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MOLECULAR GENETIC ANALYSIS OF VIRULENCE IN *MANNHEIMIA (PASTEURELLA) HAEMOLYTICA*

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1. ABSTRACT

Mannheimia haemolytica (previously known as *Pasteurella haemolytica*) is a weakly hemolytic, gram-negative coccobacillus that is an opportunistic pathogen of cattle, sheep and other ruminants. In stressed, immunocompromised animals, the organism causes a fibrinous, necrotic pneumonia, commonly called “shipping fever”. In the United States, economic losses due to shipping fever pneumonia surpass the combined cost of all other diseases of cattle. *M. haemolytica*, which is a member of the family Pasteurelleaceae, includes twelve serotypes (A1, A2, A5-A9, A12-14, A16 and A17) based on capsular antigen typing. Worldwide, serotypes A1 and A2 predominate, though all serotypes can cause disease. Serotype A1 causes pasteurellosis in cattle and has been the subject extensive study, while serotype A2 causes disease in sheep and is less-well characterized. Potential virulence factors of *M. haemolytica* have been identified and characterized by gene cloning and DNA sequence analysis. These factors include a ruminant-specific leukotoxin, an anti-phagocytic capsule, lipopolysaccharide, iron-regulated outer membrane proteins, lipoproteins, a sialoglycoprotease, a neuraminidase and two potential immunoglobulin proteases. Unlike the well-characterized leukotoxin, little is known about the expression of these other virulence factors. Attempts to dissect the mechanisms of *M. haemolytica* pathogenesis have been hindered by the lack of a robust genetic system for mutation of the organism, though new tools for genetic manipulation of *M. haemolytica* have been developed. Expression plasmids and operon fusion plasmids have been created and a series of antibiotic resistance cassettes useful for site-specific recombination have been constructed. It is anticipated that use of these tools for gene expression and mutagenesis, in combination with the soon to be released genomic sequence of a serotype A1 organism, will aid in understanding the molecular mechanisms of pathogenesis of *M. haemolytica*

and will help to drive development of new vaccines to prevent shipping fever.

2. INTRODUCTION

Mannheimia (Pasteurella) haemolytica is a weakly hemolytic, gram-negative coccobacillus that is an opportunistic pathogen of cattle, sheep and other ruminants. The most common disease presentation is an acute fibrinonecrotizing pleuropneumonia, but the organism can also cause septicemia. Pneumonic pasteurellosis, or "shipping fever", is due in large part to *M. haemolytica*. The disease kills at least one percent of North American feedlot cattle and is responsible for morbidity, decreased weight gain and loss of performance in at least an additional ten percent of these animals (1). Consequently, the disease costs the United States cattle industry at least 640 million dollars annually (2). *M. haemolytica* have been isolated worldwide, but the prevalence of disease strongly correlates with Western animal management practices that include overcrowding and transport (3, 4). In the United Kingdom, pneumonic pasteurellosis is more commonly associated with sheep (5). The organism can also cause pneumonic and septic disease in other ruminants, including goats (6), bison (7), and bighorn sheep (8). *M. haemolytica* has occasionally been linked to human infections (9, 10), but these reports are hard to reconcile within the scope of our current understanding of the organism, its species specificity and mechanisms of virulence. Occasionally "*Pasteurella haemolytica*" has made its way into medical textbooks (11) where it is confused with *Pasteurella multocida*, which can be transmitted to humans.

M. haemolytica's virulence depends, in part, on host immune status. The upper respiratory tracts of calves and sheep are colonized with *M. haemolytica* at normally low, but detectable, frequencies (12). Low anti-*Mannheimia* antibody titers, co-infection with viruses and other bacteria, and stress, caused by shipping and marketing, are all predisposing conditions (13). If innate immunity is compromised, then the organism multiplies rapidly in the nasopharynx. Bacteria can then be aspirated into the lung or can spread, via droplets, from animal to animal (14). Once in the lung, *M. haemolytica* elaborates toxic products that result in a purulent, fibrinonecrotic pleuropneumonia (15). Affected animals exhibit dyspnea, depression, cough and nasal discharge (12, 13, 16) and mortality often occurs within 48 hours following the observation of initial symptoms. In severe outbreaks, the case/fatality rate may reach 10% (17). Upon necropsy, the lungs reveal significant consolidation and multiple foci of necrosis (18). Edema and fibrin deposition on the lungs and pleura are also observed. The pathological hallmarks of the disease are the presence of degenerated neutrophils and alveolar macrophages with fibrin and blood in the alveoli, bronchi and bronchioles (19). Inflammatory mediators, such as IL-1-beta, TNF-alpha and leukotrienes, released by the neutrophils, are thought to be involved in the tissue damage that ensues. Indeed, neutrophil function is critical to the disease process since inflammation and much of the tissue destruction is reduced in neutrophil-depleted animals (20, 21).

3. CLASSIFICATION, PHYLOGENY AND EVOLUTION

The causative agent of shipping fever was first called *Bacterium bipolare multocidum* by Theodore Kitt in 1885 (22). In 1896, Flugge renamed it *Bacillus boviseptica*, which was further subdivided into separate groups that cause bovine fibrinous pneumonia (*Pasteurella boviseptica)* or hemorrhagic septicemia (now known as *Pasteurella multocida*) (23). In 1932, Newson and Cross proposed the name *Pasteurella haemolytica* for the bacterium that caused pneumonia in calves (24). *P. haemolytica* was further sub-divided into two biotypes, A and T, based on the ability to ferment arabinose or trehalose, respectively (25). In 1962, capsular serotyping was established (26), and the number of serogroups eventually reached seventeen (27). By 1985, *P. haemolytica* was excluded from the genus *Pasteurella*, based on DNA-DNA hybridization data (28). It also became clear that the trehalose fermentors represented a separate species, now known as *Pasteurella trehalosi* (29, 30). Using a combination of DNA-DNA hybridization, 16S rRNA sequencing, and biochemical typing, twelve of the biotype A organisms (serotypes A1, A2, A5-9, A12-14, 16 and A17) have been reclassified as *Mannheimia haemolytica* (27). Ironically, none of these strains ferment L-arabinose (27, 31). Multilocus enzyme electrophoresis (MLEE) also agrees with the new classification (32). This removes *M. haemolytica* from *Pasteurella senso stricto* and places the *Mannheimia* genus on a branch of a phylogenetic tree within the gamma subdivision of the Proteobacteria, whose closest relatives are *Pasteurella trehalosi, Actinobacillus capsulatus,* and *Haemophilus parainfluenzae* (figure 1).

Worldwide, *M. haemolytica* serotype A1 and serotype A2 are the most prevalent serotypes found. Serotype A1 is recognized as the most common cause of pasteurellosis in cattle, but other serotypes, such as A6 and A9 are occasionally associated with bovine disease (33). Serotype A2 is the most common cause of disease in sheep, but it is also a predominant member of the upper respiratory tract flora of healthy calves. Though *M. haemolytica* serotypes A1 and A2 can colonize the upper respiratory tracts of both cattle and sheep, they are generally, but not exclusively, species-specific in their ability to cause lower respiratory disease. Healthy cattle commonly carry *M. haemolytica* A2 in the upper respiratory tract (34), but following stress or viral infection, serotype A1 rapidly replaces A2 as the principal serotype (12). This switch is likely due to horizontal transfer from other sick animals that are actively shedding A1 organisms in nasal secretions, though capsular switching has also been suggested (35). Such serotype switching has not been reported in sheep, perhaps because all serotypes can cause disease. Nevertheless, serotypes A1, A2, A6, A7 and A9 predominate in the lamb (5).

The twelve *M. haemolytica* serotypes differ in genome structure, surface antigens and other traits, as summarized in table 1. Dr. Robert L. Davies, at the University of Glasgow, has been instrumental in revealing that *M. haemolytica* isolates are clonal and represent distinct phylogenetic lineages (36). Ribosomal RNA sequencing has shown that serotypes A1, A5-9, A12, A13,

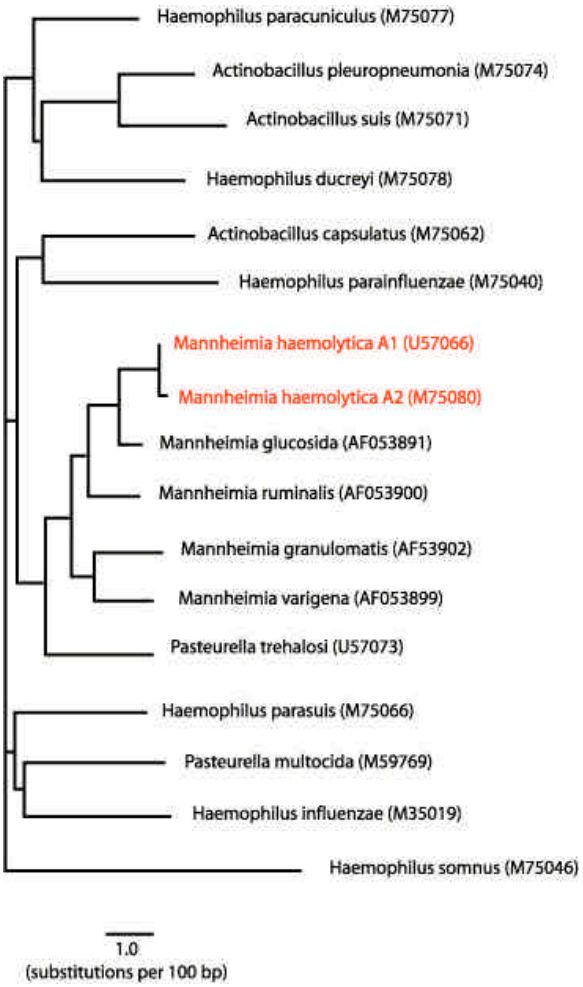

Figure 1. Phylogenetic tree of members of the *Pasteurelleaceae* generated from 16S rRNA sequences. The tree was created using the GCG GrowTree program using the neighbor-joining method of Jukes and Cantor (242). GenBank Accession numbers for the sequences used are in parentheses.

A14 and A16 have identical rRNA sequences, while the A2 sequence differs at two positions (32). Genetic analysis of serotype A17 has not been reported. MLEE analysis of 194 strains isolated from cattle and sheep from Germany, the United Kingdom and the United States, revealed that *M. haemolytica* strains could be placed in one of three distinct lineages and that these groups were associated with particular electrophoretic types (ETs), host species, capsule, lipopolysaccharide (LPS) and outer membrane protein (OMP) types (36). By MLEE analysis, the first group includes serotypes A1, A5, A6, A8, A9, A12, A14 and A16. Serotypes A7, A13, and some A2s form a second while the third group contains only serotype A2 isolates (36). More recent MLEE analysis indicates that all serotype A2 isolates belong in group 3, while serotypes A14 and A16 are likely to belong to group 2 (R. Davies, personal communication).

Analysis of OMP and LPS profiles of *M. haemolytica* strains supports the original MLEE groupings. First, three different OMP groups have been described (37). These are grouped as follows: A1, A5, A6, A8, A12; A2, A14, A16; and A7, A13. LPS profiles predict a similar, though non-identical grouping: group 1 (A1, A5, A6, A12); group 2 (A2, A8, A14, A16); and group 3 (A7 and A13) (37). In addition to these groupings, MLEE and OMP profiles show that genetically distinct populations of bovine- and ovine-adapted strains exist within each group (36, 37). For example, serotypes A1, A5, A6, A8, A9, A12, A14 and A16 are, in general, homogeneous and primarily of ovine origin. Nevertheless, bovine A1 and A6 isolates within this group can be identified because they express different OMPs than do ovine strains. These bovine-adapted clones are likely to have evolved from ovine ancestors by host-switching and adaptation involving horizontal gene transfer. Serotype A2 strains are very different from those in group 2, yet these too can be differentiated with regard to host tropism by OMP analysis. Finally, A7 and A13 strains are exclusively associated with sheep, though they express a heterogeneous group of OMPs (32). An additional observation supporting the clonal grouping of *M. haemolytica* is the possibility that the species contains a limited number of general capsular types. Despite the fact that typing sera are generally not cross-reactive (38), three structural capsule groups can be described: one that is serotype A1-like; the serotype A2 capsule; and a third that is serotype A7-like (see table 1).

The genetic bases for the clonal variation among *M. haemolytica* serotypes have not yet been studied. Whole genome analysis of different serotypes promises to reveal the genetic bases for the phenotypic differences observed in strains from different hosts and niches. Genetic polymorphisms between serotypes A1, A5, A7, A8 and A9 have been detected by I-*Ceu*I-based genomic mapping, yet the length (*ca*. 2.6 megabases) and I-*Ceu*I profiles of the genomes are very similar (39). The complete sequence of a pathogenic serotype A1 strain isolated from the pneumonic lung of a calf is being determined at the Baylor College of Medicine Human Genome Sequencing Center where data are available on a public website (www.hgsc.bcm.tmc.edu/microbial). Comparative genome analysis, using draft sequence of serotype A2 and others, is planned.

4. PATHOGENESIS AND VIRULENCE

M. haemolytica produces a number of proteins and polysaccharides that are thought to be important to its virulence. These factors have been identified by immunological and biochemical characterization of *M. haemolytica* gene products, with an emphasis on analysis of surface and secreted molecules. For most, their role in virulence has not been confirmed because of the lack of robust genetics and appropriate small animal models for *M. haemolytica* infection. Following is a brief overview of the presumptive virulence factors of *M. haemolytica*, with a focus on those of serotype A1.

4.1. Antibiotic resistance

Resistance to antimicrobial agents is widespread in *M. haemolytica*. Some of this resistance is a result of wholesale use of antibiotics as a supplement in animal feed, both for prophylaxis and growth promotion. Subtherapeutic

Table 1. Features of *M. haemolytica* serotypes (references in parentheses)

Serotype	Common Hosts	Electrophoretic Types (36)	Lineage(s) (36)	Ribotype (243)	Capsule Composition (36, 97, 98)	LPS Core Type (87)	O-Ag Type (89)	OMP Type (32)
A1	Bovine Ovine	1*, 2, 6, 7, 8	A	HA*, HB, HC, HD	→3)-ManNAc–(beta→4)-ManNAcA-(beta1→	1*, 2, 3	→3)-beta-D-Gal-p-(1→3)-beta-D-GalpNAc-(a→4)-beta-D-Gal-p-(1→	1.1, 1.2
A2	Ovine Bovine	16, 17, 18, 19, 20, 21*, 22	C, A	HA*, HB, HC*, HE, HF, HG	→NAN-(alpha→8)NAN→ (Sialic acid)	1, 3	None	2.1, 2.2*
A5	Ovine	5	A	HA, HI	A1-like?	1	A1-like	1.2
A6	Bovine Ovine	1, 9	A	HA*, HD	A1-like?	1*, 2	Identical to A1	1.1, 1.2
A7	Ovine	4,12*,13,14	A, B	HA, HC*	→3)-beta-GalNAc–(1→3)-alpha-GlcNAc-(1-p→	1, 3, 4*	A1-like	1.2, 2.1, 2.2, 3.1
A8	Ovine	5	A	HA, HD, HJ	A1-like?	1	None	1.2
A9	Ovine	3	A	HA*, HD, HK	A1-like?	1*, 3	Identical to A1	1.2
A12	Ovine	1	A	HA, HM	A1-like?	1	Identical to A1	1.2
A13	Ovine	ND	B	HN	A7-like?	4	A1-like	3.3
A14	Ovine	ND	A	ND	A1-like?	1	A1-like	2.3
A16	Ovine	ND	A	HA	A1-like?	1	A1-like	2.3

*Predominant group (when significant), by class or type. ND, not determined. Gal, galactose. GalNAc, N-acetylgalactosamine. ManNAcA, N-acetylmannosamine. NAN, N-acetylneuramin.

use of antibiotics in the feed and metaphylactic antibiotic therapy (i.e. treatment given before symptoms appear) have caused increased emergence of multi-antibiotic resistant *M. haemolytica* strains (40). Resistance genes have been found associated with small and large plasmids (41-44) and on the chromosome. The most common types of resistance encountered are to beta-lactams, tetracycline, streptomycin, sulfonamides, macrolides and sulfamethazines (40, 45, 46). Several resistance genes have been cloned and their sequences determined. The beta-lactamase encoded by plasmid pAB2 is a member of the ROB-1 family of enzymes, which are most closely related to the TEM beta-lactamases (47). These enzymes are widely distributed among the Pasteurelleaceae and ROB-1 genes have been discovered on small plasmids (*ca*. 4 kilobase) in *Haemophilus influenzae* (48) *Actinobacillus pleuropneumoniae* (49), and *P. multocida* (50). Also common is a *ca*. 4 kilobase plasmid that confers resistance to streptomycin and sulfonamide: the prototype is plasmid pYFC1 (42). Sequence analysis of the region flanking the *sulII-strA* resistance determinant on pYFC1 suggests that the resistance genes may have been associated with a mobile integron-like unit (51).

Tetracycline resistance can be conferred by one of several *tet* alleles, including *tetH* (52) and *tetB* (53) that can be associated with plasmids or the chromosome. The *tetH* allele is the most common allele obtained from *M. haemolytica* and seems to be restricted to the Pasteurelleaceae. Chloramphenicol resistance has been identified on a plasmid isolated from *M. haemolytica* strains in France (43). Somewhat ironically, chloramphenicol had been withdrawn from European veterinary use two years before the discovery of the 5.1 kilobase resistance plasmid. In the United States, chloramphenicol resistant *M. haemolytica* isolates are uncommon, perhaps because of the withdrawal of the antibiotic from use in food animals in the mid 1980s (54). Finally, it is likely that non-specific and inducible antibiotic resistance, such as the multiple antibiotic resistance (*mar*) drug efflux systems found in the Enterobacteriaceae (55), also occurs in *M. haemolytica*.

Since supplementation of animal feed with antibiotics continues, resistance in *M. haemolytica* is likely to persist. Even in the absence of feed-associated antibiotics, metaphylactic use upon arrival at the feedyard continues, and is recommended (56, 57). Recent attention has been placed on a relatively new antibiotic, tilmicosin, and its use metaphylactically. Tilmicosin is a macrolide antibiotic with good pulmonary pharmacodynamics and a low minimal inhibitory concentration against *M. haemolytica* (58). It is also beneficial because tilmicosin can cause neutrophil apoptosis in the lung, leading to decreased inflammation (59, 60). Animals treated once with tilmicosin during initial processing at the feedyard have reduced morbidity and mortality and increased weight gain, when compared to non-treated controls (56, 57, 61). It appears that tilmicosin is at least partially effective at reducing colonization and disease in calves during the initial week post-shipment when they are most susceptible to *M. haemolytica* pneumonia. Use of a drug such as tilmicosin may be considered judicious providing that efficacy is validated. Nevertheless, as could have been predicted, resistance to tilmicosin has already emerged (40). The molecular basis for this resistance has not been characterized, though resistance to erythromycin, a related macrolide, can be conferred by a single base change within the bacterial 23S rRNA (62).

4.2. Surface proteins, carbohydrates and colonization factors

4.2.1. Adhesins

Very little is known about the molecules that are involved in *M. haemolytica's* ability to colonize the pharynx and tonsillar crypts. It is likely that *M. haemolytica* encodes an adhesin that allows the bacterium to attach to respiratory epithelium. A locus encoding such an adhesin has not yet been identified, though fimbriae have been observed (63). Jaramillo *et al*. have affinity purified a 68 kilodalton (kD) rabbit erythrocyte-specific agglutinin (64). Agglutination is inhibited by N-acetyl-D-glucosamine (GlcNAc) and N-acetyl-neuraminic acid (NeuAc) or by

pre-treatment of erythrocytes with neuraminidase or protease, indicating that the host receptor is a sialoglycoprotein (64). The amino terminal sequence of the adhesion is ANEVNVYIYKQPYLI, and though the authors stated that no homologues were revealed in a database search, a variant of this sequence, ANEVNVYSYRQPYLI, occurs immediately following the signal peptide of the 35 kD *M. haemolytica* periplasmic iron binding protein, FbpA (65). FbpA interacts with the transferrin binding proteins, TbpA (100 kD) and TbpB (71 kD), to extract free iron from the glycoprotein, transferrin (66, 67). Though FbpA is periplasmic, its homologue in *Neisseria meningitidis* is "transiently" exposed on the surface of the bacterium (68). This is consistent with a multifunctional role as iron scavenger and adhesin. Nevertheless, FbpA and the adhesin differ in size (though the adhesin could be a dimeric form of FbpA) so it is unclear whether the two proteins are related in structure or in function.

Inspection of the draft *M. haemolytica* genome sequence (Highlander and Weinstock, preliminary results) indicates the presence of a *ca*. 3200 amino acid (*ca*. 340 kD) predicted open reading frame homologous to a putative filamentous hemagglutinin of *N. meningitidis* (69). Such high molecular weight hemagglutinins mediate adherence to host tissues and cells, often via RGD integrin binding motifs. Neither the *N. meningitidis* nor the *M. haemolytica* sequence contains an RGD motif, and neither sequence is it significantly similar to putative or characterized hemagglutinins described in *P. multocida* (70), *Haemophilus ducreyi* (71), or *H. influenzae* (72). Thus, the *M. haemolytica* sequence may represent a protein with a different cell or matrix tropism from the integrin-binding hemagglutinins. Additional putative adhesin-like sequences have been detected in the draft sequence; these await further analysis.

A protein called the serotype A1 specific antigen (SsaI) has also been implicated in colonization of the nasopharynx. Antibody directed against SsaI can inhibit nasal colonization by *M. haemolytica* 1 (16, 73) yet the role of SsaI in nasal colonization has not been established. The *ssa1* gene has been cloned and protein motif analysis indicates that it might possess a protease activity (74). The *ssa1* gene cross-hybridizes with sequences in serotypes A2, A5, 6, 7, 9 and 12 (75-77). Nevertheless, expression of the antigen is only observed in serotype A1 strains and only under specific growth conditions (76). Since *ssa*I expression has not been examined in the host, it is unclear whether it is expressed *in vivo* by the other *M. haemolytica* serotypes.

4.2.2. Lipopolysaccharide

LPS represents 10-25% of the dry weight of *M. haemolytica* bacteria (78), and is an important virulence factor. LPS is responsible for induction of IL-1beta and IL-8 via TNF-alpha, leading to neutrophil influx, inflammation (79, 80), and LPS damages bovine pulmonary endothelial cells (81). It has been proposed that *M. haemolytica* LPS can cause immune-mediated hypersensitivity that can exacerbate inflammation and damage by a localized Arthus or Shwartzmann reaction in the lung (82). *M. haemolytica's* leukotoxin (see below) forms high molecular weight aggregates with LPS (83) and it has often been argued that many of the leukotoxin's biological effects are due to LPS contamination (84, 85). Based on studies using purified leukotoxin and LPS, discrete activities for these molecules have been shown. *In vivo*, it is likely that LPS and leukotoxin act in synergy to cause tissue damage and inflammation. Lafleur, *et al*. have shown that LPS enhances the cytolytic activity of leukotoxin and that LPS enhances leukotoxin-dependent IL8 and TNF-alpha expression in bovine alveolar macrophages (79). Li and Clinkenbeard showed that LPS can thermally stabilize leukotoxin and enhance activity against bovine lymphoma cells (83). Similar potentiation of toxin activity has been reported for the related *Escherichia coli* hemolysin (86).

Eight different LPS types have been reported in *M. haemolytica* serotype A1 (87); these represent variants containing combinations of four core oligosaccharides and one *O*-antigen, with an identical tri-saccharide repeat (table 1) (88, 89). The *O*-antigen of serotypes A6, A9 and A12 is chemically identical to that of A1. Though the structure(s) of the *O*-antigens from A5, A7, A13, A14 and A15 have not been deduced, but they all are recognized by both anti-serotype A9 and anti-serotype A12 sera, suggesting that they may be identical (89). In contrast, serotype A2 and A8 isolates lack *O*-antigen and are rough (89, 90). Since antibody to *M. haemolytica* LPS is not completely protective (91), *O*-antigen variations may not be critical to immune evasion, though rough cells would be expected to be more susceptible to complement-mediated cytolysis.

Though the genetic basis for LPS variability in *M. haemolytica* has not yet been studied, two genes potentially involved in LPS biosynthesis in *M. haemolytica* have been cloned and characterized. A 263 amino acid reading frame encoding a peptide similar to the *H. influenzae* lipooligosaccharide biosynthetic gene, *lic2*, was identified immediately downstream of the gene encoding another putative virulence factor, the *M. haemolytica* glycoprotease (see below) (92). The LPS gene, called *lpsA*, is a potential glycosyltransferase involved in core oligosaccharide biosynthesis. A second gene involved in polysaccharide synthesis, *galE*, encoding UDP-galactose-4-epimerase, was cloned by complementation of a *galE* mutant of *Salmonella typhimurium* (93). The *galE* gene is important for virulence in a number of other pathogenic bacteria, such as *Neisseria, Salmonella* and *Haemophilus*, because it can be involved in synthesis of both LPS and capsule (94-96). *galE* mutants of *M. haemolytica* have not yet been created.

4.2.3. Capsule

Capsular antigens form the basis of the serotyping scheme of *M. haemolytica* and the structure of serotype A1, A2 and A7 capsules have been determined (table 1) (97-99). The *M. haemolytica* serotype A1 capsule is composed of mannopyranose residues, while the serotype A2 capsule is composed of sialic, or colominic, acid, identical to the capsule polysaccharide of *N. meningitidis*

and *Escherichia coli* K1 (98). Sialic acid capsules are non-immunogenic since sialic acid is a normal component of host membranes. In serotype A1, the capsule mediates serum resistance and resistance to phagocytosis (100), and anti-capsule antibodies opsonize the bacteria for uptake by macrophages (101). Nevertheless, the A1 capsule alone is not a protective immunizing antigen (102, 103).

The DNA sequence of a sixteen kilobase region that contains the *M. haemolytica* A1 capsule genes has recently been reported by Lo, *et al.* (104). The region includes twelve genes that are grouped into three regions that encode biosynthetic, transport and phospholipid modification functions. The genetic organization is like that of group II capsule gene clusters, prototypes of which are capsules from *H. influenzae* and *N. meningitidis* (105). Two of the *M. haemolytica* genes, *nmaA* and *nmaB,* encode proteins responsible for synthesis of UDP-ManNAc and UDP-ManNAcA from UDP-GlcNAc (105). Both of these sugars are found in the mature capsule (table 1). Two other genes of note are the *wbrA* and *wbrB* genes, which are homologous to capsular genes required for lipidation of the reducing ends of the polysaccharide chains (106). This was unexpected since structural analysis of the A1 capsule failed to reveal the presence of lipid and phosphorus (from phosphatidic acid) (97). The presence of lipid may have been overlooked because only a fraction of molecules are lipid substituted and the sugar-phosphate linkage is especially labile (107).

Capsule gene expression in *M. haemolytica* has not received much attention, though one study has indicated that the activities of enzymes involved in the biosynthesis of the sialic acid capsule on serotype A2 are sharply inhibited at 42° C (108). In serotype A1, capsule expression is elevated in young cultures, but expression diminishes in cultures older than 16 hours (109). Based on the arrangement of genes in the A1 cluster, Lo *et al.* suggest that the genes are organized into three or four transcriptional units, including separate, divergent promoters driving expression of the *nma* and *wbr* genes (104). Acapsular isolates have occasionally been reported (110), but the genetic bases of such have not been examined. Now that the genes have been cloned, molecular techniques are being used to create defined acapsular mutants (104).

4.2.4. Outer membrane proteins

Outer membrane proteins and lipoproteins of *M. haemolytica* may be involved in serum sensitivity (111, 112) and they are believed to be important protective antigens. Antibodies directed against some of these antigens are capable of inducing phagocytosis and complement-mediated killing (112). As a result, these surface-exposed proteins are of interest as potential vaccine candidates. As discussed previously, OMP profiles vary within and between serotypes (87) (table 1). A major OMP is the 38 kD heat-modifiable PomA protein, which is a homologue of OmpA porins from *H. ducreyi* and *E. coli* (113). Though the *M. haemolytica pomA* gene has been cloned, attempts to inactivate it have been unsuccessful (G. Murphy, personal communication), suggesting that PomA may be involved in transport of nutrients or that it may be critical to the integrity of the outer membrane. *M. haemolytica* also produces at least five distinct lipoproteins with apparent molecular weights that range from 19 to 45 kD (112, 114-116). A mutant lacking three of these lipoproteins (PlpA, PlpB and PlpD) was created by allelic exchange using the ROB-1 beta-lactamase gene from *M. haemolytica* (117). The resulting strain is more sensitive to antibody-dependent, complement-mediated lysis, and grows more slowly than wild-type in medium containing 50% fetal bovine serum (111). Since the mutant grew well in unsupplemented medium, the results indicate that the triple mutant had become more sensitive to a component or components in serum, perhaps because of membrane remodeling. Such remodeling could increase sensitivity to the complement membrane attack complex.

Beyond their roles in serum resistance and solute uptake, OMPs have been shown to have other activities relevant to pathogenesis. A preparation of purified *M. haemolytica* OMPs enhanced neutrophil adherence and chemotaxis *in vitro* (118) and caused the interferon-gamma dependent release of nitric oxide from murine peritoneal macrophages (119). In *P. multocida*, the 38 kD major porin protein has been associated with similar activities, as well as with the ability of the organism to polymerize actin (120). *M. haemolytica* has also been shown to polymerize actin (121) and this activity may correlate with the presence of a 35 kD OMP. When the strain was cured of a large streptomycin resistance plasmid, the actin polymerizing activity and the 35 kD OMP were both lost. Activity was restored when the plasmid was returned to the cured strain (Fedorova and Highlander, unpublished). Though not rigorously proven, it appears that the 35 kD OMP of *M. haemolytica* has actin polymerization activity similar to that of the 38 kD porin of *P. multocida*. Thus, as in other pathogens, *M. haemolytica* outer membrane proteins may be involved in remodeling the host cytoskeleton.

M. haemolytica produces a number of iron regulated outer membrane proteins (IROMPs) (122). These include a 77 kD protein of unknown function as well as the transferrin binding proteins, Tbp1 and Tbp2 (66, 123). These latter IROMPS are thought to be key in iron acquistion, since *M. haemolytica* does not produce siderophores (123). *M. haemolytica* bacteria grown in the lungs of cattle over-express the IROMPs, in support of their role in iron acquisition during the infection process (124). Sequence analyses suggest that the *tbpB tbpA* operon may be repressed by a protein similar to the ferric uptake regulator (Fur), since a Fur binding motif overlaps its predicted promoter (66). Additional iron-regulated proteins have been identified and characterized in *M. haemolytica*. These include the 35 kD periplasmic FbpA iron-binding protein mentioned previously (65), a 31 kD putative manganese binding protein, YfeA (125) and the *M. haemolytica* leukotoxin (126).

4.3. Toxins and extracellular enzymes

4.3.1. Leukotoxin

M. haemolytica secretes a 102 kD leukotoxin (LktA) that is a calcium-dependent cytotoxin belonging to

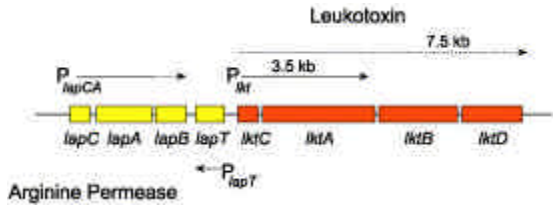

Figure 2. Open reading frame and transcript map of the leukotoxin operon (*lktCABD*) and the upstream arginine permease gene cluster (*lapCAB, lapT)*. Arrows indicate major (solid) and minor (dashed) transcripts. *P*, promoter

the RTX (repeats in toxin) family of toxins [for a review of RTX toxins, see Welch, *et al.*(127)]. Leukotoxin genes and protein have been identified in all serotypes (128) and nearly all isolates examined secrete the toxin (129). LktA is species-specific, having leukotoxic activity only against ruminant lymphoid cells (130, 131); it is also weakly hemolytic (132). Though the leukotoxin can bind to cells from a variety of species (133), cytolysis requires a specific interaction with the lymphocyte-function associated antigen 1 (LFA-1), or beta 2-integrin, on the target cell (134, 135). At high concentrations, the toxin creates pores in the cell membrane that lead to swelling and lysis (136). At sub-lytic concentrations, the toxin activates neutrophils (137), induces inflammatory cytokine production (138), invokes cytoskeletal changes, and causes apoptosis (139, 140). Combined, these activities are thought to impair primary lung immune defense mechanisms and participate in the inflammation and tissue destruction that define pneumonic pasteurellosis.

Like many other RTX toxins, the *M. haemolytica* leukotoxin is encoded by a four-gene cluster than includes the 953 amino acid structural gene, *lktA*, and genes required for its post-translational acylation (*lktC*) and secretion (*lktB* and *lktD*) (figure 2) (141). The LktA protein contains domains that are involved in pore-formation, receptor binding, calcium binding, activity and secretion (142, 143). The amino terminus contains a series of hydrophobic helices that are predicted to span the host cell membrane and cause pore formation (143). Hybrid toxin analyses, using chimeras created between LktA and the hemolysin of *E. coli,* indicate that amino acids 1 to 169 are required for target cell binding (143), though it is not clear if this binding involves a non-specific interaction with the host cell or a specific interaction with the beta 2-integrin receptor. Included within the central region of the toxin is a site for fatty acylation. Using chimeras of LktA and the *B. pertussis* adenylate cyclase-hemolysin toxin, the site of acylation was mapped between amino acids 564 and 739 on LktA (144). In the absence of this modification, the protein has no toxic effect *in vitro* (145). The carboxy-terminal domain of the protein contains a series of glycine and aspartate-rich repeats that, in other RTX toxins, have been shown to bind calcium and potentiate toxin activity (146). The carboxy-terminus contains a recognition site required for secretion by the *tolC*-dependent type I secretion system (147, 148). Toxin-neutralizing epitopes also fall within the last 229 amino acids of the protein (149).

Davies, Whittam and Selander performed an analysis of the molecular evolution of *lktA* genes obtained from *M. haemolytica*, *M. glucosida* and *P. trehalosi* strains (150). Their study revealed eight groups of allelic variants within *M. haemolytica*, and one each in *P. trehalosi* and *M. glucosida.* The variants represent combinatorial mosaics derived from up to four alleles (*lktA1, lktA2, lktA4,* and *lktA5*) that are believed to have arisen through intragenic recombination events between the three bacterial species. While *lktA4* and *lktA5* are found only in *M. glucosida* and *P. trehalosi*, respectively, recombinant toxins carrying significant blocks of *lktA4* or *lktA5* sequence are found in many ovine *M. haemolytica* strains. Such genetic exchange was surprising since *M. haemolytica, M. glucosida* and *P. trehalosi* are thought to occupy different niches and cause different diseases. Apparent horizontal transfer of entire genes was also observed. One allele, *lktA2*, is nearly exclusive to cattle, while the other alleles, carrying portions of the *lktA2* sequence, are mostly restricted to ovine hosts. Thus, while leukotoxin sequences appear to be conserved in bovine isolates, ovine strains clearly exhibit a wider range of diversity.

It is widely believed that leukotoxin is the most significant virulence factor elaborated by *M. haemolytica.* In an attempt to clarify the role of leukotoxin in bovine pasteurellosis, leukotoxin negative strains have been constructed and tested for virulence in calves. In one study, a genetically uncharacterized leukotoxin mutant (151), caused reduced mortality and smaller lung lesions than the wild-type when inoculated intratracheally (152). In another study, an *lktA* deletion mutant was tested, using bacteria delivered endobronchially (153). A similar reduction of mortality and lung lesions was observed. (153). Our group constructed and tested a mutant strain that secretes inactive leukotoxin. Using bacteria delivered transthoracically, the LD_{50} of the mutant strain increased only three-fold with respect to wild-type. The mutant strain was capable of forming lung lesions, though to a lesser extent, and was efficient at colonization of the upper respiratory tract. From these results, we concluded that other virulence factors, in addition to leukotoxin, are critical for pathogenesis (154).

4.3.1.1. *M. haemolytica* leukotoxin expression

Expression and secretion of active leukotoxin protein is regulated at the level of transcriptional initiation and elongation, protein stability (155), and by post-translation modification (156, 157). Studies have reported various environmental effects on leukotoxin "expression", but most examined levels of secreted protein only and failed to examine mechanisms of regulation. Gentry *et al.* showed that transferrin and lactoferrin enhance toxin production (158). Sun and Clinkenbeard demonstrated that leukotoxin production is increased by the addition of 1.0 mM $MgSO_4$ or 0.36 mM $FeCl_3$ to minimal medium maintained at pH 6.3 (159). These effectors may influence transcription, but they may also influence leukotoxin activation and/or secretion Early transcriptional analyses were limited to Northern blots and primer extension (156, 160, 161). Strathdee and Lo reported that leukotoxin transcription is maximal during growth at 37°C, and is

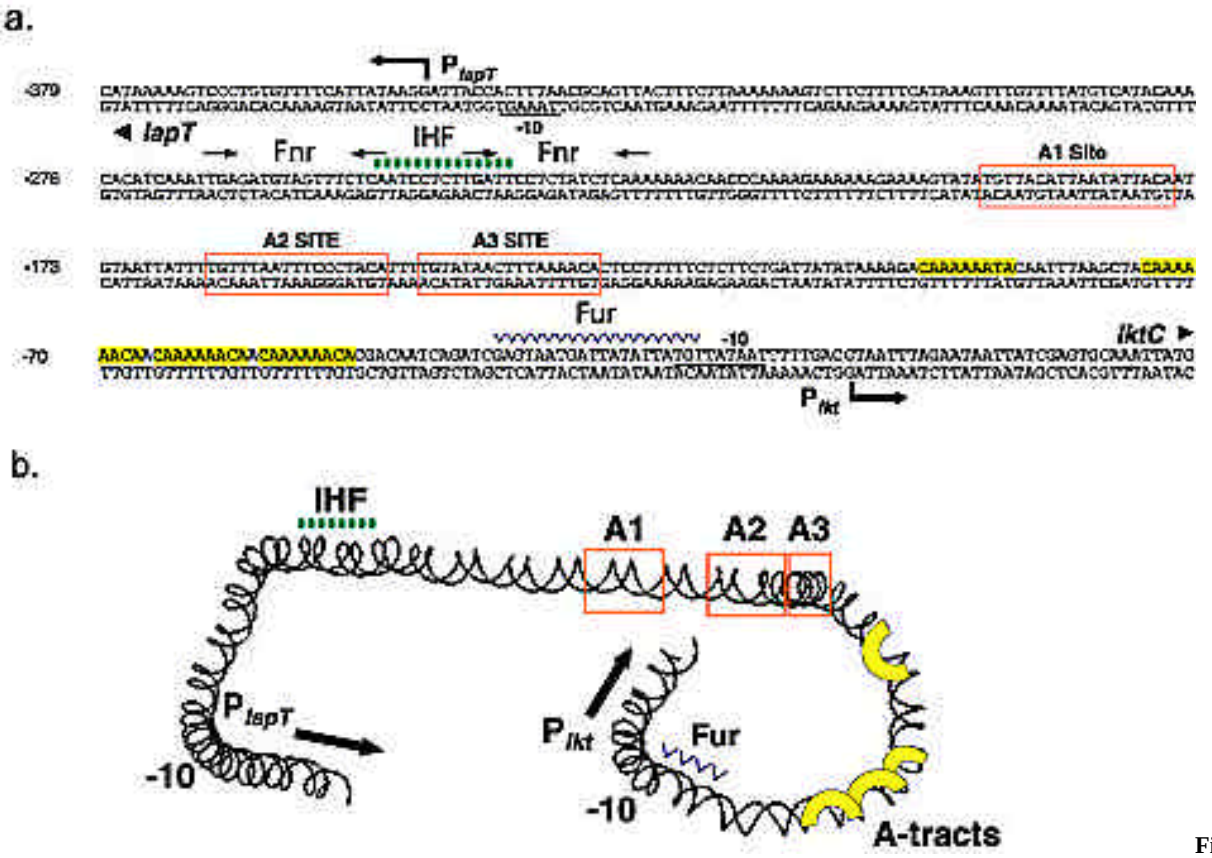

Figure 3. a. Nucleotide sequence of the *lapT-lktC* intergenic region. Promoter start-sites are indicated by the horizontal arrows and –10 sequences are underlined. The single arrowheads mark the start codons for each gene. The potential IHF binding site is indicated by the dashed lines and the putative Fnr binding sites are shown as inverted small arrows. The upstream activator protein binding sequences are boxed and the CA_6(C/T) motifs responsible for the static DNA bend are highlighted. A site for binding by Fur is indicated with the inverted carets. b. Two-dimensional model of the *lapT-lktC* intergenic region. The model was generated using the CURVATURE program (174). Motifs are highlighted as in (a).

enhanced by iron (1 mM $FeSO_4$) and by pH (7.3 vs. 6.5). We have recently quantitated leukotoxin transcription initiation using operon fusions linked to the chloramphenicol acetyltransferase reporter gene. Using this system, we have confirmed that leukotoxin transcription is modulated by temperature and, in contrast to the results of Strathdee and Lo, that transcription is repressed by iron (126).

The leukotoxin operon (figure 2) is divergently transcribed from a gene, *lapT*, which encodes a periplasmic arginine binding protein (162). Transcription of the operon begins at one of two cytosine residues 30 bp upstream of the *lktC* start codon, and proceeds through the *lktA*, *lktB*, and *lktD* genes (156). Like other RTX gene clusters, a predominant message (3.5 kilobases) corresponding to the *lktCA* transcript is observed. A minor transcript that spans the entire operon (7.5 kilobases) occurs, via antitermination, within the *lktA-lktB* intergenic region (156, 161).

We have identified a number of *cis*-acting elements that may be involved in activation and repression of leukotoxin and *lapT* transcription initiation (figure 3a). An extended -10 region, of sequence TGTTATAAT, lies 5 bp upstream from the leukotoxin start-site, but the promoter lacks a consensus -35 hexamer. Promoters that utilize extended -10 sequences usually require upstream activation (163). A potential Fur binding site (164) overlaps the -10 sequence. A static DNA bend sequence maps at nucleotides -47 to -96 with respect to the leukotoxin start-site. This bend is formed by four CA_6(C/T)A tracts, equally phased on the DNA helix, that cause the DNA to turn *ca*. 80° (165). Three repeats (A1/A2/A3), of sequence, TGT-N_{11-12}-ACA, map 5' to the DNA bend region. These motifs are similar to repeats recognized by the nitrogen fixation activator protein, NifA, from *Klebsiella pneumoniae* (166). DNA footprinting using *M. haemolytica* extracts demonstrated that a *Mannheimia*-specific protein binds the A2 and A3 repeats (167). We believe that these sequences are the targets for activator binding and that DNA looping can bring the activator protein into close proximity with RNA polymerase bound at the promoter. A near-consensus binding site for the DNA bending protein IHF (168) maps between the *lapT* promoter and the A1-A3 repeats. Binding of IHF to this region would cause a 160° bend in the DNA (169) and could influence transcription initiation at the *lapT* promoter. Additional sequence motifs of note are two potential ferric nitrate regulator (Fnr) binding sites (170) that flank the IHF binding site. These are of interest

because it has been reported that the *M. haemolytica* Fnr homologue, FnrP, enhances leukotoxin transcription in *E. coli* (171, 172). Deletion analyses in *M. haemolytica* have indicated that the A2/A3 repeats and the DNA bend are required for maximal expression of the leukotoxin promoter (126). Studies in *E. coli* indicate that the *lapT* promoter is positively regulated by IHF (173).

The *lapT-lktC* intergenic region displays a high level of DNA-directed DNA bending as modeled using the CURVATURE program (174) (figure 3b). The model shows that the static DNA bend can bring the A2/A3 repeat sequences into close proximity with the *lktC* promoter -10 region. Modeling also reveals an additional region of static bending that occurs between the *lapT* promoter and the putative IHF binding site. Both of these bends are separated by a linear domain, which imparts symmetry to the region. AT-rich DNA, such as that observed near both leukotoxin and *lapT* -35 regions, can act as an enhancer of transcription by anchoring the RNA polymerase alpha-carboxy terminal domain to template DNA (175, 176). Curved and AT-rich DNA is also a target for binding of the small histone-like protein, H-NS. H-NS is a global regulator of gene expression involved in regulation of virulence in organisms like enteropathogenic *E. coli* (177), *Vibrio cholerae* (178) and *Shigella flexneri* (179). H-NS also regulates synthesis of the *E. coli* hemolysin (180, 181). Binding of H-NS to AT-rich promoter sequences causes transcription repression by interfering with RNA polymerase binding. It also may antagonize binding of a transcriptional activator. H-NS repression is thermo-dependent. Binding is maximal at lower temperatures and decreases due to changes in promoter structure as temperature increases (182). To test the role of H-NS in leukotoxin expression, we measured transcription of the promoter in an *E. coli hns* mutant. Leukotoxin transcription was be derepressed two-fold in the mutant and repression was thermo-dependent (173). Thus, H-NS plays a role in virulence gene regulation in *M. haemolytica*.

Based on our transcriptional studies in *E. coli* and in *M. haemolytica*, we conclude that the leukotoxin promoter is regulated by both activation and repression mechanisms. Transcription may be repressed by a Fur-like protein bound to the -10 region when iron concentrations are high. In iron-depleted environments, the operon becomes depressed. At low temperatures, expression is further inhibited by H-NS protein bound to the AT-rich sequences. Such a system would keep leukotoxin transcription in check when expression might be deleterious to the survival of the bacterium (e.g. leukotoxin activation of the immune response during the commensal state). In contrast, transcription can be activated by the binding of an upstream activator protein to the A2 and A3 sites. We have preliminary evidence that the activator is part of a two-component regulatory system that responds to an additional unknown environmental cue (Marciel, Criglar, Gioia and Highlander, manuscript in preparation). Thus, the system allows for rapid derepression and activation when leukotoxin expression is advantageous to the organism (e.g. when iron stores are low and cidal leukocytes are present).

4.3.2. Glycoprotease

All serotypes of *M. haemolytica* produce a zinc metalloglycoprotease that has activity against *O*-sialoglycoproteins (183, 184). The protease is thought to act at the host cell surface to enhance adhesion and its activity *in vitro* can be potentiated by co-incubation with the leukotoxin (185). One consequence of this activity is the aggregation of platelets leading to their deposition in the alveoli of the lung (185). The sialoglycoprotein also cleaves the *O*-glycosylated glycoproteins CD34, CD43, CD44 and CD45, making it a useful reagent for epitope mapping on such cells (186). The *gcp* gene has been cloned into *E. coli* and sequenced (187). Unfortunately, the glycoprotease is found aggregated and inactive in the periplasm of *E. coli*, making purification of native, active protein difficult (188).

4.3.3. Neuraminidase

A neuraminidase produced by *M. haemolytica* has also been suggested to play a role in colonization (189). In other bacterial respiratory pathogens, neuramindases are thought to desialate salivary glycoproteins, allowing pathogenic organisms to escape defenses in the oropharynx (190). For *M. haemolytica*, it is likely that neuraminidase activity facilitates colonization of mucosal surfaces, particularly in the upper respiratory tract (191). Straus, *et al*. have shown that all serotypes of *M. haemolytica* produce neuraminidase activity and that the associated enzymes are 150 to 200 kD in size, based on gel filtration (192). The enzymes primarily cleave N-acetylneuramin lactose, but some also have activities that cleave fetuin, alpha-1-acid glycoprotein, and colominic acid (193). The draft DNA sequence of *M. haemolytica* genome includes a truncated reading frame (550 codons) with very high homology (e^{-164}; 52% identity) to the 832 codon *P. multocida nanH* reading frame (70, 194). If the missing amino terminal sequences are similar to those in *P. multocida*, then the *M. haemolytica* NanH protein would be *ca*. 95 kD in size, consistent with the molecular weight determined by Straus, *et al.* (192).

4.3.4. Immunoglobulin Proteases

Several respiratory pathogens, including *Haemophilus influenzae* (195) and *Actinobacillus pleuropneumoniae* (196) secrete proteases that cleave secretory antibodies. These proteases are serine endopeptidases that cleave monomeric IgA into Fab and Fc fragments, thus potentially enhancing bacterial colonization by elimination of local antibodies that block adherence (197). While IgA protease activity has not been identified in the supernatants of *M. haemolytica* (198), Lee and Shewen reported that partially purified culture supernatants do possess an IgG-specific protease (199). This is of interest because, in cattle, IgA predominates in the upper respiratory tract (200) while IgG is the primary secretory antibody in the lower respiratory tract (201, 202). Destruction of IgG in the lung could potentially decrease phagocytosis by alveolar macrophages thereby reducing pulmonary clearance. Though IgG cleavage may be due to glycoprotease activity (199), it is likely that a distinct IgG protease is expressed by *M.*

haemolytica. Inspection of the *M. haemolytica* draft genome (Highlander and Weinstock, preliminary results) reveals two reading frames, of 1503 and 1398 codons, respectively, that could encode IgA protease-like proteins (figure 4). The two *M. haemolytica* protein sequences are 64% identical to one another and are *ca.* 40% similar to IgA protease protein sequences from *H. influenzae*, *N. meningitidis* and *N. gonorrhoeae* (69, 203, 204). No homologue was found in the *P. multocida* whole genome sequence (70), though IgA protease production by some *P. multocida* isolates has been reported (205, 206). IgA proteases are usually encoded by a precursor peptide that is processed into three domains: the *sec*-dependent amino-terminal leader sequence, the mature protease, and an amino-terminal helper domain that permits secretion across the other membrane (204). While all five IgA protease sequences shown in figure 4 contain a conserved amino terminal signal peptide and a conserved serine residue within the active site of the mature protease, they diverge near amino acid 1000, within a region where the proteins undergo auto-proteolysis. The *H. influenzae* and *Neisseria* sequences each contain one or more P/A-P-(T/S/A)-P proteolysis sites (207) within this region, followed by a large membrane spanning domain that constitutes the helper domain. The *M. haemolytica* sequences lack the consensus proteolysis sites and are less hydrophobic in the carboxy termini than are the other IgA proteases. Based on these differences, it is plausible that the putative immunoglobulin proteases of *M. haemolytica* may be cell-associated and not freely secreted. These differences could explain the failure to identify IgA protease activity in culture supernatants.

5. SYSTEMS FOR GENETIC MANIPULATION

The common genetic tools such a plasmids, bacteriophage, transposons and antibiotic resistance genes from *E. coli* have not been useful for the manipulation of *M. haemolytica*. Means for competence-based transformation have not been discovered. Attempts to electroporate *M. haemolytica* with common cloning vectors such as the pUC-based plasmids and the IncP (e. g. RK2) or IncW (e.g. pSa) groups of plasmids have been unsuccessful (110) (Highlander, unpublished), though Frey reported that an RSF1010 derivative could be maintained in *M. haemolytica* (208). Presumably, the *E. coli* replicons fail to replicate in *M. haemolytica*, though in some cases, resistance genes used for selection may not have been expressed. None of the common *E. coli* bacteriophages, such lambda, P1, or T4, are capable of creating plaques on *M. haemolytica*, and attempts to use lambda or P1 as transducing phage have been unsuccessful. A further hindrance has been the lack of a transposon mutagenesis system for the organism; attempts using Tn*5* and Tn*10* have failed (44, Highlander, unpublished). These failures are likely the result of differences in gene expression between *E. coli* and *M. haemolytica* (209), though restriction by at least three restriction-modification systems in *M. haemolytica* also presents a significant barrier to gene transfer (210-212). As a result, it has been necessary to develop a genetic system for *M. haemolytica* using native plasmids and resistance genes.

5.1. Plasmids and resistance cassettes

M. haemolytica plasmids pAB2 (a.k.a. pPH33 or pSH10) (44, 110), and pYFC1 (42), can replicate in *E. coli.* Plasmid pAB2 can be mobilized from *E. coli* to *M. haemolytica* using the transfer functions of plasmid RP4 or pRK2013 (110). In contrast, pYFC1 cannot be mobilized because of a frame-shift mutation with one of its mobilization genes (42). Excluding the antibiotic resistance genes, pAB2 and pYFC1 are 99% homologous, yet they are compatible. A 30 bp region of non-identity between the plasmids likely delimits their replication origins. The replication and mobilization regions are also highly homologous (96-99% identity) to several other HAP plasmids, including pIG1 from *Actinobacillus pleuropneumoniae* (213) and pLS88 from *Haemophilus ducreyi* (214). Plasmid DNA of pAB2 and pYFC1, purified from either *E. coli* or *M. haemolytica*, can be electroporated back into *M. haemolytica* with equivalent frequency, indicating that the plasmids are immune to the *Mannheimia* restriction systems (215).

A series of *M. haemolytica* shuttle vectors, which replicate in *M. haemolytica* and in *E. coli*, have been created by our group and others. The plasmids were created using either pYFC1 or pAB2 as the replicon and they possess a number of different features. Using pAB2, Azad *et al.* created a mobilizable ampicillin resistant vector, pAKA16, containing a multiple cloning site. Mobilizable suicide vectors, based on pAKA16 were also created by substituting the *M. haemolytica* plasmid origin of replication with a ColE1 or R6K origin of replication (216). We have based our shuttle and expression vector system on the pYFC1 replicon, in part because we found it more easily manipulable than pAB2. Plasmids carrying a variety of resistance markers were created and three have been of particular utility (figure 5) (215). Plasmid pNF2176 is an ampicillin resistant expression plasmid that contains a multiple cloning site immediately downstream from the sulfonamide promoter. This plasmid has been used as an expression vector to complement mutations in *M. haemolytica* (145) and to express heterologous proteins such as the green fluorescent protein (Marciel and Highlander, unpublished) and Cre recombinase (154). Plasmid pNF2214 carries the *aph3* kanamycin. resistance determinant from plasmid pUC4K (Pharmacia, Piscataway, NJ) and has been useful for allelic exchange experiments where incompatible plasmids with different resistance markers are needed. A third plasmid that has been of great utility is the promoter probe vector pAM2355. This plasmid carries a promoterless chloramphenicol acetyltransferase gene downstream from a multiple cloning site, making it an operon fusion vector for transcriptional studies. Derivatives of pAM2355, carrying the leukotoxin and *lapT* promoters, have been used to quantitate transcription in *M. haemolytica* (126, 173), leading to the transcriptional model discussed previously.

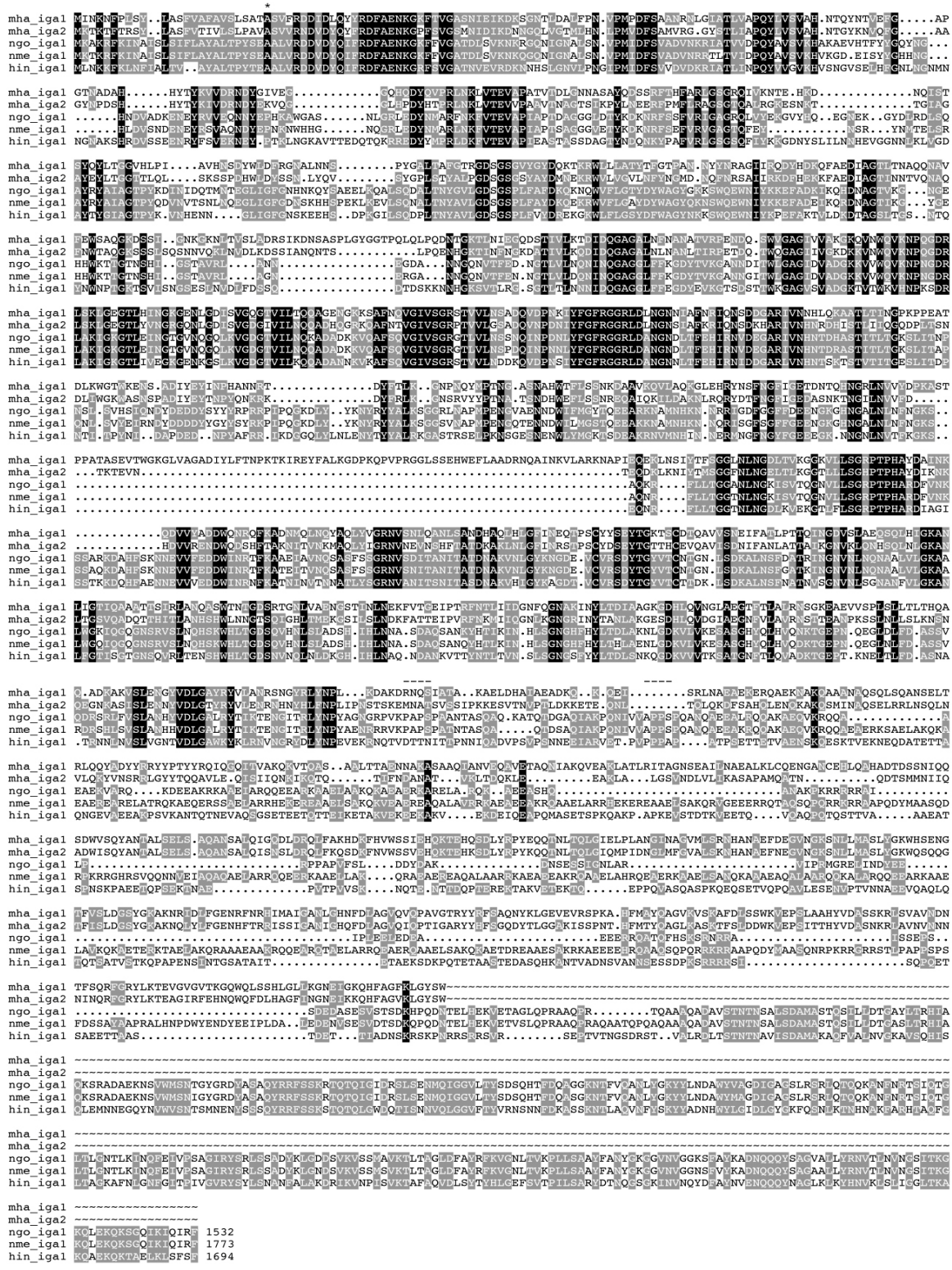

Figure 4. Alignment of IgA protease reading frames from *M. haemolytica* (mha_iga1 and mha_iga2), *N. gonorrhoeae* (ngo_iga1), *N. meningitidis* (nme_iga1) and *H. influenzae* (hin_iga1). The alignment was created using the GCG PileUp program and the figure was shaded using the BOXSHADE program. Identical amino acids are shaded black while chemically similar amino acids, or those matching in three of the five sequences are lightly shaded. The asterisk indicates the site of signal peptide cleavage, the active site serine in highlighted in yellow, and the two potential protease cleavage sites are marked with dashes over the sequences.

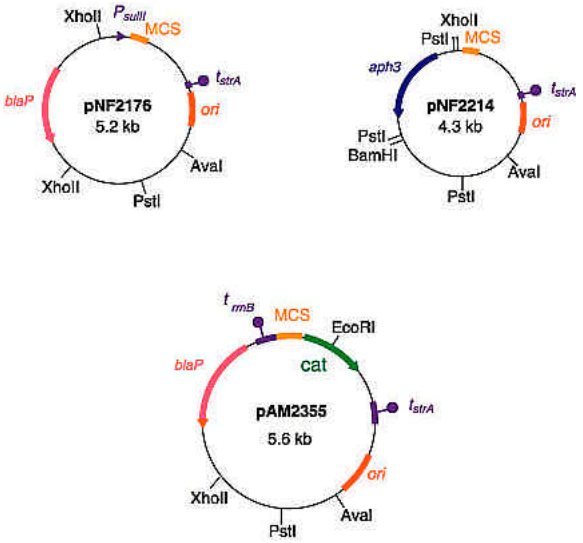

Figure 5. Partial restriction maps of *M. haemolytica* vectors. Not all restriction sites are shown. Abbreviations: *blaP*, ROB-1 beta-lactamase; *aph3*, aminoglycoside phosphotransferase, *cat*, chloramphenicol acetyltransferase; *sulII*, dihydropteroate synthase; *str*, streptomycin nucleotidyltransferase; *ori*, origin of replication; *t*, transcription terminator, *P*, promoter; MCS, multiple cloning site.

Antibiotic resistance cassettes, for use in *M. haemolytica* (figure 6), were created from a variety of sources, though we focused on *Mannheimia* resistance genes that would presumably be expressed more efficiently than similar genes from *E. coli*. The streptomycin resistance gene from pYFC1 has been particularly useful because it is resistant to the highly active *M. haemolytica* restriction-modification systems (215). The chloramphenicol cassettes are desirable because they permit selection with little or no background resistance. The cassettes in figure 6a are nonpolar. Each of these cassettes includes three stop codons in three reading frames immediately preceding the resistance gene. To restore downstream expression, the cassettes also include a ribosome binding site and start codon immediately 3' to the resistance genes. The nonpolar cassettes confer resistance only when placed downstream of an active promoter (217). Other cassettes, some carrying promoters, are flanked by *loxP* recombination sites (figure 6b**,** see below).

5.2. Allelic exchange and site-specific recombination

Using the resistance gene cassettes and the incompatibility properties of our plasmids, we developed a positive-negative selection method (218) for enhancing allelic exchange with the chromosome of *M. haemolytica.* A plasmid-free, antibiotic-sensitive cloning strain, SH1217, was created by curing a pathogenic *M. haemolytica* isolate, PHL213 (215). To perform allelic exchange, a plasmid carrying a target gene, interrupted by one of the antibiotic resistance cassettes, is introduced into *M. haemolytica,* then it is destablized by a second incompatible plasmid carrying a different resistance marker. By passaging cells for several days with selection for the second plasmid, we then identify double crossovers by replica plating. The destabilizing plasmid is then cured by treatment with novobiocin (145). We have used this technique to place CAT operon fusions into the chromosome and have insertionally inactivated a number of genes. Inactivated genes have included the type I and type III restriction genes of *M. haemolytica* (211, 212). Unfortunately, neither of the restriction mutations increased the frequency of gene transfer from *E. coli* to *M. haemolytica* (Highlander and Hang, unpublished). While other groups have introduced mutated genes into *M. haemolytica* on non-replicating ColE1-based vectors (117, 153, 219), we usually obtain mutants at a higher frequency when using the incompatibility method (145). We believe that by first establishing a mutagenic plasmid in the recipient strain, we can mitigate the problem of restriction otherwise encountered when susceptible non-replicating DNA is introduced. Briggs and Tatum used an alternate approach to overcome the restriction barrier in *M. haemolytica* (210). They cloned one of the *M. haemolytica* type II restriction modification systems into *E. coli*, then used this strain as a host to methylate the mutagenic DNA prior to transfer into *M. haemolytica*. Methylated DNA was capable of being electroporated into their recipient strain, while non-methylated DNA could not (210). No further characterization of this methylase has been reported.

To create unmarked mutations in *M. haemolytica*, we have developed the Cre-*loxP* recombination system of bacteriophage P1 (220) for use in the organism. Cassettes were created where the antibiotic resistance gene is flanked by direct repeats of the 34 bp *loxP* recombination site (figure 6b). We used this strategy to create a nonpolar insertion within the *lktC* gene of the leukotoxin operon, by first inserting the cassette into the chromosome through homologous recombination. Next, the antibiotic resistance marker was removed by expression of the Cre recombinase, expressed on plasmid pNF2442 in *M. haemolytica* (figure 6c) (154). The Cre-mediated excision of the antibiotic resistance gene is very efficient in *M. haemolytica*, so the system should be useful for creation of multiple mutations. Since excision leaves a copy of the *loxP* site in the chromosome, it should also be possible to use Cre recombinase to integrate DNA into such sites on the chromosome. This could be useful for introduction of wild-type copies into mutants, the placement of controllable promoters upstream of desired genes, or for placement of reporter gene fusions on the chromosome.

6. MOLECULAR APPROACHES TO VACCINE DEVELOPMENT

In the United States, the economic losses due to shipping fever pneumonia surpass the combined cost of all other diseases of cattle. Accordingly, methods for economical treatment and prevention are in demand. Traditional therapy for the disease has been based on the extensive use of antibiotics, including mass medication of animals, which caused an increase in the incidence of multi-drug resistant *M. haemolytica* (221, 222). Therefore, non-antibiotic prophylaxis through vaccination is more desirable. Much effort has been focused on the

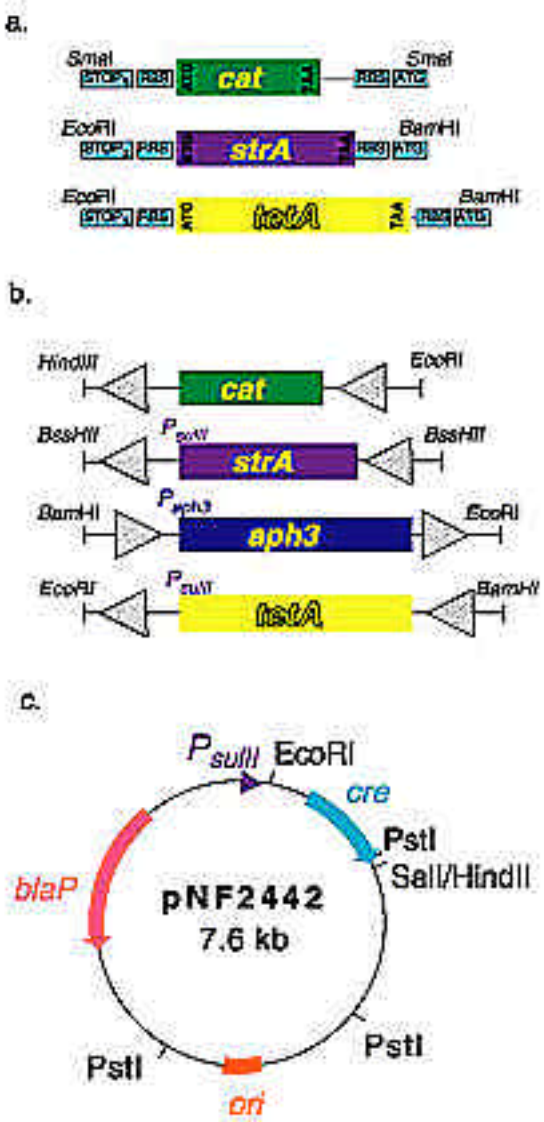

Figure 6. a. Non-polar, antibiotic resistance cassettes for gene interruption in *M. haemolytica*. b. *loxP* cassettes for gene interruption and site-specific recombination in *M. haemolytica*. The *loxP* sites are designated as arrowheads. The *loxP-cat-loxP* cassette is promoterless, while the *strA*, *aph3*, and *tetA* genes have promoters. c. Cre recombinase plasmid pNF2442. Not all restriction sites are shown. Abbreviations: *blaP*, ROB-1 beta-lactamase; *cat*, chloramphenicol acetyltransferase; *strA*, streptomycin nucleotidyltransferase; *tetA*, tetracycline resistance; *aph3*, aminoglycoside phosphotransferase, *sulII*, dihydropteroate synthase; *ori*, origin of replication, *cre*, Cre recombinase; ATG, start codon; TAA, stop codon; STOP3, three stop codons, one each in each frame; RBS, ribosome binding site; *P*, promoter.

development of vaccines to prevent shipping fever pneumonia. Several commercial shipping fever vaccines are in use (Presponse®, One Shot™, Pneumo-Star™), though data have not been published to support their efficacy under feedlot conditions [for a comprehensive review of current bovine respiratory vaccines, see Bowland and Shewen, 2000 (2)]. It appears that current vaccines provide only partial protection and some, such as whole cell bacterins, have even increased morbidity (223). As a result, shipping fever continues to be a serious animal health problem, in both the United States and abroad.

Development of effective vaccines requires an understanding of *M. haemolytica* virulence factors and antigens necessary to elicit immune protection. The protective antigens of *M. haemolytica* are expected to be the bacterial surface components such as the OMPs, LPS *O*-antigen and capsule, plus secreted molecules such as the leukotoxin. It has been shown that leukotoxin is a key protective antigen and that high titers of anti-leukotoxin neutralizing antibodies are correlated with resistance to the disease (224-226). Nevertheless, when recombinant or purified leukotoxin was tested for its ability to protect against experimental challenge, no protection was observed, indicating that leukotoxin alone is not sufficient to protect against disease (227, 228). Similarly, use of capsular polysaccharide alone, or in combination with leukotoxin was not protective (103). Use of a culture supernatant vaccine, mixed with recombinant leukotoxin, is effective in animal trials (227), suggesting that the best vaccines for shipping fever are those that contain leukotoxin plus additional supernatant-associated antigens. Others have reported success using live vaccines (229, 230), but concerns exist over the virulence of the vaccinating bacteria.

Cloned and purified proteins have been tested for their efficacy as protective antigens. In particular, the OMPs and IROMPs have been a focus for development of subunit vaccines. The cloned IROMP, TbpB, appears to be an important protective antigen that elicits antibodies that are cross-reactive with TbpB proteins from a variety of *M. haemolytica* serotypes (231). In contrast, the TbpA protein is not immunogenic (232). Screens using bovine convalescent sera have identified up to twenty-five immunogenic surface-exposed OMPs (233). Cell extracts containing mixtures of these proteins have been shown to be protective, both in cattle (234) and in sheep (235, 236). It is anticipated that additional potentially antigenic cell surface molecules will be identified by genomic and post-genomic analyses. For example, phage display can be used to identify important antigenic epitopes, by panning libraries of *M. haemolytica* peptides versus bovine convalescent serum. An interesting new concept in the development of surface-directed vaccines is the creation of bacterial ghosts, or vesicles carrying target antigens (237). Expression of the bacteriophage PhiX174 gene E in bacteria, such as in *M. haemolytica* causes cell death, leaving behind membranous ghosts (238). These ghosts contain OMPs and other surface antigens. In theory, such ghosts could also include heterologous antigens, which could be cloned and expressed in *M. haemolytica*.

Other molecular approaches to vaccine development have included the creation of attenuated

strains by mutation of specific targets. Such attenuated strains could be used as live vaccines, which are usually more effective than killed whole cell or subunit vaccines. One target for attenuation is the *aroA* gene of *M. haemolytica.* The *aroA* gene product is required for synthesis of aromatic amino acids so such strains do not replicate well *in vivo* (239). An *aroA* mutant of *M. haemolytica* was created by allelic exchange and the mutant is attenuated in a mouse septicemia model (240). A test of the strain in cattle has not been reported. A second target for attenuation is the leukotoxin gene. As discussed previously, a leukotoxin deletion strain (153) and a strain producing inactive leukotoxin (145, 154) have been constructed by allelic exchange. In theory, the strain producing inactive leukotoxin would express all of the epitopes expressed by a wild-type bacterium and could be very effective. Unfortunately, the strain still maintained significant virulence in the transthoracic challenge model (154), so enthusiasm for testing its efficacy in a calf-challenge is low. Other targets for attenuation will certainly be identified once the *M. haemolytica* genomic sequence is complete. Examples of such targets are biosynthetic genes, global regulators, and virulence gene homologues.

7. PERSPECTIVE

Mannheimia haemolytica continues to be an enigmatic organism. Our understanding of its virulence and pathogenesis has been limited, in part, because of difficulty in genetic manipulation. As a result, few strains carrying defined mutations in putative virulence factors have been tested in animals. Study of a commensal organism is also challenging because, under most conditions, the host is capable of keeping the organism, and presumably its virulence, in check. In contrast to overt pathogens, where virulence mechanisms are marked, the mechanisms of pathogenesis of commensals can be more subtle.

It is hoped that genomic sequencing of *M. haemolytica* will provide additional clues about its metabolism and pathogenesis. The limited sequence information presented here indicates that *M. haemolytica* surface proteins share features not only with other HAP organisms but with the human commensal *N. meningitidis*. This may not be entirely unexpected since the two organisms occupy similar niches in the upper respiratory tract. Though the two organisms produce very different diseases (pneumonia versus meningitis), their mechanisms of colonization may share common features, such as requirement for filamentous hemagglutinins, anti-phagocytic capsules, IgA proteases and iron scavenging proteins (241). Genomics will likely reveal new virulence genes and can be used to identify new targets for attenuation and creation of live vaccines.

Though gene transfer and genetic exchange in *M. haemolytica* continue to be challenging, we expect that the genetic tools and techniques that have been developed for *M. haemolytica* will be useful in post-sequencing genetic analyses of the organism. Efficient means of gene interruption will be needed to confirm functional properties of assigned coding regions and to assess functions of unassigned coding sequences. Development of a random transposon mutagenesis system would be ideal. Regulatable expression vectors will also be needed for complementation and for overexpression of particular gene products. Ideally, a conjugation system will be developed that will allow large regions of DNA to be transferred from one host (or serotype) to another. Such a system would greatly facilitate the comparative genomics analyses that we anticipate. If these types of systems can be developed, then a thorough analysis of *M. haemolytica* and the genetics of virulence can be undertaken.

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