA is the primary adhesin for *B. pertussis* and fimbriae are not involved in attachment by *B. pertussis*.

There have been few comparative studies of *B. bronchiseptica* attachment. This chapter describes the use of an *in vitro* attachment assay to compare attachment ability of nine different *B. bronchiseptica* strains isolated from dogs and a guinea pig. Attachment ability was also compared with fimbrial protein profiles and monoclonal antibody CF8 reactivity.

Materials and Methods

Bacterial Strains, Media, and Growth Conditions

The strains of *B. bronchiseptica* used were described in Chapter 2. Growth conditions were the same as described in Chapter 2. Colonies used in the attachment assays were picked from Bordet-Gengou agar plates and exhibited virulent phase morphology (small, domed, hemolytic in some strains).

Tissue Culture Cells, Medium, and Growth Conditions

Vero cells (African green monkey kidney cells) were provided by the University of Tennessee College of Veterinary Medicine Virology Lab. The seed inoculum was diluted in tissue culture medium so as to give individual cells rather than confluent monolayers after growth. Cells were grown for 24 hours in Dulbecco's Modified Eagle's Medium (DMEM, Gibco BRL, Gaithersberg, MD) with 10% fetal calf serum (Hyclone) in 8 chamber Lab-Tek slides (Nunc, Naperville, IL) at 37°C in an 8% CO_2 incubator. Viability of the cells was determined using a Trypan Blue exclusion assay (19).

Attachment Assay

Vero cells were washed with 200 µl of complete DMEM (DMEM medium [Gibco], with 88 units/ml penicillin G [Gibco], 88 µg/ml streptomycin sulfate [Gibco], 200 mM glutamine [Sigma, St. Louis, MO], 2 µl/l β-mercaptoethanol [BioRad], and 10% fetal calf serum [Hyclone]) three times before adding bacteria. Some experiments used incomplete DMEM (without serum). *Bordetella* exhibiting virulent phase morphology were picked from BG plates and suspended in phosphate buffered saline (PBS, 136 mM NaCl, 100 mM Na_2HPO_4, 2 mM KCl, 1 mM KH_2PO_4, 5 mM $MgCL_2$) to an optical density of 0.1 at 595 nanometers. Various dilutions of bacteria in complete DMEM were tested and 200 µl of the dilution was added to each well.

The slide containing the Vero cells and *B. bronchiseptica* was incubated for times varying from 30 minutes to 2 hours at 37°C in an 8% CO_2 incubator. After incubation, the solution containing the bacteria was removed and the cells were washed three times with 200 µl of complete DMEM. The cells were then fixed to the slide with 95% ethanol and stained. Various staining methods were tested including Giemsa stain, 1% aqueous Crystal Violet, Diff-Quik stain (Baxter, McGaw Park, IL), and a 1:2 dilution of Gram's Crystal Violet (Difco) in water. After staining, the slide was rinsed in distilled water and

the chambers removed. Bacteria attached to Vero cells were observed and counted microscopically at a magnification of 1:1000 with oil immersion. Each assay was performed in triplicate and results are reported as the mean number of bacteria attached per 50 Vero cells ± standard deviation.

Results

Research in this laboratory has focused on the involvement of fimbriae in attachment by *B. bronchiseptica*. As described in Chapter 2, strains of *B. bronchiseptica* vary in the level of serotype 2 fimbriae they produce. Strains of *B. bronchiseptica* are also variable in fimbrial protein profile. In order to compare the level of serotype 2 fimbrial expression with attachment ability, an *in vitro* attachment assay was needed. Therefore, we developed a Vero cell attachment assay similar to those previously used for *B. pertussis* to determine if fimbriae are involved in *B. bronchiseptica* attachment.

Development of Assay

Staining Conditions

In order to determine the best staining method to differentiate bacteria from Vero cells, *B. bronchiseptica* strain 110H was grown overnight on BG agar and a heavy suspension was made in DMEM. Vero cells were washed three times with complete DMEM and the suspension of bacteria was added to the wells. After incubation for 30 minutes at 37°C, the cells were stained and examined microscopically. Six different staining methods were used including Giemsa stain for one minute and for 10 minutes, a 1:2 dilution of Gram's Crystal Violet for 10 minutes, a 1:2 dilution of Gram's Crystal Violet for one minute, 1% aqueous Crystal Violet for 1 minute, and Diff-Quik stain as instructed by the manufacturer. After examination under a light microscope at a magnification of 1000x and using oil immersion, staining with a 1:2 dilution of Gram's

Crystal Violet for one minute was determined to give the best differentiation between the bacteria and the Vero cells. Slides stained with Giemsa stain or Crystal Violet for 10 minutes were stained too dark for differentiation of the bacteria from the Vero cells. The Giemsa stain for one minute and the Diff-Quik stain stained everything too light. It was possible to differentiate between cells and bacteria with the 1% Crystal Violet stain for 1 minute.

Dilution of Bacteria and Incubation Time

In order to compare attachment of *B. bronchiseptica* strains, a standard concentration of bacteria must be used for each strain. We arbitrarily chose to use a suspension of actively growing cells scraped off BG agar plates in PBS at an optical density of 0.1 at 595 nanometers. Two-fold dilutions of this suspension were made in complete DMEM and added to slides containing Vero cells to determine what dilution should be used to obtain a countable number of attached bacteria. In order to determine the best incubation time, the slides were incubated for various times ranging from 30 minutes to 2 hours in a CO_2 incubator. Slides were washed, stained with Crystal Violet, and examined microscopically. The bacteria attached to 50 Vero cells were counted (table 5). Incubation times higher than 30 minutes resulted in numbers of bacteria which were difficult to count accurately in the 1:2 dilution. At higher dilutions, the longer incubation times resulted in more bacteria being attached and still being countable. The 1:2 dilution and 30 minute incubation time were chosen to use for future assays. This incubation time is less than the generation time for *B. bronchiseptica* and therefore, bacterial multiplication during incubation is less likely to affect the number of bacteria counted in the assay.

Table 5: Effect of incubation time on *B. bronchiseptica* attachment

	Attached Bacteria[1]			
Dilution[2]	**30 min.**	**1 hr.**	**1.5 hr.**	**2 hr.**
1:2	905	2442	3288	2666
1:4	338	612	1102	1315
1:8	118	252	555	374
1:16	33	100	228	65

[1] Attached bacteria per 50 Vero cells

[2] Dilution of *B. bronchiseptica* strain 110H in complete DMEM

Standard Assay Conditions

As a result of these preliminary experiments, a standard attachment assay protocol was developed for use with various strains of *B. bronchiseptica*. Conditions chosen were use of a 1:2 dilution of *B. bronchiseptica* originally suspended in PBS to an $O.D._{595}$ of 0.1 in complete DMEM incubated with Vero cells for 30 minutes and stained with a 1:2 dilution of Gram's Crystal Violet in water for one minute. The number of *Bordetella* attached to 50 Vero cells exhibiting typical live cell morphology was determined microscopically. Dead cells are small, rounded, and stain darker than live cells. The nucleus of dead cells is often not visible due to the dark staining of the entire shrunken cell. Figure 13 shows attachment by *B. bronchiseptica* strain 110H under these conditions.

Attachment by Avirulent Phase *B. bronchiseptica*

Since attachment by *B. bronchiseptica* is believed to be mediated through the action of several adhesins which are Bvg regulated, there should be a difference in attachment ability between virulent phase and avirulent phase cells. To determine if this difference in attachment ability could be observed in this attachment assay, the virulent phase strain 110H and its avirulent phase variant were used in an attachment assay under the conditions determined above. Strain 110H attached to the cells approximately 10 times better than strain 110NH (table 6). Avirulent phase *Bordetella*, however, did adhere to the glass slide better than virulent phase strains. Almost no adherence to the slide was observed with virulent phase organisms. This adherence of avirulent phase cells to the slide provided a method of determining if strains tested in the attachment assay were in the virulent phase.

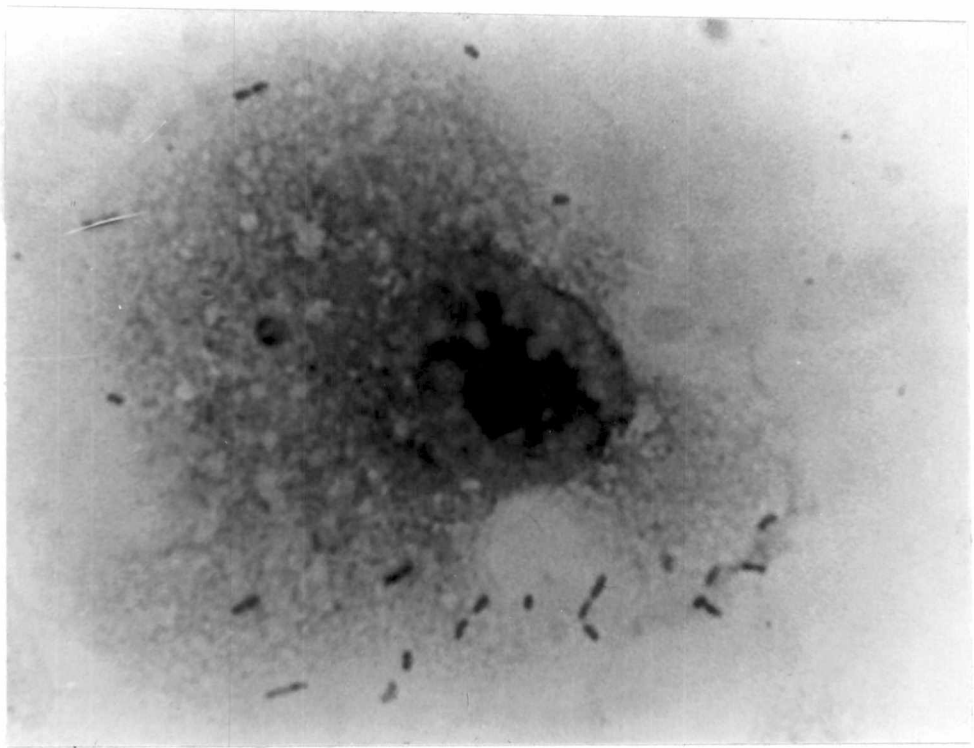


Figure 13: *B. bronchiseptica* attachment. *B. bronchiseptica* strain 110H attached to a Vero cell using the standard conditions for the attachment assay, as outlined in the text. Magnification 1000x.

Table 6: Attachment of *B. bronchiseptica* strains to living and dead cells

		Attached bacteria[1]	
Strain	**Dilution[2]**	**Live Cells[3]**	**Dead Cells[4]**
110H	1:2	1022	1125
	1:4	570	476
	1:8	67	21
	1:16	63	26
110NH[5]	1:2	178	151

[1] Bacteria attached to 50 Vero cells

[2] Dilution of *B. bronchiseptica* strains in complete DMEM

[3] Cells exhibiting live cell morphology as described in the text

[4] Cells exhibiting dead cell morphology as described in the text

[5] Avirulent phase variant of strain 110H

Attachment to Living and Dead Cells

When examining the Vero cells microscopically, some of the cells were rounded and small. These cells stained darker than the other elongated, larger cells. Cells viability was determined using a Trypan Blue exclusion assay. This assay indicated that the smaller, rounded cells were non-viable. To determine if there was a difference in attachment of *Bordetella* to living or dead cells, the number of bacteria attached to 50 live and 50 dead cells was determined (table 6). No difference was seen between the number of bacteria attached to living and dead cells with either virulent phase or avirulent phase *Bordetella.*

Attachment by *B. bronchiseptica* Strains

Eight strains of *B. bronchiseptica* originally isolated from dogs and one strain isolated from a guinea pig were tested in the attachment assay. These strains were from the three most common electromorphotypes as determined by Musser *et al* (238). As can be seen in table 7, attachment ability varied greatly between strains. Strains 17640 and 110H attached best in this assay. Interestingly, strain Romark attached at a level similar to the avirulent phase strain 110NH. There appears to be no correlation between enzyme electromorphotype and attachment to Vero cells since strains from both ET 1 and ET 6 exhibit various levels of attachment. There also seems to be no correlation between reactivity with monoclonal antibody CF8 in the pre-adsorption ELISA (Chapter 2) and attachment to Vero cells. Strain Meisel, which reacted well with CF8, attached poorly to Vero cells.

Attachment Without Serum

Four strains (110H, 17640, R-5, and 110NH) which attached at high, intermediate, and low levels were used in attachment assays performed with medium which did not contain fetal calf serum (table 8). Attachment of all strains was reduced to levels similar

Table 7: Attachment of *B. bronchiseptica* strains

Strain	Host	ET[1]	Attached Bacteria [2]
110H	Dog	1	995 ± 132
110NH[3]	Dog	1	186 ± 88
64-C-0406	Dog	1	632 ± 28
17640	Dog	6	1037 ± 170
JS 34682	Dog	6	738 ± 37
Romark	Dog	6	396 ± 30
BB Ithaca	Dog	6	264 ± 18
Meisel	Dog	6	155 ± 13
R-5	Guinea Pig	16	544 ± 21

[1] Determined by Musser *et al.* (238)

[2] Mean number of attached bacteria per 50 Vero cells in three trials ± standard deviation

[3] Avirulent phase variant of strain 110H

Table 8: Attachment of *B. bronchiseptica* strains without serum

	Attached Bacteria[1]	
Strain	**With Serum[2]**	**Without Serum[3]**
110H	995 ± 132	171 ± 79
110NH	186 ± 88	192 ± 53
17640	1037 ± 170	205 ± 52
R-5	544 ± 21	253 ± 117

[1] Mean number of attached bacteria per 50 Vero cells in three trials ± standard deviation.

[2] Assay performed using complete DMEM containing 10% fetal calf serum.

[3] Assay performed using DMEM without added fetal calf serum.

to that of the avirulent phase strain 110NH. These data indicate that attachment of *B. bronchiseptica* to Vero cells may be serum dependent.

Discussion

Bordetella attachment has been studied in experimental infections of animals, tracheal outgrowth cultures, and cells in tissue culture (90, 234, 278). Due to their widespread availability, ease of handling, and possession of cellular receptors similar to those seen on other cell types, Vero cells have been commonly used to study attachment by *B. pertussis* (127, 278). These cells are useful in initial studies of attachment before performing assays with tracheal rings or whole animals. This chapter describes the use of a Vero cell attachment assay with *B. bronchiseptica.*

Using the conditions determined in the preliminary experiments, several strains of *B. bronchiseptica* were tested in the attachment assay. An avirulent phase strain, as expected, attached poorly. Avirulent phase strains do not produce any of the *Bordetella* adhesion factors (78, 129, 296, 297, 351). Virulent phase strains of *B. bronchiseptica*, however, do produce these adhesins including FHA and pertactin (50, 194, 231, 289, 332). The strains of *B. bronchiseptica* tested attached at various levels. Strain 110H and strain 17640 attached best, exhibiting attachment of approximately 1000 bacteria per 50 Vero cells. Other strains, such as R-5 and 64-C-0406, exhibited levels of attachment intermediate between strains 110H and the avirulent phase strain.

Some strains, e.g. Meisel and BB Ithaca, exhibited attachment levels similar to that of the avirulent phase strain. Attachment at a level similar to avirulent phase strains should not be due to the presence of avirulent phase bacteria in the original suspension. *Bordetella* were grown on BG plates and only colonies exhibiting a virulent phase morphology were picked for use in the attachment assays. Also, the avirulent phase strain 110NH exhibited attachment to the slide which was not observed with any of these strains.

All of the *B. bronchiseptica* strains tested required the presence of serum in the medium for attachment at a level higher than that of the avirulent phase strain. This serum requirement supports the theory that adhesins other than fimbriae are involved in attachment. FHA and pertactin both mediate attachment through the RGD domain as well as other carbohydrate binding domains (194, 195). Fibronectin, a component of the extracellular matrix and which may be present in serum, binds to eukaryotic cells through the RGD sequence (262, 266, 270) It is interesting to speculate that these adhesins may utilize serum components to mediate attachment. Alternatively, serum components may be required for maintenance of surface receptors.

It is not surprising that the strains used in the attachment assay are variable in their levels of attachment. Most of the *B. bronchiseptica* strains tested were initially isolated from dogs. Musser *et al.* (238) found that canine strains were more variable than strains from pigs or other animals. The pre-adsorption ELISA used in Chapter 2 to measure serotype 2 fimbriae expression levels also indicated a greater variability in strains isolated from dogs than in strains from other animals. Therefore, if fimbriae are involved in attachment, then one would expect a difference between strains based on the amount of fimbriae expressed on their cell surface.

No correlation, however, was seen between attachment and monoclonal antibody CF8 reactivity in the pre-adsorption ELISA described in Chapter 2. The strain which attached best in this attachment assay, 17640, exhibited only a 66% reduction in titer in the ELISA (table 3 in Chapter 2) and some strains which attached poorly exhibited much higher percent reductions in the ELISA assay indicating that these strains possessed more serotype 2 fimbriae. For example, strain Meisel attached at a level equivalent to that of the avirulent phase strain 110NH but exhibited 89% reduction in titer in the pre-adsorption ELISA which is almost as much as strain JS 34682 (90% reduction in titer) that attached well (738 bacteria per 50 Vero cells). Therefore, it is not possible to

correlate serotype 2 fimbrial expression as measured by the pre-adsorption ELISA with attachment.

We also examined the fimbrial protein profiles of each of the strains tested in the attachment assay to determine if the presence of certain fimbrial subunits correlates with attachment ability. Monoclonal antibody CF8 recognizes an epitope present on non-denatured fimbrial polymers and does not recognize denatured fimbrial subunit proteins. Fimbrial protein profiles for each of the strains used in the attachment assay are shown in figure 5 in Chapter 2. Four major patterns of fimbrial proteins can be observed. Strain 110H possesses all three fimbrial subunit proteins in approximately equal proportions and attaches well. Strains R-5, JS 34682, and 64-C-0406 exhibit a profile with a large amount of the smallest band, an intermediate amount of the middle band, and almost none of the upper fimbrial subunit protein band. These strains all exhibit intermediate levels of attachment. Strains BB Ithaca, and Meisel each exhibit a fimbrial protein profile that shows only the lower band. The other 2 bands are not visible on the Coomassie Blue stained gel. These strains both attach poorly with strain Meisel attaching at a level similar to that of the avirulent phase strain 110NH. Strains 17640 and Romark both have a protein profile with large amounts of the lower band and smaller amounts of the other two bands. Strain 17640 attaches well. In fact, it attached better than all the other strains tested. Strain Romark, however, attached at a low level as did the other strains showing a large amount of the lower band and much less of the other two bands. Therefore, it is difficult to determine if there is any correlation between fimbrial protein profile and attachment. Further experiments utilizing ciliated cells may more clearly define this relationship between fimbrial protein profile and attachment.

In summary, a Vero cell attachment assay was developed and used to measure attachment ability of *B. bronchiseptica.* Attachment of *B. bronchiseptica* strains varied in this assay. Attachment was not correlated with monoclonal antibody CF8 reactivity as

determined by the pre-adsorption ELISA in chapter 2 but, with the exception of strain 17640, did correlate with fimbrial protein profile.

CHAPTER FOUR

CLONING AND CHARACTERIZATION OF A SEROTYPE 2 FIMBRIAE-RELATED GENE FROM *BORDETELLA BRONCHISEPTICA*

Introduction

All *Bordetella* species produce fimbriae on the cell surface in virulent phase (83, 166, 194, 366). *Bordetella* fimbriae are believed to be filaments consisting of polymers of one major subunit protein; however, some minor subunits which have accessory functions have also been found in other bacteria (83, 233, 366). In *Bordetella*, several genes are required for production of fimbriae (202, 204, 253, 358, 359, 360). Three of these genes are located in a fimbrial gene cluster immediately downstream of the FHA operon. These three genes, *fimBCD*, encode proteins involved in the regulation of fimbrial expression, transport of fimbrial subunits, and assembly of the fimbrial filament (204, 360). In addition, there has been speculation that *fimD* may encode a minor fimbrial subunit protein involved in adhesion (358). The genes for the major fimbrial subunits, *fim2* and *fim3*, are located in separate operons on the chromosome (202, 232, 317). All of these genes and the silent fimbrial genes, *fimA* and *fimX*, have been cloned and sequenced from *B. pertussis* and is also present in *B. bronchiseptica* (202, 204, 232, 253, 294, 360).

Mooi *et al.* (233) suggested that *B. bronchiseptica* carries multiple copies of the major fimbrial subunit genes, *fim2* and *fim3*, based on hybridization with probes derived from the *B. pertussis* sequences. Savelkoul (294) sequenced one copy of the *fim2* and *fim3* genes from *B. bronchiseptica* and found significant similarity with the corresponding *B. pertussis* sequences. Afimbriated mutants have been produced in *B. pertussis* to study the effect of fimbriae on attachment (234). However, due to the existence of multiple copies

of the fimbrial genes in *B. bronchiseptica*, the production of afimbriated mutants is more difficult. Therefore, in order to study the effect of fimbriae in attachment by *B. bronchiseptica*, we identified a strain (R-5) which attaches at a low level and produces low levels of serotype 2 fimbriae (Chapters 2 and 3). A cloned fimbrial gene could be introduced into this strain and its effects on attachment and fimbrial expression level observed. The serotype 2 fimbrial gene was chosen since previous experiments, described in chapter 3, indicated that the 24 kD fimbrial subunit protein may be required for attachment and this protein was determined by Western blot to be antigenically cross-reactive with serotype 2 fimbriae of *B. pertussis*. Since we did not have access to the previously cloned and sequenced *B. bronchiseptica fim2* gene, experiments were undertaken to clone the *fim2* gene from *B. bronchiseptica* strain 110H, which produces the 24 kD serotype 2 fimbrial protein at a level similar to that of the other fimbrial subunit proteins and attaches well.

Materials and Methods

Bacterial Strains, Media, and Growth Conditions

B. bronchiseptica strains used and growth conditions have been described previously in this dissertation (Chapter 2). *B. bronchiseptica* strains carrying pLAFR1-based plasmids were selected and maintained on Brucella agar containing 15 μg/ml tetracycline (Sigma) and 20 μg/ml furaltadone. *B. bronchiseptica* strains carrying pBBR1MCS were maintained on Brucella agar containing 30 μg/ml chloramphenicol and 20 μg/ml furaltadone.

E. coli strains were grown on Luria Bertani agar, Miller formula, prepared from LB Broth, Miller (Difco) with 1.5% Bacto-agar (Difco) for 18 to 24 hours at 37°C. *E. coli* strain DH-1 was grown without antibiotics. *E. coli* HB101 strains containing the plasmids pLAFR1 and pRK2013 were grown on LB agar containing 15 μg/ml tetracycline and 20

μg/ml kanamycin (Sigma), respectively. *E. coli* strains carrying pLAFR1 based plasmids with *Bordetella* inserts were grown on LB medium with 15 μg/ml tetracycline.

Chromosomal DNA Isolation

Chromosomal DNA was isolated from a two day Brucella broth culture of *B. bronchiseptica* strain 110H using the method of Meade *et al.* (220). Cells were centrifuged at 10,410 x g for ten minutes, suspended in 10 ml TES buffer (10 mM Tris HCl, 25 mM EDTA, 150 mM NaCl), and incubated with 100 mg lysozyme (Sigma) for one hour at 37°C. One milliliter of 25% (w/v) sodium dodecyl sulfate (SDS) and 0.5 ml of 5 mg/ml Proteinase K (Sigma) in 0.01 M Tris HCl, pH 8.0 (predigested at 37°C for 15 minutes) were added and the solution was incubated at 65°C for one hour. The lysate was then diluted to 30 ml with TE buffer (10 mM Tris HCl, pH 7.4, 1.0 mM EDTA) and cesium chloride was added to a refractive index of 1.399. After centrifugation at 73,950 x g for 48 hours, the chromosomal DNA was collected as the viscous fraction. The DNA was precipitated with 2.5 volumes ethanol and suspended in TE buffer.

Isolation of Plasmid DNA and Construction of Genomic Library

pLAFR1 plasmid DNA (108) was isolated by the alkaline lysis method of Birnboim (42) followed by density gradient centrifugation in CsCl as previously described (19). The chromosomal DNA was partially digested with *Eco*RI (Gibco BRL) by varying incubation time. Digestion was stopped by adding 1/20 volume of 0.25 M EDTA and incubation for 10 minutes at 65°C. Fragments ranging from 15-30 kb were isolated on an 8 ml 10-40% sucrose gradient (210). These fragments were ligated to *Eco*RI-digested, calf intestinal alkaline phosphatase-treated (19, Boehringer Mannheim, Indianapolis, IN) pLAFR1 DNA and packaged into bacteriophage lambda heads using an *in vitro* packaging kit (Promega, Madison, WI) according to the manufacturer's instructions. *E. coli* DH-1 was infected with phage from the packaging reaction (210) and

plated on LB agar containing 15 μg/ml tetracycline to select for bacteria containing the cosmid pLAFR1. 1,316 colonies were picked with sterile toothpicks and placed in wells of microtiter plates containing LB broth with 15 μg/ml tetracycline and 125 μl/ml dimethylsulfoxide (DMSO), and stored at -80°C. Plasmid DNA was isolated from transconjugants using the alkaline lysis miniprep procedure (19, 42).

Restriction Enzyme Digestion and Gel Electrophoresis

DNA restriction and modifying enzymes were purchased from Gibco BRL or New England Biolabs (Beverly, MA) and used according to the manufacturers specifications. Agarose gel electrophoresis was carried out using 1% (w/v) Seakem GTG agarose (FMC BioProducts, Rockland, ME). Gels were run at 20 volts for 20 hours at room temperature in Tris Acetate EDTA electrophoresis buffer (TAE, 210) and stained in 0.5 μg/ml ethidium bromide. Mini-gels were run at 80 volts for approximately 2 hours in Tris Borate EDTA electrophoresis buffer (TBE, 210) and stained in ethidium bromide as for other gels.

Conjugation

Conjugation involving transfer of plasmid DNA from *E. coli* to *B. pertussis* has been previously described (352). Broth cultures of *Bordetella*, *E. coli* containing the plasmid to be transferred, and *E. coli* containing the helper plasmid pRK2013 (Freidman 82) were mixed in equal volumes, incubated at 37°C for 4 hours, and plated on Brucella agar with 15 μg/ml tetracycline to select for the cosmid and 20 μg/ml furaltadone to select for *Bordetella.*

Oligonucleotide Probes and Hybridization Conditions

The entire library was plated on LB agar with 15 μg/ml tetracycline and transferred to nitrocellulose filters (210). The bacteria were lysed and the DNA denatured

by soaking the filters in 0.5 M NaOH, 1.5 M NaCl. The alkaline solution was neutralized by successive incubation of the filters in 0.5 M Tris HCl (pH 8.0) and then 2X SSC (0.3 M NaCl, 30 mM sodium citrate, 0.1% SDS). After baking at 80°C for 90 minutes, the filters were used for hybridization with an oligonucleotide probe (GAT/C GAT/C GGC ACC ATC GTC ATC ACC GGC AC), originally used by Mooi *et al.* (233), corresponding to the nucleotide sequence encoding the first ten N-terminal amino acids of the *Bordetella pertussis* serotype 2 fimbrial subunit, Fim2. The probe was synthesized by the University of Tennessee Analytical Services Facility and purified by HPLC. The probe was labeled with digoxigenin-11-dUTP (Boehringer Mannheim) and dATP by 3' tailing using terminal deoxynucleotidyl transferase (TdT, Gibco BRL) as per the instructions supplied by Boehringer Mannheim. Hybridization was carried out at 60°C overnight with 40 ng/ml probe DNA in hybridization solution (5X SSC, 1% Blocking reagent, 0.1% N-Lauroylsarcosine, 0.02% SDS). Filters were washed twice for five minutes with 0.5% SSC, 0.1% SDS at room temperature and twice at 60°C with 0.1X SSC, 0.1% SDS. Colorimetric detection of hybridization was performed using the Genius Nucleic Acid Detection Kit (Boehringer Mannheim) according to the manufacturer's instructions. Color development was performed at 37°C in the dark.

DNA was obtained by plasmid minipreps (19) from seven colonies which reacted with the oligonucleotide probe and from 3 colonies which did not react with the oligonucleotide as negative controls. This DNA was used in dot blot hybridization reactions. Two microliters of each DNA solution was dotted onto nitrocellulose strips. The DNA was denatured, baked onto nitrocellulose strips, hybridized with the probe, and detected as described above. DNA from plasmids was also probed by the Southern blotting procedure (313). Plasmids were digested with restriction enzymes, electrophoresed on 1% agarose gels, and transferred to Genescreen Plus® membranes (Dupont, Boston, MA) by electroblotting as described by the manufacturer. The membranes were probed using the same methods as described for colony blots. Dot blots

and Southern blots were also performed using an oligonucleotide probe specific for the 3' end of the *B. pertussis fim2* gene (ACT TAT GTG GGT/C TTT/C TTC GTG GTC TAC CC). This 29 base probe was designed from the *B. pertussis fim2* sequence, with some degeneracy, based on codon usage in *Bordetella*. The oligonucleotide was obtained from Oligos, Etc. (Guilford, CT).

In order to determine if pGB710 contained genes for the other known *Bordetella* adhesins, FHA and pertactin, DNA from cosmid pGB710 was hybridized with oligonucleotide probes against each end of the *B. pertussis fhaB* gene (CCT ATT TGT TGG C/TTT CAT AGA AGA G/CCC GG and ATG AAC ACG/C AAC CTG TAC AGG CTG GTC/G TT) and to each end of the *B. pertussis* pertactin gene (CCA GCT GTA G/CCG GTA GCC CGC GTG A/GAA GG and GAC TGG AAC AAC CAG TCC/G ATC GTC/G AAG AC). These probes were designed from the published sequences of *B. pertussis fhaB* and *prn* (52, 70). Oligonucleotides were obtained from Oligos, Etc. Hybridization was carried out using Genius® non-radioactive labeling and detection as described above.

Cosmid pGB710 was also used in Southern blots probed with the two oligonucleotides derived from the *fim2* gene to identify fragments for subcloning. For these blots, the cosmid DNA was digested with thirteen different restriction enzymes and run on a 1% agarose gel as described above. After electrophoresis, the DNA was transferred to BioTrans nylon membrane (ICN, Costa Mesa, CA) by vacuum blotting using an LKB VacuGene Apparatus (LKB, Bromma, Sweden). Briefly, the gel was UV irradiated for 30 seconds, and immersed in 0.4N NaOH. A vacuum of 50 psi was applied for 30 minutes to transfer the DNA from the gel to the membrane. After transfer, the membrane was exposed to UV radiation for 30 seconds to crosslink the DNA to the nylon and allowed to air dry.

Blots were prehybridized at 60°C for 2 hours in the hybridization solution described above. Oligonucleotide probes were labeled with α^{32}P dCTP by 3' tailing with

TdT using the reaction conditions described for the non-radioactive labeling. Labeled probe was separated from non-incorporated nucleotide using Nuc-Trap Push Columns (Stratagene, La Jolla, CA) as instructed by the manufacturer. Two microliters of the labeled probe was counted by Cerenkov counting using a Beckman LS7000 liquid scintillation counter. The labeled probe was added to the hybridization bags at a concentration of 40 ng/ml as for the non-radioactively labeled probe and hybridized overnight at 60°C. Blots were washed as described above and exposed to Kodak X-Omat AR film with an intensifying screen at -80°C.

Subcloning

DNA fragments of cosmid pGB710 identified as hybridizing with both serotype 2 fimbriae oligonucleotide probes were isolated from agarose gels by electroelution (19). Briefly, the bands were cut from the gel and placed into dialysis tubing washed with TAE buffer. The tubing was filled with TAE and subjected to an electric current of 20 volts for 24 hours. The polarity was then reversed and an electric current of 60 volts was applied for one minute to remove DNA from the interior side of the dialysis tubing. The liquid in the tubing was recovered and the tubing was washed with 200 µl of TAE which was mixed with the original solution. The DNA was precipitated by the addition of 1/10 volume of 3 M sodium acetate and 3 volumes of absolute ethanol.

Vector DNA's were digested with the appropriate restriction enzymes and treated with calf intestinal alkaline phosphatase as described above. Vector DNA used included pLAFR1, pBBR1MCS, pMMB207, and pMMB208. pBBR1MCS is derived from a naturally occurring *B. bronchiseptica* plasmid designated pBBR1. The multiple cloning site of pBluescript and a chloramphenicol resistance gene have been added. This plasmid was kindly provided by Marty Roop (Louisiana State University, Shreveport, LA). Plasmids pMMB207 and pMMB208 contain the *tac* promoter on opposite ends of a multiple cloning site allowing cloning of genes in each orientation for expression from

this promoter. These plasmids have been used before in *Bordetella* for expression of the adenylate cyclase toxin genes from the *tac* promoter (214). These plasmids were obtained from ATCC (Rockville, MD).

The electroeluted DNA was ligated into the vector and the plasmid was transferred into *E. coli* strain DH-5α by electroporation using a BioRad Gene Pulser Apparatus (BioRad). Cells were prepared for electroporation and electroporation was carried out using the procedure described in the BioRad Gene Pulser User's Manual. Electroporation was carried out at 80 kV with the 20 μF capacitor and the 200 Ω resistor using 0.1 cm gap electroporation cuvettes (BioRad). After pulsing, the cells were suspended in 1 ml SOC (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) and incubated for 1 hour at 37°C with shaking. After this recovery period, the cells were plated on selective medium and incubated overnight at 37°C. Subclones were screened by mini-prep and restriction enzyme analysis (19).

The 1.5 kb *Bam*HI fragment and 2.5 kb *Pvu*II fragment of pGB710 which had been subcloned into pBluescript were cut out of pBluescript using *Hind*III and *Eco*RI which cut on either side of the insert site. These fragments were ligated into the corresponding restriction enzyme sites on pMMB207 and pMMB208 producing plasmids with the *Bordetella* fragment in each orientation with respect to the *tac* promoter. Ligation, electroporation, and selection were performed as described above.

Immunological Assays

Transconjugants were used in dot blot assays. Cells were scraped off plates and a heavy suspension was made in phosphate buffered saline (PBS). Turbidity of suspensions from each strain to be tested was compared visually and adjusted to an equivalent level. Five microliters of the cell suspensions were dotted onto nitrocellulose strips, and probed

with heat inactivated (56°C, 30 minutes) monoclonal antibody CF8 at a 1:500 dilution as described in Chapter 2.

Sequencing

Single pass primer extension reactions were carried out by Lark Sequencing Technologies (Houston, TX) using standard Sanger dideoxy sequencing conditions (19). The primers used were the oligonucleotide described above which is specific for the 5' end of the *B. pertussis fim2* gene and a second primer (CCG GCA CCA TCA CCG AC) designed by Lark Sequencing Technologies based on the *B. bronchiseptica fim2* sequence from Genbank (accession number X74119).

Results

Construction of *B. bronchiseptica* 110H Genomic Library

B. bronchiseptica strain 110H produces a large amount of the 24 kD fimbrial subunit protein in fimbrial protein profiles (Chapter 2), and the presence of this protein correlates with serotype 2 fimbrial antibody reactivity (Chapter 2). In addition, the presence of this protein may correlate with attachment ability (Chapter 3). Therefore, cloning of the gene for this fimbrial protein would be useful in studying attachment. In order to clone this gene, a genomic library was produced in the cosmid pLAFR1. *B. bronchiseptica* strain 110H chromosomal DNA was isolated and partially digested with *Eco*RI. DNA fragments ranging in size from 15 to 30 kb were selected on a sucrose gradient and cloned into pLAFR1. The library consisted of 1,316 colonies with an average insert size of 22 kb. Using the formula given by Maniatis (210) and assuming the *B. bronchiseptica* genome to be approximately 5,000 kb in length, there is a greater than 99% probability of any given sequence being present in the library.

Screening for Fimbrial Related Genes

After construction of the library and selection on LB agar with tetracycline, colonies were transferred to nitrocellulose filters and probed with an oligonucleotide probe specific for the *B. pertussis* serotype 2 fimbrial subunit structural gene, *fim2*. This probe has been used previously by Mooi *et al.* (233)and reacts with a single DNA fragment from the chromosome of *B. pertussis.* Since *B. pertussis* and *B. bronchiseptica* are closely related and the fimbrial sequences are highly conserved in these species, this probe should be specific for fimbrial genes from *B. bronchiseptica* as well. Mooi *et al.* (233) found that this probe does hybridize to four to six fragments of chromosomal DNA from *B. bronchiseptica*. Each of these fragments are believed to be fimbrial genes indicating that *B. bronchiseptica* may possess multiple copies of the fimbrial genes (233). This probe reacted with seven colonies from the *B. bronchiseptica* 110H genomic library. Figure 14 shows a typical colony blot with a positive reaction visible.

However, non-radioactive DNA detection methods can be affected by proteins and other components present in colony blots (180, 221, 274, 312) and often require the use of proteinase K to prevent false positive reactions. To verify that these positive reactions were actually due to hybridization of the probe, plasmid DNA was isolated from each of the colonies giving positive reactions, dotted onto nitrocellulose strips, and again probed. A strong reaction was obtained for the DNA from five of the colonies which had appeared to give positive reactions in the colony blot, while none of the negative control DNA spots reacted (Figure 15).

Effect of Clones on Serotype 2 Fimbriae

The five cosmids which reacted with the oligonucleotide probe were transferred into *B. bronchiseptica* strains 17640 and R-5 by conjugation and selected on Brucella agar containing tetracycline and furaltadone. Strains 17640 and R-5 were determined to produce less *B. pertussis* serotype 2 cross-reactive fimbriae than strain 110H (Chapter 2).

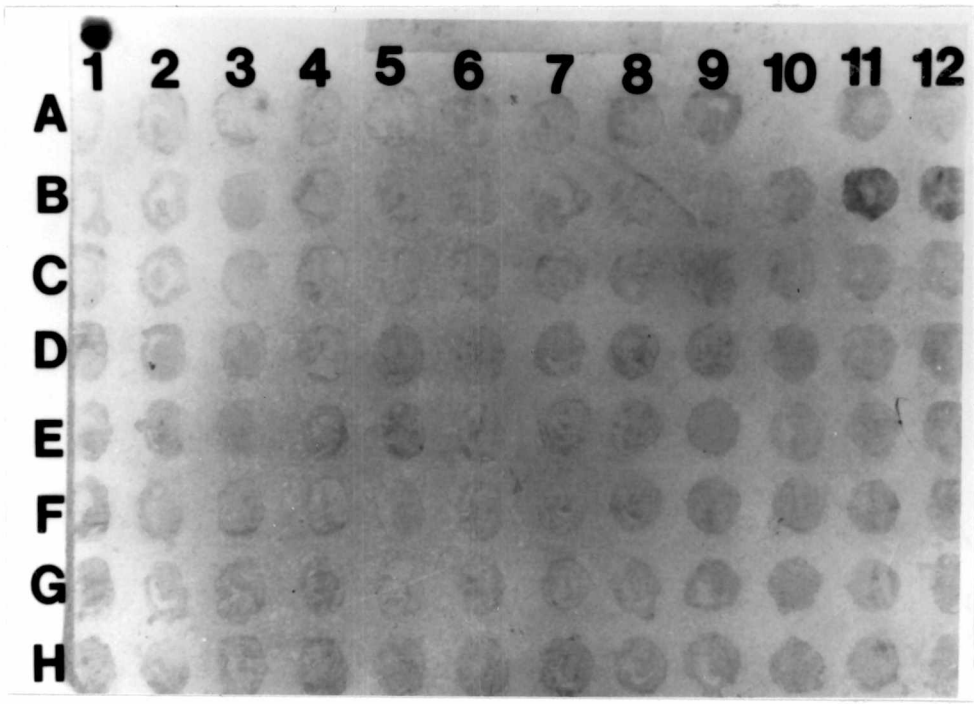


Figure 14: Colony blot of *B. bronchiseptica* strain 110H genomic library. The library was probed with a serotype 2 fimbrial subunit gene oligonucleotide probe. The upper left corner shows 110H chromosomal DNA added as a positive control. The colonies in positions B11 and B12 show positive reactions with the probe.

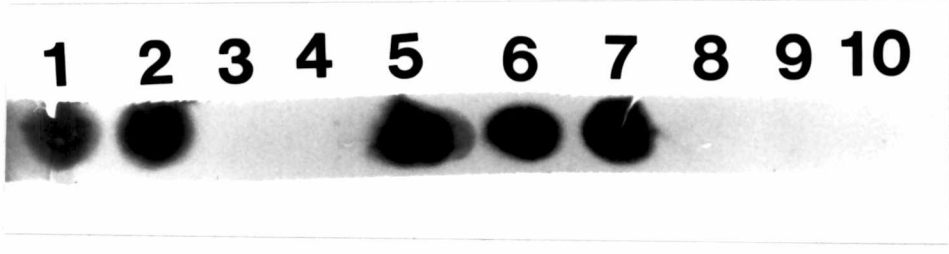


Figure 15: Dot blot hybridization with *B. pertussis fim2* probe. Two microliters of plasmid DNA isolated by the miniprep procedure were applied to each dot. Dots 1, 2, 5, 6, 7 contain clones that had previously hybridized with the probe in colony blots. Dot 1) pGB84 2) pGB612, 3) pGB18, 4) pGB811, 5) pGB710, 6) pGB85, 7) pGB611, 8) pGB12, 9) pGB211, 10) pGB126.

If the genes carried on the cosmids are serotype 2 fimbrial structural genes or genes which affect expression of serotype 2 fimbriae, then the presence of these clones in strains 17640 and R-5 should increase their reactivity with monoclonal antibody CF8 which is specific for serotype 2 fimbriae. In order to test these clones for serotype 2 fimbrial expression, dot blot immunoassays were performed with suspensions of strains 17640 and R-5 carrying the five cosmids which had reacted with the oligonucleotide probe. The parental strains 17640 and R-5 do not react with monoclonal antibody CF8 in dot blots. However, strains carrying three of the five clones did react with CF8 in each *B. bronchiseptica* strain (figure 16). The same experiment was performed using monoclonal antibody BPF2 specific for *B. pertussis* serotype 2 fimbriae and identical results were obtained (figure 17). Therefore, three of the five cosmids tested induced an increase in expression of serotype 2 fimbriae on the surface of *B. bronchiseptica* strains 17640 and R-5.

Characterization of Cosmid Clones

Plasmid DNA was isolated from each of these three clones that complemented *B. bronchiseptica* strains 17640 and R-5. The plasmid DNA was digested with *Eco*RI and used in Southern blots with the same oligonucleotide probe as was used for the original colony blots. The probe hybridized to a single 4.5 kb fragment in each of the three clones that were able to complement 17640 and R-5 (figure 18). Since the probe hybridized to a fragment of the same size in each of the three clones, it was assumed that each of these clones was from the same region of the chromosome rather than from different copies of the fimbrial gene. Therefore, only one of these clones, cosmid pGB710, was chosen for further study.

The initial reason for construction of a *B. bronchiseptica* genomic library was to clone the structural gene for the major serotype 2 fimbrial subunit, *fim2*. Hybridization

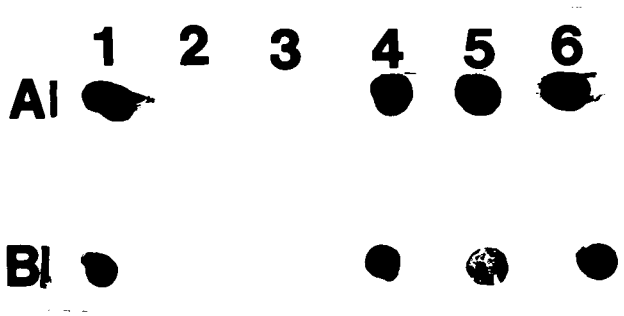


Figure 16: Dot blot with monoclonal antibody CF8. Clones which hybridized with the DNA probe were conjugated with *B. bronchiseptica* strains 17640 and R-5. Cells were scraped off plates to form a heavy suspension in PBS. Five microliters of this suspension was applied per dot. Strip A. Dot 1) 110H, 2) 17640 pGB611, 3) 17640 pGB612, 4) 17640 pGB710, 5) 17640 pGB84, 6) 17640 pGB85, Strip B. Dot 1) 110H, 2) R-5 pGB611, 3) R-5 pGB612, 4) R-5 pGB710, 5) R-5 pGB84, 6) R-5 pGB85.

Figure 17: Dot blot with monoclonal antibody BPF2. Cells were scraped off agar plates to form a heavy suspension in PBS and five microliters of this suspension was applied to each dot. Dot 1) 110H, 2) R-5 3) R-5 pLAFR1, 4) R-5 pGB710, 5) 17640, 6) 17640 pLAFR1, 7) 17640 pGB710.

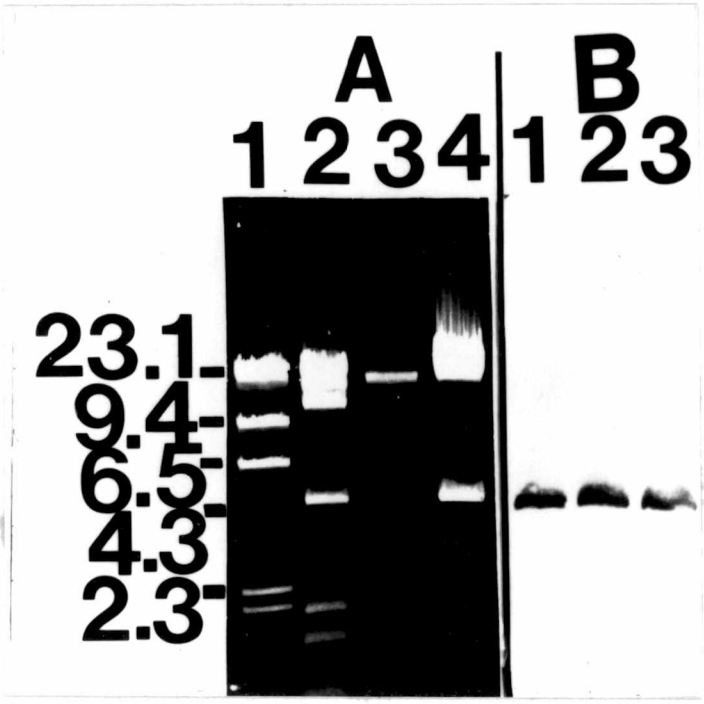


Figure 18: Southern blot with *B. pertussis* serotype 2 oligonucleotide probe. Panel A shows a representative ethidium bromide stained gel. Lane 1) Lambda *Hind*III molecular weight markers; 2) *Eco*RI digested pGB710; 3) *Eco*RI digested pGB84; 4) *Eco*RI digested pGB85. Panel B shows the reactivity with the probe detected by AP conjugated mouse anti-digoxigenin. Lane 1) *Eco*RI digested pGB710; 2) *Eco*RI digested pGB84; 3) *Eco*RI digested pGB85.

with the oligonucleotide probe and enhancement of expression of serotype 2 fimbriae indicated that these three clones may contain the serotype 2 fimbrial subunit gene. However, hybridization with a single degenerate oligonucleotide probe does not indicate that the cosmid contains this gene. Therefore, DNA from plasmid pGB710 was probed by dot blot hybridization and Southern blot with an oligonucleotide probe specific for the other end of the *B. pertussis fim2* gene. This probe hybridized to the same 4.5 kb *Eco*RI fragment as the probe for the 5' end.

Although the hybridization data with these two probes for the *B. pertussis* serotype 2 fimbrial subunit structural gene suggest that pGB710 may contain the *B. bronchiseptica fim2* gene, the large size of the *Bordetella* insert on this cosmid would not prohibit the presence of other genes on this fragment. Since we are interested in studying attachment of *B. bronchiseptica*, it was important to determine if the genes for any adhesins of *Bordetella,* other than fimbriae, are contained on this cosmid. Therefore, dot blot hybridizations were performed with oligonucleotide probes to each end of the FHA and pertactin genes of *B. pertussis*. The results of these hybridizations are shown in table 9. All four of these probes hybridized with chromosomal DNA from *B. bronchiseptica* strains 110H, 17640, and R-5 indicating that there is enough similarity between the FHA and pertactin genes of *B. pertussis* and *B. bronchiseptica* that probes designed from the *B. pertussis* sequences will detect the *B. bronchiseptica* versions of these genes. None of these probes reacted with pGB710 DNA suggesting that pGB710 does not contain the structural genes for pertactin or FHA.

Subcloning the Fimbrial Related Gene from pGB710

Experiments were undertaken to subclone the gene from pGB710 which reacts with the two oligonucleotide probes specific for the serotype 2 fimbrial subunit structural gene. Cosmid pGB710 was digested with thirteen different restriction enzymes (figure 19), and probed with both serotype 2 specific oligonucleotide probes (figure 20). Five

Table 9: Hybridization of pGB710 with probes for *B. pertussis* adhesin genes

Probe Name	Length	Specificity[1]	Hybridization[2]
FHA (N)	29 bases	5' end of *fhaB* of *B. pertussis*	–
FHA (C)	29 bases	3' end of *fhaB* of *B. pertussis*	–
Prn (N)	29 bases	5' end of *prn* of *B. pertussis*	–
Prn (C)	29 bases	3' end of *prn* of *B. pertussis*	–
Fim 2 (N)	29 bases	5' end of *fim2* of *B. pertussis*	+
Fim 2 (C)	29 bases	3' end of *fim2* of *B. pertussis*	+

[1] Gene sequence from which the probe was derived

[2] By dot blot hybridization with non-radioactively labeled probe

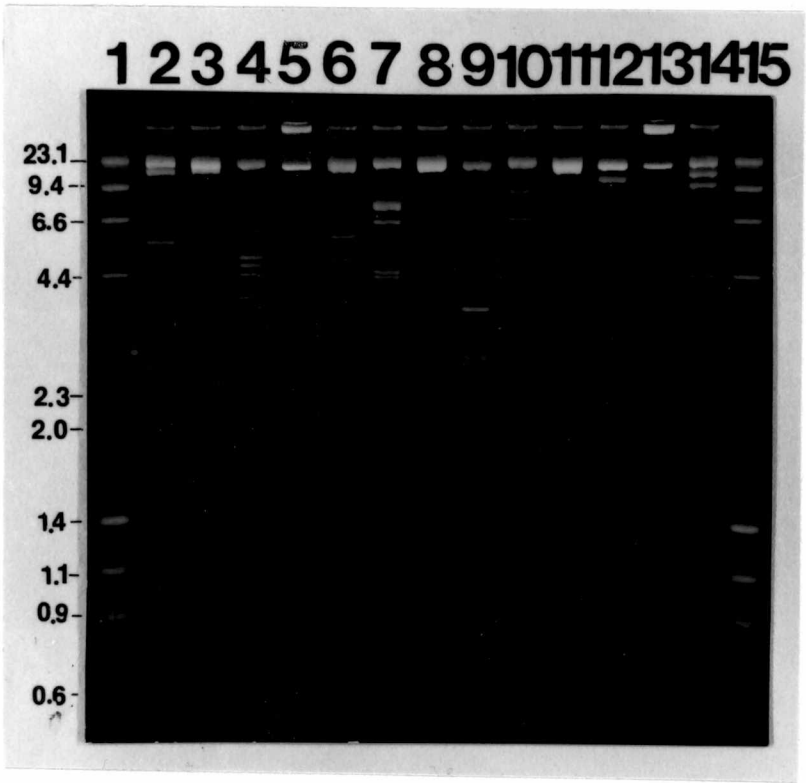
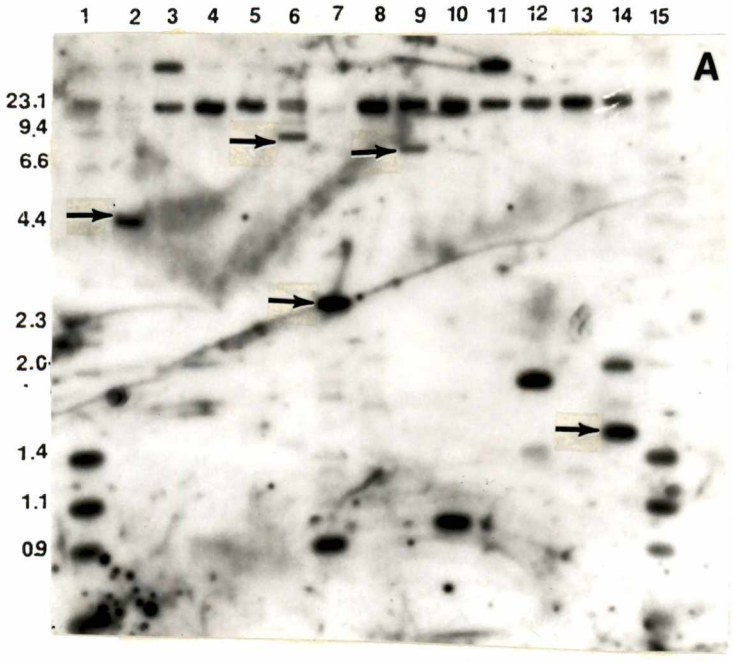
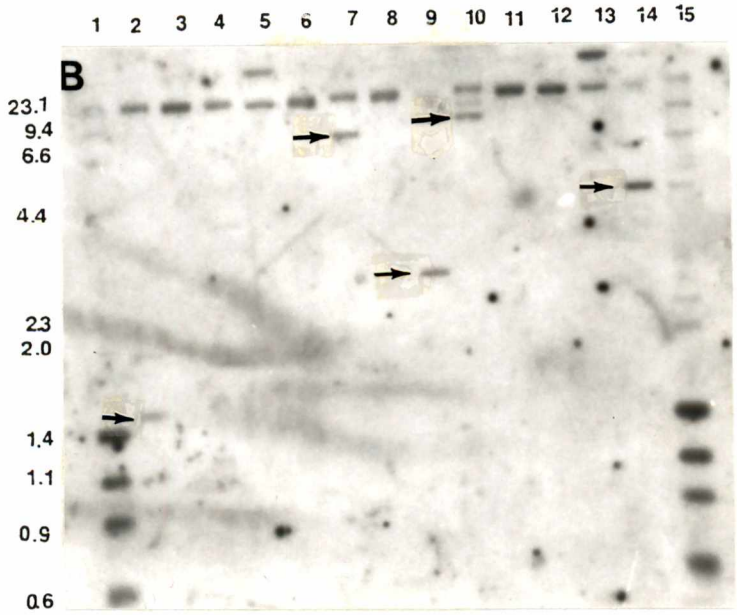


Figure 19: Restriction enzyme digest of pGB710. Cosmid pGB710 was digested with the following enzymes and run on a 1% agarose gel at 20 volts for 20 hours. Lane 1) λ *Hin*dIII, ϕX174 *Hae*III markers; 2) *Bam*HI; 3) *Cla*I; 4) *Sal*I; 5) *Apa*I; 6) *Bgl*II; 7) *Mlu*I; 8) *Nru*I; 9) *Pvu*II; 10) *Stu*I, 11) *Dra*I; 12) *Sca*I; 13) *Apa*LI; 14) *Eco*RI; 15) λ *Hin*dIII, ϕX174 *Hae*III markers.

Figure 20: Southern blot of pGB710 with *fim2* probes. Probes used were for the 5' end of the *B. pertussis fim2* gene (panel A) and the 3' end of the same gene (panel B). Bands reacting with both probes are marked with arrows. Labeled markers were also added. In both panels lanes 1 and 15 contain λ *Hin*dIII, ϕX174 *Hae*III markers; Lanes 2 through 14 contain pGB710 digested with: Panel A; Lane 2) *Bam*HI; 3) *Cla*I; 4) *Sal*I; 5) *Apa*I; 6) *Bgl*II; 7) *Mlu*I; 8) *Nru*I; 9) *Pvu*II; 10) *Stu*I, 11) *Dra*I; 12) *Sca*I; 13) *Apa*LI; 14) *Eco*RI. Panel B; Lane 2) *Apa*LI; 3) *Sca*I; 4) *Dra*I; 5) *Stu*I; 6) *Pvu*II; 7) *Nru*I; 8) *Mlu*I; 9) *Bgl*II; 10) *Apa*I; 11) *Sal*I; 12) *Cla*I; 13) *Bam*HI.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
A
23.1
9.4
6.6
4.4
2.3
2.0
1.4
1.1
0.9


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
B
23.1
9.4
6.6
4.4
2.3
2.0
1.4
1.1
0.9
0.6

fragments were identified which reacted with both probes. The three smallest fragments, a 4.5 kb *Eco*RI fragment, a 2.7 kb *Pvu*II fragment, and a 1.5 kb *Bam*HI fragment, were each isolated from gels by electroelution and cloned into pBluescript and pBBR1MCS. pBBR1MCS is derived from a naturally occurring *B. bronchiseptica* plasmid and was used so that expression in *Bordetella* could be evaluated since *B. bronchiseptica* can not maintain the plasmid pBluescript.

The fragments were also subcloned into plasmids pMMB207 and pMMB208 which contain the *tac* promoter. These plasmids have been used previously for expression of cloned adenylate cyclase toxin genes in *B. bronchiseptica* (214). These plasmids were used in order to obtain expression of the *B. bronchiseptica* fimbrial related gene in *Bordetella*. In order to ensure that each fragment was cloned into the vector in each orientation with respect to the *tac* promoter, the fragments were isolated from the pBluescript based subclones by digestion with both *Eco*RI and *Hind*III. These enzymes do not cut within the *Bordetella* insert and do cut in the multiple cloning site of pBluescript on opposite sides of the insertion site used when these fragments were subcloned into pBluescript. These fragments were ligated into the *Eco*RI-*Hind*III sites on pMMB207 and pMMB208. These plasmids have the *tac* promoter on opposite sides of the multiple cloning site in opposite orientations. Therefore by using directional cloning and both vectors, the *Bordetella* inserts can be placed under the *tac* promoter in each orientation. Table 10 lists the plasmids which were constructed and the *Bordetella* inserts which they contain. These plasmids were all introduced into *E. coli* strain DH-5α by electroporation and into *B. bronchiseptica* strains 17640 and R-5 by triparental mating. The effect of these subclones on fimbrial expression in *B. bronchiseptica* will be described in Chapter 5.

Table 10: Subclones constructed from pGB710 and pGB84

Plasmid name	Vector	Size of Insert[1]	Origin of Insert	Hybridization[2]
pGB710	pLAFR1	~ 25 kb	110H library	+
pGB84	pLAFR1	~ 20 kb	110H library	+
pBS84	pBluescript	4.5 kb	*Eco*RI fragment from pGB84	+
pGB84e	pLAFR1	4.5 kb	*Eco*RI fragment from pGB84	+
pGB711	pBBR1MCS	4.5 kb	*Eco*RI fragment from pGB710	+
pGB7B4	pBBR1MCS	1.5 kb	*Bam*HI fragment from pGB710	+
pGB7P2	pBBR1MCS	2.7 kb	*Pvu*II fragment from pGB710	+
pBS7B4	pBluescript	1.5 kb	*Bam*HI fragment from pGB710	+
pBS7P2	pBluescript	2.7 kb	*Pvu*II fragment from pGB710	+
pMMB207Bam	pMMB207	1.5 kb	*Bam*HI fragment from pGB710	+
pMMB208Bam	pMMB208	1.5 kb	*Bam*HI fragment from pGB710	+
pMMB207Pvu	pMMB207	2.7 kb	*Pvu*II fragment from pGB710	+
pMMB208Pvu	pMMB208	2.7 kb	*Pvu*II fragment from pGB710	+

[1] Determined by agarose gel electrophoresis

[2] Hybridization of insert with serotype 2 fimbriae N and C terminal probes

Restriction Mapping of the 4.5 kb *Eco*RI Fragment

Since the 4.5 kb *Eco*RI fragment of pGB710 and pGB84 hybridized with both ends of the *B. pertussis* serotype 2 fimbrial subunit structural gene, this fragment was subcloned into pBluescript. A restriction map was constructed through analysis of both single and double digestions with twelve different restriction enzymes (figure 21). Six of the enzymes, including *Hind*III, did not cut within this fragment. *Hind*III has previously been found to be unable to digest Bordetella DNA due to the presence of a *Hind*III restriction and modification system. This insert does contain a 1.5 kb *Bam* HI fragment and an approximately 2.8 kb *Pvu*II fragment which hybridize with the probes for both ends of the serotype 2 fimbrial subunit structural gene.

Sequencing of the Fimbrial-Related Gene from pGB710

Comparison of a short sequence of the gene which hybridizes with the two serotype 2 fimbrial gene specific oligonucleotide probes with the published sequence of the *B. bronchiseptica fim2* gene cloned by Savelkoul (294) would be useful to confirm that pGB710 contains the serotype 2 fimbrial subunit structural gene. Therefore, the 4.5 kb *Eco*RI fragment, cloned into pBluescript, was sent to Lark Sequencing Technologies for primer extension sequencing using the oligonucleotide specific for the 5' end of the *fim2* gene of *B. pertussis* as a primer. Lark reported that this primer bound to multiple sites on the 4.5 kb fragment making a determination of sequence impossible. Therefore, a more specific, non-degenerate primer from a region of the *B. bronchiseptica fim2* sequence slightly downstream of the site recognized by the original primer was designed by Lark Sequencing Technologies. The sequence obtained with this primer was also reported to be unreadable. The presence of more than one gene on this 4.5 kb DNA fragment with regions highly similar to the *fim2* sequence could cause these sequencing problems. Figure 22 shows the location of each primer sequence within the *B.*

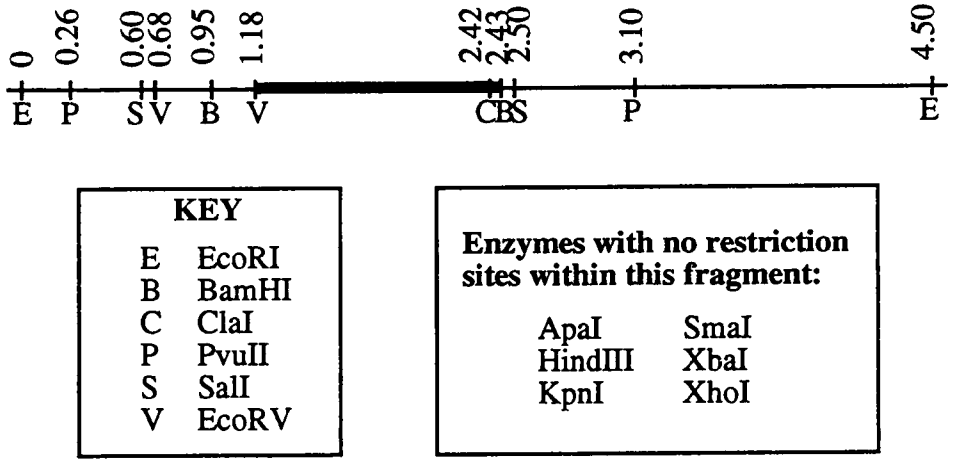


Figure 21: Restriction map of the 4.5 kb fragment from pGB84. The region hybridizing with both serotype 2 fimbriae oligonucleotide probes is indicated by a thick line. Distances on the map are in kilobases.

```
  1 gcatgcaaaa gggctgtttc ccacatcgga atcagccccc ccccccccc
 51 ccctaagacc taagatcgtg gctccataac tcttctggcg ccaagacgcc
101 cgtgttaccc atgcaagtcc ctttccaacg cgccctgccg ctgtgcttgc
151 gggccgctct ggcggccatt gcgtccgcgg cgcacgccga cgacggcacc
201 atcgtcatca ccggcaccat caccgacacc acctgcgtca tcgaggaccc
251 ggccggcccc acgcacacca aggtggtgca attgcccaag atatccaaga
301 gcgcgctggc caaggatgga gacgaagctg gccgtacgcc cttcctgatc
351 acgctcaagg actgcccctc gtccctgaac aatggcgtga aagcgtactt
401 cgagcctggg ccgaccaccg actacgctac cggcgaccta aaggcgtatt
451 cgatcgccta caacaacaac cccgccacca cccaaaatgc gatcatcgct
501 gcaagcgaag cgcagggcgt gcaaatccgc atctccaacc agaacggcac
551 caagatcccc atgggcgtgg acgccgccgc ccagaacgcc caggccttca
601 accccgtcac cgacaccgcc gacaacgcca agaagaaggt cacgttgcgc
651 tacctggcat cgtacgtaaa gaaatccggc aacattactg ccgggcaact
701 cacgacatac gtcggttttt ccatgatcta tccgtaaccc cgactcctgc
751 cctccaggca ggccaagcgg gctctcgcga tcctcccgtc ctactatcga
801 gactgagcgg cggctgttac gg
```

Figure 22: *B. bronchiseptica fim2* gene sequence. Sequence from Genbank (Accession # X74119). The sequence of the oligonucleotide probe fim2(N) is underlined. The sequence of the primer designed by Lark Sequencing Technologies is in the box.

bronchiseptica fim2 gene sequence from Genbank. The *B. bronchiseptica fim2* sequence was searched using DNA Inspector (Textco, W. Lebanon, NH) for matches to the fim2 (N) probe and the primer designed by Lark. The fim2 (N) oligonucleotide was found only once in the sequence even when up to 10 mismatches were allowed. The primer sequence designed by Lark Technologies was found only once in the *fim2* sequence when up to 3 mismatches were allowed. With 4 mismatches, this primer would recognize 3 sites in the *fim2* sequence. These data indicate that the multiple priming in the sequencing reaction was unlikely to be due to multiple recognition sites within the *fim2* sequence.

Discussion

Through construction of a genomic library from *B. bronchiseptica* strain 110H, a cosmid clone affecting expression of serotype 2 fimbriae of *B. bronchiseptica* was identified. This cosmid, designated pGB710, hybridizes with oligonucleotide probes specific for each end of the *B. pertussis fim2* gene suggesting that the cosmid may carry a copy of the *fim2* structural gene from *B. bronchiseptica*. The probe encoding the first ten amino terminal amino acids from the *B. pertussis* serotype 2 fimbrial subunit protein has been previously used by Mooi *et al* (233). Since he found hybridization of this probe to more than one fragment of chromosomal DNA from *B. bronchiseptica*, he suggested that *B. bronchiseptica* carries multiple copies of the fimbrial genes (233). It is not known if each of these copies is capable of being expressed or is expressed in *B. bronchiseptica*. There is evidence of other silent genes in *Bordetella* including the *fimA* gene which encodes a pseudofimbrial structural gene (204, 360).

In order to determine if the cosmid clone carried a full length fimbrial gene rather than a truncated fimbrial gene such as *fimA* (204, 360), a probe for the other end (the 3' end) of the gene was used in Southern blots and hybridized with the same *Eco*RI fragment as the original probe. These data suggest that the cosmid carries a full length fimbrial subunit gene or a gene with similarity to the *Bordetella* fimbrial subunit genes on

each end. It is known that *Bordetella* fimbrial genes are highly conserved. Mooi *et al.* (233) found 70% similarity between five different fimbrial genes from *Bordetella* species. In addition, Willems *et al.* (360) found that the fimbrial regulatory genes *fimBCD* also exhibit sequence similarity with the fimbrial subunit structural genes. Therefore, it would be possible for a *B. bronchiseptica* fimbrial regulatory or accessory gene to hybridize to both of these oligonucleotide probes.

Stibitz *et al.* (317) constructed a physical map of the *B. pertussis* chromosome and found that the FHA, pertactin, and fimbrial genes are widely separated on the chromosome. If the chromosome of *B. bronchiseptica* is organized similarly to that of *B. pertussis*, the genes for FHA and fimbriae or pertactin and fimbriae are separated enough that it would not be possible to clone these genes together on one cosmid. Therefore, if pGB710 contains a fimbrial structural gene, it would not be likely to contain either the gene for FHA or pertactin. In order to determine if either the gene for FHA or the gene for pertactin is present on cosmid pGB710, oligonucleotide probes for each of these genes were used. This cosmid did not hybridize with probes for either end of the FHA or pertactin gene. These probes did, however, hybridize with chromosomal DNA from *B. bronchiseptica* strains 110H, 17640, and R-5.

The exact identity of fimbrial related gene(s) on pGB710 is not known. Multiple priming which occurred in the sequencing reaction suggests that either the probes are non-specifically binding or pGB710 contains more than one fimbrial related gene. The probes used for sequencing reactions were presumably long enough to confer some specificity. The oligonucleotide designated fim2 (N) has been used by Mooi *et al.* (233) as a specific probe for serotype 2 fimbrial subunit genes in *B. pertussis* and *B. bronchiseptica*. The probe designed by Lark Sequencing Technologies was designed to be specific for the *fim2* sequence. It was a non-degenerate probe from a region slightly downstream of the fim2(N) probe sequence. Therefore, it is unlikely that both primers are binding non-specifically. Fimbrial gene sequences including those of accessory proteins

and minor fimbrial subunits are known to be highly conserved (204, 233, 360). *B. bronchiseptica* is also known to carry multiple copies of the fimbrial genes (233). In other species, fimbrial genes have been found in clusters. A fimbrial gene cluster or the presence of multiple copies of fimbrial genes with slightly different sequences may account for the results obtained in the sequencing reactions.

In summary, a cosmid containing at least one serotype 2 fimbriae related gene from *B. bronchiseptica* has been cloned in pLAFR1. Smaller fragments of this *Bordetella* insert containing sequences which hybridize with oligonucleotide probes to each end of the *B. pertussis* serotype 2 fimbrial subunit structural gene have been subcloned and a restriction map has been constructed for the 4.5 kb *Eco*RI fragment. The cosmid pGB710 does contain a gene(s) which affects expression of serotype 2 fimbriae in *B. bronchiseptica* strains R-5 and 17640 as determined by reactivity with monoclonal antibody CF8.

CHAPTER FIVE

BIOLOGICAL EFFECTS OF PLASMIDS CARRYING GENES RELATED TO SEROTYPE 2 FIMBRIAE OF *BORDETELLA BRONCHISEPTICA*

Introduction

Bordetella bronchiseptica attach to ciliated cells of the respiratory tract of animals through the combined effects of several adhesins (50, 194, 231, 289, 332). The primary adhesins of *Bordetella pertussis* are also produced by *B. bronchiseptica* (164, 248, 231). Binding properties of these adhesins, FHA and pertactin, may account for much of the attachment by *B. bronchiseptica* as well (164, 231). However, due to the wide range of species which *B. bronchiseptica* infects, adhesins not necessary in *B. pertussis* may be required in *B. bronchiseptica*. One of these adhesins may be fimbriae. The data concerning the involvement of fimbriae in attachment by *Bordetella* have- been inconclusive. Studies of *B. pertussis* mutants unable to produce fimbriae have shown that fimbriae are unnecessary for colonization of the lungs of mice (234). However, the presence of antibodies against fimbriae are protective against colonization of the respiratory tracts of mice (278).

In *B. bronchiseptica*, a correlation has been shown between the presence of serotype 2 fimbrial antigens and infection of pigs (56, Chapter 2). Strains of *B. bronchiseptica* isolated from pigs exhibited significantly higher reactivity with an antibody against serotype 2 fimbriae than strains isolated from guinea pigs (56). Funnell *et al.* (109) have also found a possible role of fimbriae in determination of species specificity. They found that afimbriated *B. pertussis* do not attach as well as fimbriated cells to baboon tracheal rings (109). These same afimbriated mutants were used by Mooi

et al. (234) and colonized mice as well as wild type strains. These data in combination with studies indicating that anti-fimbrial antibodies are protective suggest that fimbriae may be involved in the initial infection of animals, especially pigs, by *B. bronchiseptica.* Attachment to ciliated cells may be an important initial step in infection and colonization In order to study the involvement of fimbriae in attachment, a gene affecting expression of serotype 2 fimbriae was cloned from *B. bronchiseptica* strain 110H (Chapter 4).

The cosmid pGB710 carries a gene which increases expression of a serotype 2 fimbrial antigen on the surface of *B. bronchiseptica* strains R-5 and 17640 (Chapter 4). If fimbriae are involved in attachment by *B. bronchiseptica*, then one would expect this increase in fimbriae to increase attachment. Therefore, this chapter describes the effect of pGB710 on attachment by strains 17640 and R-5. This chapter also describes experiments examining the effect of pGB710 on expression of other fimbrial serotypes in *B. bronchiseptica* and the regulation of fimbrial expression through the Bvg regulatory system.

Materials and Methods

Bacterial Strains, Media, and Growth Conditions

B. bronchiseptica strains used and growth conditions have been previously described (Chapters 2 and 4). *E. coli* strain SM10 carrying plasmid pSS929 was kindly provided by Scott Stibitz (Center for Biologics Evaluation and Research, FDA, Bethesda, MD). *E. coli* strain HB101 carrying plasmid pUW2138 was kindly provided by Alison Weiss (University of Cincinnati, Cincinnati, OH). This strain was maintained on LB agar with 10 μg/ml gentamicin

Enrichment for Fimbrial Proteins

Enrichment for fimbrial proteins was performed as described in Chapter 2. One milliliter of this fimbrial enrichment was subjected to further purification using a modification of the procedure of Mooi *et al.* (233). The solution was diluted to ten milliliters in 0.05 M Tris HCl (pH 7.0), 4 M urea, 0.1% (w/v) SDS, and centrifuged at 50,000 x g for one hour. The supernatant was then centrifuged at 150,000 x g overnight. The pellet obtained after this centrifugation was washed in 4 M urea and suspended in PBS.

Conjugation

Conjugation involving transfer of plasmid DNA from *E. coli* to *B. pertussis* has been previously described in Chapter 4. pLAFR1 DNA containing various inserts was transferred by conjugation from *E. coli* DH-1 to *B. bronchiseptica* strains 110NH, 17640, and R-5. Plasmid pSS929, a pRK290 derivative bearing the same replication genes as pLAFR1, was transferred into *B. bronchiseptica* strain 110NH by conjugation using the same triparental mating and selection system.

Immunological Assays

Transconjugants were used in dot blot assays with a 1:500 dilution of CF8 ascites as described in Chapter 4. Dot blots were also performed using a 1:100 dilution of monoclonal antibodies X4B and BB05 specific for *B. pertussis* FHA and *B. bronchiseptica* pertactin, respectively. Monoclonal antibody X4B was kindly provided by Michael Brennan (FDA, Center for Biologics Evaluation and Research, Bethesda, MD) and monoclonal antibody BB05 was provided by Pavel Novotny (Wellcome Biotechnology Ltd., Beckenham Kent, U.K.).

In the Western blotting procedure, proteins were separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli (190).

Transfer to nitrocellulose was by electroblotting overnight at 100 mA followed by 200 volts for three hours, a modification of the method of Towbin *et al* (329). Blotting and detection of antigen antibody complexes was as described in Chapter 2 using a 1:1000 dilution of monoclonal antibody BPH2 specific for serotype 2 fimbriae monomeric subunits of *B. pertussis*. Monoclonal antibody BPF2 specific for conformational epitopes of serotype 2 fimbriae of *B. pertussis* (199) was used at a 1:1000 dilution on dot blots. Both of these antibodies were kindly provided by Michael Brennan (Center for Biologics Evaluation and Research, FDA, Bethesda, MD). India ink stains were performed as described by Hancock and Tsang (136). Western blots were also performed using a 1:1000 dilution of monoclonal antibody BPA10 specific for serotype 3/6 fimbriae monomeric subunits. Monoclonal antibody BPA10 was kindly provided by Michael Brennan (Center for Biologics Evaluation and Research, FDA, Bethesda, MD).

For indirect ELISA's, each *B. bronchiseptica* strain to be tested was grown for 2 days on Brucella agar plates containing appropriate antibiotics. Colonies exhibiting virulent phase morphology were used in ELISA's as described in Chapter 2.

Attachment Assay

Vero cell attachment assays were performed using the standard conditions described in Chapter 3. Each assay was performed in triplicate and results are reported as the mean number of bacteria attached per 50 Vero cells ± standard deviation.

Antibody Inhibition of Attachment

B. bronchiseptica colonies exhibiting typical virulent phase morphology were picked from BG plates and suspended in PBS to an optical density of 0.1 at 595 nm as for standard attachment assays. 1:50 dilutions of antibodies CF8, X4B, BB05, and Ra110NH were made in the bacterial suspensions and incubated at 37°C for 30 minutes. The sources and specificity of monoclonal antibodies CF8, X4B, and BB05 have been described in

Chapter 3. Ra110NH is a polyclonal rabbit serum prepared against the avirulent *B. bronchiseptica* strain 110NH in this laboratory. This serum was used as a negative control since it contains antibodies that bind to all *Bordetella* strains but does not contain antibodies against *Bordetella* adhesins or other antigens which are only expressed in virulent phase.

After incubation with the antibodies for 30 minutes, the bacterial suspension was diluted two-fold in complete DMEM and 200 µl of this dilution was added to wells containing Vero cells which had been washed three times with complete DMEM. Cells were then incubated for 30 minutes at 37°C in an 8% CO_2 incubator. After incubation, the cells were washed three times in complete DMEM to remove non-adherent bacteria, fixed in ethanol, and stained for 1 minute in a 1:2 dilution of Gram's Crystal Violet. Cells were observed and bacteria counted as for the standard attachment assay.

Recombinational Inactivation

Plasmid pUW2138 containing a gentamicin resistance gene was kindly provided by Alison Weiss (University of Cincinnati, Cincinnati, OH). The gentamicin *ori*T region of this plasmid was isolated by digestion with *Hind*III and cloned into the *Hind*III site of a pBluescript plasmid containing a 1.3 kb *Sal*I fragment from pGB710 which hybridized with the oligonucleotide probe for the 5' end of the *B. pertussis fim2* gene but not to the probe for the 3' end. Ligation into these plasmids was performed as previously described (19). This plasmid, designated pBSG1, was introduced into *E. coli* DH-5α by electroporation as described in Chapter 4. This plasmid was transferred by conjugation into *B. bronchiseptica* strain R-5 carrying cosmid pGB710 to allow recombination. Transconjugants were selected on Brucella agar containing 10 µg/ml gentamicin. Since *Bordetella* cannot maintain pBluescript based plasmids, colonies which grow on this medium must have the gentamicin *ori*T fragment in the chromosome or in the cosmid pGB710. Plasmid DNA was isolated by mini-prep (19) and electroporated into *E. coli*

DH-5α. Transformants were selected on LB with gentamicin and replica plated onto LB with tetracycline. Colonies which grew in the presence of both antibiotics were selected and plasmid DNA was isolated (19). To verify that these colonies contained recombinant plasmids, they were transferred back into *B. bronchiseptica* R-5 grown on medium containing furaltadone, gentamicin, and tetracycline. Recombinant plasmid DNA, designated pGB710R was used for Southern and dot blot hybridizations with pLAFR1, the gentamicin *ori*T fragment of pUW2138, and the 4.5 kb *Eco*RI fragment of pGB710 which hybridizes with both *fim2* probes.

Results

Effect of pGB710 on Fimbrial Subunit Expression

Cosmid pGB710 was introduced into *B. bronchiseptica* strains 17640 and R-5 by tri-parental mating and transconjugants were selected on Brucella agar with tetracycline and furaltadone. To determine if pGB710 has an affect on expression of the major fimbrial subunit proteins, fimbrial protein enrichments were obtained from strains 17640 and R-5 carrying cosmid pGB710. As can be seen in figure 23, strains R-5 pGB710 and 17640 pGB710 exhibit a fimbrial protein profile identical to that of strain 110H from which the clone was derived. This increase in fimbrial subunit protein is not due to an indirect effect of the cosmid vector since the change in fimbrial protein profile does not occur in strains 17640 or R-5 when pLAFR1 alone is present.

Since the oligonucleotide probe originally used to identify fimbrial related genes from the *B. bronchiseptica* genomic library was designed to be specific for serotype 2 fimbriae, one would expect the level of serotype 2 fimbrial subunit protein to be increased in strains carrying the cloned gene. To verify that the change in fimbrial protein profile corresponds to a change in expression of serotype 2 fimbrial subunits, a Western blot was

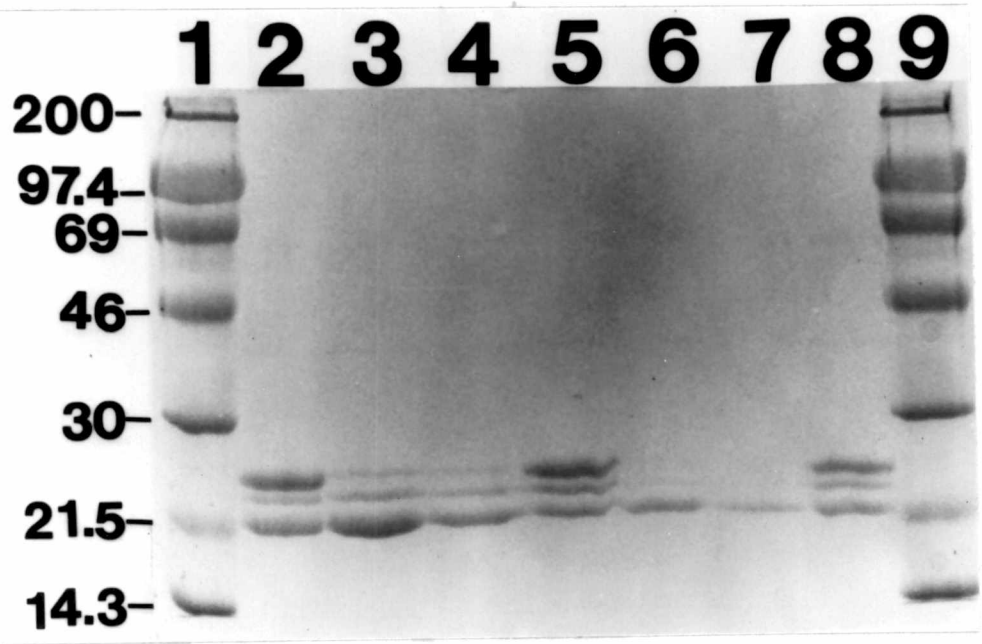


Figure 23: Effect of pGB710 on fimbrial protein profiles. Lane 1) Prestained molecular weight markers; 2) 110H; 3) R-5; 4) R-5 pLAFR1; 5) R-5 pGB710; 6) 17640; 7) 17640 pLAFR1; 8) 17640 pGB710; 9) Markers.

performed with monoclonal antibody BPF2 which is specific for the denatured serotype 2 fimbrial subunit monomer. Figure 24 shows that this antibody reacts with the 24 kD fimbrial subunit protein indicating that this protein is the *B. bronchiseptica* equivalent of the *B. pertussis* serotype 2 fimbrial subunit. This protein is present in all the strains although strains 17640 and R-5 do not produce as much of this protein as strain 110H. This same protein reacts with the antibody in strains carrying the cosmid pGB710. In the strains carrying this cosmid and in strain 110H, several bands can be observed below the 24 kD protein. These bands have been observed previously by Mooi *et al.* (233) and are believed to be degradation products . The lower amount of the 24 kD protein produced by the other strains may account for the lack of visible degradation products.

In order to quantitate the increase in serotype 2 fimbriae expression in *B. bronchiseptica* strains R-5 and 17640 when pGB710 is present and to compare this expression level with that of other strains, an indirect ELISA with monoclonal antibody CF8 was performed on these strains. As can be seen in figure 25, both *B. bronchiseptica* strain R-5 pGB710 and 17640 pGB710 react much better than the parental strains. R-5 pGB710 reacts better than 17640 pGB710 and as well as strain 110H. These data indicate that the cosmid pGB710 is increasing expression of serotype 2 fimbriae in both 17640 and R-5 but expression of serotype 2 fimbriae is being increased more in strain R-5 than in strain 17640.

In order to determine if cosmid pGB710 also has an effect on expression of serotype 3 fimbrial subunits, fimbrial protein enrichments from *B. bronchiseptica* strains carrying cosmid pGB710 were subjected to Western blotting with monoclonal antibody BPA10 specific for serotype 3/6 fimbriae monomeric subunits. Figure 26 shows that the middle 22 kD fimbrial subunit protein band reacts with the serotype 3/6 antibody. An increased amount of this protein can be observed in strains carrying the cosmid pGB710. A slight increase in the amount of this protein is also observed on Coomassie Blue stained gels of fimbrial protein enrichments from strains carrying cosmid pGB710 (figure 23).

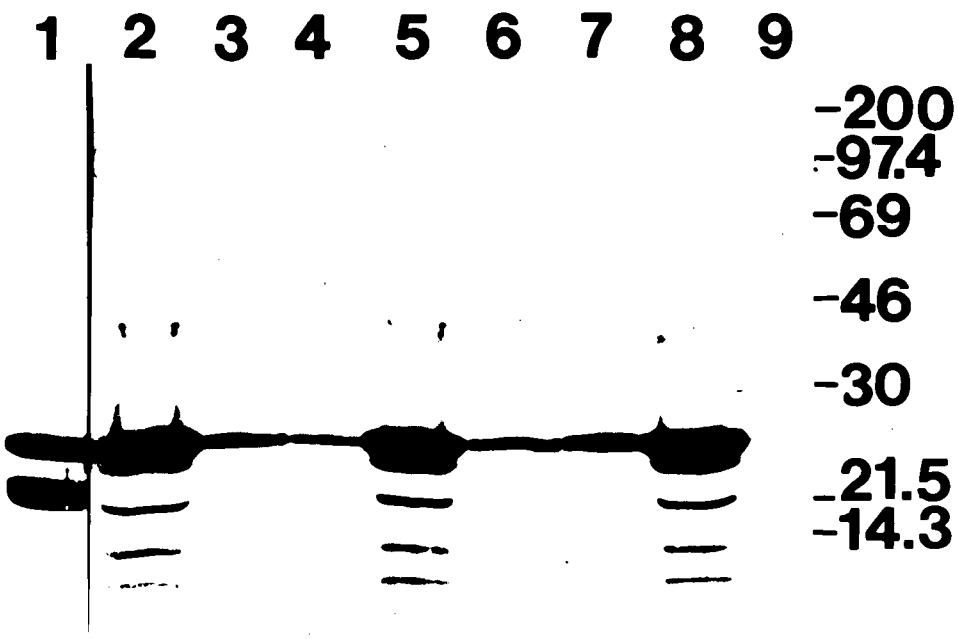


Figure 24: Western blot with monoclonal antibody BPF2. Lane 1 was stained with India ink. The rest of the blot was probed with antibody. Lane 1) 110H; 2) 110H; 3) R-5; 4) R-5 pLAFR1; 5) R-5 pGB710; 6) 17640; 7) 17640 pLAFR1; 8) 17640 pGB710; 9) Prestained molecular weight markers.

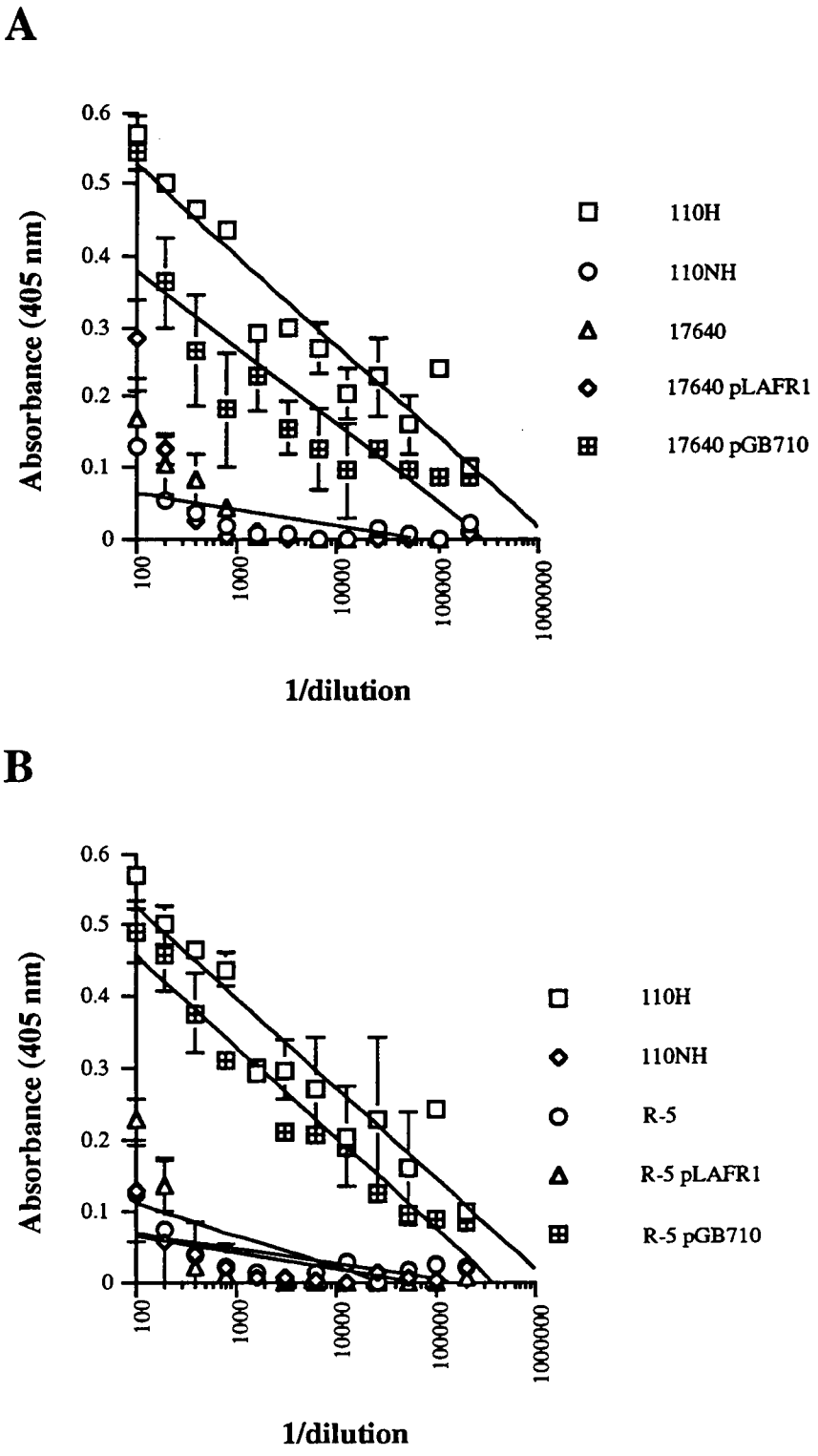


Figure 25: Reactivity of *B. bronchiseptica* strains carrying pGB710 with CF8.

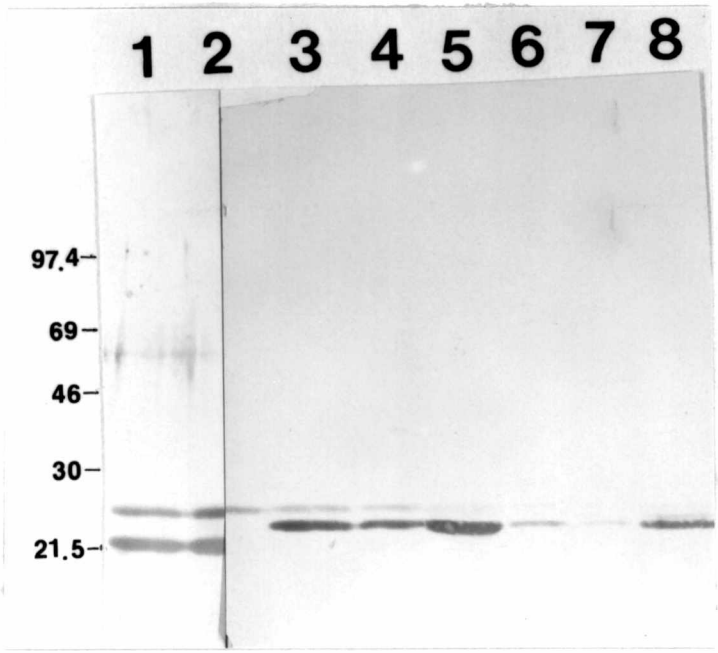


Figure 26: Western blot with monoclonal antibody BPA10. Lane 1 shows an India ink stain. All other lanes were reacted with antibody. Lane1) 110H; 2) 110H; 3) R-5; 4) R-5 pLAFR1; 5) R-5 pGB710; 6) 17640; 7) 17640 pLAFR1; 8) 17640 pGB710.

These data suggest that cosmid pGB710 may be affecting expression of both serotype 2 and serotype 3/6 fimbriae in *B. bronchiseptica* strains 17640 and R-5.

Requirement of Functional Bvg for Expression from pGB710

Bordetella fimbriae are Bvg regulated. To determine if the increase in fimbrial subunit protein expression observed in the presence of pGB710 in strains 17640 and R-5 requires the presence of a functional Bvg regulatory system, pGB710 was introduced by conjugation into *B. bronchiseptica* strain 110NH. Strain 110NH is an avirulent phase variant of strain 110H. It produces rough, non-hemolytic colonies instead of domed, hemolytic colonies as observed for virulent phase organisms (254). It also produces flagella and has reduced expression of fimbriae (193). 110NH does not react with monoclonal antibody CF8 and the three fimbrial protein bands seen in 110H are not visible in fimbrial protein enrichments from strain 110NH. Figure 27 shows that fimbrial proteins are absent in preparations from strain 110NH regardless of the presence of cosmid pGB710. Therefore, the gene carried on pGB710 which affects expression of serotype 2 fimbriae will not function in this Bvg negative strain. These data further suggest that the *bvgAS* operon is not present on the cosmid pGB710. There is, however, a 35 kD protein whose expression is increased in the presence of pGB710. The identity of this protein is not known and it may not be related to fimbriae.

It is possible, however, that other genes affecting fimbrial expression besides *bvgAS* may be mutated in strain 110NH and that these mutations are actually preventing pGB710 induced fimbrial protein expression in 110NH. To determine if there are such other mutations in 110NH, the cloned *bvgAS* genes were introduced into *B. bronchiseptica* strain 110NH by conjugation. The presence of plasmid pSS929 carrying the *bvgAS* genes caused reversion of strain 110NH to a virulent phase phenotype producing small domed colonies (figure 28). However, hemolysis was not restored. It has previously been shown that the presence of multiple copies of *bvgAS* in the cell reduces

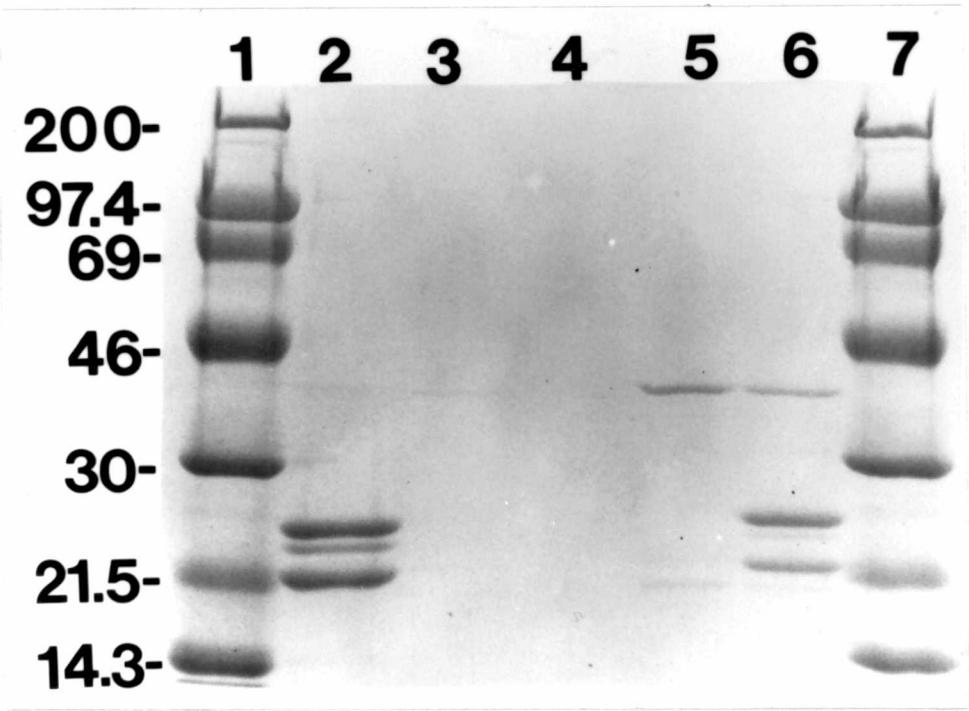


Figure 27: Fimbrial protein profile of *B. bronchiseptica* strain 110NH. Twenty microliters of each fimbrial protein enrichment were run on the gel and stained with Coomassie blue. Lane 1)Prestained molecular weight markers, 2) 110H, 3) 110NH, 4) 110NH pLAFR1, 5) 110NH pGB710 6) 110NH pSS929, 7) Markers.

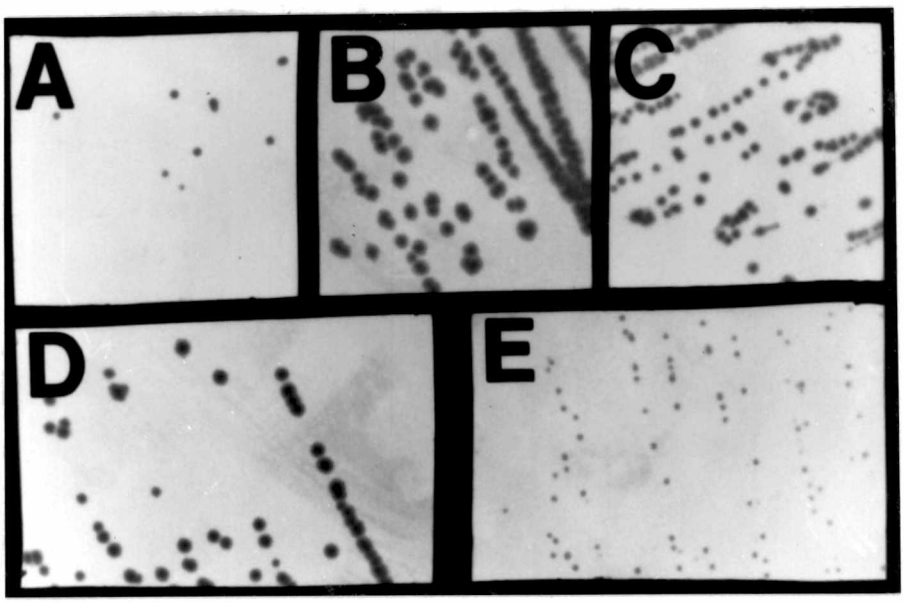


Figure 28: Colony morphology of *B. bronchiseptica* 110NH. Panel A) 110H, B) 110NH, C) 110NH pLAFR1, D) 110NH pGB710, E) 110NH pSS929. Virulent phase cells exhibit a small (≤ 1 mm), domed phenotype. Avirulent phase cells exhibit a larger, flat colony type.

hemolysis (229). Figure 27 shows the fimbrial protein profile of strain 110NH carrying plasmid pSS929. This strain exhibits a fimbrial protein profile identical to that of 110H indicating that there are not other mutations preventing expression of fimbriae in 110NH.

Attachment of Strains Carrying Cosmid pGB710

The cosmid pGB710 was introduced into *B. bronchiseptica* strains 17640 and R-5 by conjugation. Transconjugants were selected on Brucella agar plates containing tetracycline and furaltadone and used in attachment assays as previously described (Chapter 3). Results are presented in table 11. Attachment appeared to be Bvg dependent since the virulent phase strains all exhibited more attachment than avirulent strain 110NH. Strain R-5 attached only half as well as 110H and 17640. The cosmid vector pLAFR1 did not affect attachment by any of these strains. However, the presence of pGB710 caused an increase in attachment by *B. bronchiseptica* strain R-5 to a level slightly higher than that of 110H and similar to that of 17640. This increase in attachment was not seen in strain 17640.

Adhesin Genes Carried on pGB710

Since the presence of pGB710 in *B. bronchiseptica* strain R-5 caused an increase in attachment, the cosmid may be carrying a gene for a *Bordetella* adhesin. Previous experiments have shown that the cosmid pGB710 hybridized with probes for each end of the *B. pertussis* serotype 2 fimbrial subunit structural gene, *fim2*. The cosmid pGB710 was also probed with oligonucleotides specific for each end of the genes encoding FHA and pertactin and did not hybridize with probes for either of these genes (Chapter 4). In order to determine if pGB710 contains a gene affecting expression of these adhesins, a dot blot immunoassay was performed using monoclonal antibodies against *B. pertussis* FHA and *B. bronchiseptica* pertactin. Figure 29 shows that these antibodies reacted with

Table 11: Attachment of *B. bronchiseptica* strains carrying pGB710

Strain	Attached Bacteria[1]
110H	995 ± 132
110NH	186 ± 88
17640	1037 ± 170
17640 pLAFR1	1219 ± 105
17640 pGB710	1108 ± 85
R-5	544 ± 21
R-5 pLAFR1	589 ± 97
R-5 pGB710	1268 ± 40

[1] Mean number of bacteria attached to 50 Vero cells from 3 trials ± standard deviation.

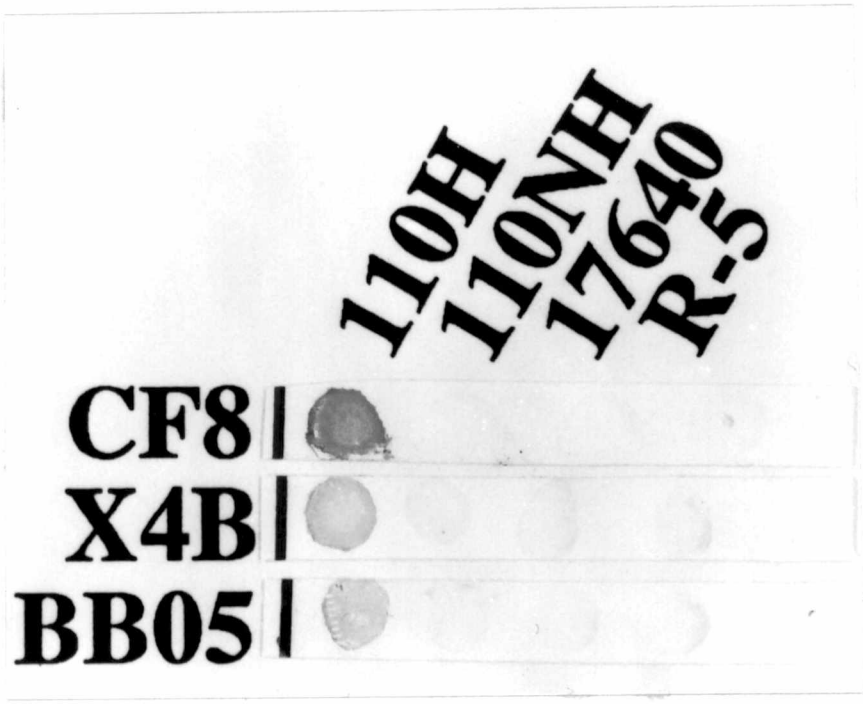


Figure 29: Reactivity of *B. bronchiseptica* strains with CF8, X4B, and BB05. Monoclonal antibody CF8 is specific for serotype 2 fimbriae; X4B is specific for FHA and BB05 is specific for pertactin. Each dot contains 5 µl of a heavy suspension of the indicated strain in PBS.

B. bronchiseptica strain 110H but did not react with strains R-5 or 17640. These data indicate that there may be antigenic differences between FHA and pertactin produced by different strains of *B. bronchiseptica.* A less likely explanation would be that these strains are not producing either of these adhesins. This lack of reactivity of strains 17640 and R-5 did not change when the cosmid pGB710 was introduced into these strains (data not shown). Since the genes carried on the cosmid were from strain 110H which reacted with these FHA and pertactin antibodies, it is not likely that these adhesins are being expressed from genes carried on pGB710.

Antibody Inhibition of Attachment

In a further effort to determine if the effect of pGB710 on serotype 2 fimbriae was causing the increase in attachment seen in *B. bronchiseptica* strain R-5 in the presence of the cosmid, antibody inhibition assays were performed. Bacterial strains were pre-incubated with monoclonal antibodies against serotype 2 fimbriae, *B. pertussis* FHA, and *B. bronchiseptica* pertactin and used in standard attachment assays. In order to minimize the effects of bacterial agglutination, a low dilution of antibody was used. The presence of excess antibody should block the binding sites lessening the incidence of binding of one paratope to the epitope on one bacterium while the other antigen binding site binds to the epitope on another bacterial cell which causes agglutination.

As a further control, polyclonal rabbit antiserum against *B. bronchiseptica* strain 110NH, an avirulent phase variant of 110H, was used. This serum recognizes shared antigens on both virulent and avirulent *B. bronchiseptica* These antibodies recognize and bind to the *B. bronchiseptica* strains but should not interfere with attachment since none of the known *Bordetella* adhesins are produced in avirulent phase. Thus, this polyclonal serum provides a control for the effects of agglutination. If *Bordetella* agglutinate under the assay conditions, then this antibody would show an effect on attachment. This serum also provides a control for the effects of antibody binding on attachment. If the presence

of antibodies on the cell surface (not specifically bound to adhesins) causes interference with attachment, then a decrease in attachment ability should be seen with antibody Ra110NH.

The results from the antibody inhibition assay are shown in table 12. The antibody, Ra110NH, did not have an effect on attachment by any of the strains tested. In addition, no clumps of cells were observed microscopically for any of the strains tested with any of the antibodies. In strain 110H, the antibodies against serotype 2 fimbriae, FHA, and pertactin each had an effect on attachment. The antibody against FHA reduced attachment to a level similar to that of avirulent phase strains. The antibodies against fimbriae and pertactin each caused only a partial inhibition of attachment by *B. bronchiseptica* strain 110H. Attachment by *B. bronchiseptica* strains 17640 and R-5, however, was not inhibited by any of the antibodies. Attachment of *B. bronchiseptica* strain R-5 carrying cosmid pGB710 was reduced, however, in the presence of monoclonal antibody CF8 which is specific for serotype 2 fimbriae. The level of attachment in the presence of CF8 was similar to that of the parental strain R-5. Attachment of *B. bronchiseptica* strains 17640 and R-5 with or without the cosmid were not inhibited by either antibody X4B or BB05. However, these antibodies were shown above not to bind to these strains.

Effect of Subclones from pGB710 on Fimbrial Expression

In Chapter 4, subclones from pGB710 were constructed which contained restriction fragments that hybridized with serotype 2 fimbriae specific oligonucleotide probes. These subclones, listed in table 10, in Chapter 4 were transferred into *B. bronchiseptica* strain R-5 by conjugation and selection on Brucella agar containing appropriate antibiotics. The *B. bronchiseptica* strains carrying the plasmid subclones were used for dot blots with monoclonal antibody CF8 which does not react with the parent strain R-5. Table 13 shows the results of these dot blots. None of the subclones, including

Table 12: Antibody inhibition of attachment[1]

	Antibody[2]				
Strain	**None**	**Ra110NH[3]**	**CF8[4]**	**X4B[5]**	**BB05[6]**
110H	966 ± 112	865 ± 134	348 ± 45	157 ± 39	431 ± 64
110NH	198 ± 81	137 ± 20	150.5 ± 8.5	154 ±12	146 ± 24
17640	1201 ± 236	1143 ± 24	1231 ± 35	1233 ± 138	1099 ± 91
R-5	527 ± 28	554 ± 11	628 ± 11	569 ± 6	590 ± 34
R-5 pGB710	1147 ± 154	898 ± 40	650 ± 27	1039 ± 11	891 ± 25

[1] Mean number of bacteria attached to 50 Vero cells in three trials ± standard deviation after pre-incubation for 30 minutes with the antibody.

[2] For all antibodies a 1:50 dilution of ascites fluid was used.

[3] Polyclonal rabbit serum against the avirulent phase strain 110NH.

[4] Mouse monoclonal antibody against *B. bronchiseptica* serotype 2 fimbriae.

[5] Mouse monoclonal antibody against *B. pertussis* FHA.

[6] Mouse monoclonal antibody against *B. bronchiseptica* pertactin.

Table 13: Effect of subclones on serotype 2 fimbriae expression

Plasmid	Expression[1]
pGB710	+
pGB84	+
pBS84	NA[2]
pGB84e	–
pGB711	–
pGB7B4	–
pGB7P2	–
pBS7B4	NA
pBS7P2	NA
pMMB207Bam	–
pMMB208Bam	–
pMMB207Pvu	–
pMMB208Pvu	–

[1] Expression of serotype 2 fimbriae related gene determined by reactivity of *B. bronchiseptica* strains R-5 and 17640 carrying the plasmid with monoclonal antibody CF8 in dot blots.

[2] NA = not applicable, pBluescript based plasmids cannot be maintained in *Bordetella.*

the pMMB207 and pMMB208 based plasmids which carry the *Bordetella* insert under the *tac* promoter, affected expression of fimbriae in *B. bronchiseptica*. This lack of expression from the *tac* promoter may indicate that the putative *fim2* structural gene on cosmid pGB710 requires the presence of other sequences on the cosmid which are not present in the subclone for expression. These other sequences could be genes for regulatory or accessory proteins. Alternatively, the fimbrial gene cloned into these plasmids may not be the right distance from promoter elements to be expressed from the *tac* promoter or may not contain a ribosome binding site which must be carried on the inserted fragment when these vectors are used.

Inactivation of the Fimbrial-Related Gene on pGB710

Since pGB710 affects expression of fimbriae and attachment by *B. bronchiseptica* strain R-5, inactivation of the gene affecting expression of fimbriae should prevent the increase in attachment observed when pGB710 is present in strain R-5 if fimbriae are involved in this effect. A plasmid was constructed containing a restriction fragment from pGB710 which hybridizes with the oligonucleotide probe for the 5' end of the *B. pertussis fim2* gene but not the probe for the 3' end of this gene. The gentamicin resistance *ori*T region of plasmid pUW2138 was also cloned into this plasmid. The plasmid was then introduced into strain R-5 with pGB710. Recombination due to a single crossover event would insert this entire plasmid into the fimbrial related gene contained on pGB710. The strategy for this recombinational inactivation is diagrammed in figure 30.

The resulting recombinant plasmid, designated pGB710R, confers tetracycline and gentamicin resistance to *B. bronchiseptica* strain R-5 and to *E. coli* strain DH-5α. Hybridization with pLAFR1, the gentamicin *ori*T fragment of pUW2138, and the 4.5 kb *Eco*RI fragment of pGB710 indicates that pGB710R contains sequences from each of the parent plasmids (data not shown). Dot blots of strain R-5 carrying plasmid pGB710 with monoclonal antibody CF8 show that CF8 does not react with R-5 pGB710R indicating

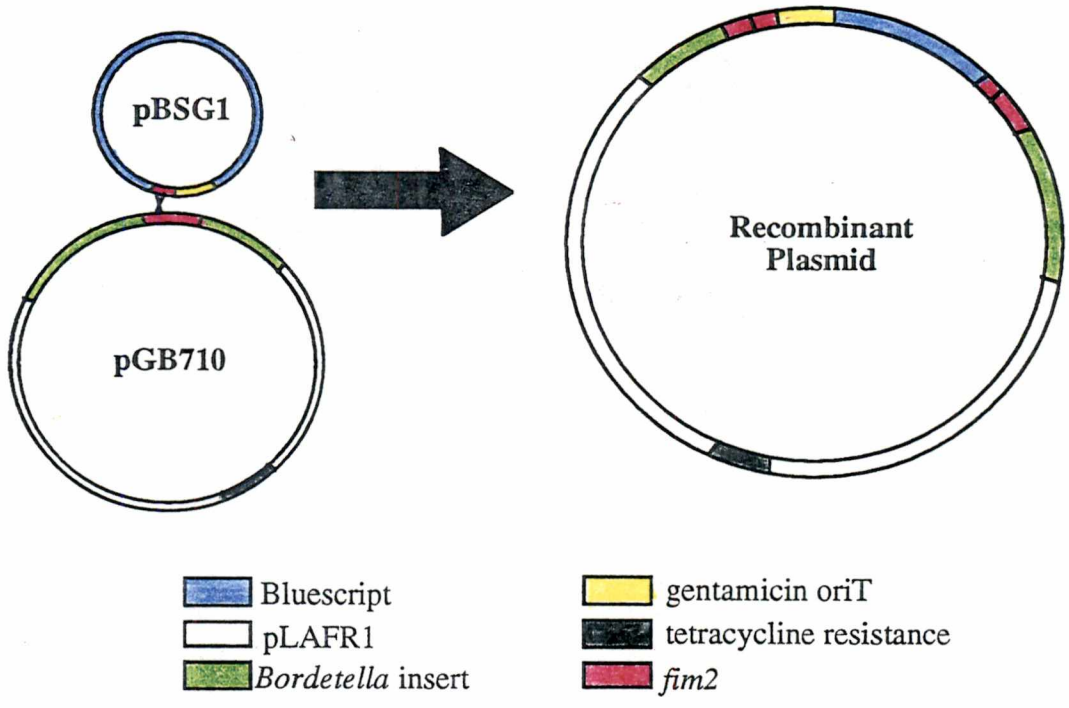


Figure 30: Recombinational inactivation.

that this plasmid no longer produces the effect on fimbrial expression caused by pGB710 (figure 31).

B. bronchiseptica strain R-5 carrying the recombinant plasmid, pGB710R, was used in *in vitro* attachment assays. The results of these assays are shown in figure 32. pGB710R attaches at a level similar to the parent strain R-5. The increase in attachment seen when pGB710 is present in R-5 is not observed with pGB710R. These data suggest that fimbriae may be involved in attachment of *B. bronchiseptica* to Vero cells since inactivation of the fimbrial related gene will prevent the increase in attachment observed when pGB710 is present in strain R-5.

Discussion

The presence of cosmid pGB710 in *B. bronchiseptica* strains 17640 and R-5 caused a change in their fimbrial protein profile to that of strain 110H from which the clone originated. Both of these strains produced more of the 24 kD fimbrial subunit protein when the cosmid was present. Western blots showed that this 24 kD band was antigenically related to serotype 2 fimbriae and since this protein has been previously identified as a fimbrial subunit protein (193), it is believed to be the major structural protein of serotype 2 fimbriae. These data suggest that the cosmid pGB710 may be carrying a copy of the *fim2* gene from *B. bronchiseptica*. However, the presence of a fimbrial regulatory gene on the cosmid could also cause the increase in production of serotype 2 fimbrial subunit proteins observed.

All fimbrial regulatory genes previously identified from *Bordetella* affect the expression of both serotype 2 and serotype 3/6 fimbriae (204, 360). Some of these fimbrial regulatory genes, *fimBCD* for example, also affect expression of the non-fimbrial adhesin FHA (204, 360). Therefore, if pGB710 carries a copy of a fimbrial regulatory sequence it would be expected to cause a change in production of the serotype 3/6 fimbrial subunit protein as well as the serotype 2 subunit. Western blots with *B.*

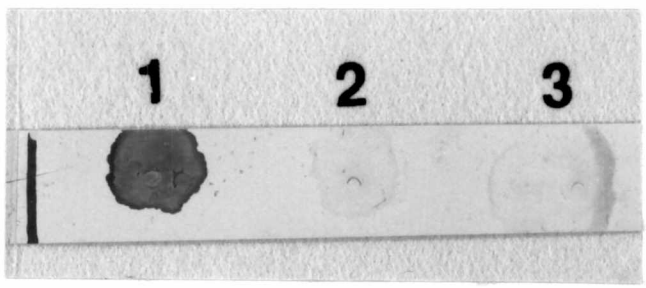


Figure 31: Reactivity of strain R-5 carrying pGB710R with CF8. Dot 1) R-5 pGB710; 2) 110NH; 3) R-5 pGB710R.

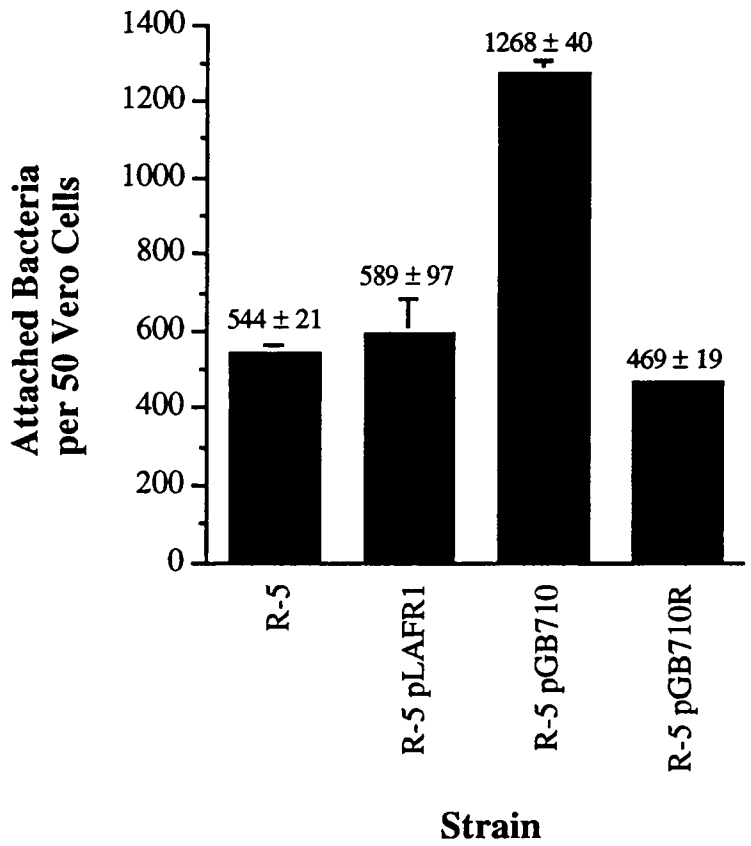


Figure 32: Attachment of strain R-5 carrying pGB710R. Assay was performed with***B. bronchiseptica*** **strain** R-5 and variants possessing the cosmid cloning vector (R-5 pLAFR1), the cloned fimbrial related gene (R-5 pGB710), and the inactivated gene (R-5 pGB710R). Results are reported as the mean number of bacteria attached per 50 Vero cells in three trials ± standard deviation.

bronchiseptica strains carrying cosmid pGB710 did show an increase in reactivity with antibody against the serotype 3/6 fimbrial subunit protein and Coomassie Blue stained gels of fimbrial protein profiles showed a slight increase in the amount of the 22 kD fimbrial subunit protein. No change was seen in the expression of FHA or other proteins.

In order to determine if the gene on cosmid pGB710 which affects serotype 2 fimbrial subunit expression requires the presence of Bvg for expression, the cosmid was introduced into the avirulent phase strain 110NH. The presence of the clone in this strain did not induce expression of the fimbrial subunit proteins. However, increased production of a 35 kD protein was observed on SDS-PAGE indicating that the cosmid may carry the gene for this protein. Mooi *et al.* (233) have previously observed a protein of this size in *B. pertussis* fimbrial enrichments. They suggest that this protein is a fimbrial accessory protein involved in transport of the fimbrial subunits or assembly of the fimbrial filament (233). The gene encoding this protein has not been identified. An increase in production of an accessory protein such as this one could cause an increased presence of fimbrial subunit proteins on the surface of the cell in the form of increased fimbrial filaments and would result in increased amounts of fimbrial subunit proteins as observed in the fimbrial protein profiles of strains 17640 and R-5 carrying cosmid pGB710. An increase in production of a fimbrial accessory protein would be expected to affect expression of both serotype 2 and serotype 3/6 fimbriae. An increase in both fimbrial serotypes was observed as a result of the presence of cosmid pGB710 in *B. bronchiseptica* strains 17640 and R-5.

In addition to causing an increase in fimbrial subunit expression in strains 17640 and R-5, cosmid pGB710 also causes an increase in attachment in strain R-5. This cosmid does not cause an increase in attachment by strain 17640 suggesting that there may be a limited number of *B. bronchiseptica* which can attach per cell. The presence of a finite number of receptors for *Bordetella* adhesins on the surface of the cells would account for this lack of an increase in attachment by *B. bronchiseptica* strain 17640. The attachment increase observed in *B. bronchiseptica* strain R-5 can be inhibited by the presence of

antibodies against serotype 2 fimbriae suggesting that fimbriae do play a role in attachment of *B. bronchiseptica.*

Monoclonal antibody CF8 reduces but does not eliminate attachment ability of strain 110H. This observation supports the hypothesis that *B. bronchiseptica* possesses multiple adhesins which act in concert to promote attachment. Interestingly, the monoclonal antibody against FHA was able to reduce attachment by strain 110H to a level equivalent to that of avirulent phase cells. Since pertactin is known to function as an adhesin and fimbriae are suspected to be involved in attachment, then one would expect these two adhesins to provide some attachment ability to strain 110H even after FHA is neutralized by an antibody. Therefore, the data suggest that pertactin and fimbriae may require the presence of FHA in order to exhibit an effect on attachment. These adhesins may actually enhance each other's functional ability. Alternatively, the antibodies against FHA may be sterically hindering access of pertactin or the fimbrial adhesin to proper receptors on the cell surface. The length and flexibility of fimbriae would, however, make it difficult for the anti-FHA antibodies to block this adhesin.

Since pGB710 contains a large *Bordetella* DNA insert, it is likely that genes other than the gene affecting expression of fimbriae are present on the cosmid. These genes could encode other adhesins of *B. bronchiseptica.* Experiments described in chapter 4 suggest that pGB710 does not contain the structural genes for FHA or pertactin. Dot blot hybridizations with *B. bronchiseptica* strain R-5 and 17640 carrying cosmid pGB710 indicated that the presence of the cosmid is not causing production of FHA or pertactin antigenically similar to that produced by strain 110H. In order to determine if the effect on attachment observed when pGB710 is present in strain R-5 is due to the fimbrial related gene instead of another gene on the cosmid, this gene was subcloned into a *B. bronchiseptica* vector, pBBR1MCS. However, none of the subclones were able to cause the increase in expression seen with the original cosmid pGB710. Therefore, larger pieces of DNA may be required in order to have the observed effect on fimbriae production.

Such a scenario is not implausible. Other *Bordetella* genes require large operons to be functional, including the FHA genes (Locht 92, Weiss 93).

Therefore, since we were unable to subclone a functional fimbrial related gene from pGB710, this gene was inactivated through insertion of a gentamicin *ori*T fragment into the gene by homologous recombination. Cosmid pGB710 containing this fragment does not increase expression of fimbriae in *B. bronchiseptica* strains as determined by dot blot with monoclonal antibody CF8. This recombinant plasmid also does not increase attachment by *B. bronchiseptica* strain R-5. These data suggest that the increase in attachment, seen when pGB710 is present in strain R-5, is mediated through the fimbrial related gene.

In conclusion, the experiments described in this dissertation suggest that fimbriae are involved in attachment and host species specificity of *B. bronchiseptica*. *B. bronchiseptica* strains isolated from pigs express more serotype 2 fimbriae on the cell surface than strains isolated from guinea pigs as indicated by increased reactivity with monoclonal antibody CF8 (Chapter 2). Although no correlation was seen between CF8 reactivity and attachment, a correlation was seen between the presence of the 24 kD serotype 2 fimbrial subunit protein and attachment (Chapter 3). In order to further explore this relationship, a gene hybridizing with probes for the major serotype 2 fimbrial subunit protein was cloned and introduced into *B. bronchiseptica* strain R-5 (Chapter 4) which did not attach well and only reacted with monoclonal antibody CF8 at an intermediate level (Chapters 2 and 3). The presence of this clone increased both CF8 reactivity and the amount of the 24 kD fimbrial subunit protein observed in fimbrial protein profiles. Attachment ability was also increased (Chapter 5). This increase in attachment could only be inhibited by antibody against serotype 2 fimbriae and not by antibodies against pertactin or FHA and could be eliminated by recombinational inactivation of the serotype 2 fimbrial gene on the cosmid pGB710 (Chapter 5). Therefore, despite evidence that

fimbriae are unnecessary for attachment in *B. pertussis* (234, 270, 278), the data suggest that fimbriae play a role in attachment by *B. bronchiseptica.*

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VITA

Eugene Burns was born July 23, 1965 in Knoxville, Tennessee. He attended public schools in Knoxville and graduated from Doyle High School in June 1983. The following September, Gene entered the University of Tennessee at Knoxville in the College Scholars Program. The College Scholars Program is an interdisciplinary program in the College of Liberal Arts (now known as the College of Arts and Sciences). In the spring of 1987, Gene completed his College Scholars research project in the laboratory of Dr. Karen Hughes in the Botany Department. This project involved development of an isolation procedure for chloroplast DNA from African violets and transformation of African violets in tissue culture using *Agrobacterium tumefaciens*. Gene was awarded the Bachelor of Arts Degree with Highest Honors in June 1987. The following September, he entered a graduate program in the Department of Microbiology at the University of Tennessee. As a graduate student, Gene taught several classes in the Department of Microbiology including Veterinary Bacteriology and Pathogenic Bacteriology. Gene's graduate research in the laboratory of Dr. David Bemis involved exploring the role of fimbriae in attachment by *Bordetella bronchiseptica*. Gene was awarded the Doctor of Philosophy Degree in May 1995 and is a member of the American Society for Microbiology.