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Bordetella bronchiseptica: A Candidate Mucosal Vaccine Vector

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To the Graduate Council:

I am submitting herewith a dissertation written by Sreekumari Rajeev entitled "Bordetella bronchiseptica: A Candidate Mucosal Vaccine Vector." I have examined the final electronic 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 Comparative and Experimental Medicine.

David A Bemis, Major Professor

We have read this dissertation and recommend its acceptance:

Robert N Moore, Stephen A Kania, Albert T Ichiki

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Graduate Studies

(Original signatures are on file in the Graduate Student Services Office.)

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A dissertation presented
for the Doctor of Philosophy Degree
The University of Tennessee, Knoxville

Sreekumari Rajeev
May 2002

Dedication

This dissertation is dedicated to my family, friends and mentors

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Abstract

Bordetella bronchiseptica colonizes the upper respiratory tract of a wide variety of mammals and is involved in diseases such as kennel cough in dogs, atrophic rhinitis in pigs and upper respiratory tract infections of laboratory animals. Studies were focused on developing a heterologous antigen expression system in *B. bronchiseptica* and to evaluate the potential of this organism as a candidate mucosal vaccine vector. Since the role of *Bordetella bronchiseptica* and *Pasteurella multocida* toxin (PMT) in the disease atrophic rhinitis of pigs was well documented, this research was focused in the possibility of developing a refined vaccine to control this disease. A promoter region related to heat shock protein genes was identified using a green fluorescent protein reporter system. This promoter drove high-level expression of GFP compared to a *tac* promoter or *B. bronchiseptica fim N* gene promoter. A non-toxic protective *P. multocida* toxin fragment and GFP were expressed in *B. bronchiseptica* in a broad-host-range plasmid vector PBBR1MCS2 under the control of the promoter region identified. Colonization kinetics, plasmid stability, and immune responses generated following intranasal inoculation of recombinant *B. bronchiseptica* were evaluated. While wild type and recombinant *B. bronchiseptica* colonized the mouse respiratory tract effectively, the plasmid was completely lost from the organism after 72 hours post-inoculation. After a single intranasal inoculation, *B. bronchiseptica* specific IgM, IgA and IgG responses were detected in serum and respiratory lavage. However, PMT-specific antibodies were not detected. Four intranasal inoculations with *B. bronchiseptica* expressing green fluorescent protein (GFP) induced a GFP-specific systemic and mucosal immune response, while

similar inoculations with *B. bronchiseptica* expressing PMT fragment did not induce a PMT-specific immune response.

This study also evaluated the immune response to a chimeric protein generated by combining a gene fragment encoding neutralizing epitopes of *Mannheimia haemolytica* leukotoxin and a fimbrial protein gene (*fim N*) from *B. bronchiseptica*. Immunization of mice with the recombinant chimeric protein elicited a significantly stronger anti-leukotoxin antibody response than comparable immunizations with fusion proteins lacking FIM N. The chimeric protein exhibited more stability. Leukotoxin is an important virulence factor in shipping fever pneumonia in feedlot cattle and is a critical protective antigen. This chimeric protein may be a candidate for inclusion in new generation vaccines against shipping fever pneumonia. The results of these studies strongly support the potential for developing *B. bronchiseptica* as a candidate mucosal vaccine vector and FIM N as a carrier protein for heterologous antigens.

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PART I

Literature Review

Introduction

Bordetella bronchiseptica is an upper respiratory tract pathogen, which infects a wide variety of host species including domestic, laboratory and wild animals and may also opportunistically infect human beings [33, 60]. *B. bronchiseptica* is involved in diseases such as kennel cough in dogs, and atrophic rhinitis in swine [60]. This organism is an adept colonizer of the upper respiratory tract of mammals and colonization is characterized by attachment to the ciliated epithelium [6, 7, 8, 9]. The attachment is achieved by the expression of adhesins such as fimbriae, filamentous haemagglutinin and pertactin [33, 81, 147].

Atrophic rhinitis is an upper respiratory tract disease of pigs characterized by degeneration and atrophy of nasal turbinate bones in market weight hogs, leading to visible distortion and shortening of the snout [20, 60, 67, 165, 169]. The infection with *B. bronchiseptica* leads to a mild reversible form of atrophic rhinitis, whereas, infection with *Pasteurella multocida* leads to a severe progressive form of atrophic rhinitis [1, 113, 165 166]. Toxins produced by *B. bronchiseptica* and *Pasteurella multocida* are involved in this disease condition [36, 37, 48, 49, 50, 91, 92, 93]. Dermonecrotic toxin produced by virulent strains of *B. bronchiseptica* can induce impaired osteoblastic differentiation and atrophic rhinitis in pigs, rabbits and mice [104, 113].

Pasteurella multocida is an important veterinary pathogen involved in diseases such as atrophic rhinitis in swine, fowl cholera in birds, hemorrhagic septicemia in cattle and other respiratory diseases in lab animals [20, 21]. *Pasteurella multocida* toxin (PMT) produced by capsular type D or type A strains of *P. multocida* is a major virulence factor in atrophic rhinitis [40, 148, 149, 184]. This toxin is a potent, intracellular, heat labile,

146kD protein that induces bone resorbtion through increased osteoclastic and impaired osteoblastic activity [66, 91, 92, 93, 98, 115, 116, 146, 151, 166]. It also causes degeneration and necrosis of liver, pneumonia, spleenic atrophy, orchitis and proliferation of bladder epithelium in experimental animals [48, 49]. PMT triggers a number of signal transuduction pathways leading to cytoskeletal rearrangement [182, 195]. The role played by *P. multocida* and *B. bronchiseptica* in atrophic rhinitis is well documented [34, 36, 37]. A mutualistic disease-causing relationship exists between these two organisms [34, 36, 37]. Colonization with *B. bronchiseptica* predisposes animals to infection with *P. multocida,* which may lead to a severe atrophic rhinitis and financial losses in the swine industry [1, 20]. PMT, itself, can also lead to a severe form of atrophic rhinitis when inoculated into experimental animals [48, 49].

Atrophic rhinitis vaccines initially consisted of different preparations of killed or live avirulent *B. bronchiseptica.* After elucidating the role of PMT in atrophic rhinitis, the use of PMT toxoid or non-toxic recombinant derivatives provided a significant degree of protection from PMT induced atrophic rhinitis and now most vaccines consist of *B. bronchiseptica* bacterin/ live attenuated vaccines and *P. multocida* bacterin and toxoid [12, 48, 49, 89, 142, 184].

Considering the synergistic role of *B. bronchiseptica* and *P. multocida* in atrophic rhinitis, further development of combined vaccines seems to be a rational approach to control this disease. A mucosal immune response to *B. bronchiseptica* will lead to immunological elimination of *B. bronchiseptica* thus preventing colonization with *P. multocida* and other respiratory pathogens. Anti-PMT-antibodies will reduce the incidence of PMT-induced atrophic rhinits.

The mucosal surfaces are the portals of entry of many pathogens and they constitute the first lines of defense against pathogens [118, 119]. Attachment properties of *B. bronchiseptica* could be effectively utilized to deliver heterologous antigens to the respiratory tract for the purpose of inducing protective mucosal immune responses against respiratory pathogens.

The major goal of this study was to explore the possibility of *B. bronchiseptica* as a candidate mucosal vaccine vector.

Specific aims of this study were:

1. To identify and isolate a constitutive promoter from *B. bronchiseptica* using a green fluorescent protein reporter system.
2. To clone and express a non-toxic *P. multocida* toxin fragment under the control of a suitable promoter.
3. To study colonization, plasmid stability and antibody response following intranasal inoculation with *B. bronchiseptica* expressing the PMT fragment.
4. To evaluate the efficacy of Fim N protein of *B. bronchiseptica* as a carrier protein for heterologous antigens.

The Genus Bordetella

Bordetellae are small, gram negative, aerobic, non-acid fast, non-spore forming coccobacilli [81, 147, 150]. All species of *Bordetella* are asaccharolytic [81, 147, 150]. They utilize amino acids and other organic acids as sources of energy [81, 147, 150]. Their optimum temperature for growth is 35-37^{o}C. The members of this genus are *B. pertusiss, B. parapertussis, B. bronchiseptica, B. avium, B. hinzi, B. holmesii,*

B. trematum and *B. petrii* [33, 81, 147, 150]. All the members of this genus are catalase positive. *B. pertussis* is the most fastidious species within the genus, and does not grow on simple media [81, 147, 150]. Other *Bordetella* species are less fastidious except *B. parapertussis* and *B. holmeseii*, which are relatively slow growing [81, 147, 150]. Nitrate reduction, urease, oxidase and motility tests can be utilized to differentiate species [81, 147, 150]. The members of this genus are closely related, genetically, and their DNAs have a G+C content of 60.2 -70% [147].

B. pertussis is strictly a human pathogen responsible for whooping cough in children,and pertussis in adolescents and elderly humans [33, 147, 188]. This is an acute, highly contagious disease in children characterized by characteristic paroxysmal coughing. *B. parapertussis* is also a human adapted pathogen and causes pertussis-like syndrome in humans [81, 147, 150]. It has also been isolated from cases of chronic non-progressive pneumonia in sheep [33, 147]. *Bordetella avium* is responsible for a condition known as coryza or rhinotracheitis in turkey poults and chickens [81, 147, 150]. This disease condition is highly contagious and predisposes birds to secondary infections [87]. *Bordetella bronchiseptica* has been associated with acute tracheobronchitis or kennel cough in dogs and atrophic rhinitis in swine [6, 7, 8, 9, 60]. This organism can also cause upper respiratory tract infection of a wide variety of mammals such as mice, rabbits, guinea pigs, cats, and horses [60].

B. hinzii, has been isolated from the respiratory tract of immunocompromised human beings, healthy turkeys, and chickens [81, 147, 150]. *B. holmesii*, has been isolated from human beings with respiratory tract infection and septicemia [81, 147, 150]. *B. trematum* has been isolated from human beings with wound infections and otitis media [33, 81,

147, 150]. *B. petrii*, the new member of this genus, was isolated from an anaerobic bioreactor [191]; however, there is very little information available on this species.

The population structure of the *Bordetella* genus has been subjected to extensive evolutionary studies. The close phylogenetic relationship of *Bordetella* with *Alcaligenes* and other environmental bacteria suggests that the ancestral *Bordetellae* were free-living and evolved to infect warm-blooded animals [156]. The 16S ribosomal RNA analysis placed *B. bronchiseptica* as the nearest relative to bacterial endosymbionts in protozoa [156]. Musser et al. obtained an estimate of genetic relatedness of 60 strains of mammalian *Bordetellae* from worldwide sources by utilizing multilocus enzyme electrophoresis of 15 metabolic enzymes [137]. Van der Zee et al. also attempted to differentiate members of this genus by comparing the electrophoretic mobilities of metabolic enzymes [190]. Multilocus sequence typing based on sequencing of a portion of house keeping genes and insertion sequences were also utilized to compare the evolutionary relationship of members of the genus [61, 156, 190]. These studies concluded that *B. pertussis, B. parapertussis and B. bronchiseptica* are subtypes of a single genomic species. *B. avium, B. holmesii, and B. hinzii* each form true genomic species [190]. It was proposed that *B. bronchiseptica* may be the evolutionary progenitor and other species may be considered as host adapted lineages of *B. bronchiseptica* [61, 156, 190]. The sequence comparison of genes encoding fimbrial subunits, adenylate cyclase toxin, pertactin, pertussis toxin and BvgAS, confirmed the close relationship between B. *bronchiseptica* and *B. parapertussis* and a more distant relationship between these species and *B. pertussis* [33, 190]. Also, human and sheep isolates of *B. parapertussis* comprise genetically distinct population [33, 190]

Gene regulation in Bordetella

Virulence gene expression in *Bordetella* spp is controlled by a two-component signal transduction system encoded by the *Bordetella* virulence gene (bvg) locus [187, 196, 197]. Some of the virulence factors controlled by this system include fimbriae, filamentous haemagglutinin, pertactin, pertussis toxin, adenylate cyclase toxin and dermonecrotic toxin. The BvgAS system is a two component signal transduction system controlled by proteins, BvgS (a sensory transmembrane protein) and a transcriptional activator BvgA (a cytoplasmic DNA binding protein) [33, 196, 197]. This locus is activated by temperatures above 37^{o}C, and low sulfate or nicotinic acid concentrations resulting in expression of virulence factors [33, 196, 197].

A second set of genes is activated when the locus is shut down by low temperature (less than 30^{o}C) or in the presence of increased sulfate or nicotinic acid concentration. Genes involved in flagella synthesis and urease expression [121, 131, 198] in *B. bronchiseptica* are negatively regulated by the *bvg* locus. However, in human isolates of *B. parapertussis* urease is repressed by *bvg* locus whereas in sheep isolates of *B. parapertussis* it is not under the control of *bvg* locus [33]. In some strains of *B. bronchiseptica*, genes involved in alcaligin biosynthesis are negatively regulated by the *bvg* locus [58]. In *B. pertussis,* five genes (*vrg*6, *vrg*18, *vrg*24, *vrg*53, *vrg*73) which involve a second intermediate regulatory locus, *bvgR* (a transcriptional repressor) were discovered [101, 126].

Phenotypic modulation was described in earlier literature as the reversible loss of virulence-associated phenotypes that happens in response to changes in environmental conditions [196, 197]. Many of the earlier descriptions of the phases (phase I to phase IV)

were based on colony morphotypes. The virulence phase was called X mode (now known as *bvg* positive). X mode is seen at 37^{o}C and in the presence of certain chemical ions such as sodium, potassium, halides, formate, and nitrate [33, 196, 197]. The avirulent phase, called C mode (now known as *bvg* negative) was seen at low temperatures and in the presence of ions such as sulfate and nicotinic acid [33, 196, 197]. Phase variation is the irreversible loss of virulent phenotype due to mutations occurring in the *bvg*-locus [33, 196, 197]. In *B. bronchiseptica*, positively regulated traits (Bvg+) were required for respiratory infection. Whereas, negatively regulated traits (Bvg-) were required for surviving nutrient limitation [30].

A recent study described another phase called *bvg* intermediate phase (Bvgi), when *B. bronchiseptica* was grown in semi-modulating conditions [31]. This phase was characterized by the presence of one subset of *bvg*+ factors with the absence of other *bvg*+ factors and the presence of factors, which are exclusively expressed in this phase [31]. Isolates with *Bvgi* phenotypes displayed reduced virulence in a rat model of respiratory tract infection and increased ability to survive nutrient deprivation [31]. Stockbauer et al. described a protein called BipA, and its gene, which was exclusively expressed, in the Bvg intermediate phase [183]. A second two-component sensory transduction system called "*ris*" has been described in *B. bronchiseptica*. This system had similarities to the *bvg* system and was up regulated at 37^{o}C and down regulated at lower temperatures or in the presence of magnesium ions [90].

Bordetella virulence factors

Colonization of *Bordetella* in the upper respiratory tract of the host is mediated through strong attachment to the ciliated epithelium. A number of adhesins such as

fimbriae, filamentous haemagglutinin, and pertactin have been recognized. Heavy colonization with production of toxins like dermonecrotic toxin, adenylate cyclase, tracheal cytotoxin, pertussis toxin, and endotoxin will lead to inflammatory responses in the respiratory tract that may produce significant clinical disease [33, 147, 150]. Major virulence factors produced by members of the genus *Bordetella* are discussed below.

B. pertussis is known to produce pertussis toxin, which is the important virulence factor in whooping cough in children [141]. *B. bronchiseptica* and *B. parapertussis* also contain the pertussis toxin gene, but do not express pertussis toxin [2, 73]. This is due to the differences in sequences in the promoter region, which make the toxin inactive [73]. Replacement of the pertussis toxin promoter region in *B. bronchiseptica* and *B. parapertussis* with that of *B. pertussis* resulted in expression of active toxin [73]

Adenylate cyclase toxin (ACT) is a member of the RTX (repeat in toxin) family of bacterial toxins [59, 63]. RTX toxins are a family of pore-forming proteins of Gram-negative bacteria, which contain glycine-aspartic acid rich repeats [52]. This toxin has bifunctional activity as a hemolysin and adenylate cyclase toxin. Adenylate cyclase and hemolytic activity are separable [157]. ACT can enter eukaryotic cells through pore formation and become activated by calmodulin, which leads to the production of unregulated cyclic AMP levels [59, 63, 75, 114, 157]. ACT is a 216 kD secreted protein and is produced by all members of the *Bordetella* genus except *B. avium* [33, 147, 157]. The major differences in amino acid sequence of adenylate cyclase toxin between *B. bronchiseptica* and *B. pertussis* were located on the carboxy terminal repeat region of the molecule [10]. Harvill et al. studied wild-type and mutants of *B. bronchiseptica* with alterations in adenylate cyclase toxin and concluded that phagocytic cells are the primary

targets of this toxin [71]. ACT is unique among the RTX toxins; in that it has enzymatic activity as well as the capacity to form an ion-permeable pore in target cell membranes. The latter causes lysis of erythrocytes, which is a colony, associated phenotype that can be easily seen on Bordet-Gengou agar containing horse or sheep blood cells [75, 157]. ACT mutants were defective in colonization [71]. The protective nature of ACT antibodies against infection suggests that this toxin is required for initial colonization [59, 97]. ACT down regulates the phagocytic activity of neutrophils and macrophages and can induce apoptosis in macrophages [10, 71]. The antigenic and protective properties of adenylate cyclase toxin of *B. bronchiseptica* are different from that of *B. pertussis* [10, 75].

Dermonecrotic toxin (DNT) is a 162 kD, intracellular, dermonecrotic, thermolabile and mitogenic cytotoxin produced by *Bordetella spp.* [138, 154, 194]. DNT can impair osteoblastic differentiation and is an important virulence factor in atrophic rhinitis in pigs, rabbits and mice [104, 113, 162]. DNT is cytotoxic for Vero cells, embryonic bovine lung cells and bovine turbinate cells [83, 178]. It induces morphological changes in cultured fibroblasts, including the assembly of actin, stress fiber formation, focal adhesion assembly, multinucleation and is a potent mitogen [83, 104, 178]. DNT deamidates the Gln 63 residue of small G proteins like Rho, Rac and Cdc42 leading to their constitutive activation and polymerization of actin [83, 95, 178]. DNT shows structural and functional homology to Cytotoxic Necrotizing Factor I of *E. coli* and deamidation activity of both these toxins are mapped to their C- terminus [110].

Tracheal cytotoxin (TCT) is a muramyl peptide released by *Bordetellae,* [74, 112] and is responsible for specific epithelial pathology in whooping cough. TCT stimulated

production of IL1 and formation of large amounts of nitric oxide result in damage to ciliated epithelial cells [33, 74, 112]. It has been suggested that TCT may also be involved loss of ciliary activity and colonization of the organism in the respiratory tract [9, 29].

Pertactin (PRN) is an outer membrane protein, and a nonfimbrial adhesin that belongs to the family of autoexporters [22, 42, 109, 111]. Autoexporter/ autotransporters are proteins, which have the ability to export themselves to the outer membrane, cleave themselves via protease activity and release final product into the medium [70]. Pertactin was initially identified in *B. bronchiseptica* as a 68kD protein [132]. It is a 69kDa protein in *B. pertussis* and a 70kD protein in *B. parapertussis* [22, 132]. Pertactin contains one RGD (arginine-glycine-aspartic acid) motif and several proline rich as well as leucine rich repeats involved in eukaryotic cell binding [33, 109]. Pertactin is also a protective antigen that is included in acellular pertussis vaccines. Although pertactin can function as a nonfimbrial adhesin and can induce a protective antibody response against *Bordetella*, its role in pathogenesis is unknown [33]. Recently genetic and phenotypic heterogeneity has been reported in strains of *B. bronchiseptica* and *B. pertussis* [135, 158]. This variation, which occurs in the repeat sequence motifs, is believed to be due to antigenic drift occurring in immunized populations.

Other *Bordetella* proteins with predicted autoexport properties include Tracheal Colonization Factor [47] BrkA [45, 163] and Vag8 [46]. These proteins share amino acid sequence homology at their C terminus, contain RGD motifs and are detected only in *B. pertussis*.

Filamentous haemagglutinin (FHA) is a high molecular weight, secreted outer membrane protein reported in *B. bronchiseptica, B. pertussis, B. parapertussis* [33, 86, 145]. FHA is a major attachment factor and protective antigen in *Bordetella* [108]. There are important domains in FHA, including a RGD motif, a carbohydrate-binding site and a heparin-binding site [68, 153, 159, 160], which are involved in eukaryotic cell binding. The RGD sequence of FHA binds to the integrin, CR3, of macrophages, which may promote phagocytosis. Attachment of FHA to ciliated cells is mediated through the carbohydrate-binding domain [153, 159, 160]. The heparin-binding domains of FHA may help in low affinity binding of the bacteria to the extracellular matrix of the upper respiratory tract [68, 122]. FHA is highly immunogenic and antibodies to FHA prevent attachment of *B. pertussis and B. bronchiseptica* [86, 96]. FHA is also a good mucosal immunogen as evidenced by high levels of anti-FHA antibodies in infected individuals [33, 86, 96, 108, 152]. In one study, FHA enhanced the mucosal immunogenicity of liposome delivered antigen administered via intranasal route [152]

Cotter et al. suggested that, FHA mediated attachment to tracheal epithelium allowed *Bordetella* to overcome constitutive mucociliary mechanisms in the trachea [32]. FHA appears to be secreted in lower amount in *B. bronchiseptica* compared to *B. pertussis* [86, 88, 108]. It was postulated that the different levels of FHA expression in these species might be due to differences in protein production, differences in cell envelope composition and structures that affect export from cells or degradation [88].

Fimbriae produced by *B. bronchiseptica* species are important virulence factors which help in adherence and colonization of the bacteria to host tissues [99]. Fimbriae are heteropolymeric filamentous appendages that are involved in attachment of many Gram-

negative bacteria. They are highly immunogenic, antigenically stable proteins that are included as components of many acellular vaccines [89]. Major fimbrial subunit genes of *B. bronchiseptica* are *fim2, fim3, fimX, fimA* and *fimN* [13, 17, 94, 133, 134, 172, 199, 200]. The fimbrial proteins of *B. bronchiseptica* and *B. pertussis* are serologically cross-reactive. Fimbrial biogenesis is a complex process involving a cluster of genes called *fimABCD* [85, 99]. These are located in the FHA operon immediately downstream to *fhaB*, the structural gene for filamentous haemagglutinin. A mutation in *FimB* can abolish the expression of fimbriae [117]. Major fimbrial subunit genes are located elsewhere in the chromosome [99]. *Fim A* encodes a major structural subunit that is nonfunctional in *B. pertussis* but is expressed in *B. bronchiseptica* [13]. Fim D, a minor fimbrial subunit produced by *B. pertussis,* is a putative adhesin and tip protein [57]. The sequence of *fimD* in *B. bronchiseptica* differs by only one base pair from that in *B. pertussis*. Mutation in *fimD* blocks the expression of major fimbrial subunits suggesting that it may have chaperone like activity [57]. The propensity for frequent deletion or addition of bases in a cytosine-rich sequence within the *Bordetella* fimbrial promoter region is a proposed mechanism for fimbrial type-switching, a phenomenon which enables *Bordetella* to express one type, two types or no fimbriae at a given point of time [200]. This may be attributed to selective pressure driven by the host's immune response or there may be differences in host receptors that induce the expression of a particular fimbrial gene. Receptors for fimbriae contain sulfated sugars like heparin sulfate, which are ubiquitous in the respiratory tract [55, 56]. Sequences similar to the heparin-binding region of fibronectin have been evaluated in *fim2* and *fim3* subunits of *B. pertussis* [55, 56]. Studies on expression of *B. pertussis* fimbriae in *E. coli* revealed that a recombinant subunit

protein was immunonologically and antigenically distinct from the native fimbriae and exhibited limited cross reactivity [192, 193]. Quaternary structure generated by subunit interaction of the fimbrial protein may be necessary for its incorporation as a protective immunogen in vaccines. Fimbriae are required for establishment of persistent colonization of the trachea in mice and may play a role in the development of a humoral immune response to *Bordetella* infection [117].

The products of Type III secretion systems are other newly identified factors involved in *B. bronchiseptica* virulence [206, 207]. Type III secretion allows Gram-negative bacteria to translocate molecules into the cytoplasm of eukaryotic cells [202]. These products interact with a variety of eukaryotic signal transduction pathways to promote bacterial-host interactions [202]. In *B. bronchiseptica* the type III secretion products are specially transcribed under Bvg+ conditions [206]. These products are highly immunogenic in rabbits and are required for in vitro cytotoxicity [33, 206, 207]. These products may also be expressed by *B. pertussis* and sheep isolates of *B. parapertussis* [33]. Yuk et al. investigated the functions of the type III secretion system in *B. bronchiseptica* by comparing the wild-type bacteria with two strains that were defective in type III secretion system [206, 207]. The mutants were defective in long-term colonization of the trachea in immunocompetent mice. Also, mutants induced high titers of anti-bordetella antibodies compared to wild-type. The authors suggested that type III secretion products interacted with components of innate and adaptive immune systems of the host and that these products were required for inducing apoptosis in macrophages in vitro and increasing the influx of inflammatory cells in vivo. This modulation of immune response may be achieved through inactivating NF-kB, a transcription factor for

regulation of genes involved in immune response [206]. Winstanley et al. investigated and confirmed the presence of a type III secretion system in animal isolates of *B. bronchiseptica* [201].

The lipopolysaccharide (LPS) molecules of members of the *Bordetella* genus differ in their structure [33]. *B. pertussis* contains a complex trisaccharide with no O antigen. Therefore, it is generally referred as lipo-oligosaccharide. *B. bronchiseptica* has a trisaccharide plus an O- antigen repeat and *B. parapertussis* has an altered trisaccharide plus O-antigen-like repeat [72]. The *wlb* gene cluster, composed of 12 genes, is required for biosynthesis and addition of the trisaccharide in *B. pertussis* and *B. bronchiseptica* and O-antigen-like repeat in *B. bronchiseptica* and *B. parapertussis* [72]. Harvil et al. investigated the effect of mutation of *wlb* locus on colonization of *B. bronchiseptica* in the respiratory tracts of mice. They observed that biosynthesis of full length LPS, by these three *Bordetellae* are essential for virulence in mice [72]. However, LPS molecules of these species play different roles in infection [72]. Changes in LPS expression in *B. bronchiseptica* is controlled by BvgAS system [189]. When resistance profiles of *B. bronchiseptica* and *B. pertussis* to various antimicrobial peptides were tested, [4] *B. bronchiseptica* exhibited significantly higher resistance to antimicrobial peptides compared to *B. pertussis*. The resistance in *B. bronchiseptica* was presumably due to the highly charged O-specific sugar side chains in its LPS [4]. It was suggested that antigenic polymorphism in the lipopolysaccharides from human and animal isolates of *B. bronchiseptica* may be due to differences in receptors of human and animal respiratory tracts [107].

Urease, a *bvg* repressed phenotype in *B. bronchiseptica,* has been another suggested virulence factor which may be involved in intracellular survival of this bacterium [120]. However, urease expression did not have any effect on the ability of *B. bronchiseptica* to colonize and persist in the mouse respiratory tract [121, 131].

Intracellular survival

Although *B. bronchiseptica* has been considered an extracellular pathogen, there have been a number of reports that demonstrate the intracellular survival of this organism. Guzman et al. demonstrated intracellular survival of *B. bronchiseptica* in mouse dendritic cells, in vitro and speculated that intracellular survival in natural infections caused by this organism, may lead to the chronicity of infection [62]. Intracellular invasion of *B. pertussis* was dependent on Bvg + phenotype and induction of apoptosis in macrophages was attributed to the adenylate cyclase toxin [5]. *B. bronchiseptica* induced macrophage killing by bvg -regulated factors [5]. Also, *Bvg* mutants of *B. bronchiseptica* showed significant survival advantage over wild type strains [5]. A study by Brockmeier and Register on intracellular survival and cytotoxicity of *B. bronchiseptica* in swine alveolar macrophages indicated that another temperature dependent regulatory mechanism, in addition to *Bvg*, may play a role in adhesion and intracellular survival of this organism [14]. However, cytotoxicity for swine alveolar macrophages was observed only in infection with virulent bvg+ strains [14]. The differential survival of *B. pertussis* and *B. bronchiseptica* in macrophages has been attributed to their differences in acid tolerance [179]. In contrast to *B. pertussis*, *B. bronchiseptica* is insensitive to an acidic PH as low as 4.5. The acidic environment of the phagolysosome contributes to increased intracellular survival of this organism. A significant TH1 response observed after

intranasal inoculation with *B. bronchiseptica* indicates that, there may be relevant intracellular phases of this bacterium during the infection cycle [64]. Chhatwal et al. suggested that, the expression of a temperature regulated acid phosphatase in *B. bronchiseptica* played a role in intracellular survival [25]. Forde et al. characterized the uptake and persistence of *B. bronchiseptica* in murine phagocytes using a bioluminescence-based reporter system [51]. In this study, *B. bronchiseptica* was mutagenized with a suicide vector carrying *lux* genes from *Photorhabdus luminescens* on a mini-Tn 5 derivative [51]. *B. bronchiseptica* was internalized by professional phagocytes in a dose-dependent manner and the bacterium survived intracelluarly for four days when a critical population size (>500:1 multiplicity of infection) was present [51]. This study also suggested that *B. bronchiseptica* may have an intracellular phase during the infection cycle.

Diseases caused by B. bronchiseptica

B. bronchiseptica had been considered a respiratory pathogen of mammals since its identification in 1910 [60]. The involvement of this organism has long been recognized in diseases such as acute infectious tracheobronchitis in dogs, atrophic rhinitis in swine, and respiratory infections of laboratory animals [60]. These diseases are mostly self-limiting, but can be chronic and some times complicated by secondary invaders [6, 7, 60]. The hallmark of infection by this organism is prolonged, efficient colonization of ciliated epithelium of the respiratory tract [9]. The ability to interfere with ciliary function is an important factor in pathogenesis of *B. bronchiseptica* infections [9].

Canine infectious tracheobronchitis, or kennel cough, is a highly contagious respiratory tract disease, affecting dogs, that is characterized by acute onset of cough [6,

7, 8, 9, 188]. Studies on experimental and natural infections of *B. bronchiseptica* in canines proved that this organism could be considered as a primary etiological agent in infectious tracheobronchitis [8]. The infection leads to moist, hacking, productive cough [7, 8, 9, 188]. Microscopic lesions were limited to the ciliated epithelial mucosa with influx of polymorphonuclear cells being a prominent feature [9]. Bacterial colonization in experimental studies reaches its maximum at seven days after aerosol exposure and slow progressive clearance occurs thereafter [8]. Many infected animals can remain asymptomatic and the disease is usually self-limiting [9, 33].

Atrophic rhinitis is an upper respiratory tract disease of pigs characterized by degeneration and atrophy of nasal turbinate bones leading to visible distortion, and shortening of the snout [60, 169]. *B. bronchiseptica* can cause moderately severe reversible turbinate atrophy [48, 49, 162]. Dermonecrotic toxin production by *B. bronchiseptica* is correlated with turbinate atrophy [113,162, 165]. Experimental infections in gnotobiotic pigs implicated that the colonization with phase I cytotoxic strains of *B. bronchiseptica* damages the nasal mucosa and predisposes to colonization by toxigenic *P. multocida* that can lead to a more severe form of the disease [162, 165]. Two recent studies on co-infection with *B. bronchiseptica* and porcine reproductive and respiratory syndrome virus (PRRS) strengthen the hypothesis that, *B. bronchiseptica* infection can adversely affect the respiratory tract defense mechanism leaving animals vulnerable to infection with secondary agents like *P. multocida* [15, 16].

There has been increased documentation of *B. bronchiseptica* respiratory tract infections of cats [181]. Clinical and experimental studies have proved that *B. bronchiseptica* can also be a primary respiratory pathogen in cats [84]. Laboratory

animals, especially guinea pigs, rabbits and rats get respiratory tract infections with *B. bronchiseptica* [60]. Infections can be insidious in such populations; seventy five percent of the rabbits in a commercial rabbitry were infected naturally with *B. bronchiseptica* without showing any disease [35]. *B. bronchiseptica* has also been associated with respiratory tract infections in horses [26, 76]. Rats and mice have been used as experimental animal models to study *B. bronchiseptica* pathogenesis [32, 33, 72, 117]. Intranasal infection with a subletha l dose of *B. bronchiseptica* leads to prolonged asymptomatic colonization in mice and rats [117].

There are well-documented cases of *B. bronchiseptica* infection in human beings [204]. They are usually associated with immunocompromised patients. In an extensive review, Woolfrey and Moody concluded that the agents might be occasionally encountered as a commensal of the respiratory tract and rarely as a pathogen in severely immunocompromised patients [204]. Animal contact may or not be a recognizable risk factor and nosocomial human to human transmission has been reported [176]. Infections in immunocompetent children are also reported [177]

Immunity to B. bronchiseptica infections

Most naturally occurring *B. bronchiseptica* infections are localized to the respiratory tract [6, 60]. Recovery from infection in dogs resulted in resistance to re-infection that lasted for 6 months [6, 60]. Vaccination with formalin killed bacteria produced high titers of serum agglutinins but did not prevent infection [7]. The dogs that recovered from experimental infection and were maintained in isolation were resistant to a subsequent aerosol challenge seven months after recovery. *B. bronchiseptica* specific sIgA in the respiratory tract has been correlated with protection [11]. Killed, parenterally, inoculated

whole-cell vaccines of *B. bronchiseptica* were successfully used to prevent death, bronchopneumonia and carrier state in lab animals [60] and to protect swine from *B. bronchiseptica* induced-atrophic rhinitis [60, 102]. A subcellular *B. bronchiseptica* vaccine containing concentrated cell wall fractions provided greater protection in swine than the whole cell vaccines [60]. A local immune response to *Bordetella* infection was achieved by a live, avirulent intranasal vaccine, which induced early protection against disease in swine and dogs. Intranasal immunization with live avirulent bacteria may reduce clinical disease and duration of shedding and may induce some degree of colonization resistance [7, 60]. Although some parenteral vaccines induced high levels of agglutinating antibodies, they did not provide protection from diseases [60]. An acellular extracted antigen vaccine protected dogs from disease and reduced shedding of *B. bronchiseptica* [41]. In dogs, parenteral or intranasal *B. bronchiseptica* vaccines may provide substantial protection from clinical signs of respiratory tract diseases, administration of both types of vaccines, in sequence, provided greatest degree of protection against the disease [41].

Gueirard et al. noted that colonization of lungs in BALB/c mice inoculated with a live, virulent strain of *B. bronchiseptica*, increased during the first 10 days and decreased thereafter [64]. A non-virulent strain in similarly inoculated mice, was cleared from lungs by 6 days. In this study, a human isolate lacking adenylate cyclase was unable to induce progressive infection of the respiratory tract of mice. Infection was also associated with an influx of leukocytes into the respiratory tract. *B. bronchiseptica*-reactive serum IgG and IgM were observed soon after infection, levels gradually increased for 14 days and remained constant for 117 days. Much of the IgM production that was produced was not

specific for *B. bronchiseptica* [64] perhaps due to triggering mechanisms unrelated to specific epitope recognition. *B. bronchiseptica* specific IgA in serum was induced soon after infection (14 days post-infection) and increased during the 117 days observation period. A cellular immune response was demonstrated to *B. bronchiseptica* and adenylate cyclase. High levels of IFNγ and IL 1-α detected in antigen-induced proliferation assays indicated that a Th1 type of immune response was produced during *B. bronchiseptica* infections. *B. bronchiseptica* infections were not accompanied by immunosuppression of cellular response to mitogens [64].

Mattoo et al. established a correlation between presence of fimbriae and effective tracheal colonization of *B. bronchiseptica* in mice and rats [117]. They demonstrated that the majority of serum IgM generated following *B. bronchiseptica* infection in rats was against fimbriae [117] and suggested that fimbriae might be involved in modulation of the humoral immune response [117]. Neither serum nor mucosal IgA response was detected in rats infected with *B. bronchiseptica*. It was postulated that absence of an IgA response might have been due to antagonistic effect of toxins produced by the type III secretion system [117]. This suggestion was supported by another study in which, mice infected with type III secretion mutants, had higher titers of anti-bordetella antibodies than mice infected with wild-type organisms [207].

Heterologous antigen expression in Bordetella spp.

Although there are several studies on expression of *Bordetella* antigens in *E. coli*, very little is known about heterologous antigen expression in *Bordetella* species. Suarez et al. expressed pertussis toxin gene from *B. pertussis* in *B. bronchiseptica* [185]. Since pertussis toxin is an essential component of acellular vaccines against whooping cough,

the aim of their study was to overcome the problems associated with slow growth and poor yields of pertussis toxin in *B. pertussis*, to facilitate improved industrial production, and to avoid bio-safety concerns of handling a human pathogen like *B. pertussis*. They constructed a chromosomal integrate of the pertussis toxin operon in *B. bronchiseptica*, that would allow inducible production of pertussis toxin from a promoter responsive to an aromatic inducer.

The first study on expression of a foreign protective antigen in *Bordetella* spp was published by Renauld-Mongenie in 1996 [161]. This study explored the possibility of using *B. pertussis* as a live mucosal vaccine vector for a protective antigen of *Schistosoma mansoni*. A *S. mansoni,* glutathione S-transferase gene was fused to the filamentous haemagglutinin gene of *B. pertussis* and the corresponding protein was expressed on the cell surface of *B. pertussis*. A single intranasal inoculation of the recombinant strain induced mucosal antibody to *Schistosoma* antigen (Sm28GST). This was the first study to demonstrate the utility of recombinant respiratory pathogens for the delivery of heterologous protective antigens. Later, in 1998, the same group of scientists created an attenuated derivative of *B. pertussis* and showed that attenuation of pertussis toxin resulted in an improved immune response to filamentous haemagglutinin and Sm28GST [128, 129]. A single intranasal inoculation of this strain induced protection against the parasite and protection against *B. pertussis* in mice. The ability of pertussis toxin-deficient and wild-type *Bordetella* to induce antibody response at a distal mucosal site was examined by Mielcarek et al. [128]. They observed that intranasal infection with *B. pertussis* produced detectable antibodies in the genital tract. Although pertussis toxin has been reported to have adjuvant properties, this study did not find any

immunomodulating effect of pertussis toxin in inducing local antibody response [128]. Administration of purified FHA by the intranasal or intravaginal route could boost the immune response generated at these sites. In another study by Mielcarek et al., priming with recombinant *B. pertussis* expressing Sm28GST and subsequent intranasal boosting with the foreign protein (Sm28GST) induced a systemic antibody response against the foreign antigen [130].

The bioluminescence gene from *P. luminescence* has been expressed in *B. bronchiseptica* and used to study the uptake and persistence of *B. bronchiseptica* in murine phagocytes [51]. A mini-Tn-5 promoter probe carrying the intact *lux* operon from *P. luminescence* was introduced into *B. bronchiseptica* and was used to create a pool of bioluminescent fusion strains of *B. bronchiseptica*. This allowed measurement of light output from the recombinant organisms without the addition of exogenous substrate.

Purified recombinant, detoxified adenylate cyclase of *B. pertussis* has also been used to deliver multiple epitopes from lymphocytic choriomenigitis virus and human immunodeficiency virus to induce a cytotoxic T cell-mediated immune response in mice [44]. The cell invasiveness and availability of permissible insertion sites are features of adenylate cyclase that may be useful for induction of protective cell-mediated immune responses against pathogens [44].

Advantages of mucosal immunization

The mucosal surfaces of the gastrointestinal, respiratory, and urogenital tracts represent the major port of entry for several human and animal pathogens [118, 119]. Mucosal exposure to foreign antigens during infection often results in development of an immune response. Therefore, mucosal immunization with specific vaccine antigens may

be more effective for immunization against infections acquired through mucosal surfaces [118, 119]. Mucosal immunizations offer several advantages compared to parenteral immunizations [24, 118, 119, 143]. They can induce mucosal as well as systemic immune responses and because of reduced direct contact with vaccine components in the systemic circulation can increase vaccine safety [24, 143]. Easy administration of multiple vaccines and reduced requirement for trained personnel make mucosal immunization strategies attractive [24, 143].

Mucosal immune system

There are many non-specific defense mechanisms that play important roles at mucosal surface. Mucus, acid, enzymes, bile, lysozyme, and lactoferrin secreted at the mucosal surface can inhibit microbes [118, 119]. Peristaltic contraction of smooth muscles, ciliary action of the epithelium, and tight junctions of the mucosal epithelium can exclude microorganisms from the mucosal surfaces and prevent invasion into deeper body tissues [103, 105, 118, 119]. Mucosal surfaces of gastrointestinal, respiratory and urogenital tracts are covered by a single layer of epithelial cells, which face an environment rich in pathogens. Some pathogens have developed effective mechanisms to colonize and invade these surfaces and, in defense, mucosal tissues are heavily populated with cells of the immune system [80, 103, 105, 118, 119]. The specialized sites where the induction of mucosal immunity begins consists of organized mucosa-associated lymphoid tissue called O-MALT and wide spread diffused mucosa-associated lymphoid tissue called D-MALT [103, 105, 118, 119]. O- MALT occurs in tonsils, respiratory tract and in gastrointestinal tract [103, 105, 118, 119]. Dendritic cells or specialized epithelial M cells capture and sample antigens at the mucosal surface [103, 105, 118, 119]. Antigen

sampling across the stratified epithelia of the oral cavity, vagina, and epithelium of the upper airways is carried out by migratory dendritic cells, which carry antigens to local O-MALT or to distal lymphoid tissue. Antigen sampling across the intestinal and bronchial epithelia is carried out by M-cells that deliver antigen to local O-MALT [103, 140]. In the airway epithelium, dendritic cells form a network with up to 700 dendritic cells /mm^2 [103, 105, 118, 119]. They represent major histocomatibility complex class II bearing, antigen-presenting cells of the airways and constitute the first line of defense against inhaled antigens. In the bronchi and in the gastrointestinal tract, M cells transport macromolecules, particles and microorganisms to the mucosal lymphoid follicles and initiate a secretory immune response [103, 105, 118, 119]. Although uptake and sampling of microorganisms by M cells lead to an immune response and eliminate mucosal infections, some viruses and bacteria exploit M cells for gaining entry into the host [103, 105, 118, 119].

Both humoral and cellular immune responses are generated at mucosal surfaces [103, 105, 118, 119]. IgA is the major antibody seen at mucosal surfaces [105]. The surface area occupied by mucosal epithelium in the body is enormous due to many macroscopic and microscopic foldings; as a result it may not be too surprising that IgA is estimated to be the most abundant immunoglobulin class found in the body (>50mg/kg bodyweight)[105]. IgA blocks the attachment of infectious agents to mucosal epithelia and provides an immune exclusion barrier in secretions against microbial pathogens, toxins, and other antigens [105, 139]. IgA can bind to lectin-like bacterial adhesins through its carbohydrate moieties and may, in this fashion, also block bacterial adhesion to receptors on epithelial cells. IgA also neutralizes viruses within the epithelial cells,

excretes antigens from sub-epithelial compartments across the epithelium into secretions, and activates complement through the alternate pathway [105].

IgA is secreted from plasma cells as dimers that bind to polymeric immunoglobulin receptors (pIgR) on the basolateral surface of epithelial cells that line the mucous membrane [105]. At the apical surface, proteolytic cleavage splits the external domain of pIGR (secretory component) and releases the dimeric IgA-secretory component complex (secretory IgA) into the mucosal secretions. In the secretions, IgA binds to antigens and prevent them from attaching to or penetrating the mucosal surfaces [105, 139, 140].

Like IgA, pentameric IgM also plays a role in preventing mucosal infections. IgM binds to the pIgR with less affinity than IgA [105]. IgG, the principle class of systemic antibody, is also seen in mucosal secretions and may enter the mucosal surface through diffusion. IgE also is considered to be a significant mucosal immunoglobulin [105]. Antibodies passively administered or actively induced at mucosal surfaces can protect mucosal surfaces from infection and invasion of pathogens. The success of oral vaccination for poliomyelitis and the abundant knowledge about mucosal immunity has created great interest in mucosal vaccination against infections that gain entry through the mucosal surfaces [105, 118].

Protein antigens applied to a mucosal surface can stimulate a mucosal immune response with secretory IgA production, a systemic immune response with IgG or IgM or development of mucosal tolerance with systemic unresponsiveness [103, 105, 143]. Tolerance is defined as loss of systemic immune responsiveness to that antigen following mucosal exposure [143]. Tolerance may be due to direct inactivation of antigen-sensitized lymphocytes via clonal deletion or anergy [143]. Other factors such as

interaction between regulatory and effector T cells, Th1 versus Th2 and γ/δ T cell receptors, bystander suppression, and tolerogenic proteins may also be involved in the induction of tolerance [143].

Vaccination strategies to induce mucosal immune response

Mucosal surfaces of the body are colonized with commensal bacteria, which may not cause any harmful effect to the host, and the maintenance of this microflora is based on a balanced immune response, which avoids an excessive overgrowth [78]. Even in individuals that are fully immunocompetent, bacterial infections of the gastrointestinal, respiratory and urogenital mucosa are among the leading problems in man and animals [78]. Parenteral vaccines are generally not very effective for inducing an immune response that prevents mucosal infections and they do not induce immune responses at mucosal sites [78]. They are usually active against invasive systemic diseases [205]. As already mentioned, stimulation of one mucosal site can lead to immune responses at distal mucosal sites [205].

Stimulation of a mucosal immune response requires efficient delivery of vaccine antigens to mucosal inductive sites. Often, enhancement of the immune response requires co-administration of adjuvants [24]. In development of vaccines against mucosal infections, it is important to define the nature of immune response that is required for protection [118, 119].

Oral delivery of vaccines has been most widely studied because of the convenience of vaccination through this route [173, 174]. Intranasal immunization will be advantageous for the control of pathogens whose route of entry is the respiratory tract because the magnitude of immune response produced will be maximum at this site [205]. Intranasal

delivery of vaccines is more effective in inducing systemic and mucosal immune response compared to other routes [205]. Mucosal immunizations with live organisms that colonize the nasal cavity may induce strong, long lasting immunity after a single inoculation [205]. Compared to oral vaccination, intranasal vaccination may have the advantages of lower dose requirement, greater stability, and less competition from colonizers [205]. Antigen will be taken up by the antigen presenting cells at the nasal mucosa (mainly dendritic cells) and will be presented to underlying nasal associated lymphoid tissue (NALT), where they induce a secretory IgA response [205], or the trapped antigen will be carried to local draining lymph node to initiate a systemic IgG response [105, 118, 205]. Recirculation and homing of memory immune cells through a common mucosal system can induce antibody response at distant mucosal sites [205]. For example, intranasal immunization with *B. pertussis* resulted in an antibody response at the genital surface [129]. Compartmentalization of the common mucosal immune system establishes a theoretic base for considering the best route to deliver mucosal vaccine [205].

Delivery of antigens by the mucosal route is associated with major problems like poor immunogenicity and susceptibility to degradation [123]. To overcome these problems, strategies such as entrapment into biodegradable microspheres, liposomes, their production by attenuated viral/ bacterial carriers or transgenic plants, or their administration with mucosal adjuvants are being used [123].

A number of adjuvants enhance the immunogenicity of antigens when delivered by parenteral route. Only a few molecules such as cholera toxin produced by *Vibrio cholerae* and heat labile toxin produced by *Escherichia coli* have been reported to act as mucosal

adjuvants [39, 79]. The potential side effects of these toxins limit their use in vaccinations [180]. Co-administration of cytokines and bacterial DNA containing unmethylated CpG dinucleotide motifs have been shown to be effective as vaccine adjuvants [100, 123].

Non-living Antigen delivery systems

Controlled delivery systems of microparticles consisting of polyesters, polylactides and glycolides are primary candidates for development of microencapsulated vaccines [123, 127]. Vaccine antigens are incorporated either adsorbed or chemically bound to the matrix [123, 127]. Incorporation of antigens into such microparticles protects the antigens from degradation and facilitates uptake of antigen and antigen presentation that leads to more efficient systemic and mucosal immune responses [123, 127]. The most important limitations of using vaccine antigens in microparticles are: stability of the antigens, the particle uptake, technical difficulties in delivery and associated toxicological issues [123, 127]. Liposomes are lipid vesicles formed when phospholipids are exposed to an aqueous environment and can act as immunoadjuvants, protect antigens, and are considered safe immunoadjuvants [123, 127]. Immunostimulating complexes (ISCOMS) are complexes built up by cholesterol, lipid, immunogen, and saponin. Administration of ISCOMS through parentral or mucosal routes can induce antigen specific immune responses [123, 127].

Live viral vectors

The natural ability of the viruses to infect target cells via specific entry mechanisms can be utilized to deliver antigens to specific cell types for antigen presentation and immunization [69]. The two types of basic live viral vectors used are attenuated viruses and replication defective or host range restricted viruses [69]. Several viruses such as

poxviruses and adenoviruses have served as vectors to deliver heterologous antigens. Vaccinia virus is the most studied viral vaccine vector [69]. Attenuated strains of Vaccinia virus expressing rabies virus glycoprotein have been used in field immunizations to protect wild animals from rabies [69]. Administration of these vectors to wild animal populations via polyurethane baits led to development of high neutralizing antibody titers against rabies virus [69]. Another commercially licensed virus vaccine vector in United States is fowl poxvirus that expresses glycoproteins of Newcastle disease virus. This vector can protect poultry from fowl pox and Newcastle disease [69].

Adenovirus vectors that can be administered orally are receiving attention. A number of antigens such as vesicular stomatitis virus glycoprotein, hepatitis B virus surface antigens, herpes simplex surface antigens have been successfully expressed in adenovirus vectors [69]. Herpes simplex virus, varicella zoster virus, poliovirus, simian immunodeficiency virus are the other viral vectors under study [69]. Replication defective mutants of poxvirus, adenovirus, and herpes simplex virus are now available and are considered to be good vaccine vector candidates because of their increased safety over wild-type and attenuated vaccine vectors [69]

Live bacterial vectors

Live multivalent vaccines using attenuated recombinant bacterial vectors have several advantages. Many proposed live vectors are administered by the oral or respiratory routes [124, 173, 174]. They are inexpensive to manufacture and, the live nature of the delivery system and the danger signals provided might make weak tolerogenic antigens more immunogenic [124, 173, 174]. Depending on the type of pathogenic mechanisms employed by a live bacterial vaccine vector, an immune response could be generated

through TH1 or TH2 pathways [124]. The specificity of different vectors to colonize different mucosal surfaces can be utilized to target an immune response at desired sites. Live antigens that express foreign protective antigens in host tissues may result in longer antigen presentation than non-living or parenterally administered preparations. Live attenuated bacterial vectors expressing heterologous protective antigens hold several attractive features compared to contemporary vaccines [124, 173, 175]. They are;

1) Unlimited cloning capacity
2) Effectiveness after single-dose vaccination
3) No requirement for addition of adjuvant
4) Non-invasive and easy administration procedure
5) Potential for producing multivalent vaccines
6) Low reactogenicity and low cost of production.

Additionally, antibiotic susceptibility allows an extra measure of safety to bacterial vectors, which is an advantage over live viral vectors [124, 173, 174, 203]. Both attenuated and commensal organisms have been successfully used as live vaccine vectors and some of them are discussed below.

Salmonella are intracellular pathogens involved in many important diseases of humans and animals. Following ingestion, salmonellae replicate in peyer's patches and disseminate via the MALT to the systemic tissues. Such infection will lead to either disease or broad-based immune responses that include systemic, mucosal, humoral and cell-mediated immunity [173, 174]. Mutants deficient in synthetic pathways involved in aromatic amino acid, purine, adenylate cyclase, and PhoP/PhoQ systems are well characterized and are efficient carriers for antigens [173, 174]. A vast number of

heterologous antigens have been expressed in attenuated salmonella including, bacterial, viral and parasitic antigens, eukaryotic proteins and cytokines [43, 173, 174]. Functional immune responses to these heterologous antigens have been demonstrated. Chromosomal integration of the genes encoding an antigen leads to stable, low level expression of the antigen. Plasmid mediated expression leads to high level expression but can result in instability of the plasmid and toxicity to the bacterial host [43, 173, 174]. Nakayama et al. introduced balanced lethal vector systems in salmonella that were characterized by the maintenance of multiple copies without the requirement of antibiotic selection [53, 54, 186]. In these systems, a plasmid that carries a vital metabolic gene which is deleted in the host bacterial strain will lead to positive selection of the plasmid bearing strain. Genes for heterologous antigens can be incorporated on the rescue plasmid. In addition, sub-cellular location [167], gene transcription, and timing of expression in eukaryotic cells [77] may influence immunogenicity of the antigen. Rapid advances in understanding of the molecular basis of pathogenesis of salmonella species have resulted in development of several candidate vaccine vector strains [23, 173, 174]. Recombinant *Salmonella typhimurium* strains evoked local as well as systemic immune response to heterologous antigens by oral, nasal, rectal and vaginal routes of immunization [82].

BCG (Bacillus Calmette Guerin), an avirulent derivative of *Mycobacterium bovis,* is widely used as a vaccine against tuberculosis in human beings [144]. While the slow growth rate of *Mycobacterium* species has impeded research, there have been tremendous developments like the discovery of shuttle plasmids (plasmids that can replicate in *Mycobacterium* and *E. coli*), gene replacement technology based on

homologous recombination with *M. smegmatis*, and antigen secretion systems [144]. Augmentation of immune responses by the well-known adjuvant activity of BCG is also an advantage. BCG has been engineered to express a variety of heterologous antigens. They include proteins from human immunodeficiency virus, tetanus toxin, pneumococcal surface protein, pertussis toxin and parasite antigens like schistosoma, leishmania, or plasmodium antigens [144]. BCG that secretes immunostimulatory cytokines have been made and tested as anti-tumor agents [144]. Since a large proportion of human beings are already immunized with BCG, the use of BCG that expresses heterologous protective antigens for control of tuberculosis and other common diseases may be realized soon.

Vibrio cholerae is a Gram-negative organism responsible for the disease, cholera. This is a non-invasive organism that induces potent long-lasting mucosal and systemic immune responses [69, 171]. The ability of this organism to adhere to the M cells has been utilized for vaccine antigen delivery to the gastrointestinal tract [69]. Attenuated strains that lack cholera toxin have been used to induce mucosal and systemic antibody responses to heterologous antigens [69, 171].

B. pertussis has been studied as a mucosal vaccine vector directed to the respiratory tract. The glutathione transferase gene from *Schistosoma mansoni* was integrated into the filamentous hemagglutinin gene of *B. pertussis* and was expressed [161]. A single intranasal immunization with the recombinant *B. pertussis* lead to immune responses to the *Schistosoma* antigen as well as to *B. pertussis* [161]. *Brucella* spp have also been considered as possible vectors for intracellular delivery of antigens [28].

Mucosal inoculation with recombinant Gram-positive bacteria such as *Streptococcus gordonii* and certain strains of Lactobacillus, which are among the endogenous microflora, have also been used to deliver heterologous antigens [155, 168]. *Streptococcus gordonii*, originally isolated from the oral cavity of humans, is capable of colonizing the oral cavity and vagina of mice. A number of antigens have been expressed in *S. gordonii* by replacing the sequences encoding the surface exposed domains of the M6 protein with foreign genes of interest [155] The secretion signal and cell wall anchoring domain of the M6 protein served to direct the export of the antigen to the surface. The foreign proteins expressed in *S. gordonii* were integrated into the chromosome by homologous recombination so that genes were stably maintained in the chromosome in vivo without any antibiotic selection.

Nonpathogenic staphylococcal species such as *S. xylosus*, and *S. carnosus*, which are widely used in meat fermentation processes, have been developed as live bacterial vectors [175]. High copy number shuttle vectors were advantageous in that they provided multiple copies of the foreign gene per cell. In these systems, the promoter, signal sequence and propeptide sequence from the *S. hyicus* lipase gene were utilized to achieve translocation through the cell membrane. However, large number of immunizations and surface display of heterologous antigens were necessary for induction of an immune response [175].

Lactococcus lactis a Gram-positive bacterium used in industry is non-invasive, non-pathogenic and does not colonize the mucosal surface. Researchers have exploited the microparticle nature of this bacterium in heterologous antigen delivery [19]. Several antigens have been expressed in *Lactococcus* using an inducible T7 RNA polymerase

system or the pTREX series of constitutive expression plasmid. When applied via a mucosal route, mucosal as well as systemic immune responses were noticed in mice.

Lactobacillus, a Gram-positive bacterium that is prominent in human indigenous gastrointestinal flora, has been used to express heterologous antigens from plasmid vectors and following chromosomal integration [168]. The potential for induction of immunological tolerance to foreign antigens has to be considered when utilizing commensal bacteria as vaccine delivery vehicles. At present, the relationship between normal flora and the host immune system is very unclear [168]. *Listeria monocytogenes*, a facultative intracellular Gram-positive pathogen, is a promising vaccine carrier for evoking a cellular immunity [65]

There are potential problems associated with the use of live bacterial vectors. Reversion to virulence is one of the major concerns associated with live bacterial vectors. Stability of attenuated phenotypes should be ensured by careful inactivation of target genes; single gene inactivation is less desirable [18, 53, 124]. Due to preexisting immunity, the vaccine dose required to trigger immune responses in endemic areas and nonendemic areas may differ. Medina et al. suggested that presentation of the vaccine in different formulations according to geographic area might solve this problem [53, 124]. The use of plasmids for expression of foreign genes may lead to plasmid instability and loss of antigen expression [18, 53, 124]. The presence of additional genes such as antibiotic markers used for positive selection is not desirable [124]. The use of low copy number plasmid vectors containing killing systems, partition function and non-antibiotic selection markers are advisable. Integration of a foreign gene into the chromosome of the host strain may lead to low level expression from a single copy gene and be insufficient

to generate an effective immune response [18, 53, 124]. Another strategy, which may be effective, is the use of in-vivo activated promoters [124]. The expression of particular vaccine antigens by non-pathogenic commensal or environmental organism may enhance their virulence in human or animal populations, and increase the environmental risk. Also, horizontal gene transfer from vaccine strains to mucosal flora or environmental organisms may pose a threat [124]. This can be prevented by incorporating conditional lethal systems [38, 136]. Prior exposure to the antigen carrier can compromise the efficacy of a vaccine candidate. As reported in salmonella, [3] utilization of different carrier strains or bacterial serotypes for preparation of vaccines or allowing an established optimal window for readministration of vaccine may eliminate this risk [124]. Host genetic factors may also modulate the type of immune response [124]. Diversity in MHC genes, and presence or absence of some genes associated with immune system can lead to differences in the type of immune responses generated [124]. It is also important to have proper balance between attenuation and immunogenicity. The dissemination of live attenuated microorganisms in the field may pose a risk of disease, especially in immunocompromised individuals and require stricter safety guidelines [69]. Vectors that are highly susceptible to antimicrobial agents are most desirable.

Conclusion

Vaccination constitutes the most cost effective tool for the prophylaxis of infectious diseases. Most pathogens gain entry into the body through the mucosal surfaces. So administration of immunogens through the mucosal route and induction of immune responses at these sites are essential. Delivery of vaccine antigens by bacterial carriers has resulted in effective humoral and cell mediated immune responses. The potential

value of live bacterial vectors for vaccination purposes was recognized by the World Health Organization (190). *B. bronchiseptica*, effectively colonizes the respiratory tract of mammals, and is an ideal candidate as a mucosal vaccine vector. Application of this system in the disease, atrophic rhinitis, is a rational approach to refining existing vaccines and could be expanded to control other diseases in animals.

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PART 2

Isolation and characterization of a promoter useful for heterologous antigen expression in *Bordetella bronchiseptica* using a green fluorescent protein reporter system

Abstract

Bordetella bronchiseptica, an adept colonizer of the upper respiratory tracts of mammals, may be selectively utilized as a live bacterial vector to deliver protective antigens to the respiratory tract. Promoters and conditions for optimal expression of foreign antigens in *B. bronchiseptica* have not been determined. In this study, a *B. bronchiseptica* promoter region (P9) related to heat shock protein genes was identified using a green fluorescent protein (GFP) reporter system. The region identified has 98% homology to the *cpn*10/60 gene of *Bordetella pertussis.* The efficiency of this promoter to drive GFP expression was analyzed and compared in four *B. bronchiseptica* strains using flow cytometry. GFP expression from the P9 promoter was also compared to that of a *B. bronchiseptica* fimbrial gene (*fim N*) promoter and *Escherichia coli* derived *tac* promoter. The P9 promoter had the highest level of activity in all *B. bronchiseptica* strains tested under normal laboratory incubation conditions using common media. The *tac* promoter was more active in *E. coli* than in *B. bronchiseptica*. The *fim N* promoter had low level activity, detectable only in *bvg* positive strains of *Bordetella bronchiseptica*. The P9 promoter may be a good candidate for expression of heterologous antigens in this species. In addition, *B. bronchiseptica* constructs with strong constitutive expression of GFP may be useful to characterize the adherence and colonization of this bacterium.

Introduction

Bordetella bronchiseptica is a respiratory pathogen, which infects a wide variety of host species including domestic, laboratory and wild animals and may opportunistically infect humans [4, 14]. *B. bronchiseptica* can express a battery of adhesins, like fimbriae, filamentous haemagglutinin, adenylate cyclase and pertactin that enable it to colonize the upper respiratory tract of various host species [10]. The ability of this organism to efficiently colonize the respiratory tract may be utilized to deliver protective antigens from other pathogens to the respiratory tract. Intranasal inoculation with *B. bronchiseptica* has been shown to induce long lasting humoral and cellular antibody responses in mice [15]. Mucosal application of vaccines induces immune responses at systemic and mucosal sites [24, 28]. A single intranasal inoculation of mice with *B. pertussis* expressing a *Schistosoma* antigen was able to induce mucosal antibody responses in the respiratory tract as well as at other mucosal sites [25, 26, 32]. Compared to the oral route of immunization, intranasal vaccine application may have the advantage of low dose requirement, greater stability and less competition from colonizers [42]. Live bacterial vectors, which can colonize the respiratory tract, are attractive candidates for this purpose [28].

Many virulence genes in the genus *Bordetella* are controlled by a two-component signal transduction system encoded by the *bvg* locus. The proteins produced by this locus, BvgA and BvgS, are responsible for phenotypic modulation in response to environmental signals like temperature and certain chemicals [10] and, the promoter region of this operon contains a cytosine repeat region that is subject to frequent mutations [3]. The significance of the *bvg* locus in infection and the effect of in vivo

conditions on its expression are not completely understood. Therefore, to study heterologous antigen expression, a strong promoter was sought that could drive constitutive expression of genes in *B. bronchiseptica*

In this study, a *B. bronchiseptica* promoter region (P9) related to heat shock protein genes was identified utilizing a promoter-probe vector carrying a promoter-less green fluorescent protein gene. This promoter induced high level expression of GFP in four different strains of *B. bronchiseptica* and was compared to GFP expression from the *Escherichia coli* derived *tac* promoter and a *bvg* regulated *fim N* gene promoter [17] under common laboratory conditions. *B. bronchiseptica* expressing GFP from P9 promoter was utilized to study in vitro adherence to Vero cells and colonization of mouse respiratory tract.

Materials and methods

Plasmids, bacterial strains, and enzymes

The origin of *B. bronchiseptica* strains has been previously described [6]. Strain 55 is a live attenuated *B. bronchiseptica* (ATCC 31437) vaccine strain. The INVF', (Invitrogen Inc., San Diego, CA) is a *lac* represser gene deficient *Escherichia coli* strain. All bacteria were grown in Luria Bertani (LB) agar or broth with appropriate antibiotic selection at 37^{o}C unless otherwise indicated. Restriction enzymes and Taq polymerase were products of PANVERA, Madison, WI. Wizard plus SV miniprep kit (Promega, Madison, WI) was used for plasmid isolation. The plasmid, pBBR1MCS2, (kindly provided by Michael E. Kovach) was used for all cloning purposes [20]. The plasmid pBBR1-KGFP (kindly provided by Stephan Kohler) was used to obtain the *gfpmut3* gene [9, 19].

DNA manipulation

All polymerase chain reactions were performed in a Gene Amp PCR system 9600 (Perkin Elmer, Narcross, GA). A 30-cycle program consisted of denaturing at 95^{o}C for 1.5 minutes, annealing at 50^{o}C for 30 seconds, and extension at 72^{o}C followed by a last cycle of 5 min extension at 72^{o}C. All primers used for PCR amplification were designed to contain restriction enzyme sites (underlined).

Promoterless *gfpmut3* gene was amplified from pBBR1-KGFP by PCR using forward primer 5'-CGGGATCCGATGAGTAAAGGAGAAGAA-3'and reverse primer 5'-CGGAATTCTTATTTGTACAATTCATCC-3' and cloned into the BamHI and EcoRI sites of pBBR1MCS2. The resulting plasmid was designated as pBBRGFP. Chromosomal DNA was isolated from *B. bronchiseptica* strain 110H using Easy DNA kit (Invitrogen Inc., San Diego, CA). It was digested with Sau3A1 and electrophoresed on an 8% agarose gel. DNA fragments ranging from 0.25-3kb were purified from agarose gel using Quiagen gel purification kit (Quiagen Inc., Valencia, CA) and ligated to BamHI digested, calf intestinal alkaline phosphatase (New England Biolabs., Beverly, MA) treated pBBRGFP. The ligation mixture was electroporated into *B. bronchiseptica* strain 110NH (Bvg^{-}, avirulent phase) using a CELL-PORATOR Electroporation System (GIBCO-BRL., Gaithersburg, MD) following the manufacturer's instructions. This strain was used to avoid the selection of *bvg*-regulated promoters. The electroporants were isolated on LB agar containing kanamycin (100µg/ml). After 3 days of incubation at 37^{o}C, cell suspensions from individual colonies were examined with a fluorescence microscope (Nikon FLUOPHOT, Garden City, New York) equipped with blue wavelength (375-490 nm) excitation filter and a 510nm barrier filter. The *tac* promoter

was amplified from pGEX-5X-1 (GST gene fusion expression vector from Pharmacia Biotech. Piscataway, NJ), using forward primer 5'-GGTCTAGACTGCACGGTGCACC-3' and reverse primer 5'-CGGGATCCTGTTTCCTGTGTGA-3' and cloned in to XbaI and BamHI sites of pBBRGFP. The resulting plasmid was designated as pBBRTACGFP.

To construct a plasmid expressing GFP under the control of *fim N* promoter, the GFP gene was amplified with the same forward primer used for constructing pBBRGFP and the reverse primer, 5'-CGGGATCCTTATTTGTACAATTCATCC-3' and cloned into a unique BamH I site in the *fim N* gene in PCR2-FIM (11). This construct was amplified by a primer specific to the 5' region of *fim N* (5'-GGAAGCTTGCCATCACCAACTTATGTG-3') and the same GFP reverse primer used for cloning pBBRGFP and cloned into the Hind III and EcoR I sites of the pBBR1MCS2 to obtain plasmid pBBRFIMGFP. All recombinant plasmids were electroporated into *B. bronchiseptica* strains 110H, 110NH, R5 and Strain 55.

Growth curve

Bacterial growth curves were determined for each strain in LB broth, Brucella broth and Stainer Scholte broth (SS broth) [35]. Each medium (10ml) was inoculated with 0.5ml of overnight culture of the *B. bronchiseptica* strains carrying recombinant plasmids at 1:20 dilutions and incubated at 37^{o}C in a rotary shaker (220rpm). At selected time points 200µl of the culture was transferred to duplicate wells of 96-well microtiter plates. The plates were read at an optical density of 592nm on an ELX800 ELISA reader (BIO-TEK instruments Inc., Winooski, VT).

Flow cytometric analysis

Bacteria containing the recombinant plasmids were grown in LB broth, Brucella broth or SS broth to late log phase at 37^{o}C and 220 rpm. One ml of the culture was pelleted by centrifugation at 14,000 rpm for 1minute. The pellet was washed 2 times in phosphate buffered saline (PBS) and suspended in PBS to an approximate OD590 of 0.2. Bacterial suspensions were analyzed in a FACSVantage SE (Becton &Dickinson, San Jose, CA) equipped with argon laser exciting at 488nm and emitting at 540nm. Forward versus side light scatter was used to gate the bacteria and the data was collected using logarithmic amplifiers. A total of 10,000 events were acquired for each sample. The mean fluorescence intensity for each sample was determined by using CELLQUEST analysis software(Becton &Dickinson, San Jose, CA). *B. bronchiseptica* strains harboring plasmid pBBRGFP were used as negative controls.

Adherence assays

Bacterial adherence to Vero cells was evaluated as previously described (17) with modifications. *B. bronchiseptica* strain 110H (Bvg $^{+}$ virulent phase) and 110NH (Bvg^{-} avirulent phase) were compared. Individual colonies from a 48 hr culture on LB agar were suspended in PBS to obtain an OD590 of 0.1 (2.5x10 ^{8} CFU/ml). One ml of this suspension was mixed with a one ml suspension of Vero cells containing 1x10 ^{6} cells/ml. This mixture was incubated for one hour at 37^{o}C with gentle mixing. The suspension was centrifuged at 1000x g for 10 minutes to remove non-adherent bacteria. The resulting pellet was washed twice in Hanks Balanced Salt (HBS) solution and suspended in 1ml HBS. Adherence of recombinant *B. bronchiseptica* to Vero cells was measured by flow cytometric analysis as described above.

In vivo plasmid stability

BALB/C mice (4-6 weeks old) were purchased from Harlan Laboratories (Indianapolis, IN) and were maintained by routine procedures in an AALAC accredited laboratory animal facility. Mice were inoculated with 50µl of bacterial suspension in PBS containing approximately $1x10^5$ CFU of *B. bronchiseptica* 110H harboring PBBRP9GFP. Mice were anaesthetized with Isoflurane (Abbot Laboratories, North Chicago, IL) and 25µl of the bacterial suspension was instilled into each nostril**.** Mice were sacrificed at 3, 6, 12, and 24 hours after inoculation. The lungs and trachea were collected and fixed in 4% paraformaldehyde for 2hrs. The slow freezing protocol as described previously [34] was followed. Briefly, after fixation, the tissues were washed in PBS, embedded in Tissue-Tek O.C.T. (Miles Inc, Elkhart, IN) and stored at 4^oC for 24 hours. The tissues were transferred to a slow freezing container with isopropyl alcohol and allowed to freeze to -70^oC. Sections were cut on a cryomicrotome (Minatome- Damon /IEC division, Needham Heights, MA), mounted in PBS and examined in a Leica SP2 laser scanning confocal microscope using 488nm argon laser line for excitation and a spectral setup for GFP to collect emission.

Results

Identification and characterization of promoter

A library of clones containing fusions with the *gfp* gene and chromosomal DNA of *B. bronchiseptica* 110H was constructed. A Bvg^- avirulent phase *B. bronchiseptica*, strain 110 NH, was used for promoter selection to avoid selection of bvg-regulated promoters. From 2500 clones examined by fluorescence microscopy, one was identified

which expressed GFP. The bacteria expressing GFP were bright green, when examined by fluorescence microscope (Figure 1, appendix). Upon restriction digestion of that clone, it was determined that the chromosomal DNA upstream to the *gfp* gene was approximately 3kb. Restriction analysis was performed using enzyme sites within the multiple cloning sites of pBBR1MCS2, flanking the *gfp* gene, to further reduce the size of the insert. The *gfp* gene with the cloned upstream promoter region was digested with EcoRV and EcoRI and the resulting 1.25 kb fragment was subcloned into the respective sites of pBBR1MCS2 to obtain pBBRP9GFP. This plasmid was used in all downstream experiments and the promoter region was designated as P9.

DNA sequencing (University of Tennessee, DNA sequencing facility) was performed in the region immediately upstream of the *gfp* gene. A BLAST search of ~250 bp of the sequence, identified this region as having 98% identity to the c*pn*10/*cpn*60 gene of *B. pertussis* [13]. There was a four base pair difference in the region immediately upstream to the -35 sequences. The gene fragment upstream to the *gfp* gene was an in frame fusion of sequence encoding the first 36 amino acids of the *cpn*10 homolog gene, along with its corresponding regulatory sequences. The predicted fusion protein contained a 4kd fragment of CPN 10 protein and intact GFP. As observed previously in *B. pertussis*, this region contains sites for σ^{32} and σ^{70} as well as a CIRCE (Controlling inverted repeats of chaperonin expression) element [13].

The plasmids pBBRP9GFP, pBBRFIMGFP, and pBBRTACGFP were electroporated into *B. bronchiseptica* strains 110H, 110NH, R5, Strain 55 and to *E. coli* strain INVF'. All strains of *B. bronchiseptica* with pBBRP9GFP showed strong green fluorescence when examined by fluorescence microscopy; whereas, the fluorescence

intensity was much lower in *E. coli* that harbored this plasmid. *E. coli* organisms carrying PBBRTACGFP were brighter than all *B. bronchiseptica* strains with the same plasmid. *B. bronchiseptica* strains carrying pBBRFIMGFP had the lowest fluorescence**.** Neither the *B. bronchiseptica* strain, 110NH, nor *E. coli* harboring this plasmid had any detectable green fluorescence.

The efficiency of P9, *fim N* and *tac* promoters to drive GFP expression was quantitated using flow cytometric analysis. LB broth, Brucella broth and SS broth were used for growing bacteria. A histogram showing flow cytometric profiles of fluorescence intensity from different promoters in *B. bronchiseptica* strain 110H is shown in Figure 2. All *B. bronchiseptica* strains consistently expressed very high levels of GFP from P9 promoter. There was no significant difference in expression of GFP between these strains; however, the activity of this promoter was 40-50 fold lower in *E. coli* than in *B. bronchiseptica* (Figure 3). In *E. coli, tac* promoter driven fluorescence (X=20) was 2-5 fold higher than in *B. bronchiseptica* strains (X=2.5-10) harboring the same plasmid. The *fim N* promoter had low level activity in *B. bronchiseptica* strains110H (X=2.5), R5 (X=2.5) and Strain 55 (X= 4.23). However, unlike the P9 and *tac* promoters, the *fim N* promoter was not active in strain 110NH (X= 1.6) and in *E. coli* (X=1.9). The intensity of fluorescence measured by FACS analysis of *B. bronchiseptica* and *E. coli* cultures expressing GFP under the P9 and TAC promoters was the same with each growth medium tested. However, when cultivated in SS broth, *B. bronchiseptica* strains 110H (X= 6.3 vs 2.5), R5 (X=5.3 vs 2.5) and Strain 55 (X= 6 vs 4.23) had increased levels of GFP expression from the *fim N* promoter when compared to GFP expression in LB broth.

There was no increase in GFP expression from *fim N* promoter in strain 110NH when grown in SS broth.

Since heat shock proteins are induced during periods of stress, the effect of stress stimuli on GFP expression from the P9 promoter was examined. Heat shock experiments were performed as described previously [38]. For all strains, the GFP expression at 25^{o} C was 4-5 times lower than at 37^{o} C. However, increasing the temperature from 25^{o}C to 37^{o}C and 25^{o}C to 42^{o}C for 20 minutes did not have any effect on GFP expression. Also, addition of hydrogen peroxide at a final concentration of 0.005 % did not have any effect on GFP expression.

Adherence assay

Adherence assays were characterized based on GFP expression from the P9 promoter. Vero cells with adherent bacteria were examined by fluorescence microscopy and flow cytometry. Consistent with our previous observation [17], using a conventional adherence assay, the Bvg $^{+}$, virulent phase strain, 110H, adhered well to Vero cells (Figure 4) but Bvg^{-}, avirulent phase, strain 110NH did not. Quantitation of the mean intensity of fluorescence by FACS analysis showed that strain110H adhered 7-13 times more than the avirulent phase, strain 110NH (Figure 5).

Plasmid stability and growth rate

The expression of GFP in overnight LB broth culture was examined with and with out antibiotic selection. All strains of *B. bronchiseptica* expressed equivalent levels of GFP from the P9 promoter under selective and non-selective conditions. In vitro stability of pBBRP9GFP was tested by continuous passage of the bacteria in non-selective medium (Tryptic Soy Agar). When bacteria were examined by fluorescence microscopy, all four

strains of *B. bronchiseptica* failed to sustain the fluorescence after five passages at 48-hour intervals (~10days) in non-selective medium. This loss of GFP expression was associated with plasmid loss, since the recombinant organism could not be recovered in antibiotic selection media. The plasmid was very stable in *E. coli,* showing green fluorescence even after 10 repeated passages on non-selective agar medium. There was no difference in growth rate of *B. bronchiseptica* strains expressing GFP compared to the wild-type organisms (Figure 6) indicating that GFP expression did not have any adverse effects on bacterial growth. The growth curves were similar for 110H, 110NH and R5; whereas a longer lag phase was observed for Strain 55. All strains also had a longer lag phase in SS broth than in LB broth or Brucella broth. At 3 hrs post inoculation, GFP expressing bacteria were visualized in cryosections of the lung and trachea by confocal microscopy. Green fluorescent bacteria were adherent to the ciliated epithelium of the trachea and bronchioles (Figure 7). In alveolar spaces bacteria were associated with cells, probably macrophages, but were not seen free in the lumen. At subsequent time periods after 3hr, fluorescent bacteria were not detected in respiratory tracts of mice that had been inoculated with *B. bronchiseptica* 110H harboring pBBRP9GFP.

Discussion

This report describes the identification and characterization of a promoter useful for heterologous antigen expression in *B. bronchiseptica* utilizing a green fluorescent protein reporter system. GFP has been successfully utilized as a reporter gene in several species of bacteria [19, 29, 36, 37, 38]. GFP expression in prokaryotes is becoming a widely used and easy tool to study various aspects of development, metabolism, gene expression and pathogenesis [36, 37]. This is the first report of utilizing this system in *B. bronchiseptica*.

In the related species, *B. pertussis*, GFP expression for the study of phagocytosis was more efficient than tagging the organism with FITC [40].

Bacterial heat shock proteins are abundantly produced throughout the life cycle of most bacteria and their synthesis is often increased during periods of stress [21]. In *B. pertussis* the CPN 60 protein exhibits immunological and structural similarities to GroEL protein of *E. coli*, which functions as a molecular chaperone [5, 7]. Complete cloning and inactivation of the *cpn10/60* homolog gene has to be done to understand the exact function of this protein in *B. bronchiseptica*. However, this study demonstrates the utility of this promoter region in heterologous antigen expression. Under normal laboratory incubation conditions, using common media, there was high level expression of GFP from the P9 promoter. Stimuli required for P9 promoter activity have not been identified. As reported in *E. coli* [1], it is possible that heterologous protein expression may itself act as a stimulus for promoter function. Recent studies indicated a role of CIRCE element as a negative regulator involved in inducing stress response [27, 44]. As suggested by Weiss et al. [13], one speculation about the presence of binding sites for both σ^{70} and σ^{32} subunits is that organisms may switch these promoter regions under stress and non-stress conditions. HSP promoters have been useful in heterologous antigen expression and immunogenicity studies with a number of bacteria [3, 38]. In spite of the concern that use of HSP as a vaccine component might lead to the generation of an autoimmune response due to the highly conserved nature of HSP's among eukaryotes and prokaryotes, microbial HSP's have been successfully employed as carrier molecules for peptide immunization [43]. The binding affinity of HSP's for peptides and their involvement in antigen processing may enhance specific immune responses [43]. The mucosal immune

response to heterologous antigens expressed in *B. bronchiseptica* from the P9 promoter and the effect of the remaining 4kD CPN peptide in the P9 promoter-GFP fusion awaits further study.

The *E. coli* based promoter *tac* is widely used in *E. coli* expression systems. In this study, GFP was expressed constitutively from this promoter since the *E. coli* strain we used, and *B. bronchiseptica* do not possess a *lac* repressor gene. The *tac* promoter has previously been utilized in *B. bronchiseptica* to study the expression of adenylate cyclase toxin [22]. Although, the level of expression of GFP from the *tac* promoter in *B. bronchiseptica* was much lower than that from the P9 promoter, heterologous antigen expression from *tac* may have value in inducing an in vivo immune response. It was observed in a *Vibrio cholerae* based heterologous antigen expression system that, the *tac* promoter was better suited for generation of an in vivo immune response than both a HSP promoter and an iron regulated promoter [16].

This study is the first to demonstrate functional activity of the *fim N* promoter in *B. bronchiseptica*. This newly reported fimbrial gene sequence variant has serologic cross reactivity with serotype 2 fimbriae of *B. pertussis* [17]. The reason for low-level expression of GFP from *fim N* promoter is currently unknown. The length of cytosine residues (C-stretch) in the promoter region plays a role in optimal transcription of *bvg*-regulated genes [34, 41]. The *fim N* gene cloned in *E. coli* has two less cytosine residues than its chromosomal counterpart [17]. Manipulation of the C-stretch may be another strategy to improve the expression of proteins from this promoter. Surface expression of heterologous antigens may have great advantage in improving antigen presentation and immune responses. Live bacterial vectors and their adhesins are attractive candidates for

improving mucosal vaccination strategies [28]. Among *B. bronchiseptica* adhesins, fimbriae are shown to have immunomodulatory effects following intranasal inoculations in mice [23]. The molecular chimerization of fim *N* with a *Mannheimia haemolytica* leukotoxin fragment induced enhanced leukotoxin neutralizing antibody response in mice immunized with this recombinant protein [30]. A recent report described the advantage of over expression of a protective antigen as a novel approach to improve vaccine efficacy [38]. The P9 promoter could be utilized to express fusion proteins of FIM N with other protective antigens. Since fimbrial proteins are abundantly produced on bacterial surfaces, chimeric fimbriae expressing epitopes from different pathogens seem to be an attractive strategy for vaccine delivery. Host species specificity of fimbriae could be selectively utilized to deliver antigens to a target species [6]. Two recent studies show the effectiveness of *B. pertussis* fimbriae as a carrier protein for conjugate vaccines [11, 31].

There was an increase in expression of GFP from the *fim N* promoter, when cultivated in SS broth, a commonly used medium for vaccine preparation [35]. A similar increase in expression of filamentous haemagglutinin when cultivated in SS broth was recently reported [18]. The potassium, sodium and chloride ion content of this mineral salts medium may be upregulating the Bvg-controlled *fim N* promoter [10].

The original pBBR1, a broad host range cryptic plasmid isolated from *B. bronchiseptica* [2], was stable without continuous antibiotic selection in *Brucella* species in vitro for >10 days and in vivo in mice for >4weeks [8, 12]. In this study, GFP expression from P9 promoter was used as an indicator of in vitro plasmid stability. The pBBRP9GFP plasmid was maintained at high to moderate levels for only 4-5 passages (10 days) in all the strains tested in the absence of antibiotic selection. Although PBBR1

was originally isolated from *B. bronchiseptica*, the genetic modifications made on the pBBR1MCS2 from the original plasmid or hetrologous antigen expression, itself, may affect plasmid stability. It is noteworthy that there was no difference in GFP expression from these recombinant strains over a period of 24 hours at 37^{o}C under selective and nonselective in vitro conditions as measured by FACS analysis.

GFP expressing bacteria were not seen in cryosections of the lung and trachea after 3 hours post inoculation. This might be the result of in vivo plasmid instability or repression of the promoter function. The manipulation and processing involved in preparing the tissue might have affected the sensitivity of the assay. A modified construct, which can stably express GFP, could be utilized for pathogenesis studies including the possible long term intracellular survival of this organism. In a subsequent study with recombinant *B. bronchiseptica* that expressed another heterologous antigen from the P9 promoter in pBBR1MCS2, only 6.5 % of the total bacteria recovered from lungs and trachea of mice retained resistance to the selective antibiotic marker, kanamycin, at 24 hours post inoculation, while colonization of plasmid-cured *B. bronchiseptica* continued as long as 56 days post inoculation. Thus, improved plasmid stability or construction of chromosomal co-integrates may be required to generate an effective immune response to heterologous antigens.

The adherence assay utilizing FACS analysis of GFP expression from the P9 promoter is a more rapid and convenient way of quantitating adherence as compared to conventional techniques. Since the adherence of *B. bronchiseptica* is an important virulence property, this assay could be effectively utilized to compare the virulence of different strains or to evaluate immune responses to vaccine preparations by attachment

inhibition assay. In conclusion, the promoters characterized in this study, may be useful for expression of different protective microbial antigens in *B. bronchiseptica*. The potential for utilizing *B. bronchiseptica* as a mucosal vaccine vector is currently under investigation.

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Appendix

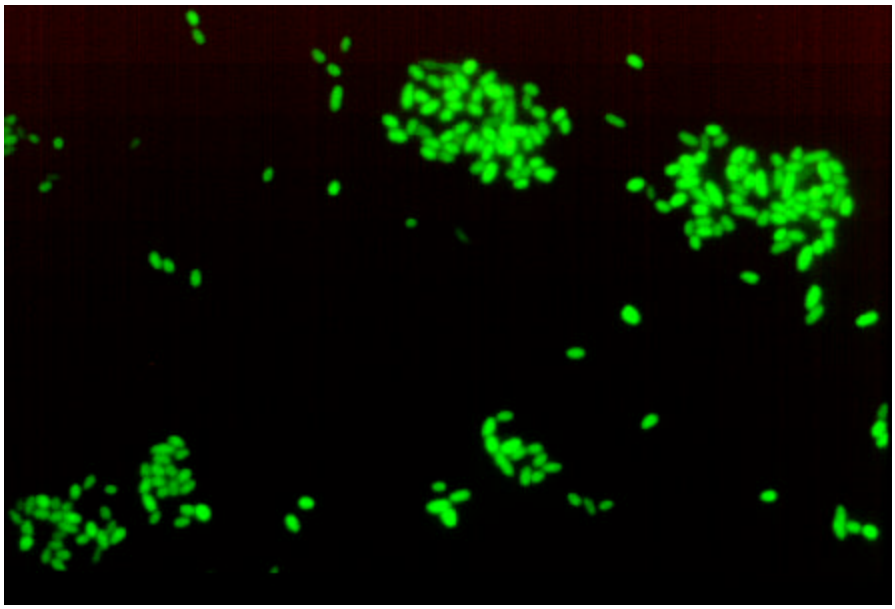
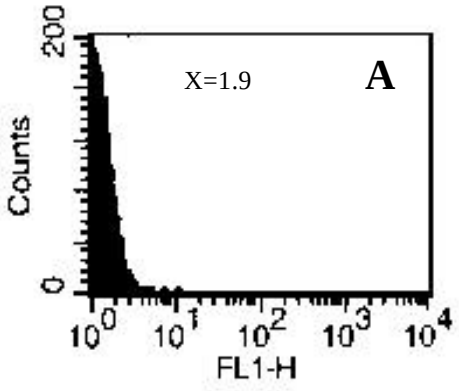


Figure 1

B. bronchiseptica strain 110NH expressing GFP from P9 promoter. Visualized by fluorescence microscopy. Magnification X1000.

Figure 2

Flow cytometric profiles of GFP expression in *B. bronchiseptica* strain 110H harboring different recombinant plasmids. (A) pBBRGFP (promoter-less), (B) pBBRP9GFP (P9 promoter), (C) pBBRFIMGFP (*fim N* promoter), (D) pBBRTACGFP (*tac* promoter). Intensity of the fluorescence on the x-axis indicates the level of GFP expression. X = Mean fluorescence intensity.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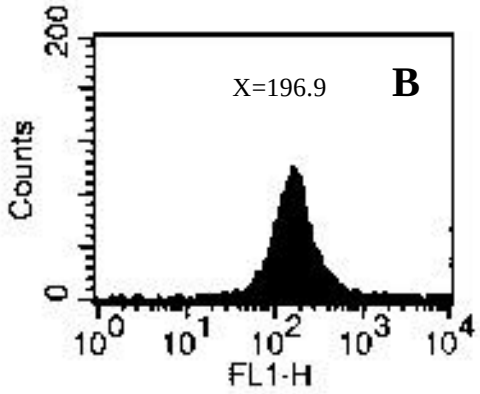
Counts
200
0
10^0 10^1 10^2 10^3 10^4
FL1-H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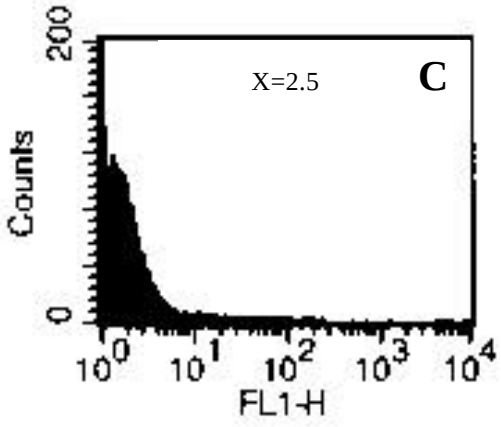
Counts
200
0
10^0 10^1 10^2 10^3 10^4
FL1-H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C
Counts
200
0
10^0 10^1 10^2 10^3 10^4
FL1-H

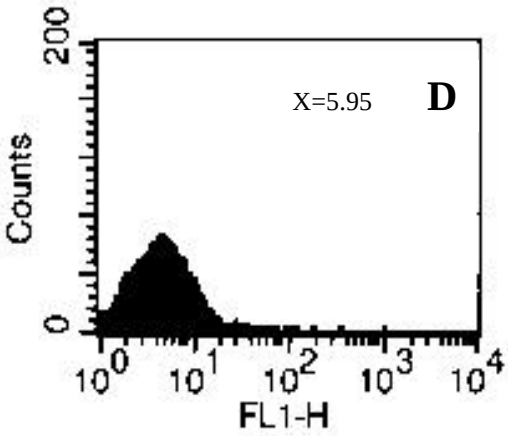
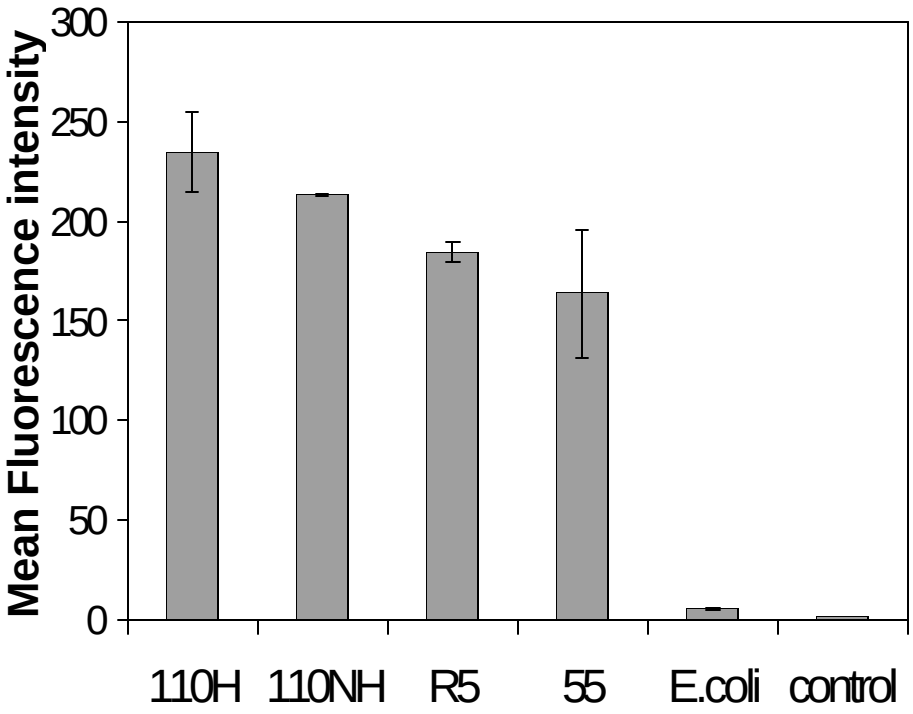

X=5.95
D
Counts
200
0
10^0 10^1 10^2 10^3 10^4
FL1-H

Figure 3

Comparison of GFP expression from P9 promoter in four different strains of *B. bronchiseptica* and *E. coli.* The cultures were grown in LB broth and processed as explained in materials and methods for flow cytometry. The intensity of GFP expression from each strain is represented by mean fluorescence intensity as calculated by CELLQUEST Software. *B.bronchiseptica* strains harboring pBBRGFP were used as negative control


Mean Fluorescence intensity
300
250
200
150
100
50
0
110H
110NH
R5
55
E.coli
control

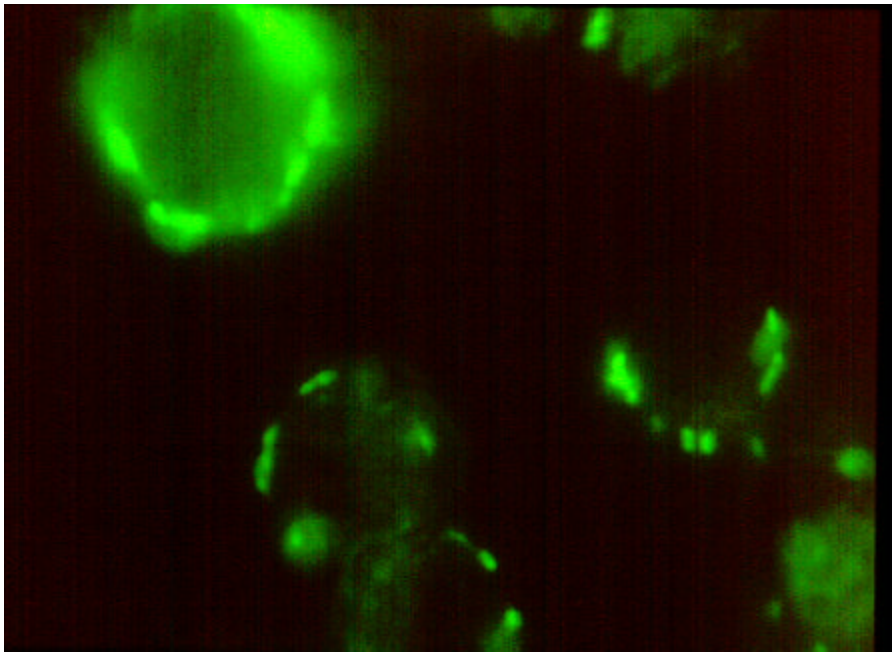


Figure 4

Fluorescence microscopic image of Vero cells with adherent bacteria.

Vero cells were treated with *B. bronchiseptica* 110H harboring pBBRP9GFP and visualized by fluorescence microscopy. Magnification X1000.

Figure 5

Bar graph showing the level of adherence of *B. bronchiseptica* strain 110H and 110NH harboring pBBRP9GFP to Vero cells. The values on y- axis indicate mean fluorescence intensity as measured by flow cytometry analysis of Vero cell suspensions treated with bacteria. Untreated Vero cells similarly incubated were used as negative controls

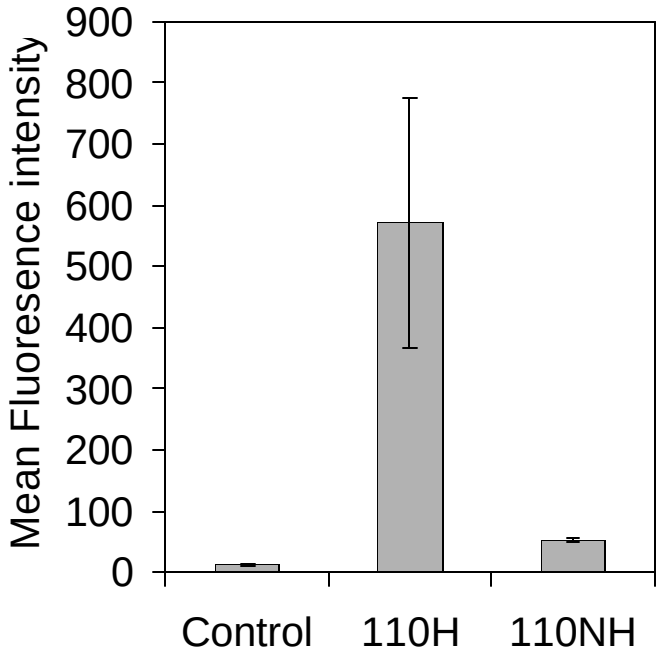

Mean Fluoresence intensity
900
800
700
600
500
400
300
200
100
0
Control
110H
110NH

Figure 6

Comparison of growth curves for wild type and recombinant *B. bronchiseptica*. Overnight cultures of *B. bronchiseptica* 110H with pBBRP9GFP and pBBRGFP were inoculated into LB broth containing kanamycin. Wild-type 110H was similarly inoculated into LB broth without kanamycin. At designated time points A590 was measured and recorded.

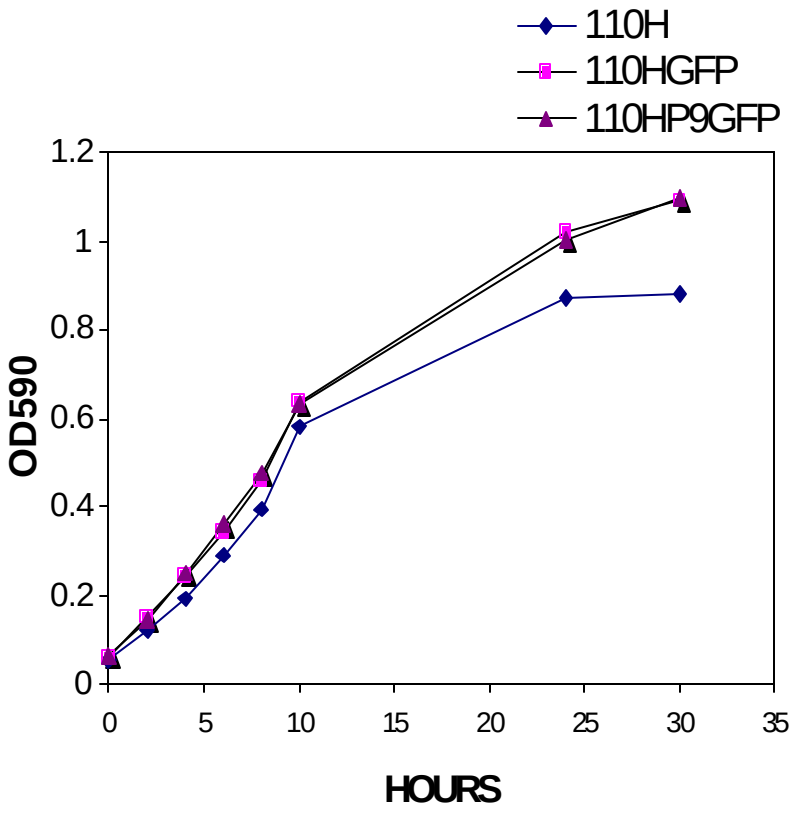
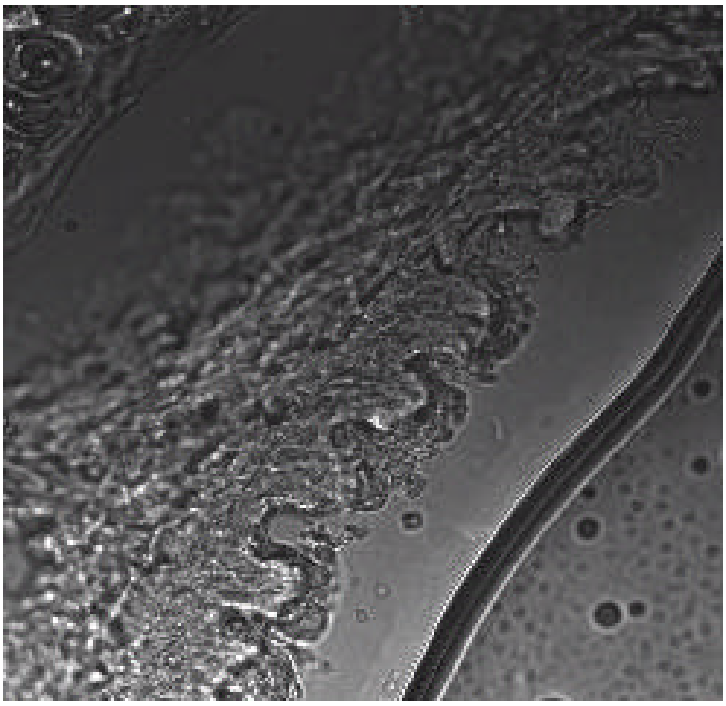

110H
110HGFP
110HP9GFP
OD590
HOURS
0
0.2
0.4
0.6
0.8
1
1.2
0
5
10
15
20
25
30
35

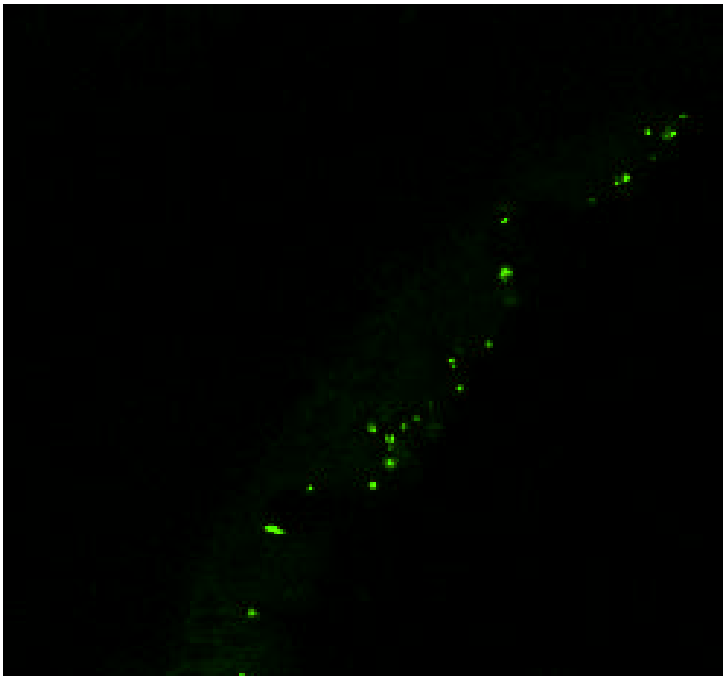
Figure 7

In vivo GFP expression in *B. bronchiseptica*. (A) Bright field image of 8µm cryosection of trachea of mouse 3 hours post inoculation with *B. bronchiseptica* 110H harboring pBBRP9GFP. (B) Corresponding confocal scanning image of section seen in A with GFP expressing bacteria attached to the ciliated epithelium. Magnification X630

A



B



PART 3

Mouse inoculation studies, Immunization of mice with recombinant *Bordetella bronchiseptica* expressing *Pasteurella multocida* toxin fragment or green fluorescent protein

Abstract

Bordetella bronchiseptica colonizes ciliated epithelium of the upper respiratory tract of a wide variety of mammals. The role of *Bordetella bronchiseptica* and *Pasteurella multocida* toxin in the disease, atrophic rhinitis, of pigs is well documented. In this study, a non-toxic, protective *P. multocida* toxin fragment (PMTCE) and green fluorescent protein (GFP) were expressed in *B. bronchiseptica* from genes cloned into a broad-host-range plasmid vector, PBBR1MCS2, and placed under the control of a promoter region isolated from *B. bronchiseptica* that appears to be a member of the heat shock protein gene family. While wild-type and recombinant *B. bronchiseptica* colonized the mouse respiratory tract effectively, the plasmid was no longer detected from the organism after 72 hours post-inoculation. After a single intranasal inoculation, IgM, IgA and IgG responses to *B. bronchiseptica* were detected in serum and broncheoalveolar lavage samples. PMTCE specific antibodies were not detected. Four intranasal inoculations with *B. bronchiseptica* expressing green fluorescent protein induced systemic and mucosal immune responses to GFP; however, similar inoculations with *B. bronchiseptica* expressing the PMTCE fragment did not induce detectable PMTCE specific immune responses.

Introduction

Atrophic rhinitis (AR) is an upper respiratory tract disease of pigs characterized by degeneration and atrophy of nasal turbinate bones leading to visible distortion and shortening of the snout [9, 28]. This disease is of major economic importance to pig breeders all over the world. Bacterial etiologies identified in this condition are toxigenic strains of *Pasteurella multocida* and virulent strains of *Bordetella bronchiseptica*, alone, or combined with environmental factors like dust, ammonia and other physical or chemical irritants [2, 6, 10, 26, 29]. *Pasteurella multocida* is an important veterinary pathogen involved in diseases such as atrophic rhinitis in swine, fowl cholera in birds, hemorrhagic septicemia in cattle and other respiratory diseases in lab animals [2]. Many capsular type D and occasional type A strains of *P. multocida* produce a potent, intracellular, heat-labile, 146Kd, mitogenic toxin (PMT), which is a major virulence factor in atrophic rhinitis [5, 6, 11]. The role played by *P. multocida* and *B. bronchiseptica* in AR is documented in a number of experimental studies [3, 4, 19, 27]. *B. bronchiseptica*, is widely distributed in swine herds with and without atrophic rhinitis. The infection with *B. bronchiseptica* can lead to mild non-progressive forms of atrophic rhinitis that do not affect growth rate [6]. Toxigenic type D *P. multocida* is the most common isolate in severe, progressive, growth retarding atrophic rhinitis [6]. Experimental inoculation of pigs with cell-free extracts of *P. multocida* or purified toxin can also lead to severe, progressive atrophic rhinitis [11]. Experimental infections in gnotobiotic pigs implicate that colonization with virulent strains of *B. bronchiseptica* damages the nasal mucosa and predisposes animals to colonization by toxigenic *P.*

multocida [3, 4]. By itself, *P. multocida* does not readily colonize the nasal mucosa. Vaccination and good management practices are the main approaches used to control this condition [6]

Considering the proposed synergistic role of *B. bronchiseptica* and *P. multocida* in AR, and the common usage of live-attenuated *B. bronchiseptica* vaccines and *P. multocida* toxoid/ bacterins to control AR, further refinements of combined vaccines are warranted. In this study, a non-toxic protective fragment of PMT and green fluorescent protein (GFP) were expressed in *B. bronchiseptica*. Colonization kinetics, plasmid stability and immune responses generated to *B. bronchiseptica*, PMT, and GFP were evaluated in mice.

Materials and methods

Plasmids, bacterial strains, and enzymes

Origin of the *B. bronchiseptica* strains used in this study has been previously described [1]. Strain 55 is a live attenuated *B. bronchiseptica* (ATCC 31437) vaccine strain. Luria Bertani (LB) agar or broth with appropriate antibiotic selection at 37^{o}C were the conditions employed to grow all bacteria unless otherwise indicated. Smith Baskerville medium was used for isolation of *B. bronchiseptica* from mouse respiratory tract samples [23]. Restriction enzymes and LA Taq polymerase were products of PANVERA, Madison WI. Wizard plus SV miniprep kit (Promega, Madison WI) was used for plasmid isolation. The plasmid, pBBR1MCS2, (kindly provided by Kovach, M. E) [12] was used for cloning and expression of the GFP gene and the PMT gene fragment. A promoter region (P9) with homology to heat shock gene promoter region of *B. pertussis* was isolated from *B. bronchiseptica* using a green fluorescent protein

reporter system. This promoter was previously used to drive high-level expression of GFP in four different strains of *B. bronchiseptica* [S. Rajeev, Dissertation, Part 2]. The PMT gene fragment (PMTCE) consisted of base pairs 1999-4055 encoding the C-terminal 685 amino acids of PMT. This fragment was amplified from genomic DNA of *P. multocida* (NCTC 12178) using LA Taq polymerase and primers 5'-CGGGATCCCGTTATTGGAAAGCCTATTGGA-3' (forward) and 5'-GGGGATCCGTTATAGTGCTCTTGTTAAGCG-3'(reverse). The PMTCE fragment was cloned into BamHI digested and calf intestinal alkaline phosphatase treated pBBRP9. The ligation mixture was transformed into *E. coli* and transformants were selected on LB plates containing kanamycin (100µg/ml). The correct insertion of the sequence was verified by restriction digestion with enzymes that cut within the PMTCE fragment. Expression of PMTCE was detected on western blots of whole cell lysates using PMT specific monoclonal antibody, Mab CRL1965, obtained from ATCC [13]. The resulting plasmid construct with PMTCE- promoter fusion was electroporated into *B. bronchiseptica* strains 110H, 110NH, R5, strain 55 using a CELL-PORATOR Electroporation system (GIBCO-BRL, Gaithersburg, MD) following manufacturer's instructions. Electroporants were selected on kanamycin plates and tested for the expression of PMTCE by Western blotting.

SDS-PAGE and Western blots

SDS-PAGE was performed using 10 % polyacrylamide gels (Ready Gels, Bio-Rad, Hercules, CA) according to standard procedures. An overnight culture of each *B.bronchiseptica* strain containing the recombinant plasmid, PBBRP9PMTCE, was adjusted to an optical density (A_{590}) of 0.5. One hundred µl of this suspension was

centrifuged at 5000 rpm for one minute. The pellet was suspended in 100µl of 1x sample buffer (Sigma Chemical Company, St. Louis, MO) and boiled for 10 minutes. Aliquots of 5µl were loaded to 10% polyacrylamide gels. The gels were electrotransferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk solution in phosphate buffered saline with 0.05%Tween 20(PBS-T) for 30 minutes and then reacted with a 1µg/ ml solution PMT specific monoclonal antibody, CRL 1965, in 1% nonfat dry milk in PBS-T for one hour. The membranes were washed four times in PBST for 30 minutes and incubated with 1/10,000 dilution of peroxidase-conjugated anti-mouse IgG (Sigma Chemical Company). After washing four times in PBS-T for 30 minutes, the membranes were developed with chemiluminescent substrate (SuperSignal ^{R} West Pico Substrate Pierce, Rockford, IL) and the membranes were exposed to CL-Xposure^{TM} Film (Pierce, Rockford, IL) following the manufacturer's guidelines.

Intranasal inoculation of mice

Four to six week-old female BALB/c mice were obtained from Harlan Laboratories (Indianapolis, IN) and maintained by routine procedures in an AALAC accredited animal facility. Groups of 3-4 mice were inoculated with ~ 1x10 ^{5} CFU of either wild-type *B. bronchiseptica* 110H or recombinant *B. bronchiseptica* 110H harboring pBBRP9PMTCE or pBBRP9GFP. Mice were anaesthetized by Isoflurane (Abbott Laboratories, North Chicago, IL) and 50µl of the inoculum was instilled into the nostrils. Mice were also similarly immunized with *B. bronchiseptica* expressing GFP from the same promoter. In a second experiment, mice, inoculated once by the intranasal route with 1x10 ^{5} CFU were

boosted three times at biweekly intervals via the intranasal route with $1x10^7$ CFU of each respective strain of *B. bronchiseptica* expressing GFP or PMTCE. An additional group of mice primed with *B. bronchiseptica* expressing GFP were given a single intranasal inoculation with 50 μg of purified recombinant GFP in PBS.

Colonization kinetics and in vivo plasmid stability

Mice were sacrificed at designated time points and the entire lungs and trachea were collected aseptically. The tissue was homogenized and serial dilutions were plated onto Smith Baskerville media with and without kanamycin. The plates were incubated for 48 hours at 37 ^oC and colony counts were performed. The percentage of kanamycin resistant *B. bronchiseptica* colonies compared to the total number of colonies recovered on non-selective media was calculated. The colonization studies were continued until the colony counts reached minimum detectable levels (56 days).

Antibody response

Mice were bled from the retro-orbital sinus on 14, 28, 42 and 56 days post-inoculation. Nasal and broncheoalveolar lavage (NBAL) were collected at the same time points using a 24-gauge teflon catheter by flushing the lung, trachea and nasal cavity with 2 ml of PBS. Pooled samples were centrifuged at 3000 rpm to remove particulate matter. Lavage samples and sera were stored at -70^oC until further evaluation. Lavage samples that were visibly contaminated with blood were discarded.

A kinetic enzyme linked immunosorbent assay (KELISA) was performed to evaluate the antibody response to *B. bronchiseptica* and PMTCE. In brief, 96-well plates (Immulon 2HB, Dynex) were coated overnight either with a suspension of *B.*

bronchiseptica (strain 110H grown on TSA and adjusted to optical density, A590, of 0.1 in PBS), purified recombinant PMTCE (10µg/ ml in PBS) or purified recombinant GFP (10 µg/ml). Plates were washed four times in PBS-T and blocked for 30 minutes with PBS-T. One hundred µl of serum (1/40 dilution) or undiluted NBAL was added to plates and incubated for 2 hrs at 37^{o}C. Plates were washed four times with PBS-T and incubated with peroxidase-conjugated secondary antibodies, anti-mouse IgG (1/2000), IgM (1/1000), and IgA (1/1000) in PBS-T. Plates were washed four times with PBS-T and developed with 100µl of substrate solution, 2,2'-azino diethylbenzothiazoline-6-sulphonic acid (ABTS, Sigma Chemical Company, St Louis MO) and hydrogen peroxide. The plates were read kinetically at A_{405} using ELx800 ELISA reader (BIO-TEK instruments Inc., Winooski, VT). The plates were read 7 times at one-minute intervals, the maximum slope (KELISA slope is directly proportional to the amount of antibody bound) was calculated using KC*junior* software (BIO-TEK instruments Inc, Winooski, VT)

Statistical analyses

The significance of differences between groups was determined by Student's *t*-tests. The data are expressed as mean +/- standard error of mean. *P* values < 0.05 were considered significant.

Results

Cloning and expression of PMTCE in *B. bronchiseptica*

Initial attempts to express a previously reported 125kD, non-toxic, N-terminal deletion mutant of PMT [21, 22] were unsuccessful. The recombinant

B. bronchiseptica harboring this plasmid exhibited marked reduction in growth in vitro and did not show any expression of this protein on immunoblots. We have identified N-terminal and C-terminal recombinant PMT protein fragments which can protect mice from lethal challenge with PMT [20]. Intraperitoneal immunization with these proteins in Freund's incomplete adjuvant elicited high level of anti-PMT antibodies and protected mice from intraperitoneal challenge with *P. multocida* toxin [20]. The C-terminal protective fragment (PMTCE) was utilized for expression in *B. bronchiseptica*.

A schematic representation of the expression plasmid is given in Figure 1(appendix). A PMT specific monoclonal antibody, CRL-1965, was used for detection of expression of this protein in *B. bronchiseptica* [13]. PMTCE expression was initially detected in electroporants of all strains of *B. bronchiseptica* and in *E. coli*. After storage at -70^{o}C, repropagation and examination, *B. bronchiseptica* strain 55 no longer produced a reactive protein band on western blot. The expected protein band of 80kD (76kD PMTCE+ 4kD from the promoter region) was seen on Western blot with chemiluminiscent substrate (Figure 2). However, the bands were not visible in Western blots developed with colorimetric substrate, indicating that the amount of protein produced was low. The amount of protein in each band was quantitated on Western blots by un-SCAN-IT TM (Silk Scientific Corporation). *B. bronchiseptica* strains 110H, 110NH, and R5 expressed 6.4µg/0.5OD/ml , 6.2µg/0.5OD/ml, and 74µg/0.5OD/ml, respectively. Each strain that expressed PMTCE and affinity purified PMTCE had a second immunoreactive band that is presumed to be a degradative fragment. Such fragments have been previously reported in purified native and recombinant PMT [20]. A third non-specific band was observed in all samples including the negative controls indicating possible cross reactivity of the

monoclonal antibody with some component in *B. bronchiseptica*. Expression of PMTCE in *B. bronchiseptica* did not affect the growth in vitro (Figure 3).

Plasmid stability and colonization kinetics

The proportion of plasmid bearing recombinant *B. bronchiseptica* in lungs and trachea of mice was determined by comparing the number of *B. bronchiseptica* CFU obtained on Smith Baskerville medium with kanamycin selection to the number of *B. bronchiseptica* CFU obtained on Smith Baskerville without kanamycin selection. *B. bronchiseptica* was not isolated from any of the uninfected mice. The percentage of kanamycin resistant colonies recovered from mice is shown in figure 4. Forty five percent of the colonies harbored recombinant plasmid at 1hr post-inoculation. This frequency gradually decreased to 0.007% at 72 hours post-inoculation. Recombinant and wild type *B. bronchiseptica* colonized the respiratory tract of mice (Figure 5). The number of organisms recovered increased until 7 days post-inoculation and gradually decreased thereafter. By day 56, the number of organisms recovered was close to the minimum detectable levels. There was no significant difference ($p<0.05$) in the total number of organisms recovered at any time point from mice inoculated with wild-type or recombinant *B. bronchiseptica*. However, colonization of wild-type and recombinant *B. bronchiseptica* can not be compared because of the rapid loss of the plasmid.

Antibody response in mice

Antibody responses generated against *B. bronchiseptica* and the heterologous antigens, PMTCE and GFP after intranasal inoculation with live *B. bronchiseptica* were evaluated by KELISA [15, 30, 31]. KELISA measures the rate of color development during the initial period of reaction, and is represented by the maximum slope, which is

directly, proportional to the amount of secondary antibody conjugate bound. KELISA performed using a single serum dilution was reported to be as sensitive and specific as determining the endpoint titer [30]. IgG, IgA and IgM levels were measured in individual serum samples and pooled NBAL samples. A single intranasal inoculation with wild-type and recombinant *B. bronchiseptica* induced marked *B. bronchiseptica* specific antibodies in the serum and NBAL (Figure 6)

B. bronchiseptica-specific serum IgM levels were detected at 14 day post inoculation and remained stable throughout the period analyzed. Serum IgG levels increased gradually in wild-type and recombinant *B. bronchiseptica* inoculated mice. The serum IgG, levels to *B. bronchiseptica* were significantly higher ($p = >0.05$) in groups inoculated with wild-type organisms compared to *B. bronchiseptica* expressing PMTCE. An increase in *B. bronchiseptica* specific serum IgA levels was also detected in the serum. In the NBAL an initial increase in IgM levels were followed by stable levels. *B. bronchiseptica* specific IgG and IgA were also observed in NBAL. The PBS inoculated controls did not produce any antibody response to *B. bronchiseptica*.

A single intranasal inoculation with recombinant *B. bronchiseptica* either expressing GFP or PMTCE did not induce detectable antibody responses to the respective proteins. In a second experiment, mice were first inoculated with $1x10^5$ CFU of *B. bronchiseptica* virulent strain, 110H or avirulent strain, 110NH expressing GFP or PMTCE by the intranasal route followed by three intranasal booster inoculations with $1x10^7$ organism at 14 day intervals.

After initial inoculation, 110H (Bvg+) infected mice became sick (characterized by raised hairs and lethargy) and recovered by third day. However, mice inoculated with

110NH (Bvg -) were normal. Even though inoculation with $1x10^5$ *B. bronchiseptica* 110H produced transient illness in mice, mice initially inoculated with $1x10^5$ *B. bronchiseptica* 110H did not suffer any ill effects following intranasal exposure to $1x10^8$ organisms 14 days later. Mice that had not received the initial exposure to $1x10^5$ organisms died within 72 hours after intranasal exposure to $1x10^7$ CFU of *B. bronchiseptica* strain 110H. Multiple inoculations with higher number of organisms were done to provide increased exposure to the heterologous antigens.

A PMT-specific immune response was not observed after exposure to the increased number of organisms. However, a GFP-specific antibodies were obtained in NBAL and serum after boosting with the increased dose of *B. bronchiseptica* 110H expressing GFP (Figure 7). Serum GFP antibodies were predominantly of IgG subclass and NBAL GFP antibodies were a mixture of IgA and IgG. Mice inoculated with avirulent 110NH and boosted similarly did not produce detectable serum antibodies to GFP. There was a slight increase in GFP-specific IgA in NBAL. When mice inoculated with recombinant *B. bronchiseptica* strain 110H expressing GFP were boosted with 50µg of purified recombinant GFP, high levels of GFP antibodies were observed in serum (Figure 8). GFP-specific response was predominantly IgG. A similar increase in GFP-specific response was not noticed in NBAL.

Discussion

Atrophic rhinitis is a disease of significant economic importance to US agriculture. Vaccines against atrophic rhinitis consist of killed or live preparations of *B. bronchiseptica* along with *P. multocida* bacterins or toxoid. Development of a safe and bivalent live vaccine with avirulent *B. bronchiseptica* that expresses protective epitopes

of PMT would represent a novel approach to control this disease. This is the first study to demonstrate expression of a foreign protective antigen in *B. bronchiseptica* and the utility of *B. bronchiseptica* as a mucosal vaccine vector directed to the respiratory tract.
A single intranasal inoculation with recombinant and wild-type *B. bronchiseptica* induced both systemic and mucosal immune responses to *B. bronchiseptica*. While IgG was the predominant antibody in the serum, IgA predominated in NBAL. In a recent report, mucosal IgA antibodies against *B. bronchiseptica* were observed after intranasal inoculation of mice with *B. bronchiseptica* and authors of this paper hypothesized that this lack of mucosal IgA response was due to the toxins secreted by the type III secretion machinery [14]

Plasmid instability leading to insufficient antigen exposure may have been the most important factor responsible for our initial inability to detect an antibody response to PMTCE and GFP after a single intranasal inoculation with recombinant *B. bronchiseptica* expressing these proteins. It was hypothesized that booster inoculations with increased numbers of organisms will provide increased exposure to the preformed foreign antigens contained in the inoculum. An antibody response was observed for GFP but not PMTCE. The inability to detect antibodies to PMTCE compared to GFP after booster inoculations might be attributed to the comparative low-level expression of PMTCE, plasmid instability or antigenic differences of PMTCE expressed in *B. bronchiseptica*. The yield of PMTCE in the *E. coli* expression system was also very low [20]. Therefore, production and purification of large quantities of PMTCE is difficult and identifying stable protective fragments of PMT for expression in *B. bronchiseptica* is worthwhile to pursue. Recently, we have shown that the molecular chimerization of Fim N protein of

B. bronchiseptica with an unstable protective fragment of leukotoxin of *Mannheimia haemolytica* augmented neutralizing antibody response to leukotoxin [24]. Stabilization of a PMT fragment by combining it with FIM N may also enhance the immune response to PMT. An intranasal dose of $1x10^5$ CFU could be given to mice without causing any deleterious effects. Increasing the number of organisms in unprimed animal's lead to 100% mortality. We could inoculate with a 100 times greater dose of *B. bronchiseptica* in previously exposed mice, without any deleterious effects. A protective immune response in mice against *B. bronchiseptica* was probably generated as early as 14 days. Four inoculations with *B. bronchiseptica* 110H expressing GFP induced significantly greater GFP-specific antibody responses in the serum and NBAL than 110NH expressing GFP. *B. bronchiseptica* 110H is a Bvg+ strain which effectively colonizes the respiratory tract where as Bvg- strains, like 110NH, do not have the capability to colonize. The difference in immune responses to GFP in 110H and 110NH infected mice suggests that, colonization played an important role in the generation of immune response against GFP. In addition to simply influencing the level of antigen that the immune system was exposed to, it is also possible, as previously suggested [14], that adhesins and other virulence factors produced by *B. bronchiseptica* colonization may have produced immunomodulating effects.

A second intranasal inoculation with recombinant GFP, after initial inoculation with *B. bronchiseptica* 110H expressing GFP induced systemic GFP-specific antibodies; whereas, control animals inoculated with GFP in PBS did not. This increase in response indicates that there was a priming effect after inoculation with *B. bronchiseptica* 110H expressing GFP, but not with 110NH that expressed GFP. Intranasal priming with

recombinant *B. pertussis* and subsequent intranasal boosting with the foreign antigen resulted in an effective systemic response against the foreign antigen [16]. Our experiment gave similar results and suggests that this mode of immunization could be utilized to generate systemic humoral immune responses against specific protective antigens.

Virulent *B. bronchiseptica* is unsuitable for field vaccination purposes. The creation of selected mutations in virulence genes like adenylate cyclase toxin and dermonecrotic toxin, along with chromosomal integration of the genes encoding the foreign antigen may be ideal. In *B. pertussis*, chromosomal integration was utilized for expression of a heterologous antigen [17, 25]. Improving the stability of the plasmid vector by construction of balanced lethal vectors as described in *Salmonella* [7] or by incorporating a post-segregational killing function without manipulation of the live host can be done [18]. While chromosomal integration may stabilize expression, it usually leads to low level expression, which can result in sub-optimum immune responses. Stable multicopy plasmid vectors can overcome this by the overexpression of antigen needed to generate an effective immune response [8].

Identification of essential protective antigens and improved delivery of vaccine components is essential for future vaccine development. Live-attenuated *B. bronchiseptica* vaccines are widely accepted in animals and molecular methods to genetically inactivate unwanted traits are available; therefore, construction of a live *B. bronchiseptica* that expresses protective PMT antigens is desirable from the standpoint of both safety and efficacy. Further manipulations of host strain, improvements in plasmid

stability and stabilization of heterologous antigen expression are essential to make this system effective.

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Appendix

Figure 1

Schematic representation of recombinant plasmid vector pBBR1MCS2 expressing *P. multocida* toxin fragment (PMTCE). Restriction sites used to clone in the fragment are indicated. P9 is a constitutive promoter cloned upstream to PMTCE in frame to drive the expression of the PMTCE.

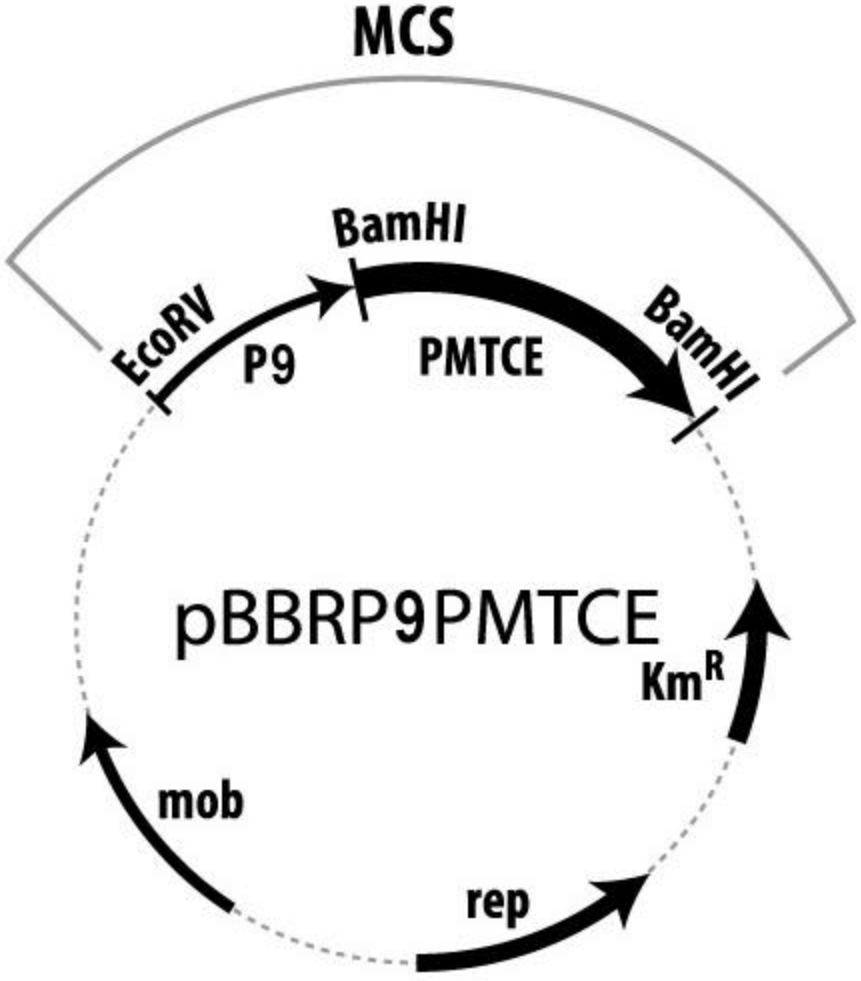

MCS
BamHI
EcoRV
P9
PMTCE
BamHI
pBBRP9PMTCE
Km^R
mob
rep

Figure 2

Expression of PMTCE in *B. bronchiseptica.*

Whole cells harboring the recombinant plasmid were subjected to SDS PAGE on a 10%polyacrylamide gel and electro- transferred to nitrocellulose membrane. Membranes were probed with PMT specific monoclonal antibody, CRL-1965 and developed with chemiluminescent substrate. Numbers on the left indicate the approximate positions of prestained molecular weight markers in kilo Daltons. Lanes 1,3, 5, and 7 are lysates of *B. bronchiseptica* strains 110H, 110NH, R5, and 55 harboring pBBRP9 without PMTCE insert respectively. Lanes 2, 4, 6, and 8 contains lysates of *B. bronchiseptica* strains 110H, 110NH, R5, and 55 harboring pBBRP9PMTCE. Lane 9 contains 50ng of affinity-purified recombinant PMTCE.

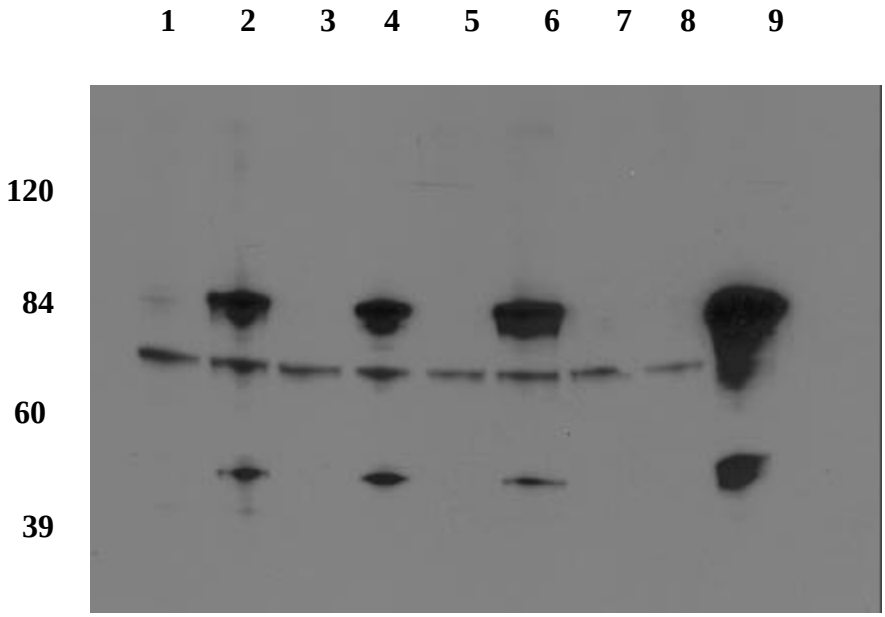

1 2 3 4 5 6 7 8 9
120
84
60
39

Figure 3

Comparison of growth curves

Overnight cultures of the recombinant *B. bronchiseptica* strain 110H harboring pBBRP9 and pBBRP9PMTCE were inoculated into LB broth containing kanamycin. Wild-type *B. bronchiseptica* was also similarly inoculated into LB broth without kanamycin. At designated time points optical densities at A590 were measured and recorded.

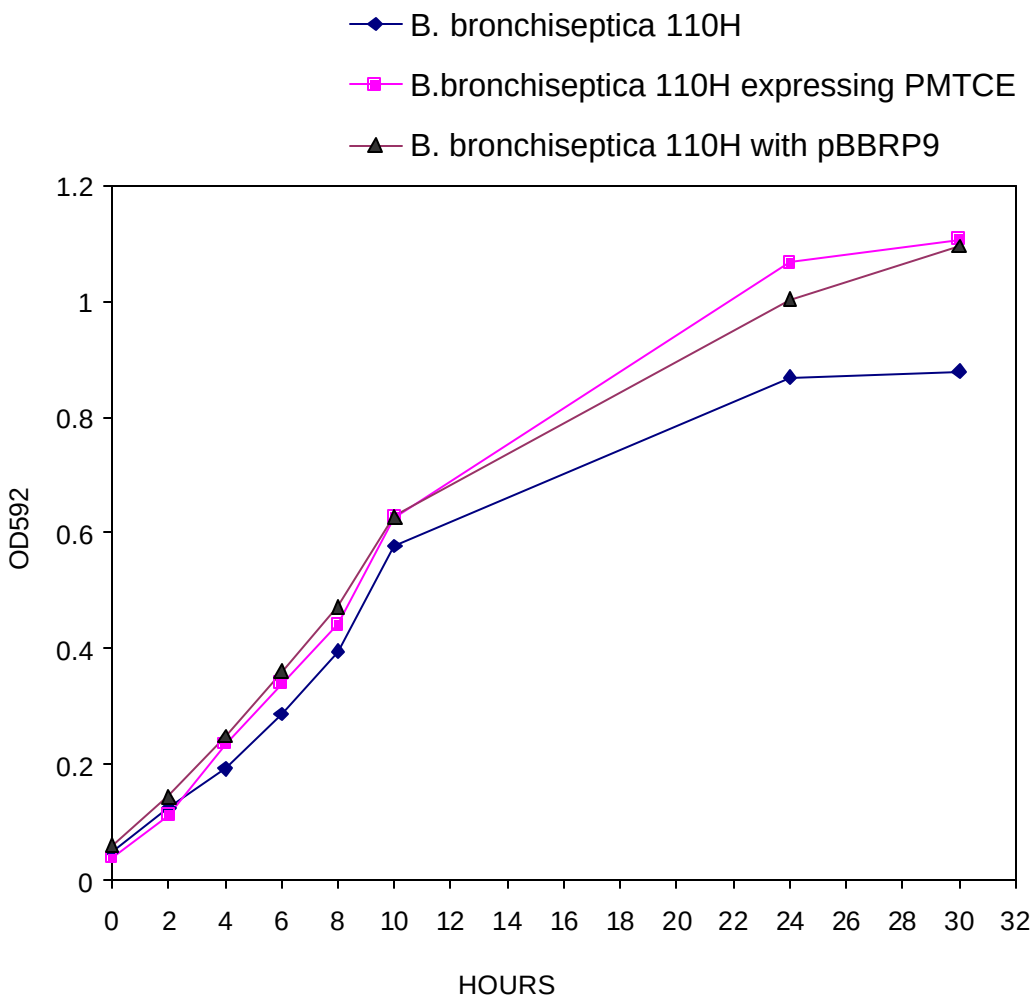

B. bronchiseptica 110H
B.bronchiseptica 110H expressing PMTCE
B. bronchiseptica 110H with pBBRP9
OD592
1.2
1
0.8
0.6
0.4
0.2
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32
HOURS

Figure 4

In vivo stability of the recombinant plasmid in *B. bronchiseptica*.

Mice were inoculated with $1x10^5$ *B. bronchiseptica* in PBS via the intranasal route. At designated time points lungs and trachea were aseptically collected and homogenized, serial dilutions were plated on to Smith Baskerville media with and without kanamycin. The percentage of kanamycin resistant colonies in relation to the total number of colonies recovered is reported.

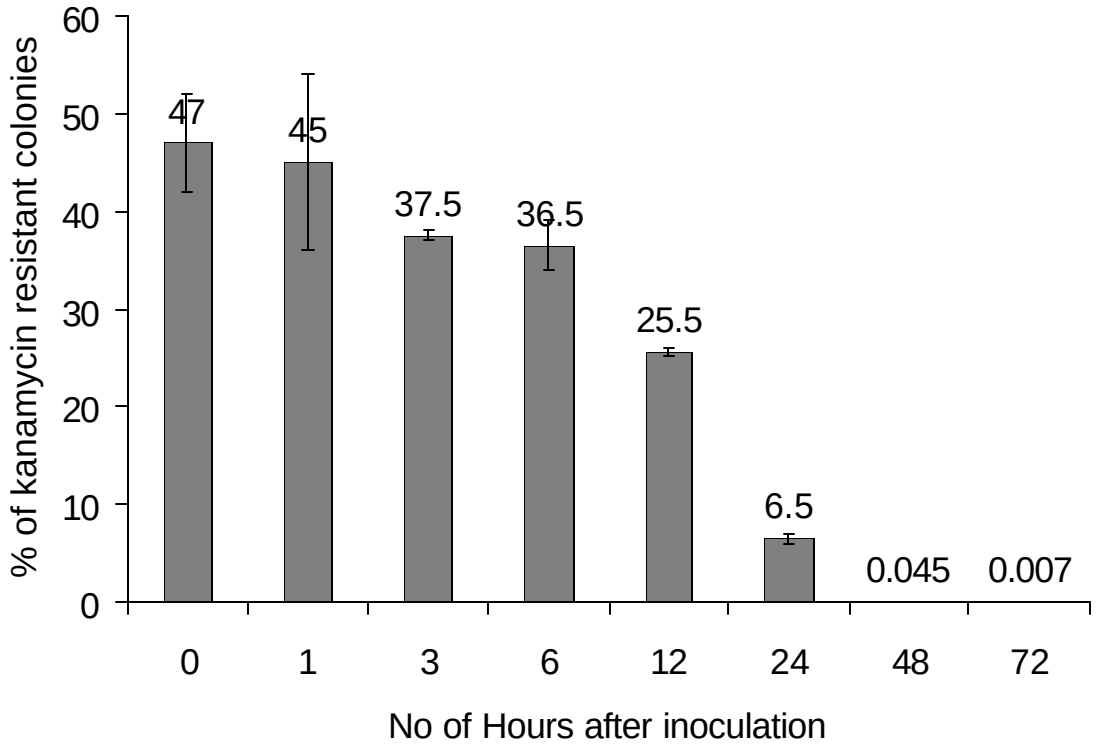

% of kanamycin resistant colonies
60
50
40
30
20
10
0
47
45
37.5
36.5
25.5
6.5
0.045
0.007
0
1
3
6
12
24
48
72
No of Hours after inoculation

Figure 5

Colonization kinetics of *B. bronchiseptica* in mice.

Mice were inoculated with $1x10^5$ *B. bronchiseptica* in PBS via the intranasal route. At designated time points lungs and trachea were aseptically collected and homogenized, serial dilutions were plated on to Smith Baskerville media. Total CFU recovered at designated time points is reported.

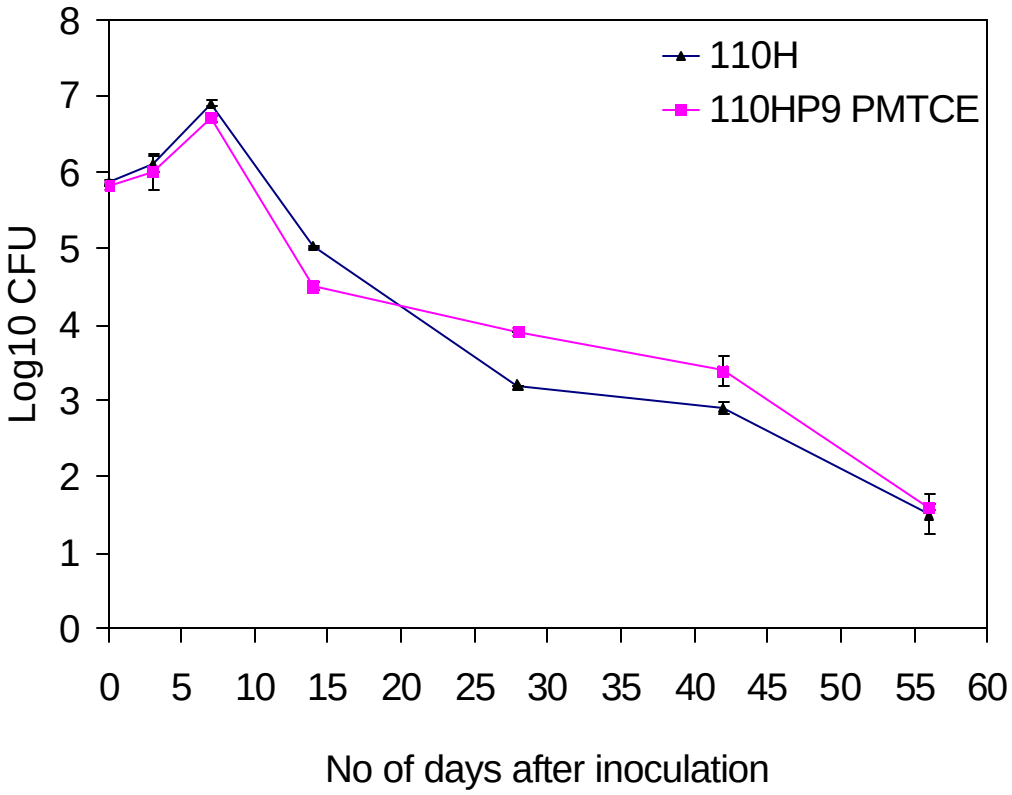

8
7
6
5
4
3
2
1
0
Log10 CFU
0
5
10
15
20
25
30
35
40
45
50
55
60
No of days after inoculation
110H
110HP9 PMTCE

Figure 6

Antibody response to *B. bronchiseptica* in mice.

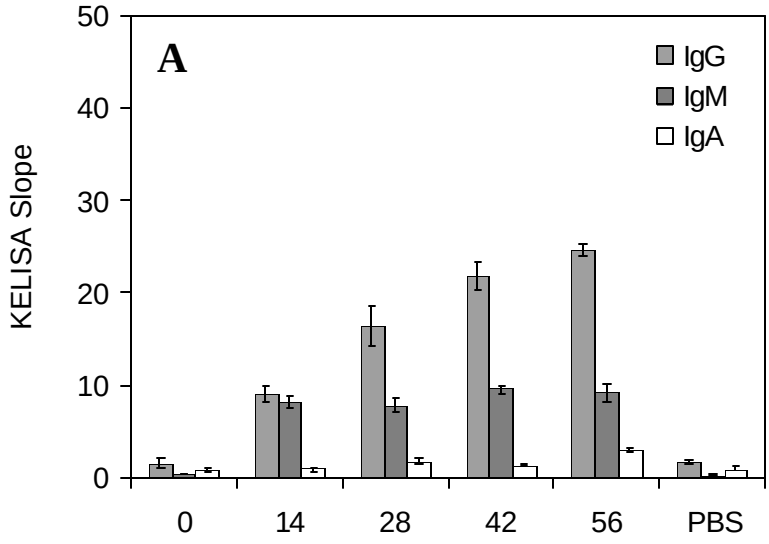
Mice were inoculated via the intranasal route with a suspension in PBS containing $1x10^5$ wild-type or recombinant *B. bronchiseptica*110H expressing PMTCE. NBAL and serum were collected at designated time points. IgG, IgM, and IgA responses were measured by KELISA and reported as KELISA slope. The day at which the samples were collected is given on x-axis. The control animals were inoculated with PBS

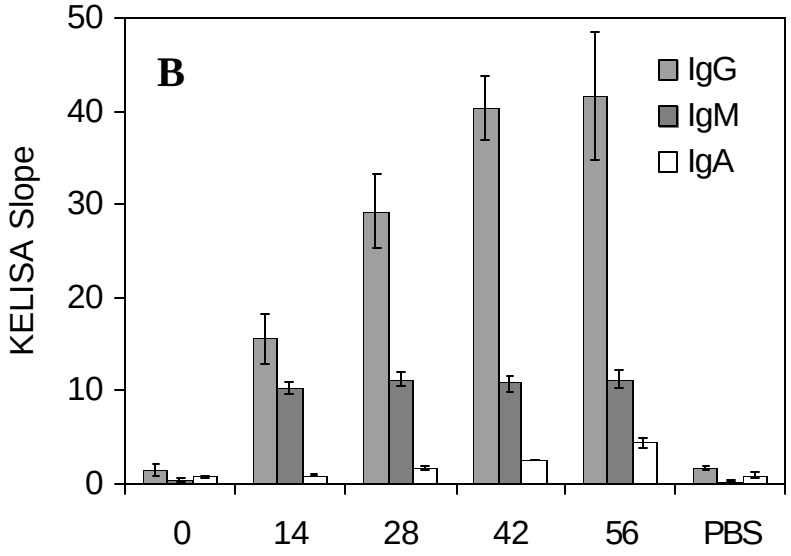
(A) Serum antibody response to *B. bronchiseptica* strain 110H in mice inoculated with recombinant 110H expressing PMTCE.

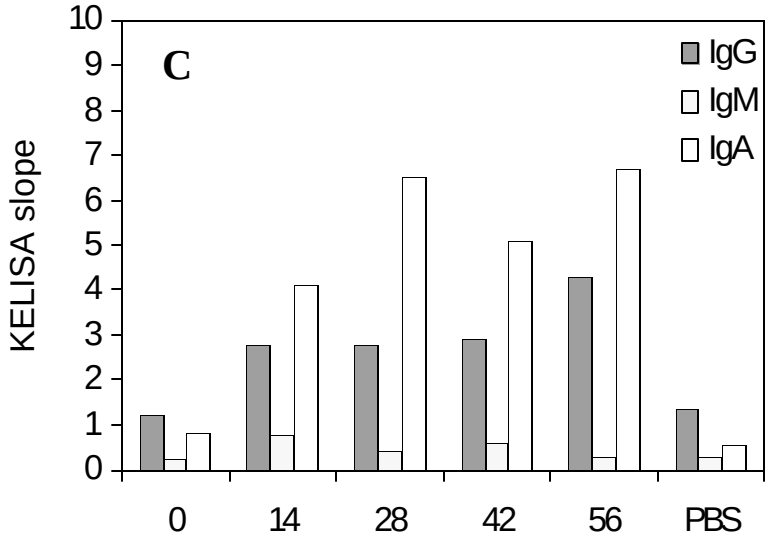
(B) Serum antibody response to *B. bronchiseptica* strain 110H in mice inoculated with wild-type.

(C) Mucosal antibody response to *B. bronchiseptica* strain 110H in mice inoculated with recombinant 110H expressing PMTCE.

(D) Mucosal antibody response to *B. bronchiseptica* strain 110H in mice inoculated with wild-type.


A
IgG
IgM
IgA
KELISA Slope
50
40
30
20
10
0
0
14
28
42
56
PBS


B
IgG
IgM
IgA
KELISA Slope
50
40
30
20
10
0
0
14
28
42
56
PBS


C
KELISA slope
10
9
8
7
6
5
4
3
2
1
0
IgG
IgM
IgA
0
14
28
42
56
PBS

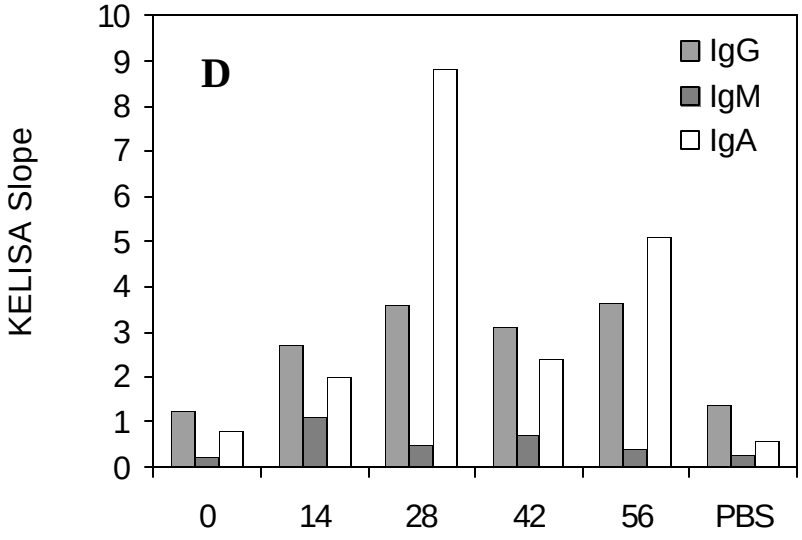
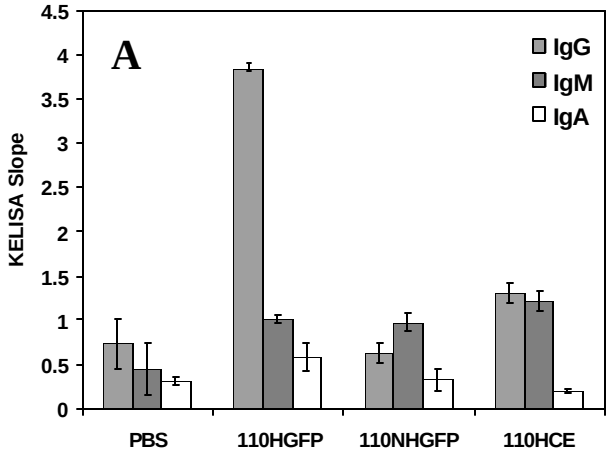

D
KELISA Slope
10
9
8
7
6
5
4
3
2
1
0
IgG
IgM
IgA
0
14
28
42
56
PBS

Figure 7

Antibody response to GFP

Mice were inoculated with $1x10^5$ recombinant *B. bronchiseptica* 110H expressing GFP, 110NH expressing GFP, 110H expressing PMTCE or PBS via the intranasal route. The mice were boosted with $1x10^7$ organisms three times at 14 day intervals. NBAL and serum was collected on the 9th day after final boosting. GFP specific IgG, IgM, and IgA response was measured by KELISA and reported as KELISA slope. (A) Serum antibody response. (B) Mucosal antibody response


A
IgG
IgM
IgA
KELISA Slope
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
PBS
110HGFP
110NHGFP
110HCE

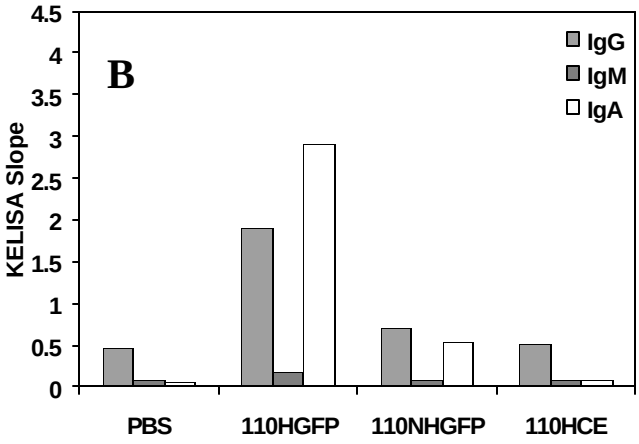
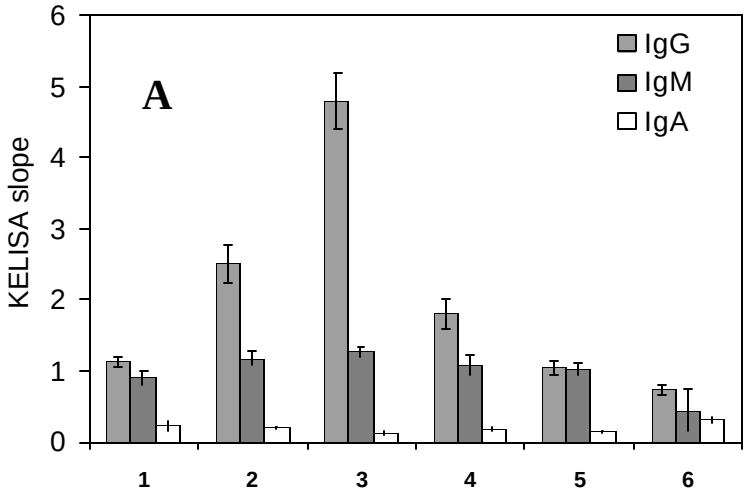
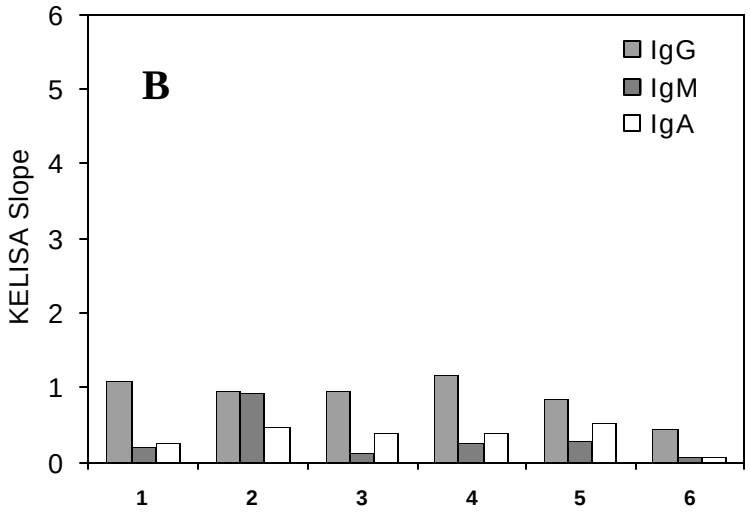

B
IgG
IgM
IgA
KELISA Slope
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
PBS
110HGFP
110NHGFP
110HCE

Figure 8

Antibody response to GFP after boosting with recombinant GFP

Mice were inoculated with $1x10^5$ recombinant *B. bronchiseptica* 110H, recombinant 110NH expressing GFP, wild-type 110H, wild-type 110NH or PBS via the intranasal route. On the 14th day post-inoculation the mice were given 50µg of GFP in PBS via the intranasal route. NBAL and serum were collected on the 9^{th} day after inoculation with GFP. GFP-specific IgG, IgM, and IgA antibodies were measured by KELISA and reported as KELISA slope. A) Serum antibody response. B) Mucosal antibody response X-axis labels, 1-strain 110H followed by GFP, 2-110NH followed by GFP, 3- 110H expressing GFP followed by GFP, 4-110NH expressing GFP followed by GFP, 5- PBS followed by GFP, 6- PBS alone.


A
IgG
IgM
IgA
KELISA slope
0
1
2
3
4
5
6
1
2
3
4
5
6


B
IgG
IgM
IgA
KELISA Slope
0
1
2
3
4
5
6
1
2
3
4
5
6

PART 4

Bordetella bronchiseptica fimbrial protein-enhanced immunogenicity of a *Mannheimia haemolytica* leukotoxin fragment

Abstract

Leukotoxin produced by *Mannheimia (Pasteurella) haemolytica* is an important virulence factor in shipping fever pneumonia in feedlot cattle and is a critical protective antigen. In this study, the immune response to a chimeric protein generated by combining a gene fragment encoding neutralizing epitopes of M. *haemolytica* leukotoxin and a fimbrial protein gene (*fim N*) from *Bordetella bronchiseptica* was evaluated. The recombinant gene was cloned in a bacterial expression vector under the control of the *tac* promoter and expressed as a fusion protein with glutathione-S-transferase (GST) in *Escherichia coli.* Immunization of mice with the recombinant protein, GST-LTXFIM elicited a significantly stronger anti-leukotoxin antibody response than comparable immunizations with GST-LTX fusion proteins lacking FIM N. The GST-LTXFIM was also more stable than GST-LTX during storage at -80^{o}C, thus alleviating a stability problem inherent to leukotoxin. This chimeric protein may be a candidate for inclusion in new generation vaccines against shipping fever pneumonia.

Introduction

Shipping fever pneumonia in feedlot cattle is a multifactorial disease that results in great economic loss in the United States cattle industry. *Mannheimia (Pasteurella) haemolytica* is the primary bacterium associated with this condition. Leukotoxin secreted by *M. haemolytica* is a ruminant leukocyte-specific, pore-forming cytotoxin of the RTX (repeat in toxin) family of toxins, and is an important virulence factor of this organism (3, 6, 8, 29, 31). Protection of cattle from shipping fever pneumonia has been correlated with the presence of leukotoxin-neutralizing antibodies (19, 20, 21, 25, 26, 27, 28). However, leukotoxin-neutralizing antibody responses induced by current vaccines are highly variable, and this disease remains an important health problem in feedlot cattle (28). Instability of native leukotoxin has led to difficulty in its purification and limited the inclusion of this antigen in vaccine preparations (7). The toxicity of native, biologically active leukotoxin may also be a concern for vaccine safety.

The gene encoding leukotoxin has been fully sequenced (12) and a number of leukotoxin- neutralizing monoclonal antibodies against linear and conformational epitopes have been mapped to peptides derived from the carboxy-terminal region of leukotoxin (10, 16). A minimal leukotoxin A gene fragment encoding carboxy-terminal aminoacids 713-939 was previously characterized and the truncated non-cytolytic recombinant protein (LTX) was recognized by three leukotoxin-neutralizing monoclonal antibodies (18). Immunization with this recombinant protein in Freund's complete adjuvant (CFA) elicited considerable leukotoxin neutralizing-antibody response in rabbits; however, the protein was unstable on storage at -80 ^{o}C (18).

Fimbriae are important virulence factors involved in attachment of many Gram-negative bacteria to host tissues (15). Fimbrial antigens are highly immunogenic and generally abundant on bacterial surfaces. We recently cloned and characterized a novel fimbrial gene, *fim N*, from *Bordetella bronchiseptica* (14), an upper respiratory tract pathogen that affects a wide variety of host animals (4, 11). Adhesins that serve colonizing functions in *Bordetella* spp. may be useful in delivering heterologous antigens and several *B. pertussis* antigens, including pertussis toxin (1, 2), adenylate cyclase toxin (23), tracheal colonization factor (5) and filamentous haemagglutinin (22) have been expressed as heterologous recombinant proteins. Fimbrial protein from *Escherichia coli* has been documented as effective carriers of heterologous antigens (15). In the course of studying the suitability of FIM N as a heterologous antigen carrier, we observed that a LTX-FIM N recombinant fusion protein enhanced the immunogenicity of LTX beyond that which had been observed with other LTX recombinant proteins. This report describes the construction and immunologic evaluation of a LTX-FIM N recombinant fusion protein.

Materials and methods

Plasmids, bacterial strains, chemicals and enzymes

Plasmids used in this study are described in Table 1. *Escherichia coli* strains, Top10F' (Invitrogen Inc., San Diego, CA) and BL21-Gold (Stratagene, La Jolla, CA) were used for cloning and protein expression, respectively. Restriction enzymes were products of Gibco/BRL (Gaithersburg, MD) and New England Biolabs (Beverly, MA). Taq polymerase was obtained from PANVERA Corporation (Madison WI). DNAse, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), and isopropyl β-D-

thiogalactopyranoside (IPTG) were purchased from Sigma Chemical Company (St. Louis, MO).

Construction of expression plasmids

Polymerase Chain Reaction (PCR) was performed in a Gene Amp PCR system 9600 (Perkin Elmer, Narcross, GA). The *fim N* gene (without putative signal sequence) was amplified from PCR2-FIM by PCR using primers ECOFWD (5'-GGGAATTCACGATCGTGATCACCGGCA -3') and XHOREV (5'-GGCTCGAGGATTATCCTTATCAAGCC -3') with engineered restriction sites (underlined). The product was digested with XhoI and EcoRI, gel purified (Gel Extraction kit, Quiagen, Valencia, CA) and cloned into pGEX-5X-1 that had been pre-digested with XhoI and EcoRI. The resulting plasmid was named pGEX-FIM. A 663 bp fragment of *M. haemolytica* leukotoxin A gene, encoding amino acids 719- 939, was amplified from pGEX-LT FUSION by PCR with primers LTBAMF (5'-GGGGATCCATAACGATATCTTTAAAGG-3') and LTECOR (5'-GGGAATTCCATTGAAGTTGGAGCCAC-3'). Engineered BamHI and EcoRI restriction sites are underlined. The PCR product was cloned into BamHI and EcoRI sites of pGEX-5X-1 to form plasmid pGEX-LTBE. To construct the plasmid pGEX-LTFIM, the EcoRI and XhoI digested *fimN* fragment from pGEX-FIM was ligated to the EcoRI and XhoI sites of pGEX-LTBE. The GST gene was amplified from pGEX-5X-1 vector using primers 5'-TATTGGAAAATTAAGGGCCTCGAGCAA -3'and 5'-TATATACTCGAGGGATTTCAAGTACTT -3', and cloned into the XhoI site of pGEX-LT FUSION downstream to leukotoxin fragment. This plasmid was called pGEX-LTGST. The 5' and 3' ends of all constructs were sequenced at the University of

Tennessee DNA sequencing facility on an Applied Biosystems 373 automated sequencer. All recombinant plasmids were introduced into *E.coli* BL21-Gold by transformation (16).

Expression and purification of the recombinant proteins

E. coli BL21-Gold cells harboring the expression plasmids were inoculated into one liter of LB broth containing ampicillin (100 μg/ml) and incubated at 37^{o}C, with rotary shaking (220 rpm), to an optical density (A_{600}) of 0.6-0.8. Plasmid gene expression was induced with IPTG (final concentration, 0.2 mM) and after two hours of further incubation, bacteria were harvested by centrifugation at 5000 x g for 10 minutes. The pellet was used immediately for protein purification or stored at -80^{o}C until further use. GST-LTX was initially purified under non-denaturing conditions according to the manufacturer's (GST Gene Fusion System, Pharmacia, Piscataway, NJ) protocol. Subsequent purification of this protein and all other recombinant proteins was performed by a modification of the procedure reported by Frangioni and Neil (9). Briefly, cells were suspended in 100ml of STE buffer (10 mM Tris-HCl, pH 8.0; 150 mM NaCl; 1 mM EDTA) with 100 μg /ml of lysozyme. Dithiotreitol (DTT) was added to a final concentration of 5 mM, and the cell suspension was incubated on ice for 30 minutes. Sarkosyl^{TM} (N-lauroylsarcosine) was added to a final concentration of 1.5%, and the suspension was sonicated for one minute to reduce the viscosity. DNAase (1200U) and Triton X-100 (final concentration 1%) were added, and the mixture was incubated on ice with intermittent shaking for one hour. After centrifugation (14,000 x g for 20 minutes), the supernatant was mixed with a one ml slurry of 50% GST sepharose (Pharmacia, Piscataway, NJ) and incubated at room temperature with gentle shaking for 4 h. The GST sepharose was washed four times with PBS (phosphate buffered saline), and bound

proteins were eluted with 6 M guanidium chloride in PBS. The eluted fraction was dialyzed overnight in PBS. Protein concentrations were determined using the Bicinchoninic Acid Assay (Sigma Chemical Company, St Louis, MO). Samples were adjusted to contain 1 mg protein /ml in PBS and stored at -80^{o}C until further use. The preparations were examined by SDS-PAGE and immunoblotting with leukotoxin and fimbriae specific antibodies.

Animals and Immunizations

Four-week old New Zealand White rabbits and 4-6 week old female BALB/c mice (Harlan Laboratories, Indianapolis, IN) were maintained by routine procedures in an AALAC accredited laboratory animal facility. Rabbits were inoculated with 500 µg of recombinant protein (1mg/ml), GST-FIM, GST-LTX or GST-LTXFIM, emulsified in an equal volume of Freund's incomplete adjuvant (IFA). Intramuscular injections were given to each rabbit on days 0, 14, 28, and 42, respectively. Blood was collected from rabbits on days 0 and 51.

Groups of 5-7 mice were immunized with recombinant proteins by intraperitoneal injection. Each animal received three 100 µl doses containing 50 µg of antigen. Antigens used for the first injections were given with an equal volume of CFA, IFA or PBS. Booster injections given on day 14, were in IFA or PBS, and final injections on day 35 were given in PBS. A control group received CFA + PBS as the first injection, followed by IFA+PBS as second injection and PBS as third injection. Four groups of 5-7 mice were inoculated with 25µg, 50µg, 75µg, or 100µg of GST-LTXFIM in IFA. Booster injections were given as described above. All mice were bled from the retro-orbital sinus

nine days after the final immunization. After serum separation, all samples were stored at -80^{o}C.

Evaluation of antibody response

A capture ELISA using mouse monoclonal antibody MAb Ltx35 (10) was performed to determine anti-leukotoxin antibody titers in rabbits. In brief, 96-well plates (Immulon 2HB, Dynex Technologies Inc, Chantilly, VA) were coated with MAb Ltx35 in PBS (10µg/ml), washed four times with PBS-0.05% Tween 20 (PBS-T) and blocked for 30 minutes at 37^{o}C in PBS-T. Native leukotoxin was prepared from culture supernatant of *M. haemolytica* as previously described (24), and was added to the wells at a previously determined optimum dilution of 1:100 and incubated for one hour at 37^{o}C. After washing four times with PBS-T, plates were incubated for 2 h at 37^{o}C with two-fold serial dilutions of serum from immunized animals. All serum dilutions were made in PBS-T. Plates were washed four times in PBS-T, incubated with secondary antibody (peroxidase conjugated goat anti-Rabbit IgG) for 1 h and developed by adding 100 µl of substrate solution containing 2,2'-azino di-ethylbenzothiazoline-6-sulfonic acid (ABTS, Sigma Chemical Company, St Louis, MO) and hydrogen peroxide. The A_{405} was measured in an EIA reader (BIO-TEK Instruments, Winooski, VT). The titers were determined as the reciprocal of the highest dilution of serum that gave an absorbance reading three times the conjugate control. For measuring antibodies in mice, leukotoxin was captured with ammonium sulfate precipitated rabbit polyclonal anti-leukotoxin serum (1/2000 dilution) and peroxidase conjugated rabbit anti-mouse IgG was used as the secondary antibody. Anti-FIM N antibodies were measured by ELISA as previously described (14).

Leukotoxin Neutralization Assays

Leukotoxin neutralization activity of serum was analyzed by a colorimetric cytotoxicity assay using tetrazolium dye (MTT) and BL-3 cells as previously described (27, 29). Initially, native leukotoxin, prepared from culture supernatant of *M. haemolytica* was titrated using BL-3 cells to determine the toxin potency. One toxin unit was defined as the dilution of leukotoxin that killed 50% of the target cells. Heat inactivated (56^{o}C for 30 minutes) serum samples diluted in RPMI-1640 were mixed with 15 units of leukotoxin (optimal concentration was determined by titration) for 30 minutes at 4^{o}C in triplicate wells of a 96 well plate. BL-3 cells (6.25 x10^{4} /well) were added to the plates and incubated for 45 minutes at 37^{o}C with 5% CO_2. MTT (10mg/ml in RPMI 1640) was added and incubated for 4 hours. The optical density (A_{562}) was measured after lysing the cells with acid alcohol (0.4N HCl in isopropanol). The percentage of leukotoxin neutralization in each sample was calculated as described by Vega et al. (30) by using the formula:

{1-[(OD of the serum treated cells - OD of leukotoxin plus serum treated cells) / (OD of untreated cell control - OD of leukotoxin treated cells)]} x100. A cut-off value of 50% neutralization was considered positive for end point determination. Neutralization titers were determined for rabbit sera using two-fold serial dilutions. All mouse sera were analyzed at single dilutions of 1:60, 1:200 and 1:600. Like dilutions from each individual mouse were tested on a single day with same batch of cells and leukotoxin.

Statistical analyses

Statistical analyses were done using Statistical Analysis Software (SAS version 8) by the General Linear Model (GLM) procedure. The significance of differences between

groups for ELISA titers and leukotoxin neutralization activity was determined by Student's *t*-tests. The data are expressed as mean +/- standard error of mean. P values < 0.05 were considered significant.

Results

Plasmid construction, recombinant protein expression and purification

The plasmids, pGEX-LTBE, pGEX-LTFIM, pGEX-FIM and pGEX-LTGST, were constructed in this study (Table 1, Appendix). Each plasmid encoded the GST gene downstream of a *tac* promoter and also had either a gene fragment (nucleotides 2673-3336) encoding the carboxy-terminal portion of leukotoxin A, the 534 bp fragment (nucleotides 90-624) of the *fim N* gene, or both. Plasmid, pGEX-LTGST, had a second copy of a truncated GST gene cloned downstream from the leukotoxin fragment. All the genes were inserted in the correct reading frame as determined by DNA sequence analysis.

Each of the plasmids expressed recombinant protein in *E. coli* BL21-Gold after IPTG induction as determined by SDS PAGE and Western blots (Figure 1, Appendix). The stained polyacrylamide gels of whole-cell lysates showed prominent bands corresponding to the predicted molecular weights of the expected recombinant proteins (48.7 kD for GST-FIM, 53.5 kD for GST-LTX, 73.2 kD for GST-LTXFIM and 74.5 kD for GST-LTXGST). Recombinant proteins, GST-LTX, GST-LTXFIM and GST-LTXGST, were recognized in Western blots by leukotoxin-neutralizing monoclonal antibodies MAb Ltx35, MAb Ltx4 and MAb Ltx2 (10). Monoclonal antibodies, BPA10, BPA5, and BPH2, specific for serotypes 2 fimbrial monomers of *B. pertussis* (kindly provided by M Brennan, FDA), reacted in Western blots with GST-FIM and GST-LTXFIM

The majority of GST-LTXFIM and GST-FIM proteins were isolated from insoluble cell lysate fractions and required purification under denaturing condition. GST-LTX was recovered from soluble cell lysate fractions and was initially purified in non-denaturing conditions. However, all protein preparations used for immunization were purified under denaturing conditions by the same procedure. Upon dialysis, GST-FIM and GST-LTXFIM formed precipitates; whereas, GST-LTX did not. Purified recombinant fusion proteins migrated to their predicted molecular weights in SDS-PAGE gels (Fig 2). The SDS-PAGE banding pattern of GST-LTXFIM was unchanged, showing no signs of degradation after storage at -80^{o}C for up to one year. On Western blots, the stored GST-LTXFIM retained its reactivity to MAb Ltx35; whereas, GST-LTX did not (Figure 3). GST-LTX produced varying patterns of low molecular weight banding, suggestive of protein degradation, in different preparations

Antibody responses in Rabbits

Groups of two or three rabbits were immunized with chimeric proteins, GST-LTXFIM, GST-LTX, and GST-FIM. Native leukotoxin and HTFIM were used as ELISA antigens. HTFIM was used instead of the homologous antigen in ELISA to avoid reactivity with anti-GST antibodies. Pre-immune sera had no detectable anti-leukotoxin or anti- HTFIM antibodies. Rabbits (n=3) immunized with GST-LTXFIM had higher average ELISA titers (70,300 +/- 17, 100) against native leukotoxin than rabbits (n=3) immunized with GST-LTX (38,900 +/- 500). However, anti-HTFIM titers were lower in rabbits immunized with GST-LTXFIM (4200 +/- 1000) than those of rabbits (n=2) immunized with GST-FIM alone (23, 000 +/-1400). Rabbits immunized with GST-FIM did not produce detectable antibodies reactive with native leukotoxin and rabbits

immunized with GST-LTX did not produce detectable HTFIM reactive antibodies. Leukotoxin neutralization titers in the BL-3 cytotoxicity assay were higher in rabbits immunized with GST-LTXFIM (340+/-130) compared to those immunized with GST-LTX (120 +/-15). The differences observed in ELISA and leukotoxin neutralization titers were not statistically analyzed due to the low sample size. Rather, the rabbit study provided preliminary data for a mouse study performed with larger groups of animals.

Antibody response in mice

The immunogenicity of LTX fusion proteins and adjuvant combinations were further evaluated in mice. The anti-leukotoxin IgG titers were determined for all individual mice (Figure 4A). All groups immunized with GST-LTXFIM produced significantly higher anti-leukotoxin antibody responses compared to groups similarly immunized with GST-LTX ($p< 0.01$). The anti-leukotoxin ELISA titers of control mice were < 20. The group of mice immunized with GST-LTXFIM in CFA had the highest anti-leukotoxin ELISA titers and was significantly different from all other groups ($P<0.01$). The anti-leukotoxin ELISA titers of mice immunized with GST-LTXFIM in IFA was not significantly different from those similarly immunized with same antigen that had been stored at -80^{o}C for six months ($p>0.05$). However, anti-leukotoxin ELISA titers of these two groups were significantly higher than GST-LTX in CFA ($p<0.05$). Anti-leukotoxin ELISA titers were significantly lower in groups immunized with GST-LTXGST in IFA than groups immunized with GST-LTXFIM either in IFA ($p< 0.01$) or CFA ($p<0.001$). The mice immunized with GST-LTX without adjuvant and stored GST-LTX had the lowest anti-leukotoxin ELISA titers.

The percentage of leukotoxin activity neutralized by each individual serum sample at a screening serum dilution of 1:60, 1:200 and 1:600 was determined. The mean leukotoxin neutralization activity of serum from individual groups of mice and the number of animals which had a neutralization activity greater than 50% are shown in Figure 4B. All the groups immunized with GST-LTXFIM induced significantly higher leukotoxin neutralization activity compared to groups similarly immunized with GST-LTX. At the 1:60 dilution, serum from 25 out of 27 mice immunized with GST-LTXFIM had leukotoxin neutralization activity greater than 50%, whereas, only 11out of 27 mice in GST-LTX groups had leukotoxin neutralization activity greater than 50%. Out of 27 mice immunized with GST-LTXFIM, 11 were positive for neutralization activity at a serum dilution of 1:200 and six were positive at a serum dilution of 1:600 (data not shown). By contrast, in mice immunized with GST-LTX, 3 out of 27 mice at 1:200 serum dilution and none at 1:600 had leukotoxin neutralization activity greater than 50% (data not shown). None of the control animals had leukotoxin neutralization activity greater than 50%. Neutralization activity was statistically analyzed for each group at the 1:60 serum dilution. There was no significant difference in neutralization activity of sera from mice immunized with freshly prepared GST-LTXFIM in CFA, IFA or PBS, and mice immunized with GST-LTXFIM that had been stored at -80^{o}C for 6 months ($p>0.05$). Mice immunized with GST-LTX in CFA had the highest anti-leukotoxin antibody response among the GST-LTX groups. There was no significant difference in leukotoxin neutralization activity between groups immunized with GST-LTX in IFA, stored GST-LTX in IFA and the control groups given CFA +PBS ($p>0.05$). The GST-LTX /CFA combination was the only GST-LTX preparation that was able to induce a leukotoxin-

neutralizing antibody response which was comparable to those produced by GST-LTXFIM antigens. Leukotoxin neutralization activities of sera from mice immunized GST-LTXGST in IFA was also significantly lower than those of sera from mice immunized with GST-LTXFIM in CFA ($p<0.01$), IFA ($p< 0.05$) and PBS ($p<0.05$). However, leukotoxin-neutralizing activity of sera from mice immunized with GST-LTXGST was not significantly higher than from those immunized with GST-LTX in CFA ($p>0.8$), IFA ($p>0.05$) or PBS ($p>0.6$). Strong correlation between anti-leukotoxin ELISA titers and leukotoxin neutralization activity was observed in groups immunized with GST-LTXFIM ($r = 0.60818$, $P = 0 .0008$, $n=27$) but not in groups immunized with GST-LTX ($r = 0.35196$, $P = 0.0718$, $n=27$). Groups of mice immunized with 25µg, 50µg, 75µg and 100µg of GST-LTXFIM in IFA, did not have significant differences in anti-leukotoxin IgG titers or leukotoxin neutralization activity (Figure 5).

Discussion

Leukotoxin is a major virulence factor in *M. haemolytica*-induced pneumonia of feedlot cattle (3, 6, 8, 29, 31). There is a significant correlation between high levels of leukotoxin-neutralizing antibodies and a reduction in pneumonic lesion scores in naturally and experimentally infected cattle (19, 20, 21, 25, 26, 27, 28). However, the instability of leukotoxin and the resulting difficulty in its purification have limited its use as an additive component in vaccines (7). This report demonstrates that immunization with a fusion protein consisting of the carboxy-terminal peptide of leukotoxin and FIM N induced high levels of leukotoxin-neutralizing antibodies in laboratory animals. Unlike GST-LTX, the chimeric GST-LTXFIM antigen was stable on storage at -80^{o} C and

induced high levels of leukotoxin-neutralizing antibody even without the use of adjuvants.

The leukotoxin neutralization assay is very sensitive; variations in leukotoxin potency and the sensitivity of target cells can greatly influence the outcome of the assay. It has been reported that the coefficient of variation of this assay, performed on separate days, ranged from 0-36% (30). In this study, the effects of day to day variations were minimized by testing the serum from each individual on a single day using a single vial of leukotoxin and the same batch of BL-3 cells. End points were determined for serial dilutions of rabbit sera. The minimal dilution at which each individual mouse sample could be compared was 1:60 and the assay at this dilution was performed twice on two different days. Leukotoxin neutralization activity was also determined at 1:200 and 1:600 serum dilutions. It was estimated that the leukotoxin neutralization titers of GST-LTXFIM immunized mice were between 60 and 600. These levels were consistent with the titers seen in rabbits and were as high as reported leukotoxin neutralization titers in cattle (26, 27, 30). In GST-LTX groups, more than half of the individual serum samples may have titers less than 60.

Mice immunized with antigens containing GST-LTXFIM consistently produced higher levels of anti-leukotoxin antibodies than mice similarly immunized with LTX antigens lacking FIM N. Inclusion of CFA in the GST-LTXFIM antigen significantly increased the leukotoxin antibody response as measured by ELISA with native leukotoxin as antigen, but did not significantly affect leukotoxin-neutralizing antibody response in the BL-3 cell cytotoxicity assay. Doses of GST-LTXFIM antigen ranging from 25µg - 100µg produced equivalent leukotoxin-neutralizing antibody responses.

These observations suggest that stringent immunization protocols (eg. high dose, and adjuvants) may not be required to elicit maximum leukotoxin-neutralizing antibody responses when the chimeric GST-LTXFIM protein is used. It was previously observed that a recombinant fusion of leukotoxin and interleukin-2 produced an enhanced lymphocyte proliferation response but did not enhance anti-leukotoxin antibody production (13).

It was observed that the LTX-FIM immunogen, regardless of the adjuvant used, consistently induced higher levels of leukotoxin- neutralizing activity in all groups and that serum samples from mice immunized with LTX-FIM immunogen neutralized at least 50% of the cytotoxicity of 15 units of leukotoxin at dilutions where other LTX immunogens lacking FIM N lost this capacity. The active toxin concentration used was intentionally chosen to be several fold higher than normally used (15 vs 1-3 units) to eliminate variability in cytotoxicity. Possibly more significant, was a much closer statistical correlation between anti-leukotoxin ELISA titers and leukotoxin neutralizing activity in sera from LTX-FIM immunized mice than in the sera from mice immunized with LTX lacking FIM. The strong suggestion is that the FIM fusion enhances production of potentially protective (neutralizing) antibodies more so than fusions lacking FIM N. A stable subunit fragment of leukotoxin, such as GST-LTXFIM might provide a basis for an ELISA with greater specificity for protective, neutralizing antibodies and would be of great aid to vaccine evaluation.

The mechanism of enhancement of anti-leukotoxin antibody production following fusion of FIM N to the carboxy-terminus of GST-LTX is unknown but may simply reflect protection of exposed LTX epitopes against degradation. In vitro and in vivo

degradation or instability of neutralizing epitopes at the carboxy-terminus of GST-LTX may explain the lower responses to GST-LTX antigen preparations. This hypothesis is supported by the observation that GST-LTX no longer reacted with MAb Ltx35, a leukotoxin-neutralizing monoclonal antibody, after storage at -80^{o}C. The frequent appearance of multiple protein bands in GST-LTX preparations is also suggestive of product deterioration. It is possible that the purification process altered the protein's conformation in a fashion that would hasten proteolytic degradation. However, fresh preparations of GST-LTX consistently reacted with MAb Ltx35. It is likely that degradation occurred at the time of processing or after thawing, since little physical or enzymatic activity would be expected during storage at -80^{o}C. The difference in the number of epitopes in these antigens does not appear to be a significant factor in these observations, since the molar proportion of LTX epitopes was approximately one third in the GST-LTXFIM antigen compared to GST-LTX. Also, the fusion of GST, a peptide of similar size to FIM N, to the carboxy- terminus of GST-LTX, did not effectively enhance the anti-leukotoxin antibody response. Therefore, it is probable that secondary or tertiary protein structure interactions in GST-LTXFIM provided greater LTX epitope exposure. These interactions may have been absent in GST-LTX or GST-LTXGST. FIM N is highly immunogenic; its hydrophobic nature and T-cell epitopes might have aided in enhanced antigen presentation and antibody response. The general insolubility and resistance of fimbrial proteins to chemical and proteolytic degradation (15) might also have predictably contributed to the enhanced immune response.

Vaccines incorporating live *M. haemolytica* organisms or whole toxin may produce febrile reactions and result in a lower generalized immune response (7). A stable

leukotoxin subunit vaccine component would seem to hold great promise for improving bovine health. The immunogenicity of LTX- FIM N recombinant protein in cattle and role of FIM N as an immunomodulatory antigen merit further study.

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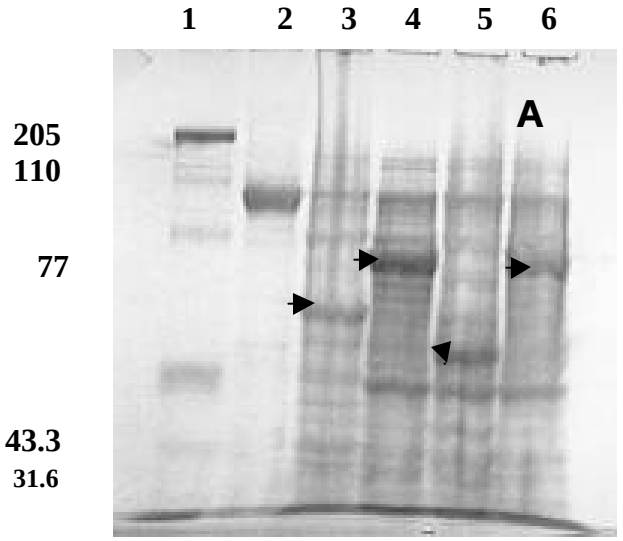
Appendix

Figure 1

SDS-PAGE and Western blot of *E. coli* whole-cell lysates harboring recombinant plasmids.

A. SDS-PAGE- Whole-cell lysates of *E. coli* (BL21-Gold) expressing the recombinant proteins were separated on a 10% polyacrylamide gel and stained with Pierce Gel Code Blue Stain reagent. Pre-stained molecular weight markers in kDa (Bio-Rad) (lane 1), Native leukotoxin (lane 2), GST-LTX (lane 3), GST-LTXFIM (lane 4), GST-FIM (lane 5), GST-LTXGST (lane 6). Arrows indicate prominent bands corresponding to the predicted molecular weight of expected recombinant proteins.

B. Western blot- Proteins were electro-transferred on to a nitrocellulose membrane and probed with anti-leukotoxin monoclonal antibody, MAb Ltx35.


1 2 3 4 5 6
205
110
77
43.3
31.6
A

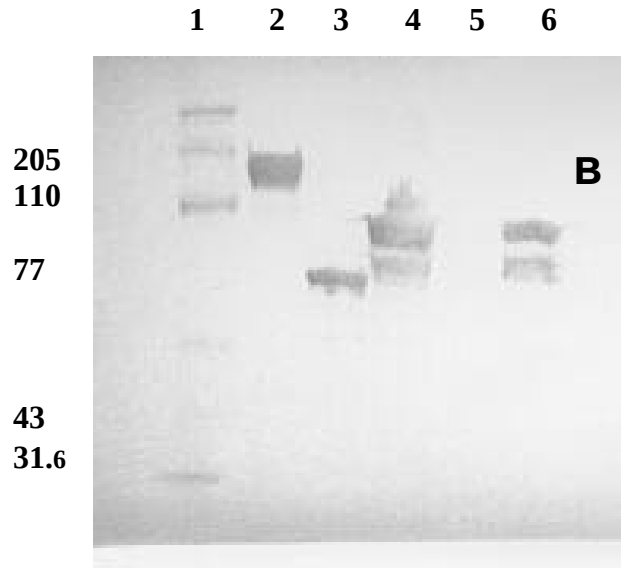

1 2 3 4 5 6
205
110
77
43
31.6
B

Figure 2

SDS-PAGE of recombinant proteins used for immunization.

Recombinant proteins were purified by affinity chromatography. Approximately 15μg of each protein was electrophoresed on a 10% polyacrylamide gel and stained with Pierce Gel Code Blue Stain reagent. Pre-stained molecular weight markers in kDa (Bio-Rad) (lane 1), GST-LTXFIM (lane 2), GST-LTX (lane 3), GST-FIM (lane 4).

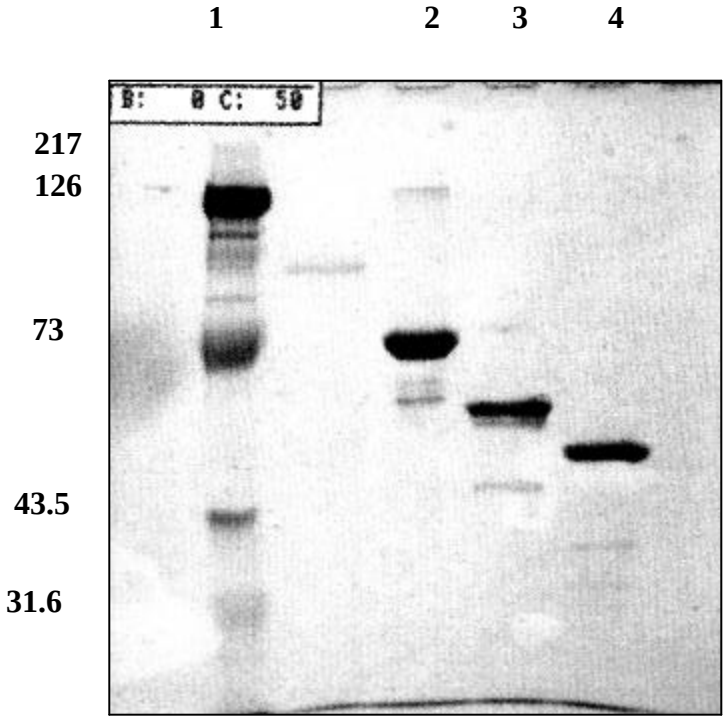
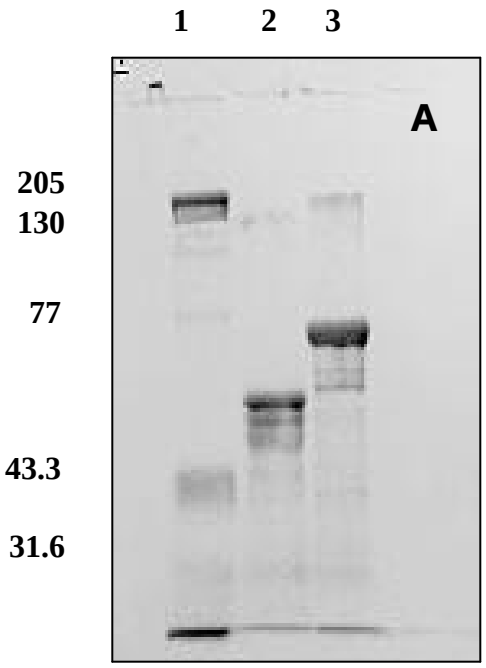

1
2
3
4
217
126
73
43.5
31.6
B: 0 C: 50

Figure 3

SDS-PAGE and Western blot of recombinant GST-LTX and GST-LTXFIM stored at -80^{o}C for 1 year.

A. SDS-PAGE -Approximately 15μg of each protein was electrophoresed on a 10% polyacrylamide gel and stained with Pierce Gel Code Blue Stain reagent. Pre-stained molecular weight markers in kDa (Bio-Rad) (lane 1) GST-LTX (lane 2) GST-LTXFIM (lane 3)

B. Western blot. The proteins were electro-transferred to nitrocellulose and probed with anti-leukotoxin antibody MAb Ltx35.


1
2
3
A
205
130
77
43.3
31.6

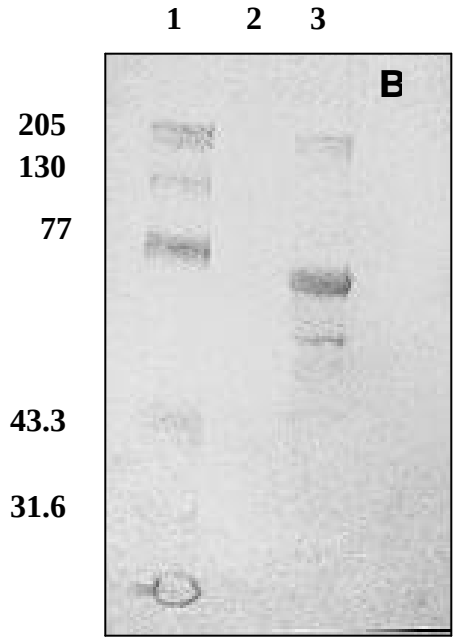

1
2
3
B
205
130
77
43.3
31.6

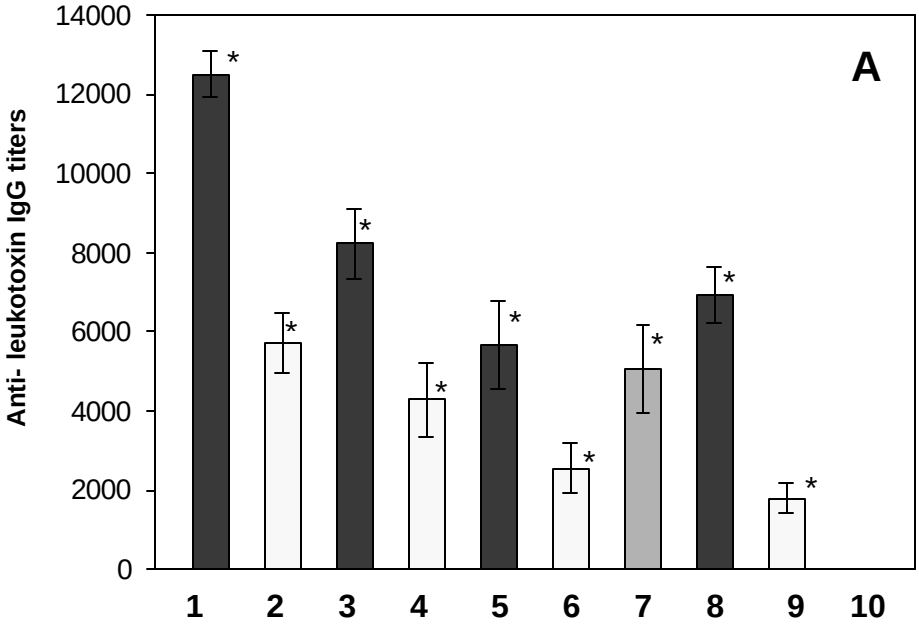
Figure 4

Antibody response of individual groups of mice immunized with recombinant proteins Mice were immunized with three successive injections of antigens on days1, 14 and 35 as described in text. (1). GST-LTXFIM in CFA, (2). GST-LTX in CFA, (3). GST-LTXFIM in IFA, (4). GST-LTX in IFA, (5). GST-LTXFIM in PBS, (6). GST-LTX in PBS, (7). GST-LTXGST in IFA, (8). GST-LTXFIM in IFA (stored), (9). GST-LTX in IFA (stored), (10). Control (CFA+PBS). The composition of first immunization was as given above. The second dose of antigen had similar adjuvant composition but CFA was substituted with IFA, and antigen alone in PBS was used for third dose in each group. Dark bars represent LTX with

FIM N, while lighter bars represent LTX immunogens without FIM N. The significance of differences in responses from immunized groups was analyzed by Student's *t*-test. A *p*-value <0.05 was considered significant. Presence of asterisk indicates the values are significantly different from control animals. (* = p< 0.001., ** = p< 0.01., *** = p<0.05).

A. Mean anti-leukotoxin IgG ELISA titers +/- SEM is reported**.**

B. Leukotoxin neutralization activity is expressed as the percentage of neutralization of 15 units of leukotoxin by a 1:60 dilution of serum measured on two different days. The mean +/-SEM is reported. The number shown above each bar is the number of mice that had greater than 50% leukotoxin neutralization activity out of the total number tested.


A
Anti- leukotoxin IgG titers
14000
12000
10000
8000
6000
4000
2000
0
1
2
3
4
5
6
7
8
9
10

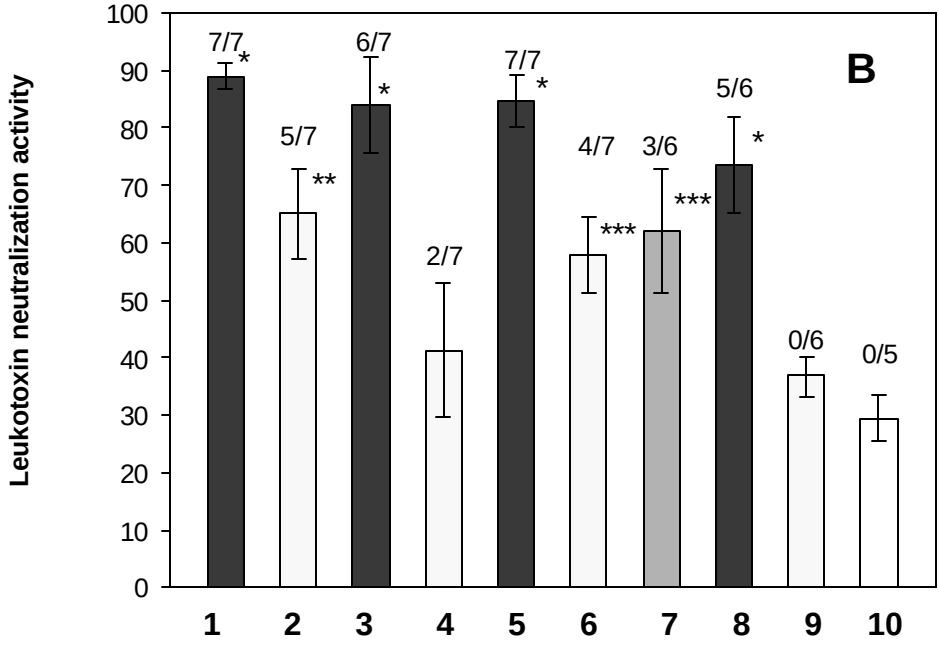
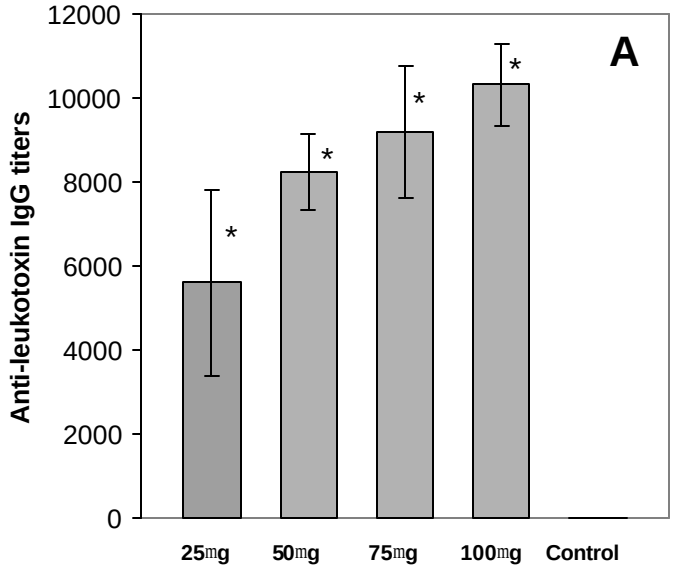

B
Leukotoxin neutralization activity
100
90
80
70
60
50
40
30
20
10
0
7/7
5/7
6/7
2/7
7/7
4/7
3/6
5/6
0/6
0/5
1
2
3
4
5
6
7
8
9
10

Figure 5

Effect of GST-LTXFIM antigen dose on anti-leukotoxin antibody response in mice. Mice were inoculated I.P. on days 0, 14, and 35 with GST-LTXFIM. The first two doses were in IFA and last in PBS. The significance of differences in responses from immunized groups was analyzed by Student's *t*-test. A *p*-value <0.05 was considered significant. Presence of asterisk indicates the values are significantly different from control animals. (* = p< 0.001., ** = p< 0.01., *** = p<0.05).

A. Mean anti-leukotoxin IgG titers +/- SEM.

B. Leukotoxin neutralization activity is expressed as the percentage of neutralization of 15 units of leukotoxin by a 1:60 dilution of serum. The mean +/- SEM is reported.


A
Anti-leukotoxin IgG titers
12000
10000
8000
6000
4000
2000
0
*
*
*
*
25mg
50mg
75mg
100mg
Control

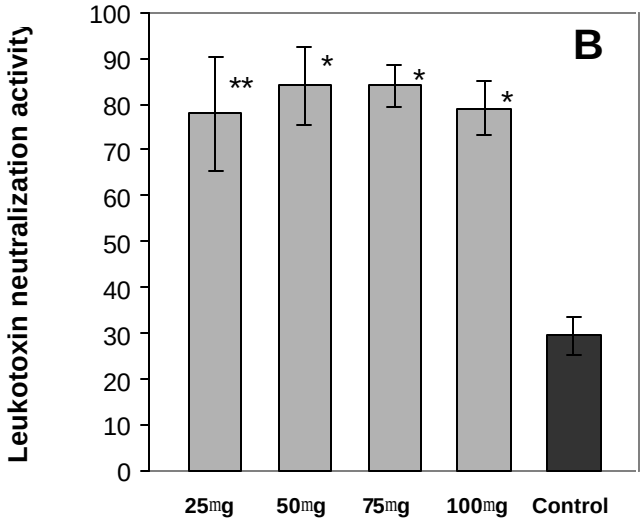

B
Leukotoxin neutralization activity
100
90
80
70
60
50
40
30
20
10
0
**
*
*
*
25mg
50mg
75mg
100mg
Control

TABLE 1. Descriptions of plasmids used in this study

Plasmid	Description
PCR 2.1	TA Cloning vector (Invitrogen) Ampicillin resistance
pGEX-5X-1	GST gene fusion expression vector (Pharmacia).
PCR2-FIM	*fim N* gene with regulatory and coding sequence in EcoRI and HindIII sites of PCR 2.1 (14)
pGEX-FIM	*fim N* gene coding sequence without the putative signal sequence in EcoRI and XhoI sites of pGEX-5X-1 (this study) (GST-FIM)[A]
pGEX-LT FUSION	Leukotoxin A gene nucleotides encoding carboxy-terminal amino acids 713-939 in EcoRI and XhoI sites of pGEX-4T-1 (16)
pGEX-LTBE	Leukotoxin A gene nucleotides encoding carboxy-terminal aminoacids 719-939 from pGEX LT FUSION in BamH I and EcoRI sites of pGEX- 5X-1 (this study) (GST-LTX)[A]
pGEX-LTFIM	Leukotoxin A gene fragment in BamHI and EcoRI sites and *fim N* gene in EcoRI and XhoI sites of pGEX-5x-1.(this study) (GST-LTXFIM)[A]
pGEX-LTGST	GST gene fragment cloned downstream to LTX in XhoI site of pGEX-LT FUSION. (This study) (GST-LTXGST)[A]
pPROEXTM-HT-FIM	*fim N* gene without the signal sequence in pPROEX^{TM} HT (Gibco/BRL) (HTFIM)[A] (14)

Letters in the parenthesis with superscript A indicate the name of the protein expressed by the plasmids.

PART 5
General Summary

Summary

The first part of this research work was focused on identifying constitutive promoters suitable for heterologous antigen expression in *B. bronchiseptica*. This investigation resulted in isolation and characterization of a promoter region related to heat shock gene family. The promoter was active in vitro and was able to induce high-level expression of GFP. The *taco* promoter, which is an *E. coli* derived promoter, was also active in*B. bronchiseptica.* Low-level activity of *Fim N* promoter was detected in Bvg+ strains of *B. bronchiseptica* indicating that this promoter was Bvg-regulated. The low level activity of this promoter was enhanced by growth in a minimal salt media and may be further enhanced by manipulating the C-stretch region of the promoter. The promoter we identified belonged to the family of heat shock genes. These genes are usually induced under stress conditions. However this study did not identify any induction condition for this promoter region.

The plasmid used to express heterologous antigen in *B. bronchiseptica* was originally isolated from *B. bronchiseptica*. However, the reconstructed plasmid was not stable in vivo or in vitro under non-selective condition. The foreign DNA engineered into the plasmid and heterologous antigen expression may be the factors contributing to plasmid instability. Identification of moderately active promoters, construction of more stable plasmids, and chromosomal integration of the foreign gene can be pursued

In the second part of the study, we expressed a non-toxic protective *P. multocida* toxin fragment (PMTCE) in *B. bronchiseptica* under the control of the promoter we identified. However, the level of expression of this protein was very low. Intranasal inoculation with *B. bronchiseptica* expressing PMTCE did not induce a PMT-specific

antibody response. Mucosal and systemic antibody response was generated against *B. bronchiseptica.* However, four intranasal inoculations at 14 day intervals with *B. bronchiseptica* expressing GFP induced a mucosal and systemic immune response against GFP. Factors like differences in the antigenicity, low level expression and plasmid instability might have contributed to the difference in immune response to these two heterologous antigens expressed by *B. bronchiseptica*. Improvement in plasmid stability and antigen expression is required to improve the system.

The third part of this study evaluated the immune response to a chimeric protein created by fusing the FIM N protein of *B. bronchiseptica* and an unstable protective fragment of M*annheimia haemolytica* leukotoxin. Immunization of mice with this chimeric protein elicited stronger anti-leukotoxin antibodies than similar immunization with protein, which lacks the FIM N. Also, the molecular chimerization improved the stability of the leukotoxin fragment. This protein may be an efficient vaccine candidate to prevent *M. haemolytica*-induced shipping fever in feedlot cattle. Adhesins are excellent targets for vaccine development and they have been explored as potential vehicles for delivery of heterologous antigens. FIM N protein may be an effective carrier for heterologous antigen delivery in *B. bronchiseptica*. However, the immunomodulatory property of FIM N needs further investigation.

Atrophic rhinitis is a disease of significant economic importance to US agriculture. The involvement of *B. bronchiseptica* and *P. multocida* in this disease condition is well documented. This study provides basis for the development of an improved, single component vaccine against atrophic rhinitis. However, optimization of antigen expression to induce an effective immune response is required. Further genetic modification to

inactivate the important virulence factors of *B. bronchiseptica* has to be pursued. Since *B. bronchiseptica* can colonize the respiratory tract of wide variety of host species, potential for applying this model to other species and diseases is great. Utilizing the benign colonization of *B. bronchiseptica* to deliver a protective antigen will be a potent way to induce a mucosal immune response at the respiratory tract mucosa.

VITA

Sreekumari Rajeev was born in 1963 in Kerala, India. She completed her high school education at St Mary's High School, and her Pre Degree in St Michael's College, Cherthala, Kerala. She graduated from the College of Veterinary Medicine, Kerala in 1986 obtaining a BVSc &AH degree. She joined the Animal Husbandry Department of the Government of Kerala and worked for 10 years. In 1997 she joined in a graduate program in Comparative and Experimental Medicine at the University of Tennessee. She completed board certification in Veterinary Microbiology (Bacteriology and Virology) in 2000 and became a Diplomate of American College of Veterinary Microbiologists. She received her doctoral degree in May 2002. Sreekumari Rajeev plans to pursue a career as Veterinary Bacteriologist at the Animal Disease Diagnostic Laboratory of the Ohio State Department of Agriculture.