Exploring the Cell Surface: Identification and Characterization of Lipoproteins in *Mycoplasma mycoides* subsp. *mycoides* Large Colony, *Mycoplasma mycoides* subsp. *capri*, and *Mycoplasma capricolum* subsp. *capricolum*

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by

TARA R. MARTIN

Dr. Michael Calcutt, Dissertation Supervisor

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List of Abbreviations

*Mmy*LC – *Mycoplasma mycoides* subsp. *mycoides* Large Colony

*Mmy*SC – *Mycoplasma mycoides* subsp. *mycoides* Small Colony

Mmycapri – *Mycoplasma mycoides* subsp. *capri*

Mcap – *Mycoplasma capricolum* subsp. *capricolum*

Mccp – *Mycoplasma capricolum* subsp. *capripneumoniae*

*M*BG7 – *Mycoplasma* sp. Bovine group 7

Vlc/*vlc* – Variable lipoprotein of Large Colony (strain GM12)

Vsl/*vsl* – Variable surface lipoprotein (Large Colony strain GM648-13)

Vsc/*vsc* – Variable surface lipoprotein of *capri*

Chapter 1 - Introduction

Mollicutes

The bacteria comprising the class *Mollicutes* (trivial name mollicutes) were originally thought to be viruses due to their small size and the inability to culture them in basic laboratory media (134). In depth studies have since shown that mollicutes are related to the Gram-positive eubacteria family *Bacillaceae*, and are descended from a *Clostridium*-like predecessor of the *Lactobacillus* lineage (123,233). Mollicutes have small genomes, ranging from 540 to 1300 kb (62,193), and it is thought that this minimal size is a result of reductive evolution (243). As a result of extensive gene loss, these bacteria exist as obligate parasites, as they are not able to synthesize amino acids, fatty acids or purines (78,172,174), necessitating very rich, complex media for cultivation in a laboratory setting (94). In addition to the loss of certain nutrient anabolic pathways, these bacteria have also lost the capability to synthesize a cell wall. In fact, *Mollicutes* is derived from the Latin for 'soft skin' referring to the lack of a cell wall surrounding the bacterium; only a single limiting membrane (174) separates the cell interior from the host environment. Members of this class are also renowned for the chronic nature of infection, as well as being refractory to antibiotic therapy (24,108,180).

There are several unique genetic characteristics of mollicutes beyond their small genome size and lack of genes needed to synthesize essential nutrients.

Mollicutes have a characteristically low genomic G+C content, ranging from 24-33% (62,79) overall but this varies within certain regions of the chromosome. An increased G+C content is found in conserved genes that depend on nucleic acid secondary structure, such as rRNA and tRNA genes, and a higher A+T content is seen in intergenic regions (62,79,174). Due to the low G+C content, mollicutes preferentially use A-U rich codons (79,150,151), and there are fewer glycine, proline, alanine, and arginine residues found in conserved proteins (79,174). In addition the codon UGA has been reassigned to encode the amino acid tryptophan as opposed to signaling for translational stop as seen in most other bacteria including *Escherichia coli* (87,245). The alternate usage of the UGA codon makes expression of mycoplasmal proteins in common bacterial expression vectors problematic, resulting in truncation of any protein containing UGA tryptophan codons.

In the absence of a cell wall, the cell surface of mollicutes is the crucial interface between bacterium and host, not only to obtain essential nutrients, but also for colonization and avoidance of the host immune system. Over two-thirds of the mass of the single limiting membrane is comprised of proteins (174). This includes transmembrane proteins such as P1 of *Mycoplasma pneumoniae* (86), polytopic proteins which have been identified in the complete genomes of *M. pneumoniae* and *Mycoplasma genitalium* (62,79,162), as well as multiple lipoproteins (38,46). A large number of lipoprotein genes have been found in completed mollicute genome sequences, ranging between 5-8.5% of annotated ORFs (62,79,136). This is high when compared to Gram-positive bacteria such

as *Bacillus subtilis*, in which approximately 2.8% of ORFs are predicted to be lipoprotein genes (223), as well as Gram-negative bacteria, with only 1% of ORFs encoding lipoproteins in *Escherichia coli* K-12 (177).

Surface proteins, including lipoproteins, serve a multitude of functions on the mollicute cell surface. Multiple adhesins, some lipoproteins (77,113,249) and somewhat surprisingly, common housekeeping genes (7,80), have been identified on the cell surface of various mollicute species that are important if not essential for the initiation of colonization (42,145). In addition to adherence, several surface expressed proteins have been found to affect the host immune system. MALP-2 of *Mycoplasma fermentans* and MALP-H of *Mycoplasma hyorhinis* are both potent macrophage stimulators (146,147). A lipoprotein in *Mycoplasma pulmonis* has been shown to protect the cell from complement killing (202), while a family of size-variable lipoproteins in *M. hyorhinis* protects against growth inhibition by host antibodies (37). It has also been shown that M161Ag of *M. fermentans* is able to induce the cytokine interleukin-10 in human monocytes *in vitro* as well as kill undifferentiated myelomonocytic cell lines hindering the host immune response to bacterial infection (171). IL-10 is known to induce a toleragenic response as opposed to inflammatory response in the host (116). It is speculated that all of these surface proteins function to increase the survival of the bacteria in its host organism.

Mollicutes cause significant diseases in a variety of hosts despite their reduced genome and parasitic lifestyle. Common human pathogens include *M. pneumoniae*, a causative agent of atypical pneumonia (34,128), and the closely

related organism *M. genitalium,* which causes non-gonnococcal urethritis, which is considered to be an emerging sexually transmitted disease (93,112). *Mycoplasma hyopneumoniae* infection leads to porcine enzootic pneumonia (105), which has a significant economic impact on the global pork industry (208,217). An important poultry pathogen, *Mycoplasma gallisepticum,* causes respiratory disease in chickens that results in the loss of over $11 million a year (137-139). A lethal flesh-eating disease in alligators is the result of colonization by *Mycoplasma alligatoris* (27). Several agriculturally important plant diseases are also the result of infection by mollicutes including Aster yellows disease, caused by *Candidatus* Phytoplasma asteris (111), and citrus stubborn disease, caused by *Spiroplasma citri* (127). Closely related to the spiroplasma branch of the mollicutes is the *Mycoplasma mycoides* phylogenetic cluster (Fig 1-1) that contains two very significant animal pathogens, *Mycoplasma mycoides* subsp. *mycoides* Small Colony (*Mmy*SC) and *Mycoplasma capricolum* subsp. *capripneumoniae,* the causative agents of Contagious Bovine Pleuropnemonia (216) and Contagious Caprine Pleuropneumonia (126), respectively. The focus of this dissertation is the analysis of additional *M. mycoides* cluster members that are primarily caprine pathogens.

Mycoplasma mycoides cluster

The *Mycoplasma mycoides* cluster is a group of phylogenetically closely related ruminant pathogens (215). These organisms infect bovine or caprine species

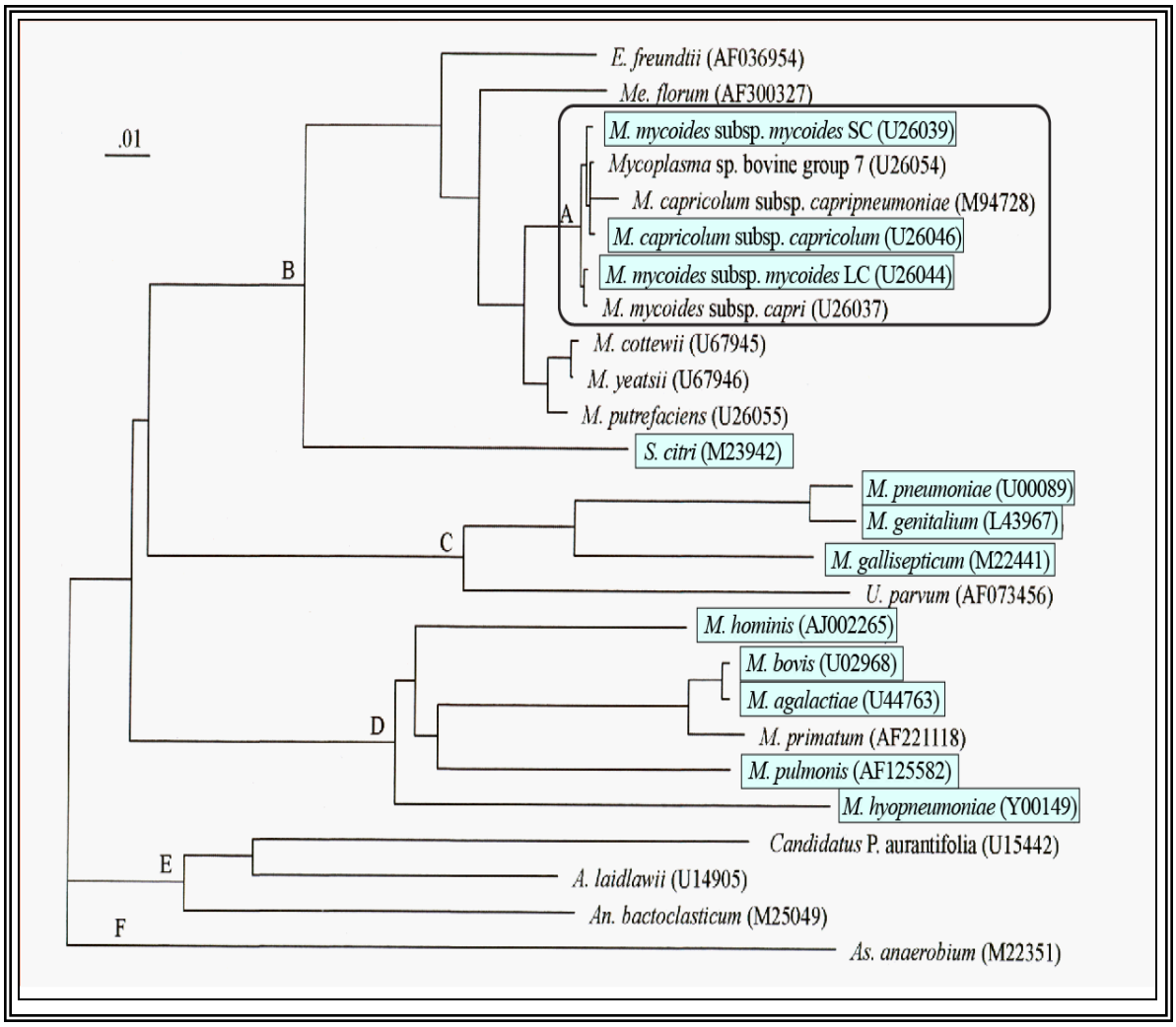


Figure 1-1. Phylogenetic Tree of Selected *Mycoplasma.* Phylogenetic relationship of representative *Mycoplasma spp.* based on 16S ribosomal RNA sequence. The *Mycoplasma mycoides* cluster is highlighted in the box. Species for which genome sequences have been released, in either complete or shotgun reads, are highlighted in blue. Adapted from A. Persson, PhD Dissertation, 2002.

leading to a wide range of disease (Table 1-1). The close genetic relatedness and conserved gene synteny seen in the completed genome sequences of members of the cluster is distinctive for such a large number of mollicutes, as many phylogenetic groups in this class are more deeply branched (Fig. 1-1). The cluster is divided into two main sub-clusters, the *capricolum* and the *mycoides* subgroups, based on multiple phylogenetic analyses (124,165,222). The *capricolum* subgroup contains *M. capricolum* subsp. *capricolum* (Mcap), *M. capricolum* subsp. *capripneumoniae* (*Mccp*), and *Mycoplasma* sp. Bovine group 7 (*M*BG7). The *mycoides* subgroup contains *Mmy*SC, *Mycoplasma mycoides* subsp. *mycoides* Large Colony (*Mmy*LC), and *Mycoplasma mycoides* subsp. *capri* (*Mmycapri*). It should be noted here that despite infecting the same ruminant host, the two bovine infecting cluster members, *Mmy*SC and *M*BG7, are in distinct phylogenetic branches. While host specificity is fairly stringent for each mycoplasma, there is a degree of enzootic transmission of bacteria seen between these animals although it may not result in disease (154,182).

The most notorious member of the cluster is *Mycoplasma mycoides* subsp. *mycoides* Small Colony. *Mmy*SC was the first mycoplasma species identified, and serves as the type species for the genus (21,156). This mycoplasma is the causative agent of Contagious Bovine Pleuropneumonia (CBPP), and is the only bacterial pathogen on the A List of diseases reportable to the World Organization of Animal Health (1). CBPP is a highly contagious disease resulting in tissue necrosis and fluid buildup in the bovine lung. In the case of acute CBPP, symptoms include loss of appetite, agalactia, fever, labored

Species	Host	Disease
*Mmy*SC	bovine	Contagious Bovine Pleuropneumonia (CBPP)
*Mmy*LC	caprine	mastitis, arthritis, pneumonia, agalactia, septicemia
Mmycapri	caprine	mastitis, arthritis, pneumonia, agalactia, septicemia
Mcap	caprine	mastitis, arthritis, pneumonia, agalactia, septicemia
Mcpp	caprine	Contagious Caprine Pleuropneumonia (CCPP)
BG7	bovine	mastitis, arthritis, pneumonia, septicemia

Table 1-1. *Mycoplasma mycoides* cluster members, hosts, and diseases.

breathing and death. Infection results in up to a 75% mortality rate in as little as three weeks (9). *Mmy*SC was eradicated from the United States in 1893 and is currently not present in the Western Hemisphere (26). There were several outbreaks of CBPP in Europe in the 1990s, including the countries of Portugal and Spain, and the disease is also found in parts of Asia, especially India and China (26). *Mmy*SC is endemic in areas of sub-Saharan Africa causing massive economic loss estimated to be between 768,000 and 14,987,000 Euros per country per year (212). Detailed action plans to control and eradicate *Mmy*SC infection have been outlined by numerous agencies (8,9,212), but political turmoil and economic hardships in many sub-Saharan African nations have made these action plans difficult to implement (129,212). The recent outbreaks in parts of Europe, the increase of global trade over the past few decades and the devastating impact on African nations highlights the need for better diagnostic and control methods for infection with *Mmy*SC.

Current control methods for CBPP include vaccination, herd culling, and slaughter of infected animals (8). Both vaccines are comprised of live, avirulent strains of *Mmy*SC, but this has proved to be problematic. There have been reports of injection site reactions which are due at least in part to the vaccine strain reverting to a virulent phenotype (219). Experimental infection of cattle with the vaccine strain can lead to development of CBPP as well (130). Vaccination strategies can be successful, but a very strict protocol and greater than 80% coverage of animals must be enforced, which is not feasible on the entire continent (125). There have been several reports of decreased efficacy of

the vaccine in recent years (187,221) and multiple groups have been working on the development of new vaccines (51,118,125). The discovery of immunogenic epitopes specific to *Mmy*SC, as well as to other members of the cluster, has proven difficult but is essential to diagnosis and vaccine development.

Rapid and accurate diagnosis is difficult for members of the *M. mycoides* cluster due to the cross-reactivity of antibodies (40,164,167,186,220) and a high conservation of DNA sequence, making PCR identification problematic (39,166). This can make it more difficult and time consuming to appropriately treat infection in a herd as well as to monitor the prevalence and spread of these agriculturally significant diseases. Although there is a close genetic and antigenic relatedness amongst these bacteria, each strain is largely associated with one specific host, although this is not absolute, and causes varying degrees of disease (126,216). Clearly it is desirable to determine unique markers for each species in this group as well as to understand the factors responsible for host specificity and disease severity, and many comparative techniques have been utilized, including both genomic and proteomic approaches, to achieve this.

Small subunit ribosomal DNA sequencing is the current basis for distinguishing between the species and sub-species of the *M. mycoides* cluster (181,244) (Fig 1-1), but new approaches coupled with the ever growing number of completed genome sequences have led to the development of several newer diagnostic tests. Suppression-subtractive hybridization performed using *Mcap* and *Mmy*LC allowed for the isolation of a DNA segment that is specific for all strains of *Mcap*, including multiple field isolates (122). Real-time PCR has also

been adapted to help distinguish between bacteria in this group, but originally was only able to distinguish between the two main sub clusters, *M. mycoides* and *M. capricolum* (60). A newer real-time PCR assay has been developed that can identify *Mmy*SC and *Mccp* specifically, bypassing the need to cultivate these fastidious pathogens and confirm the identity of the species before treatment, management, and reporting of an outbreak (119). Unfortunately this level of accurate and rapid detection does not extend to all members of the *M. mycoides* cluster (119,122).

The research presented in this dissertation focuses on three specific species of the *M. mycoides* cluster that infect caprine hosts. *Mmy*LC, *Mmycapri* and *Mcap* are important pathogens in many parts of the world. These mycoplasmas are included among the causative agents of Contagious Agalactia, making them reportable to the World Organization for Animal Health (1). Economic losses due to Contagious Agalactia have been estimated in excess of US $30 million in European countries surrounding the Mediterranean (153). In the Central Valley of California, caprine mycoplasmosis has been one of the leading causes of morbidity and mortality in affected goat herds, with one outbreak of *Mmy*LC in young kids having a morbidity and mortality rate greater than 90% (43), while a second outbreak lead to the destruction of an entire 700-head herd (45). In some countries where *Mmy*LC infection is endemic, such as France and Spain, there are surveillance programs monitoring outbreaks and collecting epidemiologic data in hopes of controlling disease (12,175,214).

Infection with any one of the three species listed above can lead to a wide range of disease presentations including pneumonia, mastitis, agalactia, polyarthritis and septicemia (159,220). As might be predicted, disease is more severe in kids than in mature goats due to the septicemic manifestation. Infection with *Mmy*LC has been experimentally shown to have a 73% mortality rate in kids, with most dying 7-14 days after exposure (44). The major histological changes included interstitial pneumonia, synovial lining necrosis, and in some cases histiocytic meningitis (44). Infection with *M. capricolum* leads to similar but more severe pathological and histological findings (44). In another study in which kids were experimentally infected with *Mmy*LC, *Mmycapri* and *Mcap,* death was reported in all animals 1-5 days after inoculation. Histopathology found acute interstitial pneumonia and arthritis and immunohistochemical analysis confirmed the septicemic state of affected kids (179).

One of the current taxonomic topics in the field of Mycoplasmology is the classifications of *Mmy*LC and *Mmycapri.* It has been suggested that these two mollicutes be condensed into one taxonomic group, *mycoides* subspecies *capri* (230). One-dimensional SDS-PAGE profiling of total cell protein between numerous strains of *Mmy*LC and *Mmycapri* resulted in mixed subspecies groups being formed and the conclusion that these two subspecies were indistinguishable from one another (110). Comparison of the LppA protein sequences showed that there was a higher degree of homology within these two species compared to other closely related mycoplasma, and that PCR primers

specific for *lppA* from *Mmy*LC and *Mmycapri* would not amplify the equivalent region in other cluster members but could not discriminate between the two subspecies (140). Based on the analysis of a segment of *rpoB,* all cluster members were distinguishable except *Mmy*LC and *Mmycapri* (230). These data all suggest that subspecies *Mmy*LC and *Mmycapri* should be classified together as *Mycoplasma mycoides* subsp. *capri.*

One confounding factor in the study of disease determinants in the *M. mycoides* cluster is the paucity of genetic tools. There are a large number of insertion sequences in *Mmy*SC (235), as well as *Mmy*LC, while there are none in *Mcap*, suggesting there may be differences in the propensity of these strains to take up foreign DNA. However, *Mcap* does harbor an integrative conjugative element (ICE) that has been shown to be capable of actively excising out of the chromosome (98). There have been ICEs identified in other members of the cluster, including *Mmy*LC and *Mccp* (M. Calcutt, unpublished results), as well as numerous other species of mollicutes (31,203,227); it has been suggested that these units may be involved in horizontal gene transfer (203). The only native plasmids that have been identified in the *M. mycoides* cluster have been found in *M*BG7 (53) and *Mmy*LC (56,100). Shuttle vectors derived from the *Mmy*LC plasmdis have been shown to undergo recombination in mollicutes to delete the *E. coli* origin of replication (101). The *M. mycoides* cluster plasmids are actually three of the four known mycoplasma plasmids, the fourth having been discovered in *Mycoplasma yeatsii*, a close relative of the cluster, and characterized by Dr. Bethany Kent at the University of Missouri (98). The construction of *oriC*

plasmids has been useful for some genetic manipulation, including the deletion of the *lppA* gene in *Mcap* (92).

Antigenic Variation and Contingency Loci

In order for bacteria to flourish they must be able to survive the dynamic environment of their host organism. This includes both the host innate and humoral immune responses which must be evaded for colonization and persistence of infection. There are a myriad of ways which bacteria have evolved to deal with this, including host immunomodulation (88,226), host cell invasion (148,149), and molecular mimicry (33,141). One strategy employed by many pathogens is changing what is expressed on the cell surface, known as antigenic variation. This can occur in two different ways: 1) a phenotypic moderation resulting from gene expression regulation in response to the host environment, such as a stress response (163) or 2) a genotypic alteration via mutation that is present in the offspring of the progenitor cell creating variation within the population (144). One way pathogens achieve genotypic alteration is via phase variation, which is a high frequency, heritable, and reversible stochastic switching of cellular genetic expression (Fig. 1-2). The rate of spontaneous mutation is on the order of 10^{-8} to 10^{-11} whereas the rate of switching for a phase-variable gene is around 10^{-2} to 10^{-5}, approximately one million times more frequent than random genetic mutation (144), allowing for the rapid accumulation of phenotypic variants within the population.

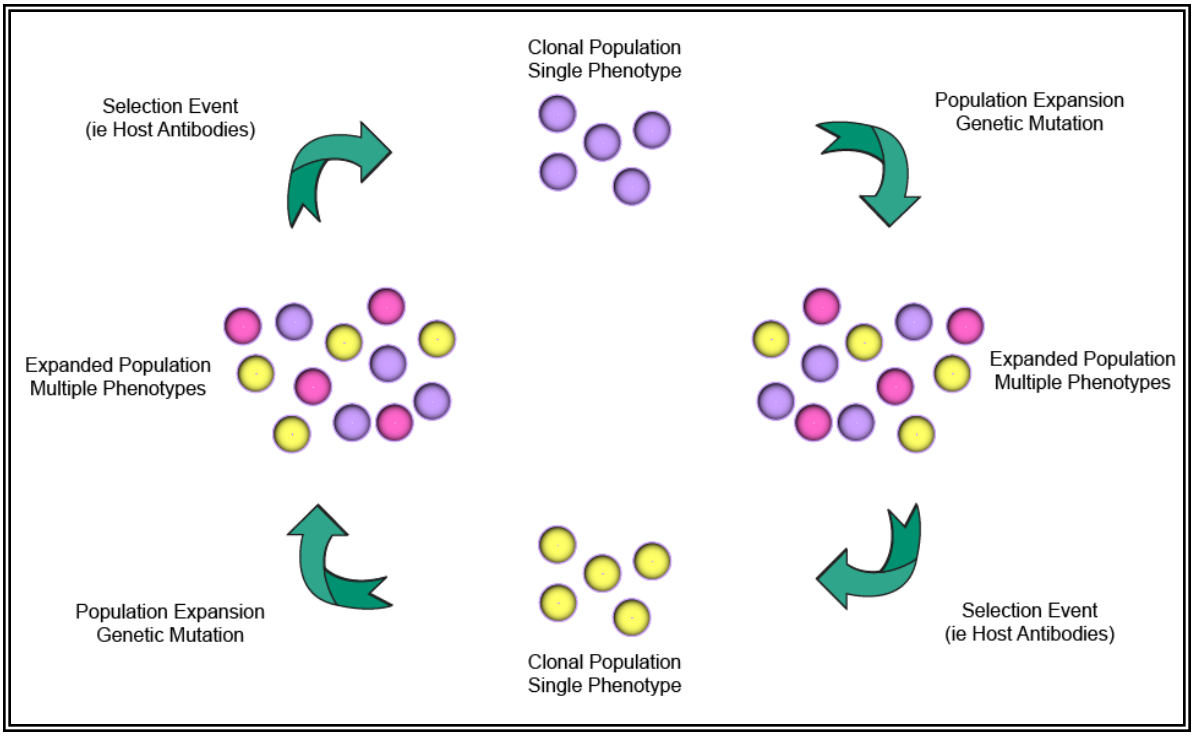


Figure 1-2. Process of Phase Variation Within a Bacterial Population. If a clonal population is expressing the same phenotype, allowing that population to grow and expand will result in a variation of phenotypes observable in the population. This is a result of genetic mutation that is heritable and reversible. In the event of selective pressure, a majority of the bacterial population will be cleared, but due to variation a small subpopulation will survive to propagate and again begin to expand and phase-vary.

Phase variation was first described in 1922 in the case of two antigenically different flagella being expressed in *Salmonella enterica* (11). Since then, multiple systems have been identified and characterized across diverse bacterial species including *E. coli* (161), *Salmonella typhimurium* (91) and *Burkholderia mallei* (205). Phase variation has been described as a way to evade the host immune system by changing surface-expressed epitopes recognized by host antibodies (83,158), aid in niche adaptation (188,234), mediate the switch between virulent and avirulent variants within populations (59,135) and to enable the formation of biofilms in *Pseudomonas aeruginosa* (50). Diverse mechanisms responsible for antigenic variation include DNA inversion, in which recombination flips different ORFs in and out of frame behind the active promoter (67,194,248), promoter inversion, which involves changing the orientation of the promoter to lead to active or inactive transcription (3,4,35), and slip-strand mispairing at simple sequence repeats.

Simple sequence repeats (SSRs) are understood to control phase variation via slip-strand mispairing during DNA replication (144). The location of the SSR is critical and is frequently in a region controlling transcription or translation, therefore causing an insertion or deletion that changes the expression profile of the corresponding gene. For fimbriae variation in *Haemophilis influenzae*, this insertion/deletion occurs in a TA dinucleotide repeat found in the promoter, changing the spacing of the RNA polymerase binding sites through which transcription occurs (225). The SSR can also occur outside of the promoter region as it does in *Bordetella pertussis*. A polyC tract alters the

distance between the activator and RNA polymerase binding sites, controlling transcriptional activity of the promoter (237). Phase variation at the level of translational control is seen in *Haemophilis influenzae* for the *mod* gene. A tetranucleotide repeat found within the ORF is subject to insertion and deletion, resulting in a change of reading frame that will either produce a full-length protein or a truncated version (48). *mod* expression also influences the activity of many downstream genes in what has been termed a phasevarion (206).

The availability of an ever increasing number of complete genome sequences has emphasized the extent of SSRs in bacterial species (144). SSRs are also some of the most conspicuous phase-variable mechanisms, aiding in the identification of hypervariable regions in bacterial chromosomes. These hypervariable, or contingency, genes have evolved to have a higher mutation rate than housekeeping genes so that phenotypic variation is increased, thereby increasing likelihood of survival (143). Richard Moxon coined the term contingency locus to describe a region of hypervariable DNA that controls high-frequency, heritable, stochastic genotypic switching enabling the pathogen to be prepared for different contingencies that occur in the host environment (144). Contingency loci have been identified in diverse pathogens including *Yersinia pestis* (184), *Campylobacter jejuni* (117), *E. coli* (178), *Neisseria gonorrhoeae* (207) and the *M. mycoides* cluster (164,240). The contingency loci in the cluster encode putative lipoprotein genes that phase vary via a change in the number of TA dinucleotide repeats found in the promoter region (164,240).

Bacterial Lipoproteins

Bacterial lipoproteins are anchored in a cellular membrane via a lipid modification at the N-terminus. Collectively these proteins perform diverse functions within the bacterium and the only commonality is a conserved prolipoprotein signal sequence which consists of approximately 20-30 amino acids, mostly hydrophobic in nature, then ending in a lipobox (89). The lipobox is a highly conserved motif comprised of the last four amino acids of the signal sequence that is essential for correct protein processing; the consensus sequence for eubacteria is [LVI][ASTVI][GAS][C] in which the cysteine is invariant (13,121). Lipoprotein genes are translated into pro-lipoproteins containing the N-terminal signal sequence which directs the protein through the membrane and is proteolytically cleaved. The conserved cysteine residue is first modified with diacylglycerol via diacylglyceryl transferase, and then the signal sequence cleaved by signal peptidase II (Fig. 1-3) (13). In most bacterial species the NH_2 group on the N-terminal cysteine is acylated by an apolipoprotein transacylase resulting in a triacylated mature lipoprotein (13). To date there have not been any genes encoding transacylase homologues identified in mollicutes (62,78,79,174). Furthermore, most experimental evidence supports lipoproteins in mollicutes as being diacylated (109,160,195). Despite the lack of a recognized

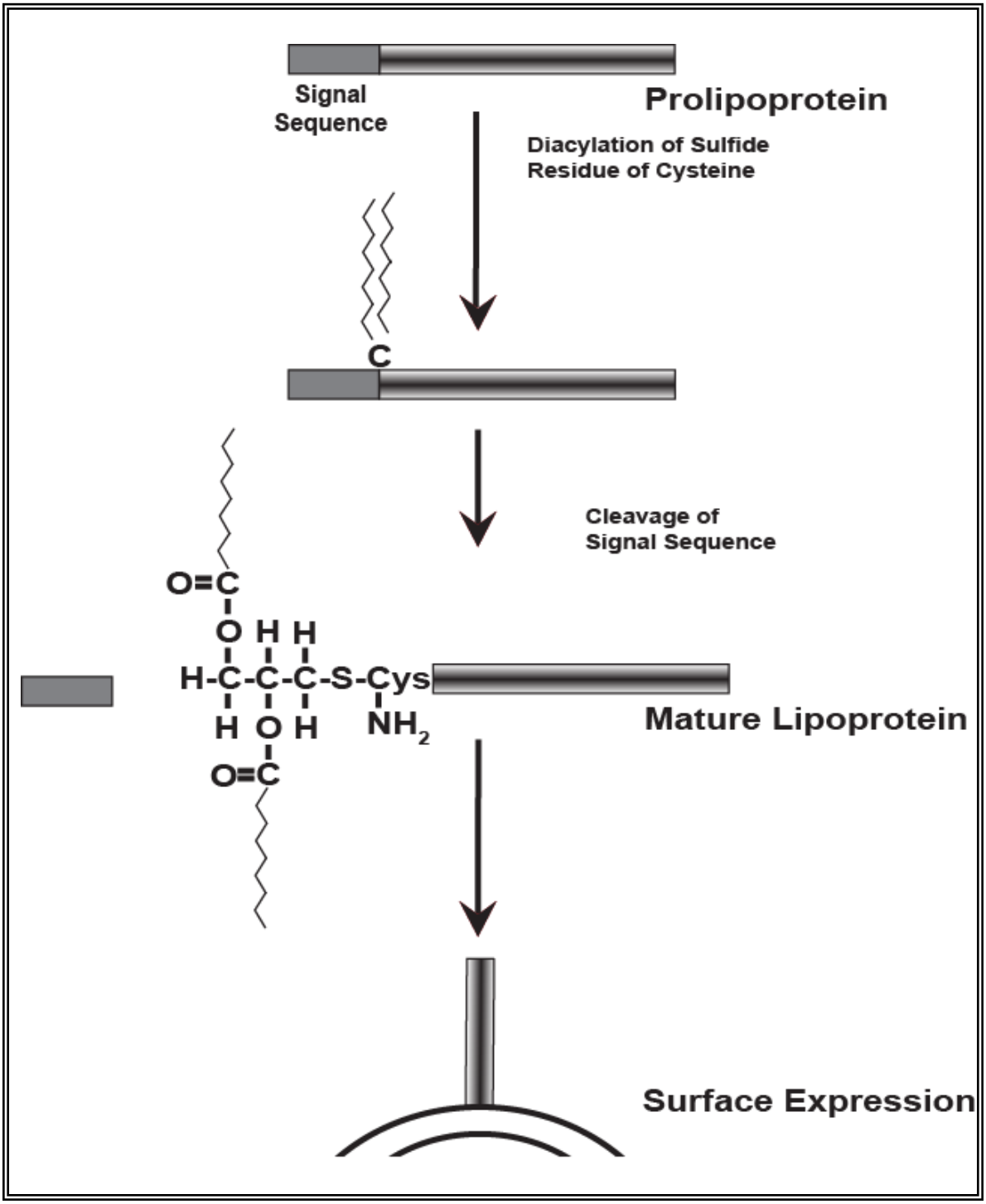


Figure 1-3. Processing of Bacterial Lipoproteins. Lipoprotein genes are translated as prolipoproteins which contain a conserved signal sequence. The signal sequence is cleaved at a conserved cysteine residue which is then modified with diacylglycerol and trafficked to the cell membrane where the lipid moieties serve as an anchor allowing the protein to be expressed on the cell surface.

enzyme to add the third acyl group to the mature lipoprotein, there have been triacylated proteins identified in both *M. genitalium* and *M. pneumoniae* (197,199). It is possible that there is an enzyme that performs the final N-acylation, but that is shares little sequence homology to known apolipoprotein transacylases.

The first lipoprotein was described in *E. coli*, the murein lipoprotein (MLP), one of the most abundant cell envelope proteins in this bacterium (25). The MLP is an outer membrane protein that is essential to keep the outer membrane associated with the cell by binding peptidoglycan (211,232). Since 1969, lipoproteins have been shown to be ubiquitously distributed throughout the prokaryotic kingdom and to serve a number of different roles for the bacterial cell. Multiple lipoproteins serve as adhesins, such as PsaA of *Streptococcus pneumoniae,* DpbA and DpbB of *Borellia burgdorferi* which bind decorin (68) and the P29 adhesin from *M. fermentans* (113). Other lipoproteins are part of transporter systems, helping the cell acquire nutrients from the environment. OpuAC is the ligand-binding protein for glycine betaine uptake in *B. subtilis* (96). A major superfamily facilitator pump in *Mycobacterium smegmatis* requires the presence of LprG in order to function (58). In *Mycoplasma hominis*, not only is the substrate-binding protein of an oligopeptide permease, OppA, essential for transport, but has also been shown to function as an ecto-ATPase and an adhesin (81). Lipoproteins are also important for the correct function of the pilus in *P. aeruginosa* (103) and *Neisseria meningitidis* (14). Lipoproteins perform a wide variety of functions on the exterior of the bacterial cell.

Lipoproteins on the cell surface of mollicutes face a different environment to those found in Gram-negative and Gram-positive bacteria. The majority of lipoproteins in Gram-negative bacteria species are exported into the periplasmic space between the inner and outer membranes where they are then anchored into the outer membrane facing the periplasmic space (20). The Lol system is required for the correct export and localization of these proteins in Gram-negative bacteria (152). In Gram-positive bacteria, there is no outer membrane, and therefore no periplasmic space, so lipoproteins are typically located on the cell membrane facing the host environment (210). The outer surface of the Gram-positive bacteria however is covered with a thick peptidoglycan layer which, while porous, still offers a protective layer surrounding the bacterial cell (210). Mollicutes do not have an outer membrane or a peptidoglycan layer, leaving lipoproteins expressed on the single limiting membrane fully exposed to the extracellular milieu. Lipoproteins of mollicutes do not contribute to the structural stability of the outer cellular components as seen in Gram-negative bacteria (25). It appears that lipoproteins are essential to the survival of mollicutes as signal peptidase II mutants have not been recovered from large-scale genome transposon insertion experiments (63,85,236). This is very different from other bacteria in which signal peptidase II mutants have a reduced virulence, but are still viable (46,176,191).

While lipoproteins are used in a number of ways to help bacteria colonize the host, they are also potent immune activators. Many organisms' immune systems have evolved to be able to recognize pathogen associated molecular

patterns, or PAMPs, and in mammals this function is performed by Toll-like receptors. Each TLR recognizes a specific pattern, such as LPS or single stranded DNA, and the TLR responsible for the recognition of the acylated portion of a lipoprotein (which can be exposed as bacterial cells are lysed) is TLR2 (5). Several groups have reported that lipoproteins from varied mollicute species are able to activate the host immune system via multiple TLRs; this activation is measured by the induction of Nuclear-Factor Kappa B (NF-κB). In *M. pneumoniae*, a diacylated lipoprotein subunit of F_OF_1-ATPase induced NF-κB through TLR1, TLR2, and TLR6 (198). *M. pneumoniae* lipoproteins N-ALP1 and N-ALP2 activate NF-κB via TLR1- and TLR2-dependant pathways, as did a synthetic triacylated peptide, leading the authors to believe that these two proteins may be triacylated (197). In the closely related bacterium *M. genitalium*, protein MG149 followed this trend by signaling through TLR1 and TLR2 and is also predicted to be triacylated (199). MG309, also from *M. genitalium*, activated an inflammatory response via TLR2/TLR6 which is proposed to be through specific amino acids as opposed to the lipoprotein modification (133). The MALP lipoprotein in *M. fermentans* stimulates NF-κB production through TLR2 signaling (155,195) . Apoptosis in a human cell line was induced by NF-κB activation mediated by the activation of TLR2/TLR6 when the cells came into contact with MALP-2 (90). It is clear from these recent results that characterization of the host immune response to mycoplasma lipoproteins is important to the understanding of bacterial survival, the ability of these pathogens to cause

chronic infections, and will lay the foundation for improving current vaccines for effectively controlling infection.

Mycoplasma Pathogenesis

One focus of research on bacterial species is how these microbes cause disease. There is a myriad of determinants that leads to disease manifestations in the host organism including colonization and virulence factors. Adhesins are critical players in bacterial colonization and collectively have been found to recognize an array of host proteins. In *E. coli* IHE 3034, FimH adheres to type I and type IV collagens (169); Fbp68 of *Clostridium difficile* binds fibronectin (76); and the housekeeping protein glyceraldehyde-3-phosphate dehydrogenase is expressed on the cell surface of *M. genitalium* where it is able to bind mucin (7). Classical virulence factors such as toxins (115,200), cytolysins (10) and invasins (32), while common in other bacterial species, are either rare or have not yet been identified in mollicutes.

The list of virulence factors of *Mollicutes* is relatively short for a group of pathogens with a relatively large number of available genomic sequences. Due largely to the lack of genetic systems and good animal models, very little is known about the actual mechanisms of disease causation in these bacteria. A common theme in the literature is the suggestion that host tissue damage is a by-product of host inflammatory responses (174). No classical virulence factors, such as pathogenicity islands or virulence plasmids, have been identified in any

mollicute species making the etiology of disease in these bacteria a 'black box.' This complicates effective prevention and treatment of mollicute infection and raises the question how some mollicutes are able to cause serious, chronic disease.

The currently known repertoire of virulence factors for mycoplasma includes adhesins, capsules, host immunomodulators, enzymes, metabolic by-products and two toxins (Table 1-2). Adhesins are essential in the initiation of colonization and subsequent infection of the host. Many examples have been identified in mollicutes including P1 (114) and P30 (41) of *M. pneumoniae,* MgPa (142) and P140 (29) of *M. genitalium*, and PvpA of *M. gallisepticum,* which in addition, is a phase-variable lipoprotein (19). Multiple mycoplasma species have a capsule (173), including *Mmy*SC and *Mccp* which is composed of a polysaccharide (84,185). *Mycoplasma pulmonis* is able to form a biofilm that protects the bacterium from killing by host complement and the anti-microbial molecule gramicidin (201). A family of size-variable lipoproteins, the Vlps, of *M. hyorhinis* also protects from complement killing when the expressed lipoprotein contains a larger number of tandem repeats (37). Hydrogen peroxide is a by-product of glycerol metabolism, a common metabolic pathway in bacteria, but has been shown to be a virulence factor in both *Mmy*SC and *M. pneumoniae* inducing inflammation and cell death (71,168). Sialidase and hyaluronidase are both essential spreading factors in *M. alligatoris* that aid with the rapid dissemination and explain the necrosis associated with infection with this species via degradation of extracellular matrix proteins (28). Recently a human

Species	Virulence Factor	Role
M. pneumoniae	CARDS TX	ADP-ribosylating and vacuolating toxin
M. fermentans	Macrophage Activating Lipoprotein (MALP) Plasminogen binding	Stimulates macrophages Aids in dissemination of bacteria
Mycoplasma hyorhinis	Vlp	Size-variable; longer protein confers protection from host complement
*Mmy*SC	H_2O_2 production	Causes inflammation and cell death in host
Mycoplasma pulmonis	Biofilm formation	Protection from host complement
Mycoplasma arthritidis	*M. arthritidis* mitogen (MAM) Mycoplasma Immuno-dominant Antigen (MIA)	Superantigen Size-variable; longer protein associated with less virulent phenotype possibly due to epitope masking or increased recognition by host immune system
Mycoplasma alligatoris	Sialidase Hyaluronidase	Break down of extracellular matrix proteins

Table 1-2. Known Virulence Factors in *Mycoplasmas* Excluding Adhesins and Capsules.

surfactant-A binding protein, community-acquired respiratory distress syndrome toxin (CARDS TX), was identified in *M. pneumoniae* (95). CARDS TX was shown to have ADP-ribosylase activity in cell culture and organ culture leading to vacuolization, cell rounding, and disruption of the cell monolayer (95). While there may be very few classical virulence factors identifiable in the genomes of these organisms outside of adhesins, mollicutes have evolved pathogenic mechanisms to aid in the colonization and infection of the host environment.

Even less is known about the mechanisms of virulence in the *M. mycoides* cluster compared with other members of the class *Mollicutes*. The pathology of infection for members of this cluster is varied, and except for what is known about *Mmy*SC, relatively little is known. *Mmy*LC, *Mmycapri, Mcap,* and *M*BG7 have been shown to form a biofilm under specific laboratory conditions, but the nature and/or function the biofilms is unknown (132). Despite initially reporting that *Mmy*SC does not form a biofilm (132), without the liquid/air interface *Mmy*SC was able to bind to cell culture flasks (131). The adherent cells demonstrated a significant increase in resistance to osmotic and heat shock, oxidative stress, as well as survival for much longer than planktonic cells, but this was highly variable between strains (131). To date there are no characterized adhesins for any member of the *M. mycoides* cluster. *M*BG7 is able to bind and activate plasminogen, but this was shown via an *in vitro* assay and a specific protein was not identified (22). No pathogenicity islands, virulence plasmids, or classical toxins have been identified in the available genome sequences of *Mmy*SC, *Mcap,* or *Mmy*LC. Despite this seeming lack of virulence factors, *Mmy*SC is able

to induce apoptosis in bovine leukocytes, although the mechanism for this has yet to be determined (49).

Comparison of African and European strains of *Mmy*SC revealed the first virulence factor in the *M. mycoides* cluster. African strains of *Mmy*SC are more virulent than strains responsible for the recent European outbreaks of CBPP (228). Genomic comparisons of *Mmy*SC African strain Afadé and European isolate L2 revealed that one factor in this difference was the deletion of an 8.84-kb genomic segment in a proposed glycerol ABC transporter in the European isolate compared to the African strain (229). The deletion included the lipoprotein gene encoding LppB (228). Further *in vitro* studies suggested that it was the production of H_2O_2 that was responsible for the difference in virulence between the African and European strains (229), but this has yet to be shown *in vivo*. H_2O_2 is a by-product of glycerol metabolism, a common metabolic pathway in many bacterial species, and it is the premise of part of this work that in *Mcap* (as well as other mycoplasmas) this pathway has been utilized for virulence which may aid in the release of nutrients into the extracellular milieu.

This work has focused on identifying and characterizing lipoprotein genes and gene products from *Mmy*LC, *Mmycapri,* and *Mcap* to better understand the basis for disease causation in the *M. mycoides* cluster. Comparison of the contingency loci of these organisms will help determine the extent of intra- and interspecies variation, allowing for better diagnostic tools and aiding in the identification of potential vaccine candidates and/or disease determinants. In

addition, the groundwork has been laid to characterize the function of the lipoprotein LppB in virulence of *Mcap*. Understanding the role of LppB on the cell surface will enable a better understanding of virulence factors, and will be the first report of the function of a lipoprotein in this cluster. Together these studies will advance the knowledge of underlying molecular and virulence mechanisms in the pathogenic species that constitute the *M. mycoides* cluster.

Chapter 2 – Materials and Methods

Bacterial Strains, Plasmids, Media, and Growth conditions

Mycoplasma

Four mycoplasma strains were used in this study. *Mycoplasma mycoides* subsp. *mycoides* Large Colony strain GM12, *Mycoplasma mycoides* subsp. *mycoides* Large Colony strain GM684-13, and *Mycoplasma mycoides* subsp. *capri* type strain PG3 were gifts from Dr. Mary Brown (University of FL). *Mycoplasma capricolum* subsp. *capricolum* Kid (ATCC 27343) was obtained from the American Type Culture Collection (Manassas, VA). Wild-type strains were grown in modified Hayflick medium (242) (21 g PPLO broth [Difco Laboratories, Detroit, MI], 5 g D-glucose, 20% [v/v] heat-inactivated horse serum [Invitrogen, Grand Island, NY], 10% [v/v] yeast extract [Invitrogen], 4 ml 0.5% [w/v] phenol red [Sigma-Aldrich, St. Louis, MO] and 500,000 U penicillin G in a final volume of 1L, pH 7.8) or on modified Hayflick agar (Hayflick medium supplemented with 1% [w/v] Noble agar [Difco]) in a 37°C incubator. *lppB* Tn4001 transposon insertion mutant *Mycoplasma capricolum* subsp. *capricolum* 157 was provided courtesy of John Glass (J. Craig Venter Institute). It was grown in modified Hayflick medium

supplemented with 2 ug of tetracycline/ml at 37°C. Cultures were grown until a red to orange/yellow color change was observed in the medium indicative of acid production demonstrating bacterial growth.

Escherichia coli

E. coli strains DH10B and DH5α (Invitrogen, Carlsbad, CA) were used for initial plasmid transformations. *E. coli* strain Rosetta (Invitrogen) was used for all protein over-expression. *E. coli* was grown in Luria-Bertani (LB) (10g bacto-tryptone, 5 g yeast extract, 10 g NaCl in volume of 1 L, pH 7.5) broth overnight in a shaking 37°C incubator, or plated on LB agar and incubated overnight in a 37°C incubator supplemented with appropriate antibiotic for each plasmid vector. Plasmid vectors pZErO-2 (Invitrogen) (50 µg kanamycin/ ml), pET-20b (Novagen, Darmstadt, Germany) (100 µg ampicillin/ml), and pET-41a (Novagen) (50 µg kanamycin/ml) are all commercially available. pMAL-C2 (100 µg ampicillin/ml) was courtesy of Kim Wise (University of Missouri). The plasmid pMC03 was provided courtesy of Alain Blanchard (106).

Genomic and Plasmid DNA Preparation

Mycoplasma cultures were grown in 10 ml Hayflick medium until medium had changed from red to orange, then pelleted via centrifugation at 8,000 x g for

10 min. Genomic DNA was purified using the DNeasy Tissue Kit (QIAGEN, Valencia, CA) following the protocol for Gram-negative bacteria. DNA was eluted from the mini-prep column with 75 µl of double distilled H_2O (ddH_2O). *E. coli* cultures were started from a single colony grown overnight in 5 ml of LB broth, then recombinant plasmid purified using the Qiagen Mini-Prep Spin Kit (QIAGEN) according to the manufacturer's instructions.

Southern Blot Analysis

Genomic DNA was digested with EcoRI, HindIII, MunI, or BglII (Fermentas, Glen Burnie, MD) and separated via electrophoresis on a 0.7% (w/v) agarose gel. The gel was then submerged and shaken in i) depurination solution (250 mM HCl) for 10 min, ii) denaturing solution (0.5 N NaOH, 1.5 M NaCl) for 30-60 min, and iii) neutralization solution (1.0 M Tris-HCl pH 8.0, 1.5 M NaCl) for 30 min. DNA was then transferred via capillary transfer to a positively charged nylon membrane (Roche Diagnostics, Indianapolis, IN) and UV crosslinked using a UV Stratlinker 2400 (Stratagene, La Jolla, CA) on the auto setting. The membrane was incubated in pre-hybridization solution (5X SSC [diluted from 20X SSC (3 M NaCl, 0.3 M sodium citrate, 1 mM EDTA)], 1% [w/v] Blocking Reagent [Roche], 0.1% [w/v] N-lauroylsarcosine, 0.2% [w/v] sodium dodecyl sulfate [SDS]) for one hour at 55°C. A labeled oligonucleotide probe (Appendix I) was created using 10 pmol of primer, 1 mM digoxigenin-11-ddUTP (Roche) and the

Terminal Transferase Kit (Roche) according to the manufacturer's instructions. Ten µl (5 pmol) of labeled probe was added to 25 ml pre-hybridization solution and the membrane incubated at 55°C overnight. Two 5-min washes in 2X SSC 0.1% (w/v) SDS were followed by two 15 min washes in 0.5X SSC 0.1% (w/v) SDS, both at 55°C. The membrane was then equilibrated in maleic acid buffer (100 mM maleic acid, 150 mM NaCl, pH 7.5) plus 0.3% (v/v) Tween 20. The membrane was then blocked in maleic acid buffer with 1% (w/v) blocking reagent for 60 min at room temperature. The same blocking solution was used to incubate the membrane with Anti-DIG-alkaline phosphatase (Roche) at a 1:10,000 dilution for 30 min and then the membrane was washed two times, 15 min each, in maleic acid buffer plus 0.3% (v/v) Tween 20. The membrane was then equilibrated in detection buffer (100 mM Tris-HCl, 100 mM NaCl, pH 9.0) and detection was completed using 7.5 µl CDP-STAR (Roche) diluted in 1.5 ml detection buffer applied to membrane for 5 min. The membrane was then exposed to Kodak XAR-5 X-Ray film and developed using a Future 3000SV Automatic Film Processor (Fisher Scientific, Geneva, IL).

Colony Hybridization

Genomic DNA libraries were created as described above and plated out on LB agar plates supplemented with 50 µg kanamycin/ml. Plates were incubated overnight at 37°C, and then placed in refrigerator for 1 hour. One Nylon Membrane for Plaque and Colony Hybridization (Roche) was prepared for

each plate by cutting small notches for realignment with plate after completion of blot. The membranes were then laid on the plate surface for 5 min at room temperature; during this time marks were made on plate surface to aid in membrane alignment. Each membrane was then lifted off the plate and set colony side up on chromatography paper that had been saturated in denaturation solution (0.5 N NaOH, 1.5 M NaCl)) for 15 min. The membranes were then transferred to new chromatography paper saturated in neutralization solution (1 M Tris-HCl pH 7.5, 1.5 M NaCl) colony side up, for 5 min. Lastly the membranes were placed, colony side up, on chromatography paper saturated with 2X SSC for 15 min and then crosslinked as described above for Southern hybridization. Membranes were washed in 3X SSC 0.1% (w/v) SDS for 1 h at 68°C in a shaking water bath. The colony side of the membrane was then gently wiped with a Kimwipe to remove all cellular debris. Next, membranes were sealed in a bag, then 15 ml of pre-hybridization solution (12.5 ml 20X SSC, 1 ml 10% [w/v] SDS, 0.5 ml 10% [w/v] N-lauroylsarcosine, 5 ml 10% [w/v] blocking reagent) was added and incubated 1 h at 50°C in a shaking water bath. The used solution was then emptied and replace with 15 ml of fresh pre-hybridization solution, then 50 pmol of dig-labeled oligonucleotide probe was added and the membranes were incubated at 50°C overnight. Washes were done twice for 5 min using 2X SSC, 0.5% (w/v) SDS and twice for 15 min using 2X SSC, 0.1% (w/v) SDS, all at 50° C. The membranes were then washed for 1 min at room temperature in maleic acid buffer plus 0.3% (v/v) Tween 20. Blocking of the membranes was done with a 10% (v/v) solution of 10% (w/v) blocking solution in maleic acid buffer at room

temperature for 1 h. Anti-digoxigenin-AP-Fab Fragments (Roche) were diluted 1:5000 in blocking solution, added to the membranes, and incubated for 30 min at room temperature. The membranes were then washed twice in maleic acid buffer with 0.3% (v/v) Tween 20 for 15 m at room temperature. Alkaline phosphate buffer was used to equilibrate the membranes for 2 min at room temperature and then probes detected with 200 µl of NBT/BCIP (Roche) in 10 ml of alkaline phosphate buffer. Containers with membranes were kept in the dark until developed.

PCR

General PCR

All PCR was performed with a 200 model Peltier Thermal Cycler (T & M) or Eppendorf Mastercycler Gradient Thermal Cycler (Fisher Scientific). PCR was carried out using the Expand Long Template PCR System (Roche Applied Science) or Phusion Polymerase (Finnzyme) except where noted. Expand Long Template (Roche) reactions consisted of 0.2 µM of forward and reverse primers, 10-20 ng DNA, 3.75 U Expand Long Template Enzyme Mix, 200 µM deoxynucleoside triphosphates (dNTPs) and 1X Expand Long Template Buffer 3 (2.75 mM $MgCl_2$). The cycling conditions were as follows; one cycle at 94°C for 30 s, 54°C for 15 s, 68°C for 1-6 min; 30 rounds at 94°C for 30 s, 54°C for 15 s, and 68°C for 1-6 min plus 10 seconds per round. Phusion (Finnzyme) reactions

contained 0.2 µM of forward and reverse primers, 10-20 ng DNA, 1 U Phusion Polymerase, 200 µM dNTPs, and 1X Phusion FX Buffer. The cycling conditions were as follows: one cycle at 98°C for 1 min, 35 10-s cycles at 89°C, 54°C for 20 s, extension at 72°C for 30s/1kb of predicted amplicon length, and one cycle at 72°C for 10 min. Amplicons were purified using the QIAquick PCR Purification Kit (QIAGEN), eluted with ddiH_2O, and size was determined by resolving 3 µl of the PCR product on a 0.7% (w/v) agarose gel and comparing bands of DNA to a 1-kb DNA Ladder (Promega, Madison, WI). DNA concentration was determined using a NanoDrop Spectrophotometer 2000C (Thermo Fisher Scientific, Wilmington, DE).

Inverse PCR

Inverse PCR was performed as follows: 300 ng of genomic DNA was digested with an appropriate restriction endonuclease for 1 h at 37°C and heat-inactivated at 80°C for 20 min. Seven µl (43 ng) of digested DNA was added to 1 µl 10X ligase buffer, 1 µl T4 DNA Ligase (Invitrogen), and 1 µl ddH_2O and incubated overnight at 16°C. Oligonucleotide primers were designed to amplify outwards from the known sequence. PCR was then performed using the aforementioned Phusion protocol.

Multiple Mutation Reaction (MMR) PCR

Mutagenic primers were designed to have a melting temperature at least 4 degrees higher than external primers with the desired mutation near the middle of the oligonucleotide sequence. The master mix consists of 1x MMR buffer (20 mM Tris-HCl pH 8.5, 3 mM $MgCl_2$, 50 mM KCl, 0.5 mg/ml BSA, 0.5 mM NAD^+), 10 pmol of each oligonucleotide, 100 ng DNA, 1 U *Pfu* polymerase, 3 U Ampligase (Epicentre, Madison, WI). The cycle program, one cycle 95°C for 5 min, 26 cycles of (95°C for 30 s, 57°C for 30 s, 65°C for 6 min), was performed in the Eppendorf Mastercycler Gradient Thermal Cycler. Amplicons were separated by agarose gel electrophoresis, purified, and then sequenced to ensure the desired mutation was obtained.

DNA Cloning and Sequencing

For genomic DNA libraries, DNA and vector pZErO-2 (Invitrogen) were digested with EcoRI, HindIII, MunI, or BamHI (Fermentas). Digested DNA was then ligated into the appropriately linearized vector at 16°C overnight (500 ng DNA, 100 ng pZErO-2, 1 µl 10X ligase buffer, 1 µl T4 DNA Ligase (Invitrogen), and 1 µl ddH_2O). The ligation mixture was transformed into *E. coli* DH10B or DH5α cells (Invitrogen) via heat shock (30 min on ice, 45 s at 42°C, 2 min ice),

recovered in 1 ml SOC medium (Invitrogen) for 1 hour, then plated on LB agar plates containing 25 µg of kanamycin per ml agar. Colonies were expanded in LB medium containing 25 µg of kanamycin per ml overnight and plasmid DNA isolated as above. Inserts were sequenced using M13- and SP6-based vector primers provided with the pZErO-2 kit (Invitrogen). DNA primer walking was used to obtain the remaining sequence in each insert. To amplify regions from unsequenced strains, oligonucleotides based on the conserved flanking housekeeping genes were designed and used as primers (Appendix I) in a PCR reaction using either Expand Long Template or Phusion protocols. Amplicons were purified using the QIAquick PCR purification kit (QIAGEN) and sequenced using the amplifying primers. Primer walking was used to obtain the remaining sequence of each insert.

DNA sequencing of PCR amplicons and recombinant constructs was completed at the DNA Core Facility at the University of Missouri-Columbia, using BigDye Terminator chemistry on a PE Biosystems 3730 capillary DNA sequencer (Applied Biosystems, Foster City, CA).

Sequence analysis and comparisons

Initial analysis, assembly and DNA comparisons of *Mmy*LC GM12, *Mmy*LC GM648-13, and *Mcapri* were performed using the GCG Software Package (Genetics Computer Group, Madison, WI). Sequence comparisons

between *Mmy*SC and *Mcap* genomes and generated sequence data were performed with BLASTn available through the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov). Open reading frames were called based on BLASTn results, and later confirmed using Geneious software available at http://www.geneious.com (54). Protein alignments shown in this work were carried out using the Geneious Alignment program at default parameters, and DNA alignments were done utilizing the Mauve plug-in for Geneious at default parameters.

Antibody Production

For the production and isolation of polyclonal antibodies used to detect VlcA, VlcB and VlcC, first each gene was amplified using the Expand Long Template (Roche) protocol. Each oligonucleotide was engineered to contain BamHI or HindIII restriction endonuclease sites for directional cloning into the pMALC-2 vector. Each purified amplicon was digested with BamHI and HindIII for 30 min and the pMALC-2 vector was digested for 1 hour at 37°C. The DNA was purified using the QIAquick PCR Purification Kit (QIAGEN), then ligated into the pMALC-2 vector at 16°C overnight using T4 DNA Ligase (Invitrogen). The ligation mix was then transformed into *E. coli* strain DH10B, transformants picked and expanded overnight, and plasmid was purified, then sequenced to ensure

correct sequence. Cultures were grown in Rich Medium + glucose (10 g tryptone, 5 g yeast extract, 5 g NaCl, 2 g glucose in total volume of 1 L) supplemented with 100 µg ampicillin/ml medium to an OD_{600} of 0.5. Induction of protein expression was performed using isopropylthiogalactoside (IPTG) to induce the *lac* promoter at a final concentration of 0.3 mM and incubation continued at 37°C for 2 hours. Samples were taken of pre-induction and post-induction cultures and analyzed via 10% (w/v) SDS-PAGE analysis. Once induction was verified, cells were lysed using B-Per Protein Extraction Agent (Pierce Thermo-Scientific, Rockford, IL). Protein purification was performed according to New England Biolabs suggested protocol. Briefly, 15 ml of amylose resin was packed into a 2.5 x 10 cm column and set up for gravity flow of lysed cells. Column was equilibrated with 8 volumes of column buffer (20 mM Tris-HCl pH 7.5, 200 mM NaCl, 1 mM EDTA), then crude extract was allowed to flow over the column at a rate of approximately 1 ml/min. The column was then washed with 12 volumes of column buffer to remove any non-bound protein. Elution was performed with column buffer plus 10 mM maltose. Three ml fractions were collected and analyzed for protein content via dot blot and amido black staining. Mouse injections, anti-sera collection, and enzyme-linked immunosorbent assay (ELISA) screening of polyclonal antibodies were performed by Mark Foecking (University of Missouri).

A synthetic peptide was used to produce monoclonal antibodies to VlcH. The peptide sequence GDKSQQKAPDKKDEKKDIEKSKKEE was prepared on a model 432A peptide synthesizer (Applied Biosystems) using standard 9-

fluorenylmethylcarbonyl protection chemistry by Fabio Gallazi (University of Missouri). Murine monoclonal antibodies were produced and isolated at the University of Missouri-Columbia Cell and Immunology Core Facility and screened via ELISA (240). Briefly, peptides were conjugated with N-[6-(biotinamido)hexyl]-3-(2-pyridyldithio)propionamide (biotin-HPDP), immobilized on microtiter plates that were coated with avidin, and then incubated successively with monoclonal antibody, secondary horseradish peroxidase (HP)-conjugated antibody, and 2,2-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) to quantify binding that was monitored by optical density at 410 nm (OD410).

Protein Over-Expression

Cloning into Protein Expression Vectors

Amplicons from the *vlcA, vlcB,* and *vlcC* genes cloned into pMALC-2 were used for cloning into the pET-41a vector. Similar primers were designed for the amplification of *vlcH*, containing BamHI and HindIII sites for directional cloning. *lppB* primers were designed for directional cloning into pET-20b, pET-41a, and pMALC-2. Amplicons and vector were digested and ligated as described above. Constructs were transformed into cells of the *E. coli* Rosetta (Invitrogen) expression strain using heat shock, and plated on LB agar plates supplemented with 25 µg kanamycin/ml. Plasmid DNA was collected and sequenced to ensure that no mutations were present. Cultures were grown in LB at 37°C to an OD_{600} of 0.6. Induction was performed using IPTG at a final concentration of 0.4 mM

and incubation continued at 37°C for 2 hours. Samples were taken of pre-induction and post-induction cultures and analyzed via SDS-PAGE analysis. Duplicate gels were also run and stained using Coomassie blue to visualize total protein. Staining was performed by fixing the gel (25% [v/v] isopropanol, 10 % [v/v] glacial acetic acid in ddH_2O) for 30 min, then staining with Coomassie blue (50 ml glacial acetic acid, 120 mg Coomassie blue in total volume of 500 ml) for 1 h. Destaining was achieved by washing the gel in 10% (v/v) glacial acetic acid until desired staining was achieved.

Auto-induction for Protein Expression

Auto-induction was performed as previously described by Studier *et al*. (209). Two to three bacterial colonies containing the appropriate cloned gene were inoculated into 250 ml of complex medium (1% [w/v] tryptone, 0.5% [w/v] yeast extract, 25 mM Na_2HPO_4, 25 mM KH_2PO_4, 50 mM NH_4Cl, 5 mM Na_2SO_4, 2 mM $MgSO_4$, 10 µM Fe^{+3}, 54 mM glycerol, 2.8 mM glucose, 5.6 mM α-lactose). Cultures were incubated overnight with shaking at 37°C, then cells were lysed using a French Press (15000 psi) and protein analyzed as described below in Western Blot Analysis.

Protein Expression Analysis

Colony Immunoblotting

For colony immunoblots, approximately 10^3 mycoplasma cells were plated on Hayflick agar and allowed to grow until colonies became visible to the naked eye, usually 48-72 hours. Plates were then refrigerated for at least one hour. A nitrocellulose membrane was laid on top of the colonies and allowed to sit for 5 min. The membrane was then lifted and incubated in blocking medium (3% [w/v] bovine serum albumin in phosphate buffered saline [PBS]). Anti-sera or monoclonal antibody was then diluted 1:500 in PBS, and the membrane incubated at 4°C overnight. The membrane was washed three times for 5 min with PBS, and then incubated with a horseradish peroxidase-conjugated goat mouse-specific antibody (MP Biomedicals, Durham, NC) for 2 hours at room temperature. The membrane was again washed three times for 5 min with PBS and developed by incubating membrane with detection agent (12 mg 4-chloro-1-napthol dissolved in 4 ml methanol, 20 ml PBS, 20 µl 30% [v/v] H_2O_2). The reaction was halted by washing in ddiH_2O after color development, usually 2-10 min. Counterstaining was performed using Ponceau-S (0.1% [w/v] Ponceau-S in 5% [v/v] acetic acid) which non-specifically stains protein to allow visualization of colonies that did not bind antibody on the cell surface.

Western Blot Analysis

Protein analysis was performed on either total cellular protein or on Triton X-114 phase-extracted protein samples. For total cellular protein of mycoplasma, cells from 3-6 ml of culture were pelleted, and for *E. coli,* cells from 1.5-3 ml of culture were pelleted. The cell pellet was resuspended in 2X SDS-PAGE loading buffer (1.25 ml 1 M Tris HCl pH 6.8, 4 ml 10% [v/v] SDS, 2 ml glycerol, 200 µl β-mercaptoethanol, 0.02 mg bromophenol blue), and boiled for 5 min. Ten µl of protein samples was loaded into each well of a 10-12% (w/v) SDS-PAGE gel and run at 100 V for 2.5 hours in Tris-Glycine Buffer (25 mM Tris, 250 mM glycine, 0.1% [w/v] SDS, pH 8.3). Protein was then transferred to a nitrocellulose membrane at 25 V for 2 hours using the X-Cell II Blot Module (Invitrogen) and incubated in blocking solution (3% [w/v] BSA in PBS) at 4°C overnight. The membrane was washed 3 times for 5 min each in PBS, then incubated with the primary antibody or anti-serum overnight at 4°C. The membrane was then washed, 3 times for 5 min in PBS, and incubated in the HRP-conjugated goat anti-mouse-specific antibody (Imgenex, San Diego, CA) (1:500 dilution in 3% [w/v] BSA in PBS) for 2 hours at room temperature. The wash was again repeated, and the blot developed as described above for the colony immunoblot.

Triton-Phase Extraction

Triton X-114 extractions were performed using 10 ml of mycoplasma culture, and all reactions carried out in a 1.5 ml microcentrifuge tube. Mycoplasma culture was centrifuged at 6000 x g for 10 min at 4°C. The cell pellet was washed 3 times in 10 ml of cold PBS and resuspended in 900 µl of cold PBS. One hundred µl of 10% (v/v) Triton X-114 in PBS was added, the mixture incubated at 4°C with gentle mixing for 30 min, and then pelleted via centrifugation at 12,000 x g for 3 min at 4°C. Supernatant was then removed, incubated at 37°C 5 min and again centrifuged at 12,000 x g for 3 min at 4°C. The upper aqueous (AQ) phase was then transferred to a new tube and washed as follows: 10% (v/v) TX-114 was added to the AQ phase to a final concentration of 1%, vortexed, incubated on ice for 5 min, incubated at 37°C for 5 min, centrifuged for 3 min at room temperature, and upper AQ phase transferred to a new tube. This was repeated a total of three times and the final AQ phase was analyzed via Western blot. The TX-114 pellet was washed 3 times as follows: 900 µl of PBS was added to pellet, vortexed, incubated on ice for 5 min, incubated at 37°C for 5 min, centrifuged for 3 min at room temperature, and the supernatant discarded. After the last wash, the TX-114-extracted pellet was resuspended in 100 µl of 2X SDS-PAGE loading buffer and analyzed by Western blot analysis.

RNA Purification and Reverse-Transcriptase PCR

Mycoplasma cultures were grown in 10 ml of Hayflick medium until color had changed from red to orange. Cells from three to six ml of culture were then pelleted in 1.5 ml RNase-free microcentrifuge tubes at 6000 x g for 10 min. RNA extraction was performed using the RNeasy Kit (QIAGEN) per the manufacturer's instructions. All steps were performed using RNase-free disposable plasticware and reagents. RNA was aliquoted and stored at -80°C until use.

For reverse transcriptase experiments, total RNA from mycoplasma cells was used. 50 ng of RNA was incubated with 20 pmol of primer in a total volume of 5 µl at 70°C for 5 min. This was then incubated on ice for 5 min, and kept on ice until added to the reverse transcription reaction. A master mix was created using 6.1 µl RNase-free H_2O, 4 µl RT buffer, 1.5 mM $MgCl_2$, 10 mM dNTPs, 1 U RNase inhibitor, and 1 µl ImPrompt-II reverse transcriptase (Promega) per sample. All experiments included a sample with no reverse transcriptase to rule out DNA contamination of RNA. Fifteen µl of master mix was added to 5 µl of RNA/primer mix and the extension reaction was carried out on an Eppendorf Mastercycler Gradient Thermal Cycler as follows: 70°C for 5 min, 4°C for 5 min, 25°C for 5 min, 42°C for 60 min, 70°C for 15 min. Product was then amplified via PCR as described previously and visualized via agarose gel electrophoresis.

RNase Protection Assay

Probes for RNase protection assays (RPA) were synthesized using the MaxiScript Kit (Ambion, Foster City, CA). Primers (Appendix I) were designed so that a T7 promoter would be present on the strand complementary to the RNA of interest and then amplified via PCR using the Phusion protocol. Each transcription reaction included 1 µg template DNA, 1X provided buffer, 10 mM each of ATP, CTP and GTP, 6 mM UTP, 4 mM biotinylated UTP, and 1 U T7 RNA polymerase. The mixture was incubated at 37°C for 1 hour, and then DNase treated using the TURBO DNase Kit (Ambion). Probes were separated on a 6% (w/v) PAGE-UREA gel running at 200 V for 30 min. Bands were visualized via UV shadowing, excised from the gel, and eluted in the buffer provided with the kit at 37°C overnight. All probes were stored at -20°C.

Positive control transcripts were made to ensure the assays were working correctly. Primers were designed to amplify a region of DNA directly upstream of the AUG start codon for both *gtsA* and *lppB.* A T7 promoter sequence was added to the strand opposite that used for the probes to create a complementary RNA strand that would hybridize to each probe. *In vitro* transcription was performed as above, except there was no biotinylated-UTP added to the reaction mix and that the RNA was not gel purified.

The RPA was completed using the RPA III Kit (Pierce). Reactions contained 1-50 µg of total RNA from *Mcap*, and a 2x molar excess of probe.

Control reactions were set up using yeast RNA from the kit (T7 MaxiScript [Ambion]) or positive control RNA from T7 *in vitro* transcription. Probes and RNA were co-precipitated with 0.5 M NH_4OAc (final concentration), and then 2.5 volumes of 100% ethanol were added and mixed thoroughly. Tubes were incubated at -20°C for 15 min, centrifuged for 15 min at 4°C at 8,000 x g, then the supernatant was removed and discarded. Ten µl of Hybridization Buffer was added to the pellet, tubes were vortexed briefly, incubated at 95°C for 4 min, then hybridized overnight at 42°C. The probe/RNA mixture was subjected to RNase A/T1 (1 U) digestion at 37°C for 30 min (one yeast RNA sample did not have RNase added). Probe/RNA hybrids were precipitated using provided buffer, centrifuged at 4°C for 15 min at 8,000 x g, and the supernatant was removed and discarded. Pellets were resuspended in gel loading buffer and separated at 180 V for 30 min on a 6% (w/v) TBE-UREA PAGE gel (Invitrogen) alongside 1 µl of BrightStar Biotinylated RNA marker (Ambion). RNA was transferred to a positively-charged nylon membrane using the X-Cell Blot Module (Invitrogen) at 100 V for 30 min, then UV crosslinked at 120 mJ/cm^2.

Detection of the RPA was carried out using the RPA III SuperSignal Chemiluminescent Detection Kit (Pierce). Briefly, the membrane was incubated in Blocking Buffer for 15 min, then in Conjugate Solution containing strepavidin for 20 min. The membrane was washed four times in provided Wash Buffer, equilibrated in provided buffer, and then covered with the detection substrate solution for 5 min. Bands were detected by exposing the membrane to Kodak

XAR-5 X-Ray film and developed using a Future 3000SV Automatic Film Processor (Fisher Scientific).

Mycoplasma Transformation Using Electroporation

Mcap or *Mmy*LC GM12 cells were grown in 30 ml of Hayflick medium until the color change from red to yellow-orange had occurred. Cells were pelleted at 8,000 x g for 7 min at 4°C, then washed three times in 3 ml of freshly prepared HEPES-sucrose Buffer (0.28 M glucose, 8 mM HEPES, pH 7.4). After the last wash, the cell pellet was resuspended in 1 ml HEPES-sucrose buffer, 400 µl transferred into UV-sterilized Molecular BioProducts electroporation cuvettes (2 mm gap) and 5-20 µg of plasmid DNA was added. A BioRad Gene Pulser, Pulse Control, and Capacitance Extender units were utilized for electroporation. The settings used for electroporations were 2.0 KV, 25 µF, and 200 Ω. After electroporation, cells were resuspended in 600 µl 37°C Hayflick medium and allowed to recover for 2-4 hours at 37°C. Cells were then plated on Hayflick agar supplemented with 2 µg tetracycline/ml and incubated at 37°C. Colonies of pMCO3 positive control transformants were usually seen within 2-5 days.

Construction of *lppB* Cassettes

lppB Deletion Cassette

Primers (Appendix I) were designed to amplify 1 kb of DNA upstream and downstream of *lppB*, including the start and stop codon of the ORF. The primers also contained cloning sites for directional cloning into the pMCO3 vector, as well as PstI sites for the cloning of the *tet* resistance gene into the construct. Primers (Appendix I) were also designed to amplify the *tet* gene from pMCO3 and add PstI sites to either flank. The spiralin promoter was upstream of the gene to allow for expression in mycoplasma species, so directional cloning of the *tet* gene was not of issue. Amplicons were purified and digested as previously described, then ligated into the digested pMCO3 vector as previously described. Recombinant vector was transformed into *E. coli* DH10B using heat shock, and transformants selected on LB agar plates containing 10 µg tetracycline/ml. Colonies were selected and expanded in LB broth supplemented with 10 µg tetracycline/ml overnight at 37°C. Plasmids were purified and sequenced to ensure correctness.

lppB Complementation Cassette

Primers (Appendix I) were designed to amplify the synthetic *lppB* gene for cloning into pMCO3. Splice overlap extension PCR (SOE PCR) (196) was used to add the spiralin promoter upstream of both *lppB* and the gentamicin gene

(amplified from the pGP353 vector (69)). Primers were designed to have a 15 to 20-nucleotide overlap on each fragment at the overlap site. Amplicons were separated on a 0.7% (w/v) low melt agarose gel and then excised. The first step of the reaction is a primerless reaction, containing 28.5 µl ddH_2O, 10 µl HF Buffer (Phusion), 10 mM dNTPs, 5 µl of each gel slice to be joined, 0.5 µl Phusion enzyme run on the following program: 94°C 2 min and 10 cycles of (94°C 15s, 56°C 20 s, 72°C 1 min). When that program had finished, 15 pmol of each outside primer, and another 0.5 µl of Phusion enzyme were added and the spliced product amplified as follows: 94°C 2 min and then 35 cycles of (94°C 15 s, 62°C 20 s, 72°C 2.5 min) and a final extension at 72°C for 3 min. The amplicon was then purified and used in subsequent cloning steps. PstI sites were included in the primers designed for final amplification, and cloning performed as described previously. Gentamicin resistant colonies were picked and expanded in LB broth, recombinant plasmid purified, and sequenced for accuracy.

Chapter 3 – *M. mycoides* Cluster Contingency Loci Comparisons

Introduction

The bacteria in the *M. mycoides* cluster are genetically and antigenically closely related. Homology between conserved housekeeping genes ranges between 80-97% identity (data not shown), and a high degree of synteny is seen between the complete genome sequences of *Mmy*SC strain PG1 and *Mcap* strain Kid (240). Alignment of the *dnaA* region of *Mmy*SC, *Mcap*, and *Mmy*LC was completed to illustrate the conserved nature of the gene organization and high homology of DNA sequence in these bacteria (Fig 3-1). Rapid diagnostic tests for the species in this cluster are difficult due to the high degree of cross-reactivity of antibodies (40,167) and the high DNA sequence conservation complicates PCR identification (39,166). Despite this close relatedness, there is a difference in host specificity and disease severity caused by the organisms in the cluster. It is not feasible to use genetic approaches in the host animal, necessitating the investigation of the genomic makeup of the *M. mycoides* cluster members. It is anticipated that these comparisons will disclose determinants that are responsible for host specificity and disease severity.

The diseases caused by infection with the mycoplasma in the cluster are not only serious, but economically important on a global scale. Contagious

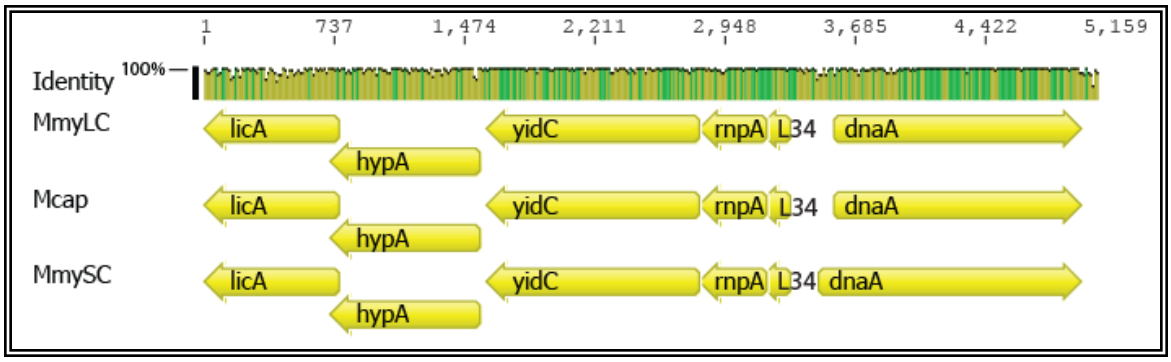


Figure 3-1. Alignment of *dnaA* Region of 3 Mycoplasma Strains. A DNA alignment of *Mmy*LC strain GM12, *Mcap,* and *Mmy*SC is shown. This is an example of the high level of conservation in DNA sequence as well as gene synteny found in members of the *M. mycoides* cluster. Yellow arrows represent open reading frames; green bars signify 100% identity, olive bars signify percent identity less than 100% (represented by height of the bar). Numbers are nucleotide coordinates.

Bovine Pleuropneumonia and Contagious Caprine Pleuropneumonia, caused by *Mmy*SC and *Mccp* respectively, are responsible for the loss of millions of dollars a year in Sub-Saharan African countries. Contagious Agalactia leads to the loss of over $30 million in European countries surrounding the Mediterranean alone (153). These losses represent not only loss of livestock, but also loss of food, milk, and skins as well as losses due to restricted trade as a result of control measures in response to infection.

The importance of the diseases caused by members of the *M. mycoides* cluster mandates a better understanding of the pathogenesis of these bacteria. Over the past decade, multiple comparisons of the mycoplasma in this group have been performed. A large scale comparison of protein profiles via one dimensional SDS-PAGE confirmed the taxonomic relationships of the *M. mycoides* cluster described previously (73). It was found that a genetic deletion in a putative glycerol uptake system was the notable difference between the highly virulent African strain Afadé and the less virulent European isolate L2 (228). Multiple analyses have been performed looking for homologues of proteins that have been identified on, or are predicted to localize to the cell surface, especially lipoproteins. Polyclonal antibodies to a major 72-kDa lipoprotein antigen (P72) in *Mmy*SC (36) led to the identification and characterization of P67 of *Mycoplasma* sp. Bovine Group 7 (64), and a DNA probe specific for *p72*, the gene encoding LppA, identified the LppA genes via Southern hybridization in both *Mmy*LC and *Mmycapri* (140). LppB, which will be discussed in more detail in Chapter 3, has also been identified in multiple species of the *M. mycoides*

cluster, including *Mmy*SC and *Mmy*LC (228). A third lipoprotein, LppC, was identified in *Mmy*SC, and while Southern hybridization showed the presence of similar DNA in *Mmy*LC, *Mmycapri,* and *M*BG7, antibodies directed against LppC did not recognize proteins in any species other than *Mmy*SC (167). Western blots performed with antibodies to the C-terminal portion of the *Mmy*SC LppQ protein recognized proteins in *M*BG7, *Mmycapri,* and *Mccp* (2). The aforementioned comparisons of the lipoprotein repertoire in the *M. mycoides* cluster have all reinforced the idea of similarity between these bacteria, whilst only finding a few differences that may have little utility for diagnostic purposes.

The research presented herein focused on *Mmy*LC and *Mmycapri*. As detailed above, the two lipoprotein genes identified thus far in *Mmy*LC and *Mcapri* have been shown to be present in multiple cluster members. This made the report on the cluster distribution of the phase-variable *vmm* gene of particular interest (164). A probe to *vmm* hybridized to DNA from all members of the *M. mycoides* cluster except *Mmy*LC type strain Y-goat and *Mmycapri* type strain PG3 (164). This suggested a possible way in which to differentiate these two bacterial groups from other members of the cluster. The absence of a *vmm* homologue in both of these groups also raised the question of whether phase-variable proteins would be present in *Mmy*LC and *Mcapri*, and if so, how different are they from other members of the cluster? The completed genome sequences of *Mmy*SC strain PG1 and *Mcap* strain kid, which became available at the initiation of this project, provided the foundation to examine the genetic makeup of *Mmy*LC and *Mmycapri* in a more comprehensive and efficient way.

With the availability of two complete genome sequences, global comparisons of the chromosome became possible. The comparisons of the *Mmy*SC PG1 and *Mcap* kid genomes revealed several pertinent features. As mentioned previously, there is a high degree of gene synteny, with the genes present in one chromosome present in the same relative location in the second. There is also high homology found between the DNA sequence, however several regions of diversity were apparent, two of which have been previously described (240). As expected from previous hybridization data (164), comparisons confirmed that there was a lipoprotein gene in *Mcap* that showed homology to *vmm* (240). Further examination of the genome revealed that several lipoprotein genes were present that shared homology with *vmm,* termed the *vmc*s. These genes are also capable of undergoing phase-variation by a similar mechanism to *vmm* (240). Despite the similarities, there were differences in the ORFs of the lipoproteins in the *vmc* loci, including the ability of several of the genes in *Mcap* to undergo size variation (240). Closer examination of the chromosomal alignment revealed the existence of several contingency loci (loci that contain phase-variable genes that can change the surface protein repertoire to increase the likelihood of survival of any given contingency) encoding putatively phase-variable lipoprotein genes (Fig 3-2). The regions of variability were flanked by highly conserved housekeeping genes in both the *Mmy*SC and *Mcap* genomes. This enabled the design of probes to hybridize to *Mmy*LC and *Mmycapri* DNA. The conservation in the flanking regions between *Mmy*SC and *Mcap* also brought about the hypothesis that contingency loci

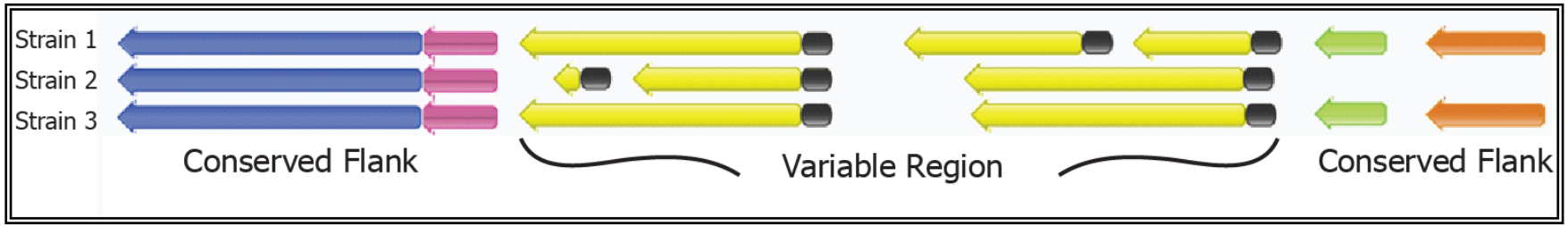


Figure 3-2. Schematic Representation of Contingency Locus Alignment in *M. mycoides* cluster. These loci are flanked by conserved housekeeping genes (blue, pink, green, orange arrows). The yellow arrows represent putative lipoproteins that show potential to phase vary via a simple TA dinucleotide repeat in the promoter regions (black ovals).

may be present in corresponding regions of the chromosome in some, if not all, members of the *M. mycoides* cluster, including *Mmy*LC and *Mmycapri*.

This work was undertaken to determine the presence and extent of variation in the poly[TA] tract contingency loci of *Mmy*LC GM12, *Mmy*LC GM648-13, and *Mmycapri*. At the time this study was undertaken, there were only two lipoprotein genes identified in *Mmy*LC and *Mmycapri*, LppA and LppC both with significant homology to lipoproteins identified in other members of the *M. mycoides* cluster (140,228). Since there was no hybridization of the *vmm* probe (164), it was not known: i) if there were contingency loci in the genomes of *Mmy*LC or *Mmycapri*? ii) if these loci did exist, was phase-variation controlled via a poly[TA] tract? and iii) how divergent were the genes present in these loci?.

Results

Framework of the Contingency Loci

The five contingency loci in this study were identified and characterized from *Mmy*LC strain GM12, *Mmy*LC strain GM648-13, and *Mcapri* strain PG3 using either PCR or genomic DNA libraries coupled with DNA sequencing as described in Materials and Methods (pg. 31-34). Primers used for these experiments are listed in Appendix I. The *Mmy*LC isolates used in this study are two available strains isolated in the United States, and PG3 is the type strain of

Mmycapri. Oligonucleotides were designed based on short identical sequences found within the conserved housekeeping genes flanking each locus identified by comparison of the complete genomes of *Mmy*SC and *Mcap*kid. After these data had been generated, a shotgun sequence of *Mmy*LC GM12 became available (02/07). All previously obtained sequences were compared to the sequence available through NCBI, and were found to be 100% identical.

Five contingency loci were identified in *Mmy*LC GM12 and *Mmycapri.* Sequence analysis revealed a conserved framework for these contingency loci in all five mycoplasma strains examined, with a few exceptions that are discussed below. The loci are flanked by conserved housekeeping or conserved hypothetical genes exhibiting 87.8 to 98% sequence identity at the DNA and protein level (Fig. 3-3). Comparisons showed that there was no conservation of DNA sequences, except for the promoter regions, in the contingency locus intergenic regions. The flanking gene ORFs represent the boundaries of each contingency locus and the loci have been named according to one of the flanking housekeeping genes: SgaT for the ascorbate-specific PTS system enzyme IIC/IIB showing homology to the SgaT permease of *Streptococcus suis*, Gap for glyceraldehyde-3-phosphate dehydrogenase, S21 for the 30S ribosomal protein S21, LicA for the phosphotransferase system lichenan-specific IIA component homologue, and Nag for the putative N-acetylglucosamine transporter component.

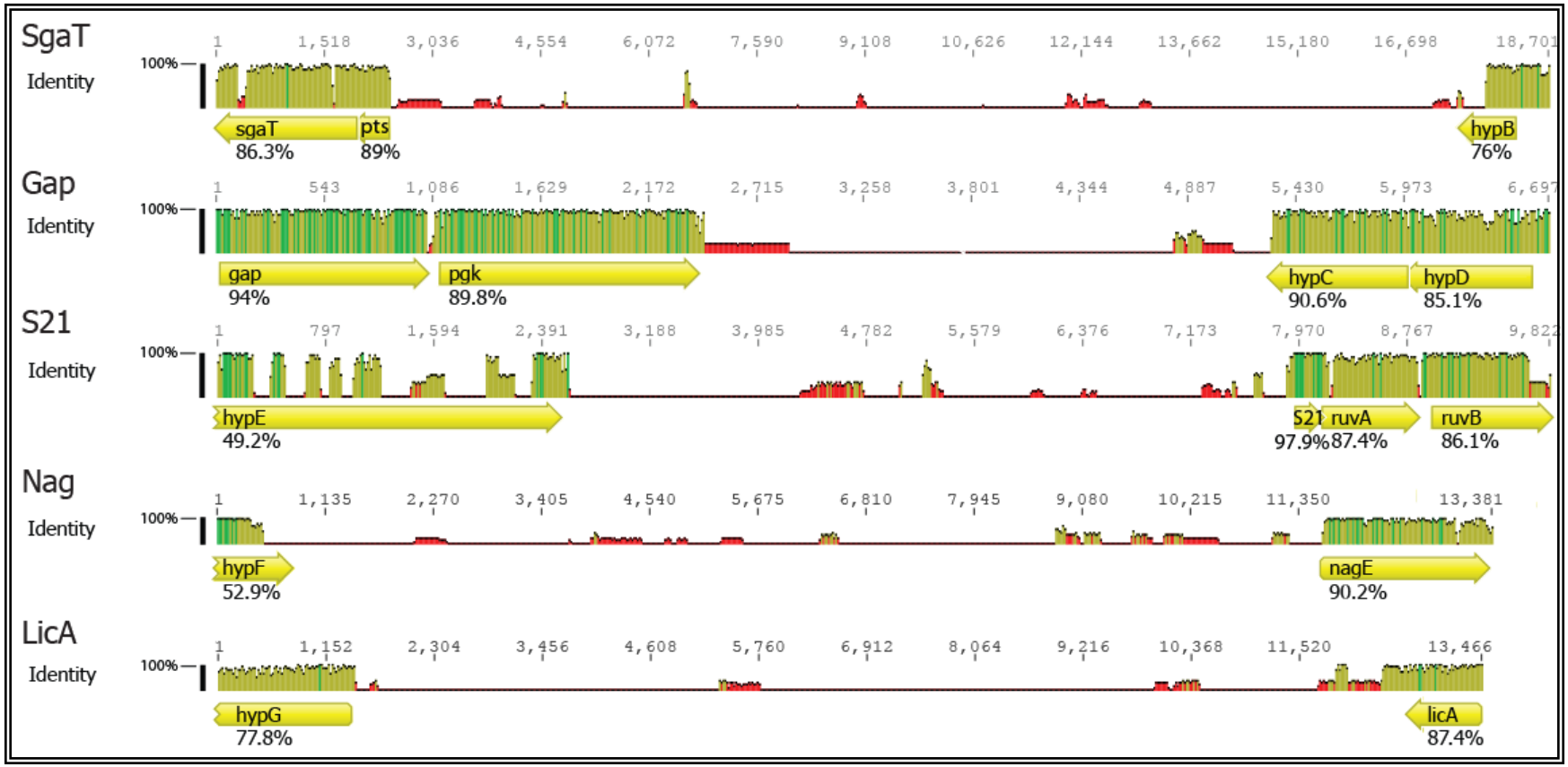
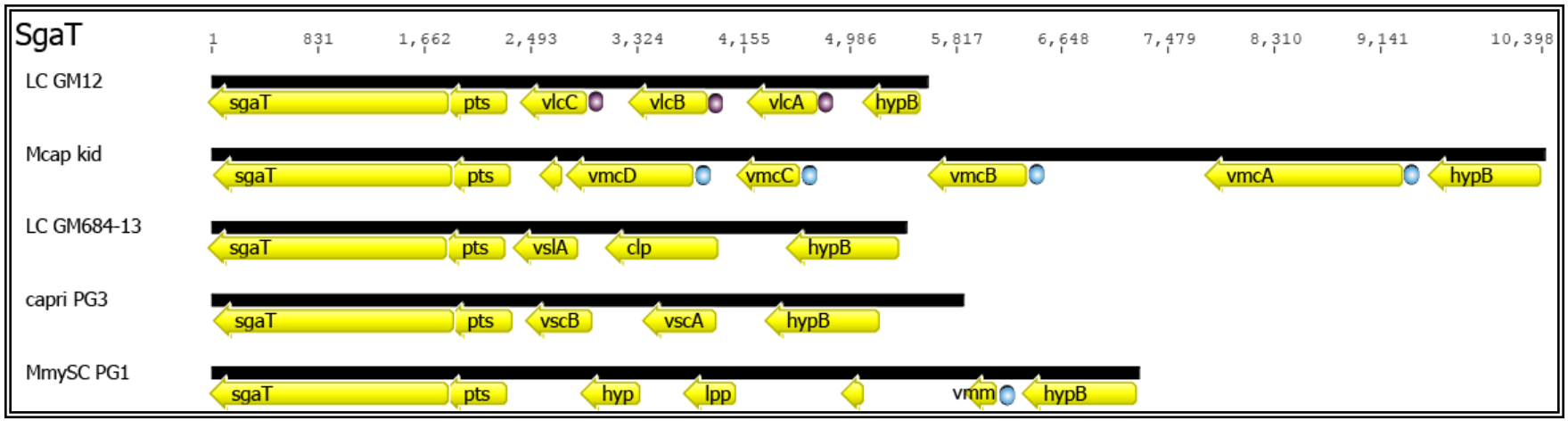
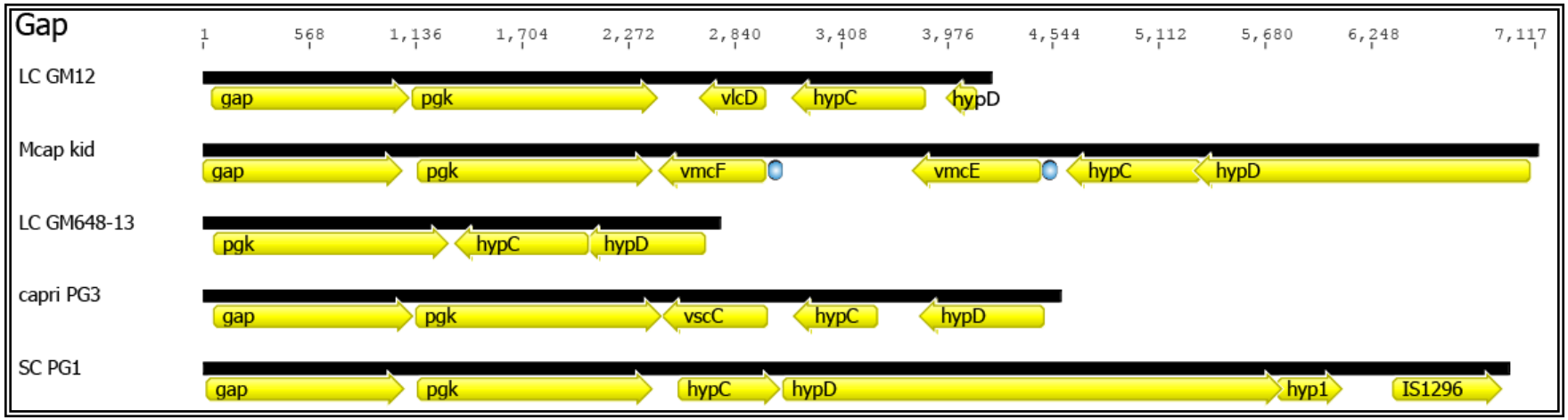


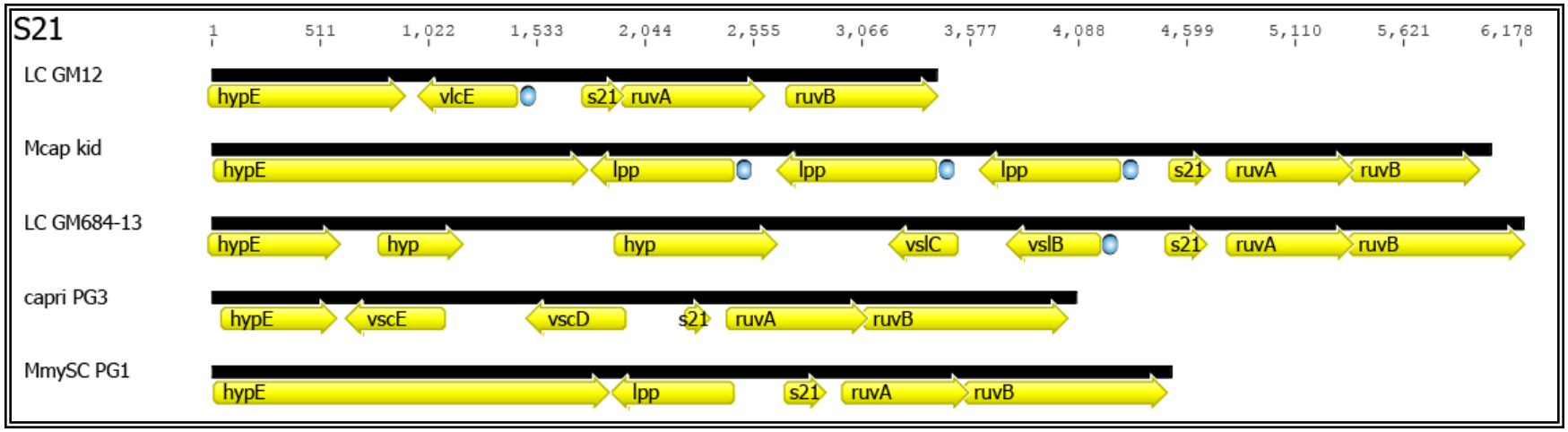
Figure 3-3. Conservation of Flanking Genes. Contingency loci from all 5 evaluated strains were aligned, and the results are shown above. The yellow arrows show the open reading frames of the conserved genes. Numbers below ORFs are percent identity at the DNA level found between all strains included in alignment. Numbers above are the nucleotide coordinates corresponding to the largest locus out of the 5 strains.

There are several reasons these particular loci warranted further investigation: i) the existence of divergent DNA separating these conserved flanking genes in multiple mycoplasma species, ii) the presence of open reading frames encoding putative lipoprotein genes and iii) the presence of a poly[TA] tract between the -35 and -10 boxes of the predicted promoter region preceding a majority of the lipoprotein ORFs. These promoters have been shown to control the phase-variable expression of lipoproteins in both *Mmy*SC and *Mcap* and are found upstream of all but nine of the 28 newly identified lipoprotein genes discussed here (Fig 3-4). These promoters will be discussed in further detail in Chapter 4.

The contingency loci are not clustered on the genome, but are dispersed around the chromosomes of *Mcap* and *Mmy*SC PG1; analysis of the draft sequence revealed that this is also the case for *Mmy*LC GM12, and although it is currently unknown for *Mmy*LC GM648-13 and *Mmycapri* PG3, the available data suggest that the same pattern will be seen. Gene synteny is also observed surrounding each contingency locus (Fig 3-3) in all five mycoplasmas examined herein. There is one notable exception to this synteny occurring in the *Mmy*SC Gap region. The ORFs for glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase are present as seen in the upstream flanking position (Fig 3-4), but the downstream flanking ORFs found in *Mcap*, *Mmy*LC strains, and *Mcapri*, are not found in *Mmy*SC. *Mmy*SC also differs in three other regions by the inclusion of multiple ORFs between the conserved flanks,


SgaT
1
831
1,662
2,493
3,324
4,155
4,986
5,817
6,648
7,479
8,310
9,141
10,398
LC GM12
sgaT
pts
vlcC
vlcB
vlcA
hypB
Mcap kid
sgaT
pts
vmcD
vmcC
vmcB
vmcA
hypB
LC GM684-13
sgaT
pts
vslA
clp
hypB
capri PG3
sgaT
pts
vscB
vscA
hypB
MmySC PG1
sgaT
pts
hyp
lpp
vmm
hypB


Gap
1
568
1,136
1,704
2,272
2,840
3,408
3,976
4,544
5,112
5,680
6,248
7,117
LC GM12
gap
pgk
vlcD
hypC
hypD
Mcap kid
gap
pgk
vmcF
vmcE
hypC
hypD
LC GM648-13
pgk
hypC
hypD
capri PG3
gap
pgk
vscC
hypC
hypD
SC PG1
gap
pgk
hypC
hypD
hyp1
IS1296


S21
1
511
1,022
1,533
2,044
2,555
3,066
3,577
4,088
4,599
5,110
5,621
6,178
LC GM12
hypE
vlcE
s21
ruvA
ruvB
Mcap kid
hypE
lpp
lpp
lpp
s21
ruvA
ruvB
LC GM684-13
hypE
hyp
hyp
vslC
vslB
s21
ruvA
ruvB
capri PG3
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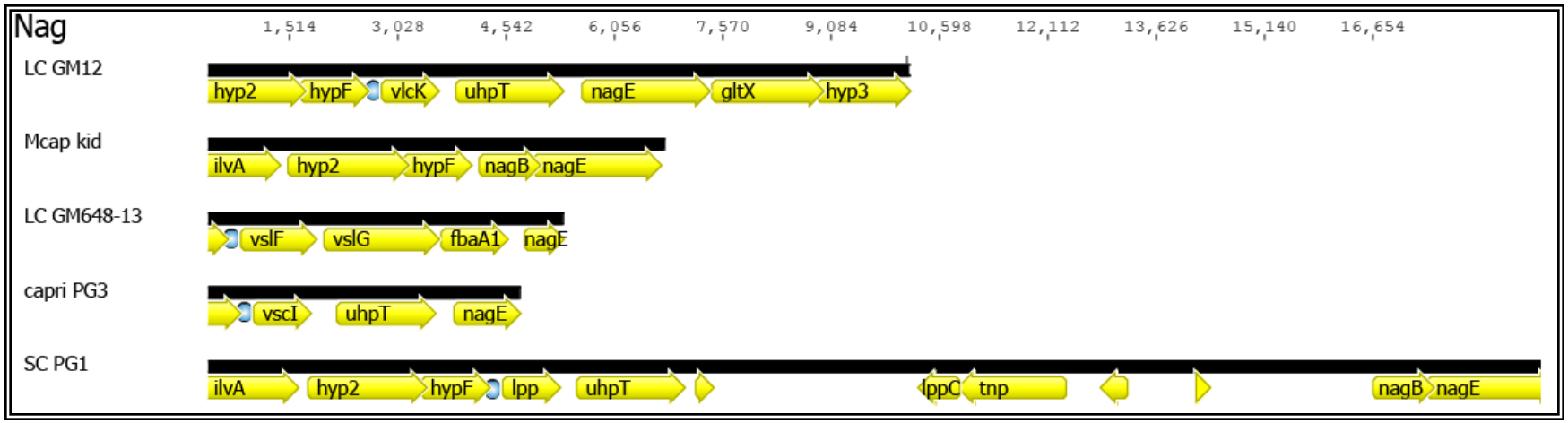
MmySC PG1
hypE
lpp
s21
ruvA
ruvB


Nag
1,514
3,028
4,542
6,056
7,570
9,084
10,598
12,112
13,626
15,140
16,654
LC GM12
hyp2
hypF
vlcK
uhpT
nagE
gltX
hyp3
Mcap kid
ilvA
hyp2
hypF
nagB
nagE
LC GM648-13
vslF
vslG
fbaA1
nagE
capri PG3
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ppC
tnp
nagB
nagE

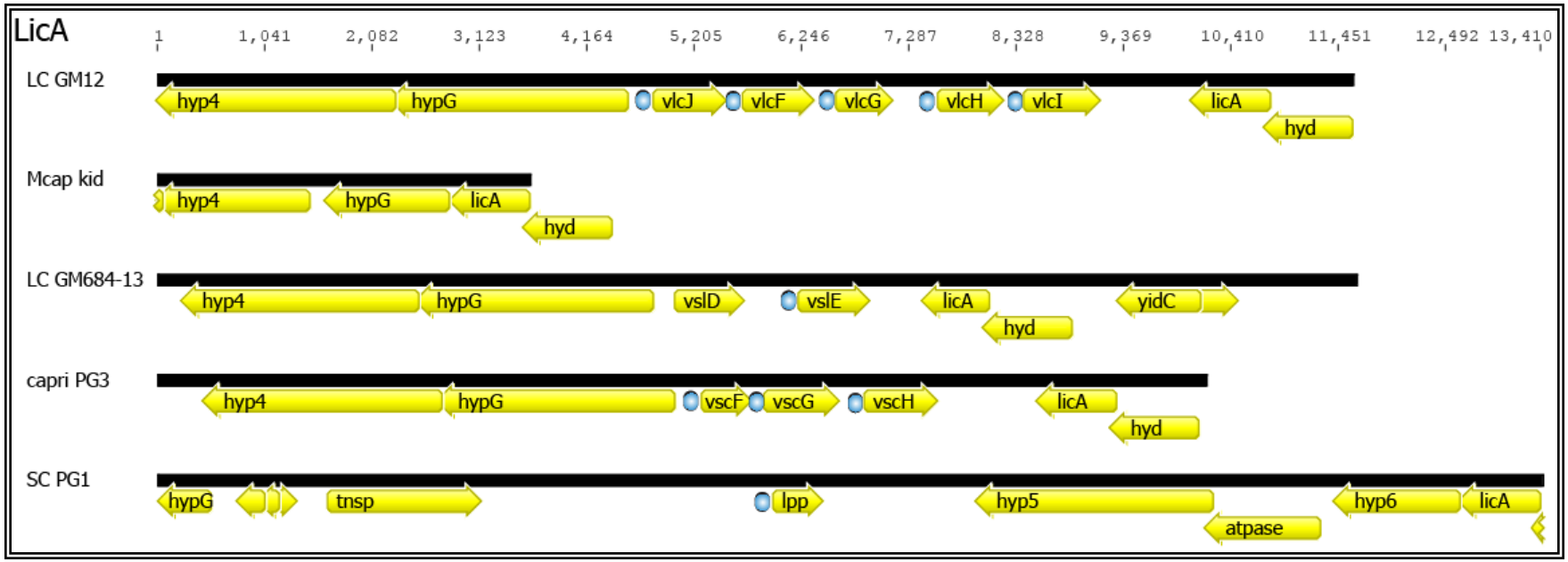


Figure 3-4. Regions of Genome Used for Comparisons. Shown are the unaligned sequences from all five strains used for each of the five alignments. The numbers along the top represent number of nucleotides, solid black lines are the sequence, and yellow arrows represent open reading frames. The blue ovals indicate a poly[TA] tract, the purple ovals represent interrupted poly[TA] tracts. This illustrates the variation that occurs between the conserved flanks. This also shows to scale the distance between open reading frames in each strain.

most noticeably in the LicA and Nag regions, as well as in the SgaT region. These ORFs are associated with insertion sequences (IS), and may reflect rearrangements precipitated by the movement of IS elements. Although *Mcap* lacks IS units, there are a large number of insertion sequences found in the *Mmy*SC genome constituting 13% of the genome (235), when compared to other sequences of the *M. mycoides* cluster.

Comparison of the Five Contingency Loci

While the frameworks surrounding the five contingency lipoprotein loci are well conserved, there is a variable amount of similarity found within the sequences between the housekeeping genes. The number of lipoprotein genes varies in each locus, both between strains and species in the *M. mycoides* cluster. There are examples of 'empty sites' both in *Mcap* and *Mmy*LC GM648-13 which do not contain lipoprotein genes between the flanking housekeeping genes (Fig 3-4). This study focused on those loci found to contain putative lipoproteins in *Mmy*LC GM12, *Mmy*LC GM648-13, and *Mcapri* PG3 for the sake of a more global cluster comparison that will provide clues to cluster divergence.

Sequence and bioinformatic analyses revealed multiple loci and multiple putative lipoprotein genes in each strain. In *Mmy*LC GM12, five loci were identified containing a total of 11 lipoprotein ORFs, while the same number of loci in *Mcapri* were identified, but contained only nine lipoprotein ORFs. *Mmy*LC GM648-13 has seven lipoprotein ORFs encoded within four loci. Comparisons of

the 27 newly-identified putative lipoprotein genes showed a highly conserved lipoprotein signal sequence (Fig. 3-5), containing the bacterial lipobox consensus sequence [LVI][ASTVI][GAS][C] (13). Overall there is not significant conservation of the mature lipoproteins found within these mycoplasmas, with the exceptions discussed below. A search for nucleotide as well as protein similarity in GenBank has shown that the identified ORFs have no similarity to previously identified proteins outside of the *M. mycoides* cluster, nor do they have recognizable motifs to provide clues to the function beyond the lipoprotein signal sequence. All putative lipoproteins are predicted to be small, ranging between 10.97 and 24.09 kDa in all but the Nag region, and a majority of the putative lipoproteins are basic, with only eight being acidic and one with a pI of 7.36 (Table 3-1). There are trends seen in the isoelectric points of the putative proteins of each region: the SgaT region contains mostly acidic proteins, the Gap region contains only basic proteins with very similar isoelectric points, the S21 locus proteins are varied, those in the LicA region have isoelectric points between 9 and 10, and in the Nag region the proteins are not only all basic, but are larger than those of any other locus (Table 3-1). Protein size predictions are between 26.69 and 59.20 kDa, the largest being found in *Mmy*LC GM684-13.

The Nag region is distinct compared to the other contingency loci in several ways. As mentioned above, the predicted lipoprotein genes are larger than in the other loci, especially in *Mmy*LC GM648-13. In the Nag locus there is also a high degree of sequence conservation seen between organisms in the

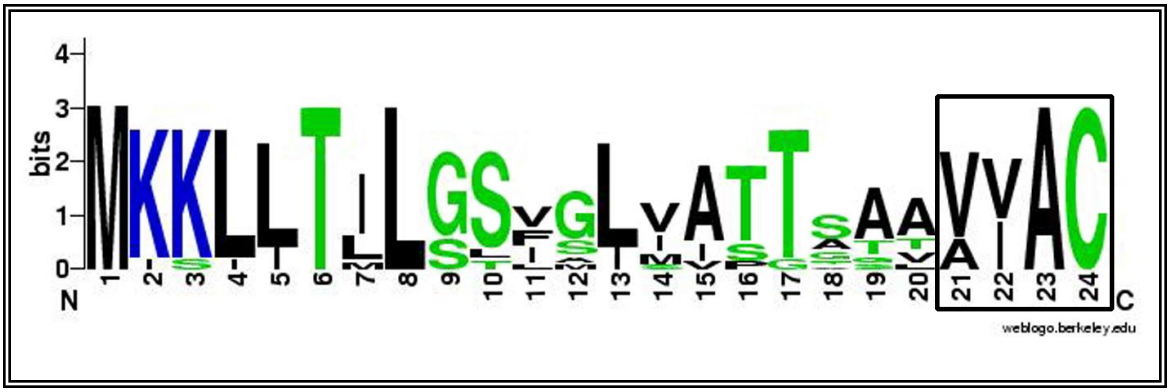


Figure 3-5. Sequence Logo of Lipoprotein Signal Sequences in Identified Lipoproteins from *Mmy*LC GM12, *Mmy*LC GM648-13, and *Mmycapri*. This illustrates the most common amino acids seen at each position in the lipoprotein signal sequences of the Vlc, Vsl, and Vsc proteins. This also illustrates how the lipobox signal (outlined in black box above) aligns with the conserved sequence seen for all bacteria [LVI][ASTVI][GAS][C]. The bit score represents the frequency and conservation of an amino acid at each position with a maximum score of 3, and amino acid position is given below. Created using weblogo.berkeley.edu.

Lipoprotein	Isoelectric Point	Size (kDa)	Locus
***Mmy*LC GM12**			
VlcA	5.2	18.396	SGAT
VlcB	9.31	19.861	SGAT
VlcC	4.03	16.31	SGAT
VlcD	10.68	11.48	GAP
VlcE	9.06	16.008	S21
VlcF	9.31	22.939	LICA
VlcG	6.8	16.891	LICA
VlcH	9.78	21.328	LICA
VlcI	9.89	24.094	LICA
VlcJ	9.93	23.789	LICA
VlcK	9.18	26.740	NAG
***Mmy*LC GM648-13**			
VslA	4.91	15.662	SGAT
VslB	10.11	15.367	S21
VslC	4.6	10.996	S21
VslD	9.81	22.277	LICA
VslE	9.68	22.783	LICA
VslF	10.00	36.006	NAG
VslG	8.5	59.201	NAG
Mmycapri			
VscA	10.13	18.433	SGAT
VscB	4.04	16.266	SGAT
VscC	10.2	19.261	GAP
VscD	5.52	16.179	S21
VscE	8.46	16.079	S21
VscF	6.83	15.267	LICA
VscG	7.36	23.319	LICA
VscH	9.2	23.008	LICA
VscI	9.18	26.687	NAG

Table 3-1. Summary of Isoelectric Points and Predicted Size of the Putative Lipoproteins of *Mmy*LC GM12 (Vlc), *Mmy*LC GM684-13 (Vsl), and *Mmycapri* (Vsc).

putative lipoprotein genes (Fig. 3-6). VlcK, VslF, and VscI have strong protein sequence conservation, and this extends to include MSC_0117 from *Mmy*SC (Fig. 3-7). Based on sequence homology and linkage of this lipoprotein gene to this locus, it is possible that this lipoprotein may act as a substrate-binding protein related to the Nag locus. *Mmy*LC GM648-13 differs from the other mycoplasma in this study in several ways. VslF shares a high degree of similarity (approximately 70% identity) to the lipoproteins identified in the other three strains in this region, but there is an 81-amino acid insertion in this protein (Fig. 3-7) between amino acids 172-254 accounting for its length of 322 amino acids versus the 239 amino acids of VlcK, VscI, and MSC_0117. The insertion is not a repeated unit, which is a common method of size variation in phase-variable lipoproteins. Except for this insertion the VslF sequence aligns with VlcK, VscI, and MSC_0117.

*Mmy*LC GM684-13 also encodes a second lipoprotein gene in this locus; *vslG* encodes a 499-aa protein with a predicted size of 59.20 kDa. This is the largest putative lipoprotein found in any of the contingency loci in *Mmy*LC strains or *Mmycapri* with the next largest being VslF at 36 kDa, also in the Nag region of *Mmy*LC GM648-13. BLAST analysis showed that VslG contains significant homology (43% identity) with a putative lipoprotein, MCAP_0561, found within the Integrative Conjugal Element of *Mcap* (ICEC). This mobile element belongs to a family of ICEs in mycoplasma species that was first discovered in *M. fermentans* (31). Since then several ICEs have been identified in mycoplasma species, including *Mcap* (98), *M. hyopneumoniae* (227) and *Mmy*LC

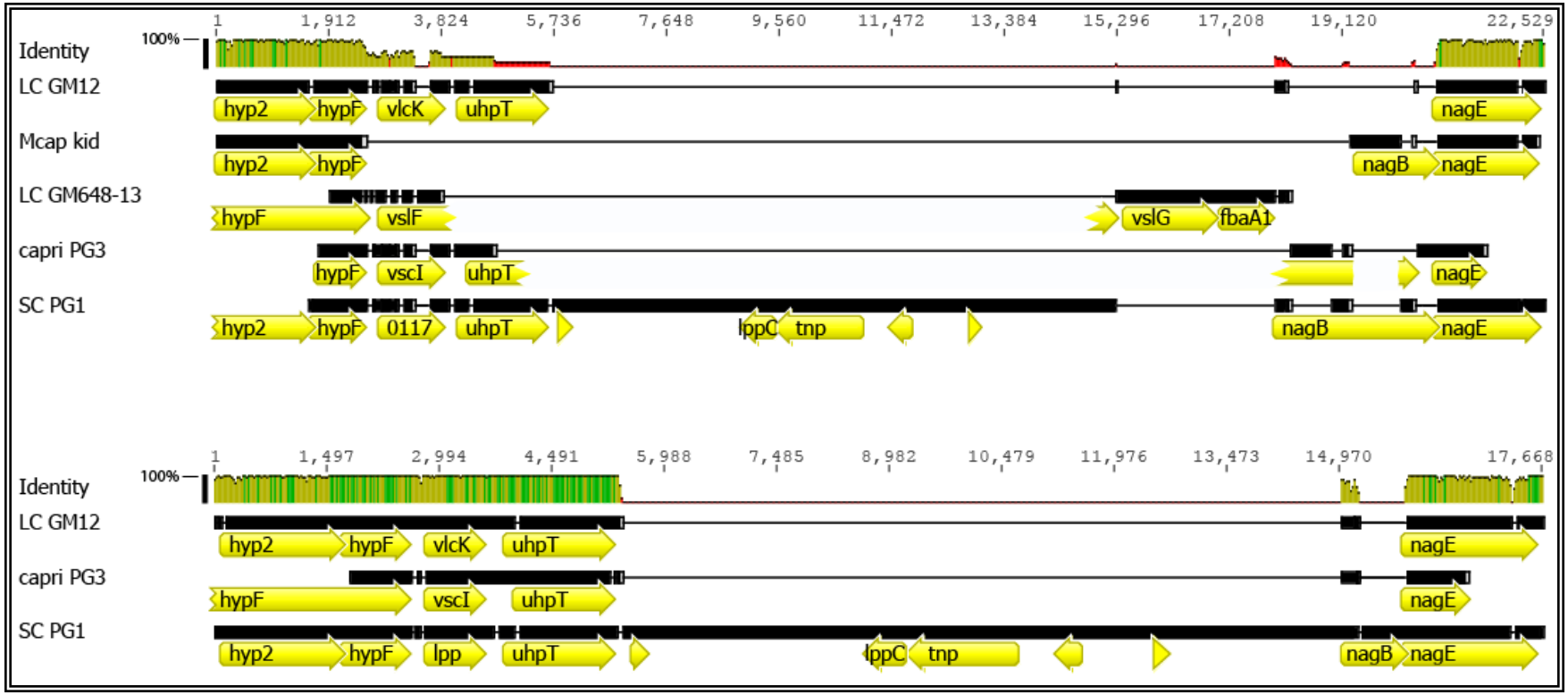


Figure 3-6. Alignments of DNA Sequences in the Nag Contingency Locus. The top alignment includes all five strains examined. Conservation of flanking genes can be seen on both ends of the locus. Identity is represented by the height and color of the bars across the top, green bars being full height and representing 100% identity. Thick black lines represent DNA sequence, thin black lines represent introduced gaps. The bottom alignment shows the high degree of homology of the Nag region, including the lipoprotein genes, between *Mmy*LC GM12, *Mmycapri*, and *Mmy*SC. There is a large insertion into this locus in *Mmy*SC associated with a transposase gene, and an ORF encoding *nagB* (putative glucosamine-6-phosphate deaminase) that is not seen in *Mmy*LC GM12 or *Mmycapri* but is present in *Mcap*.

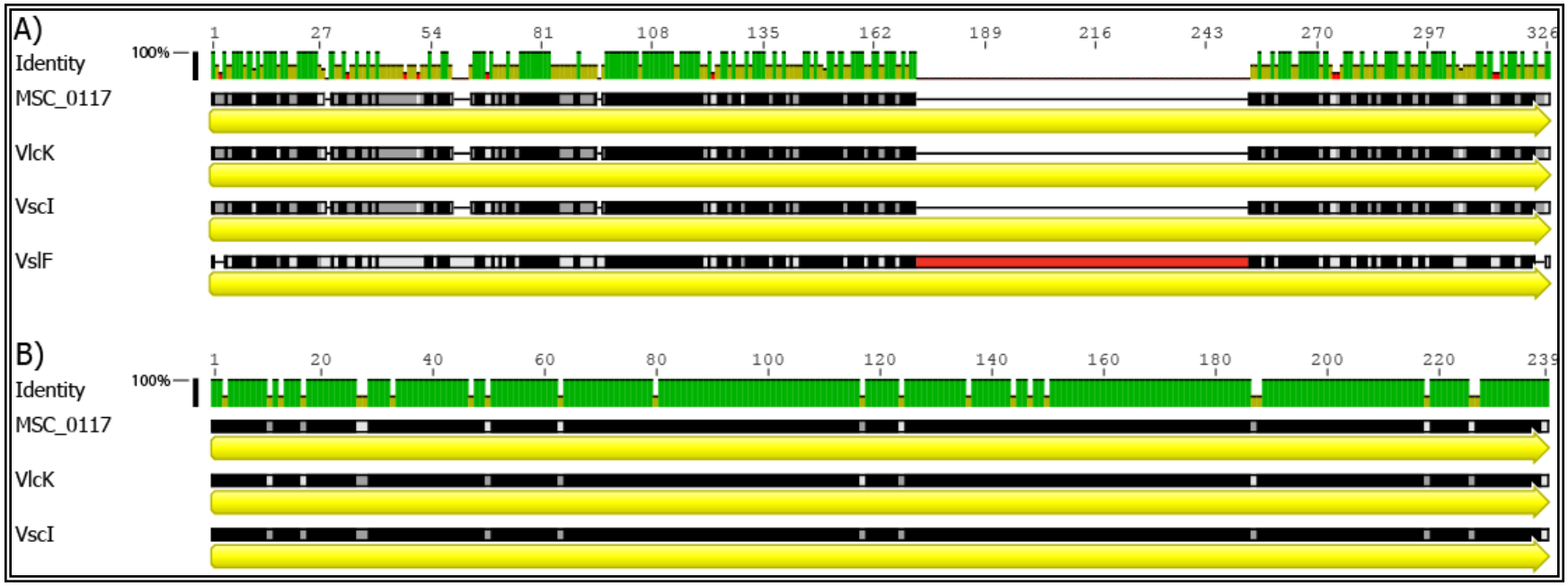


Figure 3-7. Alignment of Conserved Putative Lipoproteins of the Nag Contingency Locus. A protein alignment of the putative lipoproteins from the Nag locus showing a high degree of amino acid sequence conservation. A) Four proteins showing conservation from *Mmy*SC, *Mmy*LC GM12, *Mmycapri,* and *Mmy*LC GM684-13. Green bars represent identical amino acid residues, olive bars represents 75% identity, and red bars represents 50% identity. The red horizontal bar in VslF represents the 81 amino acid insertion relative to the other three strains. B) Alignment of the lipoproteins found in *Mmy*SC, *Mmy*LC GM12, and *Mmycapri*. Yellow arrows represent the protein, thick black line the amino acid sequence, the think black line gaps in the protein sequence introduced by alignment. Green bars represent identical residues and olive bars represent identical residues in two of the three sequences. Grey shading in the black horizontal bars above the yellow arrow signifies the identical residues in the corresponding protein, while the white spaces represent the unique amino acids.

(Mutangadura and Kent, unpublished results). This raises the possibility that VslG is not part of the contingency locus, but represents a truncated ICE. Of the *M. mycoides* cluster members for which sequences are available, *Mcap* is the only isolate that lacks lipoprotein genes in the Nag locus. It is possible that this may be specific to the *capricolum* subcluster, but this will be undetermined until more *Mcap* isolates are analyzed.

In the LicA region the lipoprotein genes show overall a 45% identity in pairwise comparison, which decreases to 33% when the signal sequence is excluded (Fig. 3-8). This is higher than the protein identities seen in the SgaT, Gap, and S21 regions. The alignment shown in Figure 3-8 highlights four regions beyond the signal sequence that are more conserved than the overall alignment. Comparisons within the LicA locus of each strain show a similar degree of identity, in the range of 45-49%, as well as the same four regions that share a higher identity.

When the deduced lipoprotein ORF coding sequences were compared in pair-wise combinations, a higher degree of similarity was seen between several lipoprotein ORFs in MmyLC and Mcapri PG3 than in the overall comparison (Fig.3-9) as would be expected based on the close phylogenetic relationship of these isolates. In the Gap region there is significant conservation of the DNA sequence in the location encoding VlcD of MmyLC GM12 and VscC of Mcapri PG3 (85.2% identity). This is reflected in the 88.0% identity seen between VlcD and the N-terminal 100 amino acids of VscC. Although there is a total of 12 bp

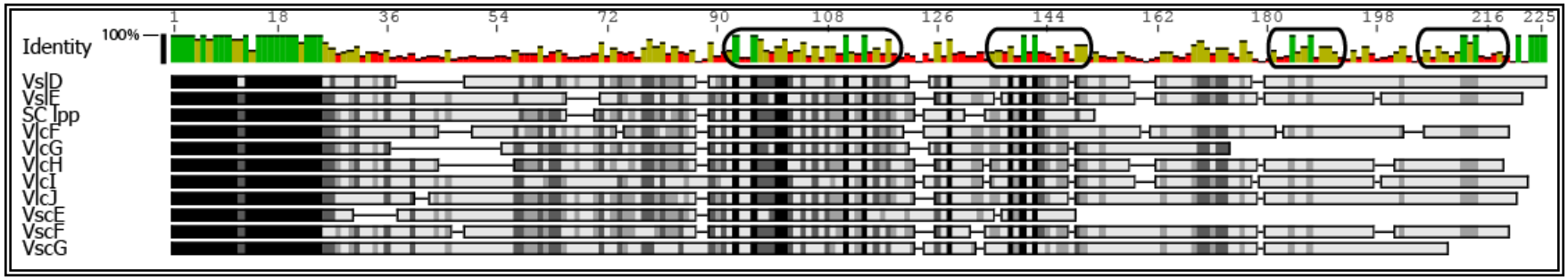
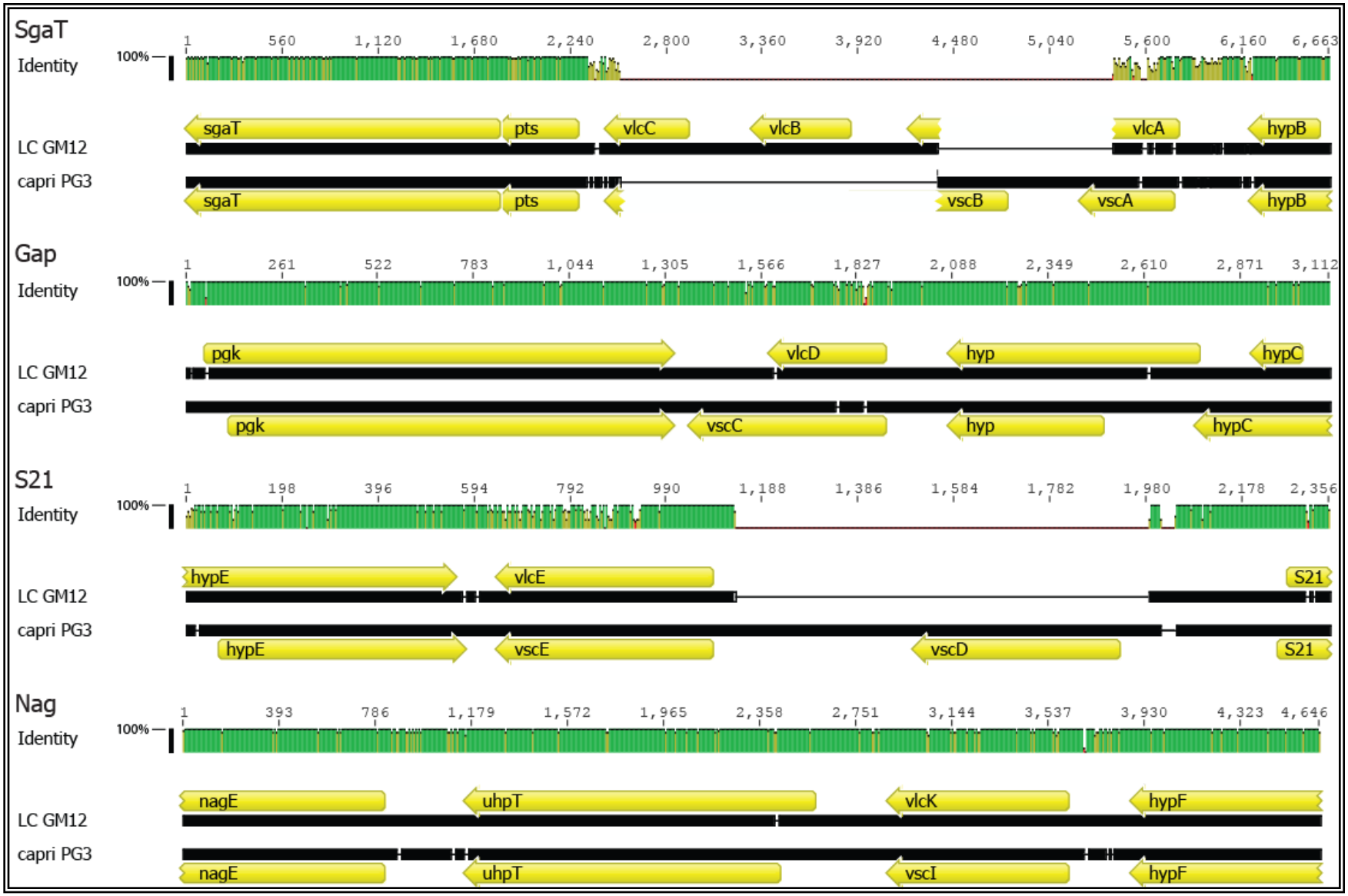


Figure 3-8. Alignment of Putative Lipoproteins of the LicA Locus. This alignment shows the conservation of the signal sequence as well as four regions that show a higher degree of conservation than the rest of the protein sequence (in black circles). Shading of the sequence indicates conservation, the darker the shading, the more identical residues. Solid thin lines indicate gaps introduced into sequence by alignment. Overall identity is 45%, excluding the signal sequence identity decreases to 33.3%.


SgaT
Identity
100%
1
560
1,120
1,680
2,240
2,800
3,360
3,920
4,480
5,040
5,600
6,160
6,663
LC GM12
sgaT
pts
vlcC
vlcB
vlcA
hypB
capri PG3
sgaT
pts
vscB
vscA
hypB
Gap
Identity
100%
1
261
522
783
1,044
1,305
1,566
1,827
2,088
2,349
2,610
2,871
3,112
LC GM12
pgk
vlcD
hyp
hypC
capri PG3
pgk
vscC
hyp
hypC
S21
Identity
100%
1
198
396
594
792
990
1,188
1,386
1,584
1,782
1,980
2,178
2,356
LC GM12
hypE
vlcE
S21
capri PG3
hypE
vscE
vscD
S21
Nag
Identity
100%
1
393
786
1,179
1,572
1,965
2,358
2,751
3,144
3,537
3,930
4,323
4,646
LC GM12
nagE
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hypF
capri PG3
nagE
uhpT
vscI
hypF

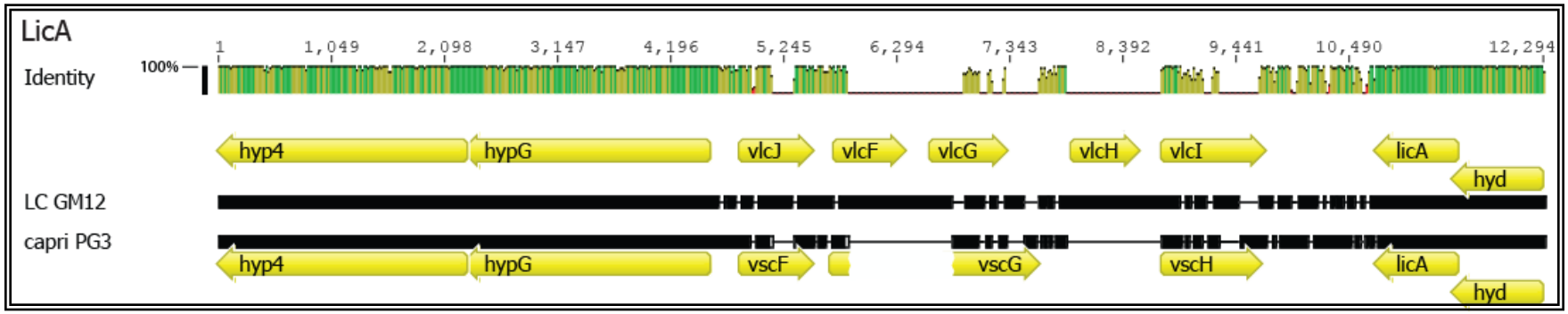

Figure 3-9. Alignment of *Mmy*LC GM12 and *Mmycapri* Contingency Loci. DNA alignments showing the high level of sequence conservation found between these two strains, especially in the Gap, S21, and Nag loci. Yellow arrows represent open reading frames, thick black lines represent DNA sequence, and thin black lines represent gaps introduced by alignment. Identity is represented by the height and color of the bars across the top, with green representing 100% sequence identity.

deleted in the *Mcapri* PG3 N-terminal sequence compared to *Mmy*LC GM12, a single nucleotide deletion in the *vlcD* ORF results in a predicted protein product that is truncated by 70 amino acids compared with VscC (Fig. 3-9). A comparison of the *vlcE* and *vscE* ORFs revealed 85.4% and 75.3% identity at the nucleotide and amino acid level respectively. Analyzing the entire S21 locus, *Mmy*LC GM12 and *Mcapri* PG3 share only 57.6% identity in nucleotide sequence. This is largely due the *vlcD* ORF present in *Mmy*LC GM12 which is not present in *Mmycapri.*

*Mmy*LC GM12 and *Mcapri* PG3 are both lacking in lipoproteins containing internal repeats in the five contingency loci, in contrast to *Mcap* which contains several in these same regions. These blocks of repeated nucleotides within the protein-coding region have been shown to be responsible for the size variation of lipoproteins in several mycoplasma species (23,183,249), purportedly via slip-strand mispairing during DNA replication. One ORF in the *Mmy*LC GM648-13 SgaT region, labeled *clp* (Fig 3-4), does have repeated blocks of sequence showing the potential for size variation. The blocks are 27 nucleotides long and translate into the 9-amino acid repeat PKGTEGKDA. There are 19 consecutive repeated blocks; BLAST analysis revealed similarity to collagen-like proteins Scl3C and SclF in *Streptococcus equii.* The repeat region of *clp* is 32% identical and 46% similar to the repeat units found in the *S. equii* proteins. However there is a frameshift in the signal sequence (region was sequenced twice on each strand) of *clp* so it is predicted that this gene, if expressed, would not be translated as a lipoprotein gene.

Discussion/Conclusions

The *M. mycoides* cluster is well-known not only for the agriculturally important diseases caused by these organisms, but also for the close genetic and antigenic relatedness that can confound diagnosis (39,40,165,166). There is a high degree of cross-reactivity between antibodies raised to specific mycoplasma species and other mycoplasma species that are either closely related (99,142,164) or that share hosts (190). With the availability of two completed genome sequences from the *M. mycoides* cluster, it is possible to look for potential differences on the genomic level and extend this assessment to other members of the cluster.

Comparison of five genomic loci in four sub-species of the *M. mycoides* cluster has shed light on differences found in these genomes that should facilitate efforts to better differentiate between species in this group of organisms. Prior to this research only the lipoproteins LppA (140) and LppB (228) had been identified in *Mmy*LC and *Mmycapri*, which are also present in *Mmy*SC and *Mcap*. Furthermore, a probe to *vmm* did not hybridize to DNA from *Mmy*LC Y-goat or *Mmycapri* PG3 (164). This work identified 27 lipoproteins in *Mmy*LC GM12, *Mmy*LC GM648-13, and *Mcapri* that overall show little to no homology with any lipoproteins found in *Mmy*SC or *Mcap*. Importantly variation in the lipoprotein repertoires studied here is seen within a species, as shown by the unique ORFs, such as *vlcH* and *vslG* identified in *Mmy*LC GM12 and *Mmy*LC GM648-13 respectively. This variation mirrors what has been shown in antigenic comparisons of *Mmy*LC field isolates, confirming that *Mmy*LC is a very diverse

subspecies (47). These data complement those findings as the previous studies did not determine the identities of the variable antigens.

Recently it has been proposed by two groups that the *Mmy*LC and *Mmycapri* subspecies should be combined into a single species and named *Mycoplasma mycoides* subsp. *capri* (124,230). This is based on multiple phylogenetic analyses including sequences of rRNA (165), *rpoB* (230), and lipoproteins (222) as well as protein profiles (110). Comparisons of the contingency loci in this study support this suggestion. The S21 and Gap regions of *Mmy*LC GM12 and *Mmycapri* showed significant sequence homology in the putative lipoprotein as well as the flanking housekeeping genes (Fig. 3-8).

While overall there is a considerable difference in the putative lipoprotein genes identified when compared to the high degree of similarity found in flanking genes, there are notable exceptions. The Nag region in four of the strains displays a high degree of conservation of the putative phase-variable lipoprotein encoded between the flanking genes, but is an “empty site” in *Mcap*. This could indicate that this contingency locus originated in a common ancestor of the *Mycoides* sub-cluster; confirmation of this would require comparison of the corresponding locus in *Mccp* and *M*BG7, as well as of multiple *Mcap* isolates, as there could be a deletion of this lipoprotein in *Mcap* strain kid. It is also possible that this lipoprotein serves as a substrate binding protein for the Nag transporter. The Gap and S21 loci in *Mmy*LC GM12 and *Mmycapri* also share a high degree of similarity as discussed above. These similarities are most likely a reflection on the relatedness of these organisms, reinforcing the subclusters found in other

phylogenetic analyses (124,165,222), as opposed to disease determinants or host specificity since *Mmy*SC is a bovine pathogen that causes a highly contagious pneumonia.

These comparisons of the contingency loci present in five strains of mycoplasma reveal a variable repertoire of proteins that may be expressed on the cell surface. While the function of these putative proteins remains unknown, it is quite possible that they are involved in bacterial survival due to the phase-variable nature of the predicted promoters. The possibility for combinatorial expression of these lipoproteins on the cell surface could be involved with evasion of the host immune system. This study verified that there were indeed lipoproteins that show the potential to phase vary in *Mmy*LC and *Mcapri* despite the lack of hybridization in these species of the *vmm* probe. Sequence data collected here is being used to help track and analyze epidemiological data collected in France, where *Mmy*LC is endemic. The ability to compare large regions, if not entire genomes, of the *M. mycoides* cluster has enhanced the ability to identify these differences to narrow the search for disease determinants. The next step is to determine the function of those lipoproteins identified in this study that share little to no homology with other lipoproteins, shedding light on how these organisms cause differential disease in either bovine or caprine hosts. This pangenomic approach can be useful in identifying virulence factors, as well as evolutionary relationships. Pangenomic comparisons have been completed in six *E. coli* strains, including a human commensal strain (170) , but the number of available genome sequences in the genus *Mycoplasma* would provide the most

comprehensive coverage of a bacterial group and could shed light on not only the relationship of the genus, but insight into the evolution of bacteria as a whole.

Chapter 4 – Phase Variation of Lipoprotein Expression

Introduction

Phase variation describes the ability of a gene to be expressed or not expressed through a heritable, reversible mutation in the DNA (143). This is a strategy employed by multiple pathogenic species, including fungi, protozoa, and bacteria (57,70,204), to increase phenotypic variation within a population (143,144). Phase variation does not give 'instant' results in that it is not an immediate response to an external signal affecting transcription in the cell that receives the signal, as is the case for a feedback loop or stress responses (74,213), but it does increase the number of phenotypes present to enhance the likelihood of survival in a dynamic host environment (144). Phase variation has been shown to be responsible for several different survival mechanisms including niche adaptation in *Helicobacter pylori* (188), evasion of host antibodies in *E. coli* (148), and biofilm formation in *Pseudomonas aeruginosa* (50).

The occurrence of phase variation is well documented in *Mycoplasma* species for multiple families of lipoprotein genes (Table 4-1). Two main mechanisms control this phenomenon; site-specific DNA inversion (17,61,66,82) and slip-strand mispairing at simple sequence repeats (SSRs) (65,164,231,240).

Mycoplasma Species	Lpp Family	Element Involved With Variation
*Mmy*SC	Vmm	Poly[TA] tract promoter (164)
Mcap	Vmc	Poly[TA] tract promoter (240)
*Mmy*LC GM12	Vlc	Poly[TA] tract promoter
M. gallisepticum	pMGA	Poly[GAA] tract 5' non-coding region (65)
M. bovis	Vsp	Site-specific promoter inversion (120)
M. hyorhinis	Vlp	Poly[A] tract promoter (247)
M. pulmonis	Vsa	Site-specific DNA promoter inversion (17)
M. agalactiae	Vpma	Site-specific promoter inversion (66)
M. hominis	Vaa	Poly[A] tract (250)
M. fermentans	P78	Poly[A] tract (218)
M. penetrans	Mpl	Site-specific promoter inversion (82)
M. synoviae	Vlh	Multiple gene conversions (157)
M. arthritidis	Maa2	Poly[T] tract promoter (231)

Table 4-1. Described Phase-Variable Proteins of *Mycoplasma*.

Slip-strand mispairing during DNA replication leads to either an insertion or deletion of repeat units that affect transcription (164,240) or translation of the associated ORF. The work in this dissertation focuses on a TA dinucleotide SSR found within the putative promoter region of lipoproteins in contingency loci that controls gene transcription.

In the case of the characterized phase-variable promoters in the *M. mycoides* cluster, phase variation occurs via a stochastic insertion/deletion event changing the number of TA repeats in the promoter region of the gene (164,240). In *Mmy*SC and *Mcap*, the poly[TA] tract is present between the predicted -35 and -10 boxes, which match the consensus sequence for an *E. coli* promoter demonstrated to be the recognition/binding site of RNA polymerase (75,164,240). A change in the length of this tract has been shown to correlate with gene expression in both *Mmy*SC and *Mcap*; a spacing of 17 base pairs, 10 TA repeats, leads to gene expression and any other number effectively turns the gene 'OFF' (164,240). The poly[TA] tract promoter has been shown to be responsible for the phase-variable expression of one lipoprotein in *Mmy*SC and several lipoprotein genes found in the SgaT as well as the Gap regions of *Mcap* (164,240). This type of promoter is not found upstream of every lipoprotein gene in each region, but it is present upstream of 17 of the 27 newly identified putative lipoprotein ORFs in *Mmy*LC GM12, *Mmy*LC GM684-13, and *Mmycapri*.

The predicted advantage of the phase-variable expression in this group of mycoplasma is the potential for combinatorial expression leading to surface variation. Relatively large intergenic spaces separate the putative lipoprotein

ORFs in the contingency loci (ranging from 193-421 base pairs) as seen previously in *Mcap* contingency loci (240), making it unlikely that the lipoprotein genes would be expressed in operons. The presence of putative phase-variable promoters upstream of individual ORFs, as well as the size of intergenic spaces between the putative lipoprotein ORFs suggests that each putative lipoprotein in the identified contingency locus undergoes variation independently of other contingency gene ORFs in the locus or chromosome. Based on the work presented in Chapter 3, and confirmed by the shotgun genomic sequence, *Mmy*LC GM12 contains 10 lipoprotein genes with putative phase-variable promoters each with the potential to be either ON or OFF. If each of these is able to vary independently, that would give the potential for 2^{10} variants, or 1,024 different possible combinations of lipoproteins on the cell surface. Currently there are no functions known for any contingency gene in the *M. mycoides* cluster, but it is predicted that variable expression of the lipoprotein genes in these loci would increase variation within the population, allowing a higher likelihood that subpopulations would survive selective contingencies.

With the discovery of the five contingency loci in *Mmy*LC GM12 discussed in Chapter 3, it was of great interest to determine the functionality of the poly[TA] tract promoter in this organism. This type of promoter was previously shown to be functional in both *Mmy*SC (164) and *Mcap* (240) to control phase-variable expression of lipoprotein genes in other contingency loci, but it was unknown if it was active in *Mmy*LC. Based on sequence analysis, the putative promoter of *vlcH* was predicted to be functional and phase-variable, so

studies were undertaken to determine if VlcH was expressed on the cell surface and able to phase vary, based on the spacing of the poly[TA] tract promoter.

Results

Phase-Variable Expression of Variable lipoprotein of Large Colony H (VlcH) of *Mmy*LC GM12

The LicA contingency locus of *Mmy*LC GM12 contains 5 putative phase variable lipoproteins and is the largest of the five loci identified in this strain. During the initial sequence analysis of this region, it was predicted that the poly[TA] tract promoter of *vlcH* was in the 'ON' conformation based on the 17-bp spacer when compared to the characterized promoters of both *Mmy*SC and *Mcap* (Fig. 4-1). Murine antibodies were raised against a 25-amino acid synthetic peptide (Materials and Methods pg. 38) of the mature VlcH lipoprotein. The synthetic peptide was shown to be divergent from other putative lipoprotein amino acid sequences via protein alignment using BLASTp and Geneious. It was also predicted to be hydrophilic based on Kyte-Doolittle analysis (104). Antibodies were used to screen colonies for surface expression of VlcH through colony immunoblotting (164), a technique which allows the visualization of antibodies binding to proteins expressed on the cell surface of bacterial colonies via a colorimetric assay. Initially a laboratory stock of *Mmy*LC GM12 was expanded in culture, then plated, colonies lifted and protein detected with

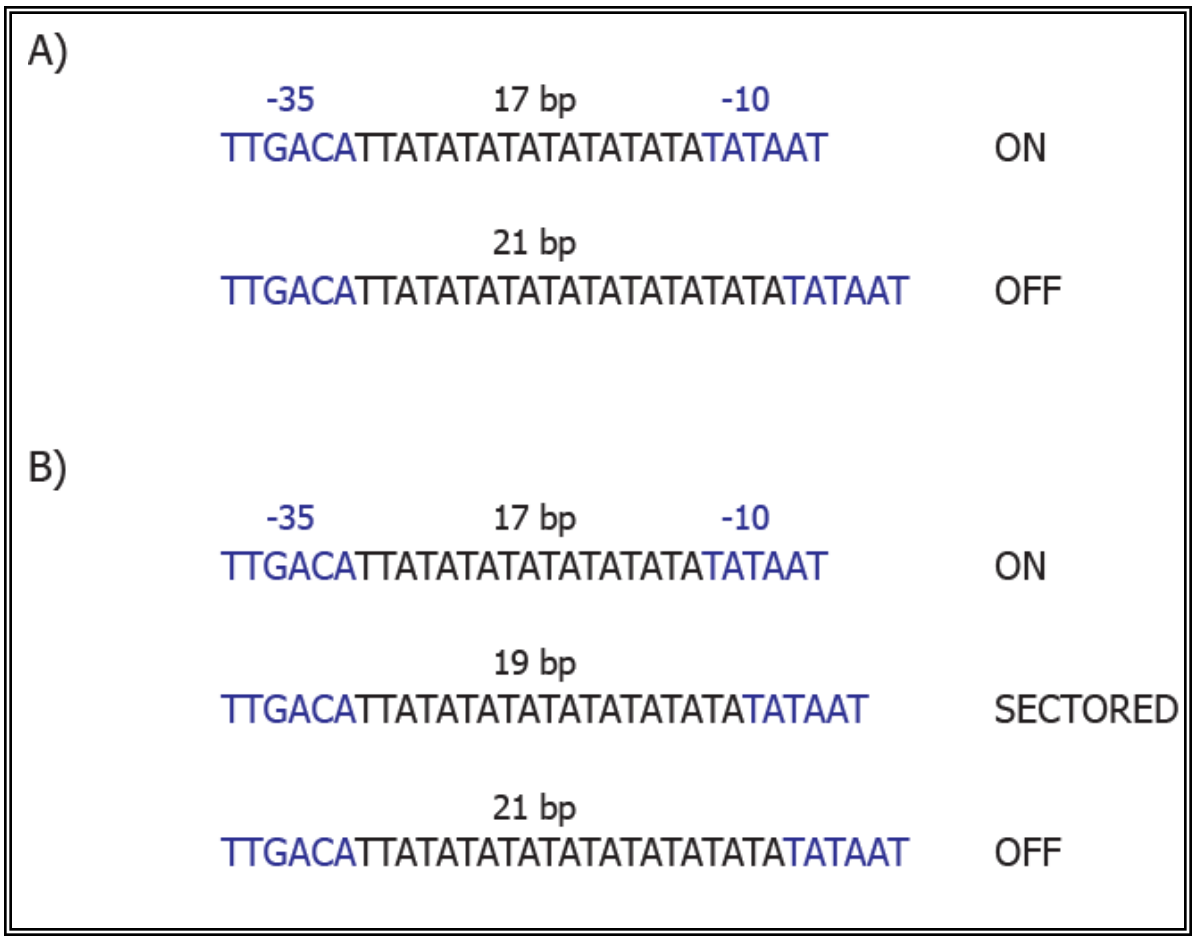

Figure 4-1. Poly[TA] Tract Promoter Spacing Of Characterized Phase Variable Promoters in *M. mycoides* Cluster. A) The promoter spacing and colony phenotype of *vmm* from *Mmy*SC (164). B) The promoter spacing and colony phenotype of *vmc*s from *Mcap* (240). Blue nucleotides represent the -35 and -10 regions of the promoter.

antibodies. Pre-immune control sera did not dectect colonies, but two phenotypes were observed using the specific antibodies; colonies expressing VlcH on the cell surface (purple colonies from antibody recognition) or those not expressing VlcH on the cell surface (pink colonies from the counter-stain Ponceau-S) (Fig. 4-2) at approximately a 1:3 ratio.

Four colonies of *Mmy*LC GM12 were selected for each phenotype and expanded in 6 ml liquid Hayflick medium. PCR was used to amplify the region upstream of the *vlcH* ORF from genomic DNA obtained from the expanded clonal isolates, and the DNA sequence determined. The length of the poly[TA] directly corresponded to the expression of VlcH on the cell surface in all eight samples (Fig. 4-3); a 17-bp spacer was seen in 'ON' isolates, and a spacer of either 19- or 15 bp was observed for those isolates that were 'OFF'. Samples from the expanded clonal populations were also replated and analyzed for VlcH surface expression as described above. Eight colonies expressing a 'switched' phenotype (four 'OFF' colonies from an original 'ON' colony and four 'ON' colonies from an original 'OFF') were again selected, expanded, and replated. This procedure was repeated two more times to generate a lineage of switching phenotypes and demonstrate the correlation of the length of the poly[TA] tract with protein expression. All PCR amplicons generated from VlcH expressing isolates had a nucleotide spacing of 17 bp between the -35 and -10 box as shown in Figure 4-3. A 19-bp spacing was the most common spacing seen in those colonies that were not expressing VlcH (7 out of 9, none of which were sectored), although spacings of either 15- or 21 bp were also observed.

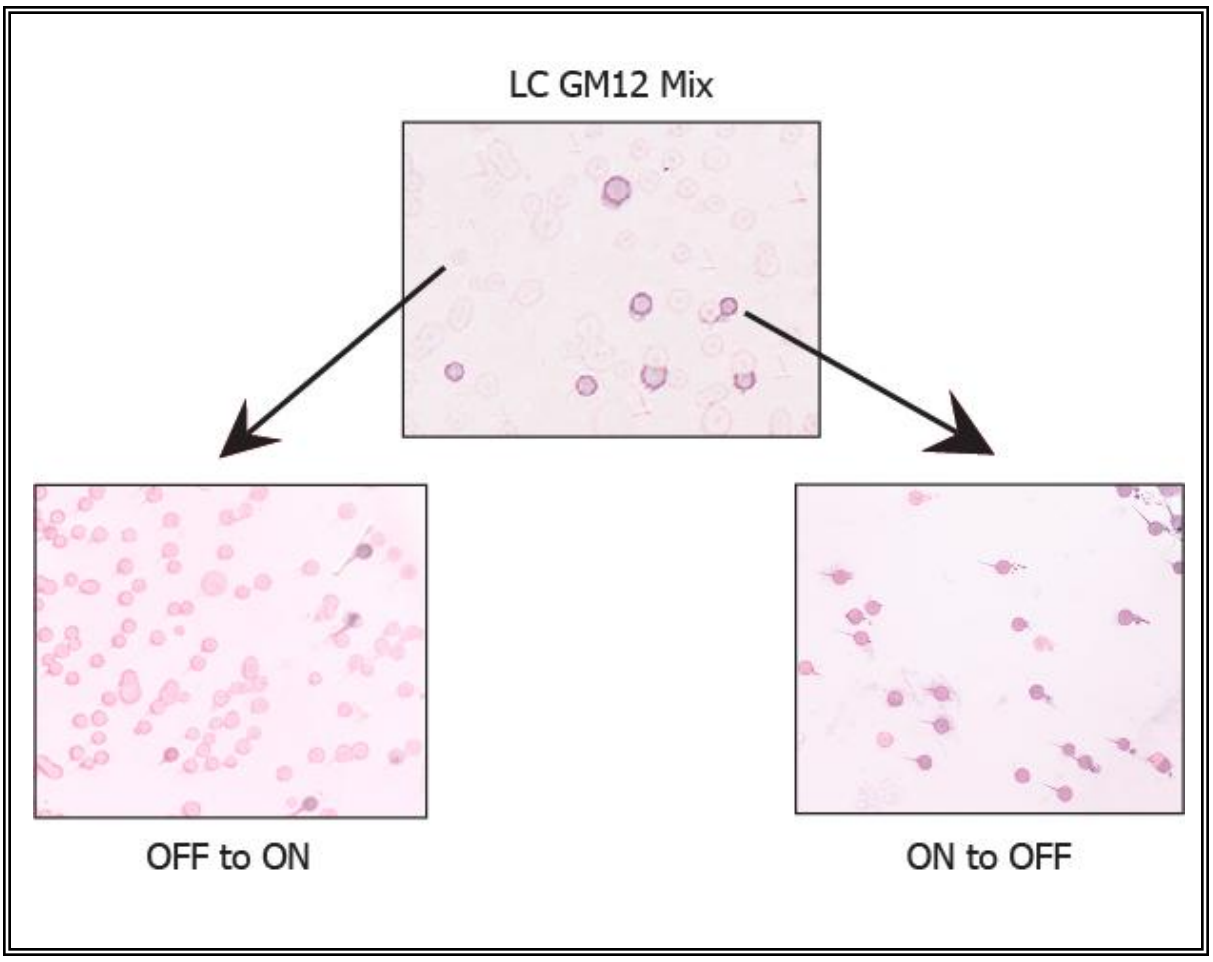

Figure 4-2. Variable Expression of VlcH on Cell Surface of *Mmy*LC GM12. Colony immunoblots showing recognition of VlcH expressed on the cell surface via monoclonal antibody. This figure represents a single negative and a single positive colony that were picked from the original population, expanded in medium, replated, and again a colony immunoblot was performed. In the lower left panel, the majority of the population retains the parental phenotype of 'OFF', but three colonies have switched to 'ON'. In the lower right panel the same is seen, except the major phenotype is 'ON' and the switched phenotype is 'OFF'. Purple colonies are positive for VlcH expression via monoclonal antibody recognition, pink colonies are counter-stained with Ponceau-S.

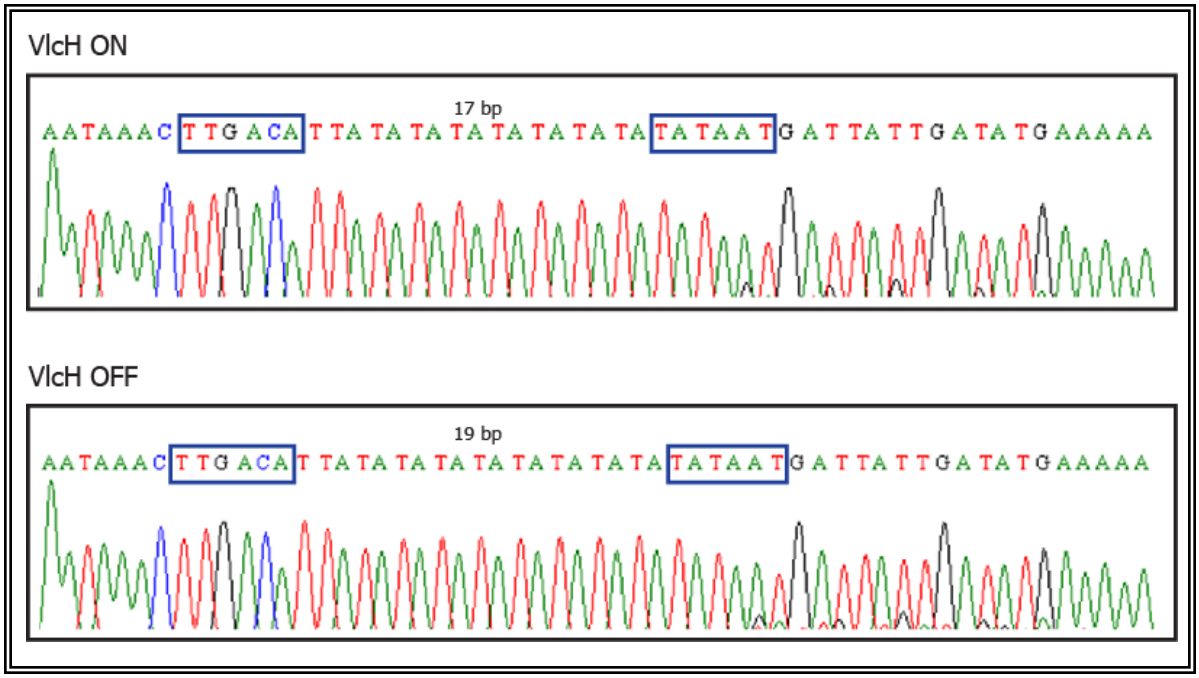

Figure 4-3. Length of the Poly[TA] Tract Corresponds to Expression of VlcH. Chromatograms showing the sequencing results of amplicons from the promoter region of *vlcH* in 'ON' and 'OFF' isolates. All colonies that were positive for VlcH expression via colony immunoblot had a spacing of 17 nucleotides between the -35 and -10 boxes (outlined in blue). Colonies that did not stain positively for VlcH expression had a spacing other than 17 nucleotides, the most common being 19 as shown above.

To confirm the surface localization of VlcH as seen in the colony immunoblot analysis, membrane and cytosolic proteins were separated via Triton X-114 detergent extraction. This technique partitions membrane-associated proteins to the detergent phase while hydrophilic proteins are localized to the soluble fraction (241). Western analysis showed that protein from the expanded colonies shown to express VlcH on the cell surface via colony immunoblot had a 21-kDa band (corresponding to the predicted size) recognized by the anti-VlcH antibody in the Triton X-114 fraction lanes (Fig. 4-4). No detection of protein was observed in the soluble fractions. A low amount of staining is detected in Triton X-114 lanes (Lineage 1 off lanes, and Lineage 2 off lane) containing protein from negative cultures. This may be explained by the necessary expansion of the clone to collect enough protein for Western analysis. This led to some phenotypic switching in the mycoplasma population due to the predicted high frequency of the phase-variation of approximately 1:100 (unpublished data) as determined via colony immunoblot for VlcH. This is the first report of a phase-variable lipoprotein in any strain or isolate of *Mycoplasma mycoides* subsp. *mycoides* Large Colony.

Analysis of Atypical Poly[TA] Tract Promoters in *Mmy*LC GM12

The SgaT contingency locus is the corresponding locus to the locus in which *vmm*, encoding the first phase-variable lipoprotein in the *M. mycoides* cluster, was identified and characterized in *Mmy*SC (164). In the report

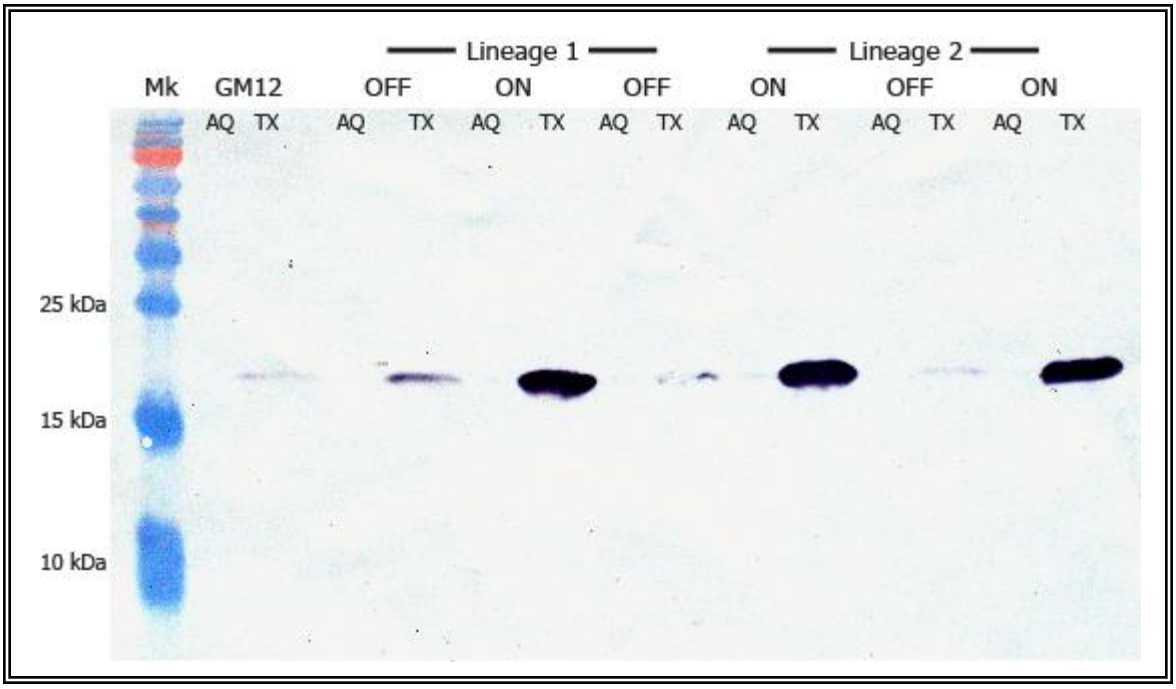

Figure 4-4. Western Analysis of VlcH Lineages. Two lineages are represented: Lineage 1 is from an original 'OFF', Lineage 2 is from an original 'ON' colony, each pair of lanes (AQ, TX) represents one passage. Triton X-114 (TX) and aqueous (AQ) protein phases of *Mmy*LC GM12 proteins were analyzed for expression of VlcH using murine antiserum. Protein was only recognized in the TX-114 phase, supporting the surface localization of VlcH. Faint bands found in 'OFF' TX phases are likely a result of high frequency switching and clonal expansion.

characterizing *vmm*, a Southern hybridization showed the presence of *vmm* homologues in all cluster members except *Mmy*LC type strain Y-Goat and *Mmycapri* type strain PG3 (164). Once available, the *Mcap* genome sequence was searched via BLASTn algorithm for the *vmm* gene homologue. This analysis revealed two regions of homology in both the SgaT and Gap regions in *Mcap* (240), initially identifying the first contingency locus present in multiple members of the *M. mycoides* cluster. Isolation and sequencing of the SgaT locus in both *Mmy*LC GM12 and *Mmycapri* PG3 revealed the presence of multiple putative lipoprotein genes that differed from those previously identified. Also it was observed that the lipoprotein ORFs were preceded by poly[TA] tracts as seen previously for *vmm* and the *vmc*s (164,240).

Three putative lipoprotein open reading frames were identified in the *Mmy*LC GM12 SgaT locus and designated *vlcA, vlcB, and vlcC.* The ORFs showed no significant homology to *vmm,* the *vmc*s, or any other entries in GenBank, with the exception of the conserved signal sequence of multiple lipoproteins. Each of these ORFs is preceded by a putative promoter that contained an interrupted poly[TA] tract (Fig. 4-5A). The putative promoters for *vlcA* and *vlcB* are identical, but the putative *vlcC* promoter is different from that of *vlcA* and *vlcB*, but also contains an interruption. In addition to the interruption, the spacing between the -35 and -10 is 18 bp, as opposed to the 17-bp (+/- 2-4 base pairs) spacer seen in previously described *M. mycoides* cluster poly[TA] tracts (164,240). It is anticipated that phase variation of each lipoprotein is

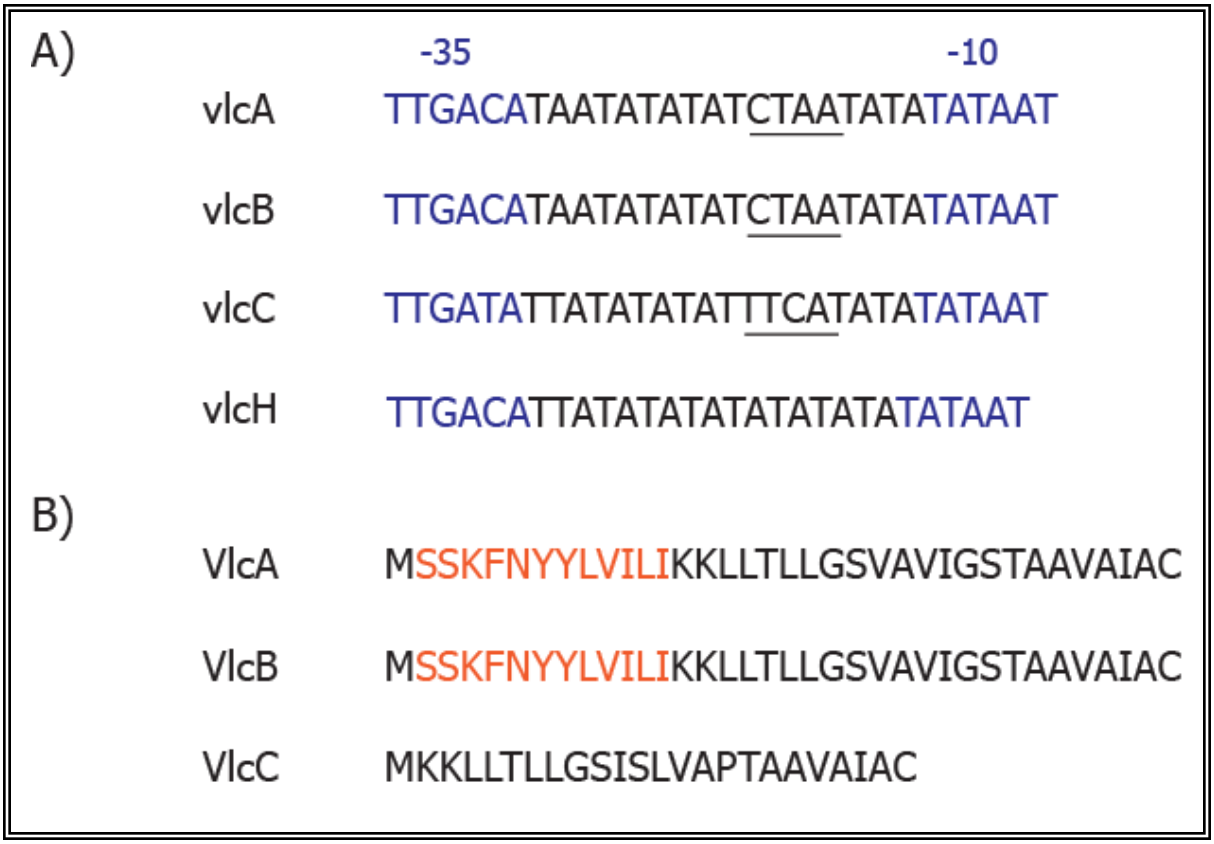

Figure 4-5. Unique Features of VlcA, VlcB and VlcC in *Mmy*LC GM12. A) Putative promoter regions of *vlcA, vlcB* and *vlcC* compared to the phase-variable promoter of *vlcH*. The spacing seen in *vlcA, vlcB,* and *vlcC* is 18 bp as opposed to the 17-bp spacer in the promoter region for *vlcH*. The poly[TA] tracts of these three ORFs are interrupted in comparison to that of *vlcH.* The -35 and -10 boxes are represented by the blue nucleotides, the disruptive nucleotides are underlined. B) Lipoprotein signal sequences of VlcA, VlcB, and VlcC demonstrating the 12-amino acid insertion in the signal of VlcA and VlcB. Inserted amino acids are shown in orange.

unlikely, or perhaps occurs at a much lower frequency than with non-interrupted poly[TA] tract promoters due to the additional nucleotides disrupting the TA repeat. Another unique feature of both VlcA and VlcB is a 12-amino acid insertion between the methionine codon that is predicted to code for translational start and the highly conserved lysine residue that follows in most other *M. mycoides* cluster lipoproteins found in the contingency loci (Fig. 4-5B). This insertion is identical between *vlcA* and *vlcB*, and shows no homology to other lipoprotein signal sequences. It is possible that this is the result of a single G to T transition changing the methionine to an isoleucine, and therefore extending the signal sequence back to the next methionine codon. Since signal length is generally conserved (121,192) it is unknown what affect, if any, this extension will have on the expression, processing, or trafficking of these putative lipoproteins.

In an attempt to determine expression on the cell surface, fusion proteins of VlcA, VlcB, and VlcC were constructed to allow the production of specific antibodies. Taking advantage of the lack of UGA tryptophan codons present in these ORFs, fusions were made between the maltose binding protein and the N-terminus of each lipoprotein gene in the pMALC-2 vector (113) and over-expressed in *E. coli*. The fusion proteins were then purified on an amylose affinity column. Each of the fusion proteins was then used to immunize mice to produce polyclonal antibodies to VlcA, VlcB, and VlcC. All antisera recognized MBP and the specific protein to which it was raised in ELISA analysis of fusion protein, whereas preimmune sera did not. The ELISA assays included purified protein along with purified protein supplemented with purified MBP to compete

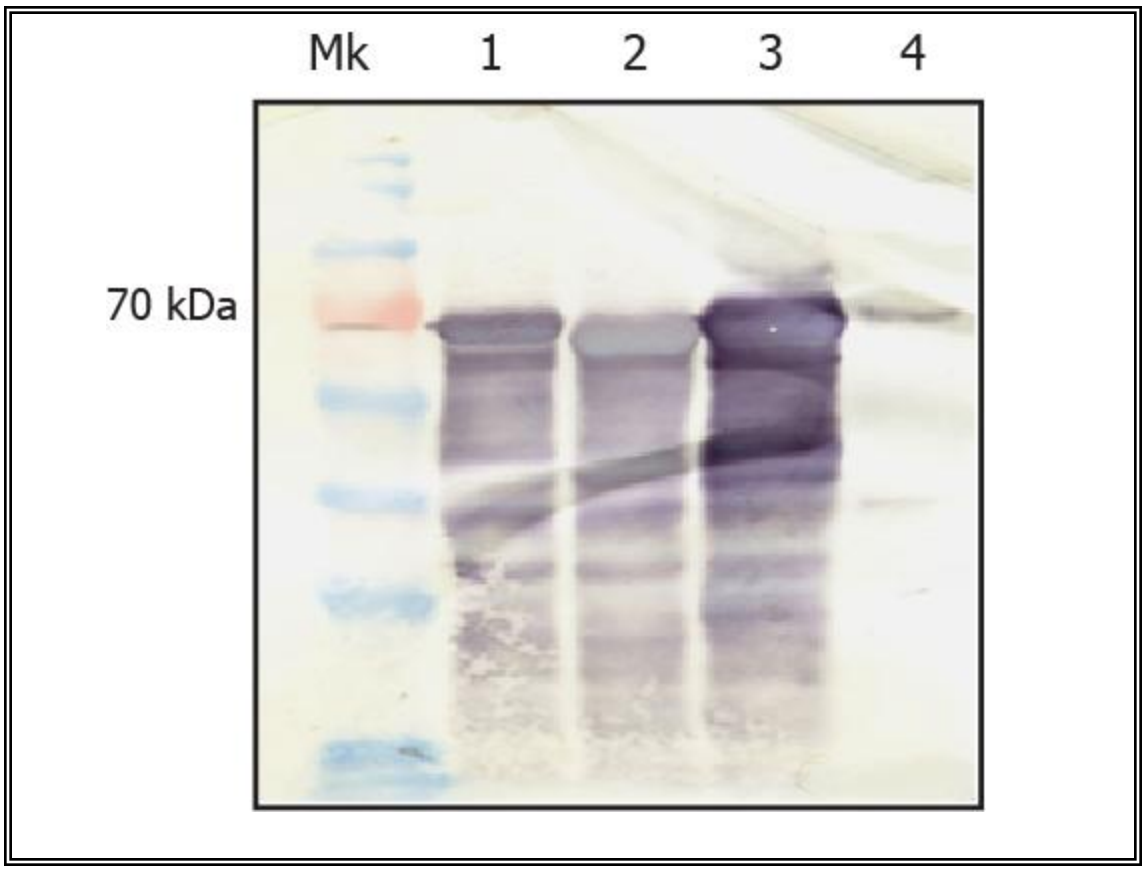

Figure 4-6. Western Analysis of MBP-VlcA Recognition by Polyclonal Antibodies. Purified MBP fusion protein was separated via electrophoresis on a 10% (w/v) SDS-PAGE gel along with GST-His_6 tagged protein and analyzed with polyclonal anti-sera. Band recognition in Lanes 1-3 are due to MBP specific antibodies. The antibodies used for this western were specific for MBP-VlcA and representative of the results achieved for all three fusion proteins. Mk is the protein ladder, Lane 1 is MBP-VlcC, Lane 2 is MBP-VlcB, Lane 3 is MBP-VlcA, and Lane 4 is GST-VlcA-His_6.

out antibodies specific for MBP and demonstrate specificity of antibodies for the protein of interest, as did Western analysis run with the same set of samples (Fig. 4-6). Having determined specificity of generated anti-sera, colony blots and protein extracts from *Mmy*LC GM12 were analyzed. Despite recognition of the fusion proteins, colony immunoblot and Western analysis of total cell protein of *Mmy*LC GM12 did not detect expression of VlcA, VlcB, or VlcC protein. To further test the antibodies that were generated, *vlcA, vlcB,* and *vlcC* were cloned as translational fusions to a GST tag, as well as a C-terminal HIS_6 tag, into the pET-41a vector for over-expression in *E. coli*. Following induction with IPTG, cell pellets were collected and total protein was run on an SDS-PAGE gel. Western analysis using the MBP-Vlc fusion antisera did not detect any protein in the VlcA, VlcB, or VlcC over-expression lanes either. It is unknown if the absence of protein detection was due to an inactive promoter, or if the absence of protein was attributable to post-transcriptional processes. Reverse-transcriptase PCR was used to determine if mRNA transcripts of any of the three ORFs were being produced. Analysis showed that *vlcA, vlcB,* and *vlcC* are all transcribed under laboratory growth conditions (Fig. 4-7).

Discussion/Conclusions

Phase variation is a common pathogenic strategy to deal with an ever changing external environment (57,158,188). Use of this tactic extends to include the cell wall-less, parasitic *Mycoplasma* (15,218,240). Several bacteria in the *M.*

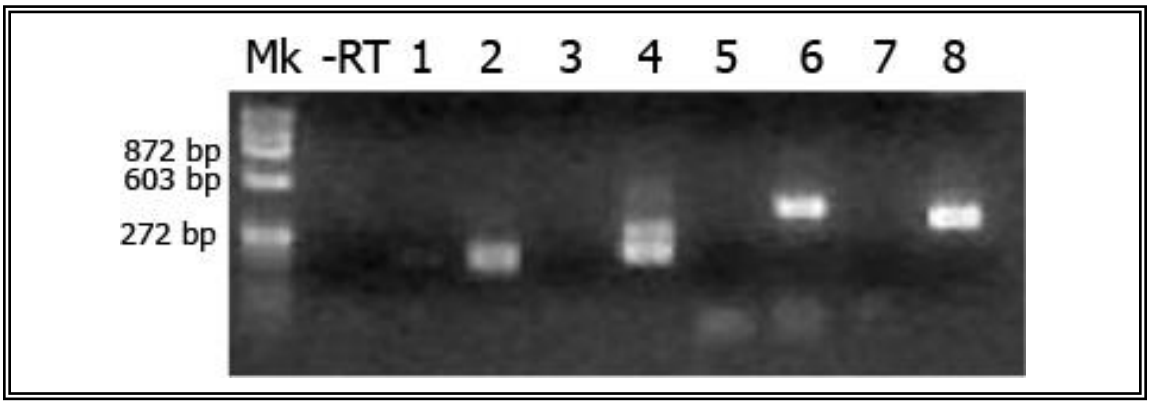

Figure 4-7. Detection of Transcripts of *vlcA, vlcB* and *vlcC.* Reverse-transcriptase PCR was used to amplify mRNA from putative lipoprotein genes in the SgaT locus of *Mmy*LC GM12. *rpsU* encodes the ribosomal subunit S21 and is included as a positive control. Lane Mk – marker, lane -RT – no reverse-transcriptase, lane 1 – *rpsU* water control, lane 2 – *rpsU*, lane 3 – *vlcA* water control, lane 4 – *vlcA,* lane 5 – *vlcB* water control, lane 6 – *vlcB,* lane 7 – *vlcC* water control, lane 8 – *vlcC*.

mycoides cluster have been shown to use a simple sequence repeat in the promoter region to mediate this variable phenotype. *vmm* of *Mmy*SC is the prototypic phase-variable lipoprotein in the *M. mycoides* cluster (164), and it was found that phase-variable lipoproteins in *Mcap* utilize identical promoters to achieve an analogous phenotypic change (240). Expression of these previously characterized lipoproteins depends on the number of dinucleotide repeats in the poly[TA] tract between the -35 and -10 hexamers in the promoter; a spacing of 17 nucleotides leads to transcription and subsequent translation of the downstream gene and any other spacing effectively turns the gene 'OFF' (164,240). The putative lipoprotein designated VlcH of *Mmy*LC GM12 was identified as the first phase-variable lipoprotein in *Mmy*LC. Sequence data showed that the spacing in the promoter region corresponded to expression of VlcH and that the promoter spacing for 'ON' and 'OFF' variants was consistent with the characterized promoters in *Mmy*SC and *Mcap*.

The fact that identical phase-variable promoters have been characterized within three distinct members of the *M. mycoides* cluster raises the possibility that this mechanism of antigenic variation may be common to all cluster members. Poly[TA] tracts have not been associated with phase variation in any other mollicute genome, so the TA SSR may be specific for this phylogenetic group. Despite the difference in host tropism and disease severity in *Mmy*SC vs. *Mcap* and *Mmy*LC, the poly[TA] phase-variable promoter is conserved. The presence of these promoters in multiple members of the cluster and in species that are found in two different phylogenetic sub-clusters, is another illustration of

the close relatedness of these organisms and the importance of comparative analyses to identify distinguishing characteristics of these *Mycoplasma* species.

The detection and identification of interrupted TA dinucleotide repeats of *vlcA, vlcB,* and *vlcC* was unanticipated and of interest. As shown in Chapter 3, the flanking housekeeping genes in this region are highly conserved in different members of the cluster, between 85-89% identical; however, there are some distinctive attributes about the putative lipoproteins and the promoters associated with them in *Mmy*LC. Firstly, it is unknown if these genes are able to undergo phase-variable expression by expansion/contraction of the promoter region due to the 'extra' nucleotides contained within each promoter tract. It is possible that slip-strand mispairing could still occur in regards to the TA repeats 5' of the interruption, but seems unlikely with no more than three in a row as this number of repeats occurs greater than 500 times in the *Mmy*LC genome sequence. Secondly, the spacing between the -35 and -10 RNA polymerase binding sites is 18 base pairs in each of the three promoters. For *vlcH,* as well as *vmm* and the *vmcs,* the active promoter spacing is 17 base pairs which could mean that *vlcA, vlcB,* or *vlcC* may have decreased or altered expression. However, there has been no detailed characterization of promoters in *Mycoplasma*, so it is unknown what other spacer lengths are compatible with active transcription. Initial results presented herein suggest that a spacing of 18 base pairs allows for transcription of genes due to the fact that transcripts for *vlcA, vlcB,* and *vlcC* were amplified via RT-PCR. Lastly, it is unknown how the interrupted TA tracts arose in *Mmy*LC GM12. Promoters like these are not found in the SgaT region of *Mmy*LC

GM648-13 or *Mmycapri*, or in any other region of any of the cluster members examined herein. It is possible that this represents a recent duplication and subsequent divergence of the coding sequence, but a more global examination of the SgaT region in multiple strains of *Mmy*LC would paint a clearer picture of the distributions and functionality of the promoters seen in the SgaT region of *Mmy*LC GM12.

Another unique feature of the lipoprotein genes in the SgaT locus of *Mmy*LC GM12 is a possible insertion seen in the signal sequences of both *vlcA* and *vlcB*. The extra 36 nucleotides translates into an additional 12 amino acids extending the 5' hydrophilic region present in the lipoprotein signal sequence, but the affect this may have on the signal peptides is unknown. As mentioned above, this may be the result of a G to T transition. This extended signal sequence may be the reason that VlcA and VlcB were not detected on the cell surface or in total cellular protien. However it is possible that the signal is recognized and processed in the same way as other lipoproteins as the lipobox matches the consensus sequence observed for the other mycoplasma lipoproteins. There may be a very low amount of VlcA, VlcB, or VlcC that is transcribed and/or translated, as would be the case if the 18-bp spacer represents a weak promoter. The extended signal sequence of VlcA and VlcB may lead to a misfolded protein and rapid degradation of the product, but this would not account for the lack of detection of VlcC which does not have extra amino acids in the signal. It may be necessary to either express these lipoproteins in another organism or mutate the promoter region to study the

effects of the extended signal sequence on protein processing and expression on the cell surface.

The studies presented here have led to several advancements in our understanding of mycoplasma. VlcH is the first phase-variable lipoprotein described in the subspecies *mycoides* Large Colony. The murine antibodies produced through this study will be useful to screen field isolates to generate epidemiological data in endemic areas such as France or Spain, in addition to the sequence information discussed in Chapter 3. These antibodies were also used in verifying the first report of genome transplantation in mycoplasma in which the genomic DNA of an *Mcap* cell was replaced with the genomic DNA of *Mmy*LC GM12, effectively changing the cell into *Mmy*LC GM12, with variable expression of VlcH on the cell surface (107).

The phase-variation of *vlcH* via a poly[TA] tract promoter suggests that this is a common mechanism of antigenic variation employed by the *M. mycoides* cluster, helping to further our understanding of how these bacteria may cause disease. Further studies of the interrupted poly[TA] tract of the *Mmy*LC GM12 SgaT region will allow for a better understanding of mycoplasmal promoters, such as what spacing between the -35 and -10 hexamers is required for transcription. Taken together, these data have added to the understanding of the molecular mechanisms involved with antigenic variation in the *M. mycoides* cluster.

Chapter 5 – Characterization of lipoprotein LppB from *Mycoplasma capricolum* subsp. *capricolum* strain kid

Introduction

Very little is known about virulence mechanisms of mycoplasmas. Despite fourteen completed genome sequences, there are very few defined virulence factors identified such as toxins. Multiple adhesins have been identified in mollicutes, including proteins that bind to plasminogen (22,246), mucin (7) and heparin (30,239) but to date, no specific adhesin has been identified for any member of the *M. mycoides* cluster. It has been reported that some component of *M*BG7 membrane protein fractions is able to bind and activate plasminogen, but no specific protein was shown to be responsible for this activity (22). Several virulence factors have been identified in mollicutes, including two toxins (95,224), necrosis-inducing enzymes (28), biofilm formation (132,201), antigenic variation (as discussed in Chapter 4) and H_2O_2, the by-product of a metabolic pathway (71,168).

Two recent reports included data that a by-product of glycerol metabolism, hydrogen peroxide (H_2O_2), plays a major role in the ability of mycoplasma to cause disease in the host organism *in vitro* (71,168). The release of H_2O_2 from the mycoplasma cell is related to, if not responsible for, the hemolytic activity of *Mmy*SC (18,229). The production of H_2O_2 by *Mmy*SC is significantly impacted by

two factors: i) the presence of the putative glycerol transport locus (*gts)* identified by sequence homology to other bacterial glycerol transporters (229) and ii) the metabolic enzyme L-α-glycerophosphate oxidase (GlpO) (168). The *gts* locus in *Mmy*SC has been the focus of study since an 8.84-kb deletion in this region was detected when comparing the less virulent European isolate, L2, to the fully virulent African strain Afadé (228). This locus includes four genes predicted to comprise an operon; *gtsA*, the proposed ATP binding cassette*, gtsB*, a proposed permease*, gtsC,* a proposed permease and *lppB,* the proposed substrate-binding protein. The loss of three genes, *gtsB, gtsC* and *lppB,* in all European strains of *Mmy*SC, is correlated with the attenuated phenotype (229). The strains containing the deletion were shown to produce less H_2O_2 *in vitro* and exhibit less cytotoxicity towards bovine nasal epithelial cells (18).

In addition the putative transporter, GlpO catalyzes the oxidation of glycerol-3-phosphate into dihydroxyacetone phosphate, and H_2O_2 is a toxic by-product of that reaction (168). Interestingly, there are no identified genes for catalases or dismutases in the *Mmy*SC genome (235), the products of which work to protect the cell from oxidative damage. This raises the paradox of production of a reactive oxygen species such as H_2O_2 without a way to protect against the damage, which would most likely negatively impact bacterial viability. Interestingly, GlpO contains two transmembrane domains and the use of antibody binding studies found that GlpO is localized to the cell surface (168). The antibodies were also used to block the activity of GlpO on the cell surface, and this inhibited the production of H_2O_2 (168). The current model for H_2O_2 as a

virulence factor is that glycerol is imported into the cell via the GtsABC transporter, is phosphorylated to become glycerol-3-phosphate, and during the oxidation process catalyzed by GlpO, H_2O_2 is produced at the cell surface and released into the host extracellular environment (168) (Fig. 5-1). These data suggest that H_2O_2 plays an important role in virulence, necessitating the characterization of the pathway responsible for H_2O_2 production in the mycoplasma cell.

The lipoprotein gene that is associated with the *gts* operon, *lppB*, was the second lipoprotein gene to be identified in the *M. mycoides* cluster (228). It is present in all members of the cluster either by sequence analysis or immunological assay (52,228), except for the previously mentioned European strains of *Mmy*SC (228). This gene is also associated with the *gts* operon in more distantly related mollicutes, including *M. agalactiae* (203), *M. hyopneumoniae* (227) and *M. pulmonis* (63), despite a low level of synteny seen overall in mycoplasma genomes. LppB contains a conserved lipoprotein signal sequence at the N-terminus, suggesting that it is processed and expressed on the cell surface (52,228). The close association of *lppB* with *gtsA, gtsB,* and *gtsC* suggests that these genes may be part of an operon all associated with glycerol transport.

Many transport systems in Gram-positive bacteria have lipoproteins that serve as substrate binding proteins (SBPs) on the cell surface as opposed to in the periplasmic space where most SBPs are located in Gram-negative bacteria (20). In *Bacillus subtilis* the lipoprotein OpuAU is the glycine betaine binding

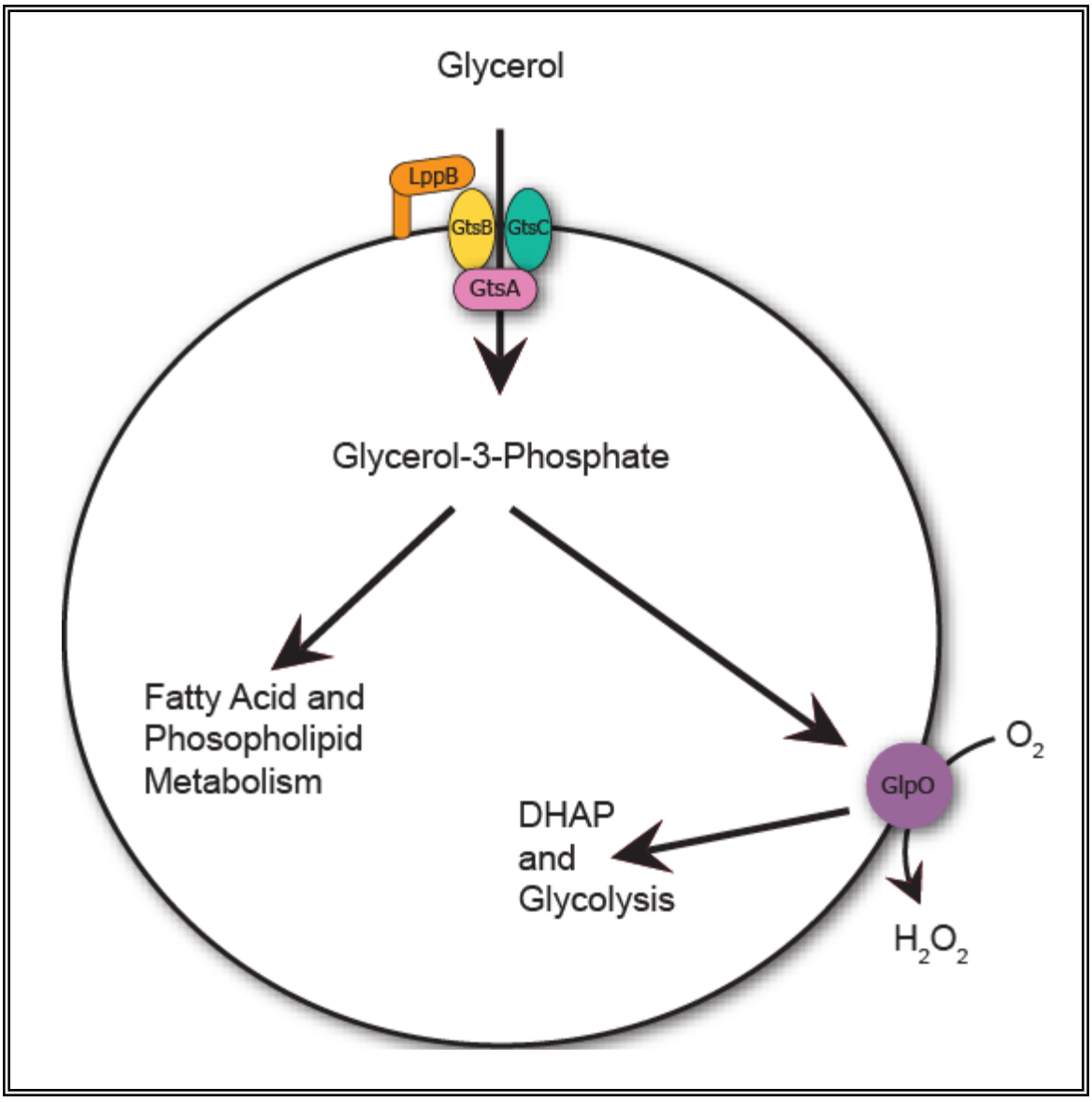

Figure 5-1. Glycerol Metabolism in *Mmy*SC. This is a representation of the glycerol metabolism pathway as it pertains to import and virulence in *Mmy*SC. Glycerol is imported into the mycoplasma cell via the GtsABC transporter, phosphorylated, and then oxidized by GlpO. It is predicted, due to the surface localization of GlpO that the by-product H_2O_2 is released outside of the mycoplasma cell. This schematic includes LppB on the cell surface where it is predicted to act as the substrate binding protein for the GtsABC transporter.

protein of the OpuA transporter (97). *Staphylococcus aureus* contains a lipoprotein, GmpC, that binds dipeptides for import into the cell (238). Three highly homologous lipoproteins in *Streptococcus pneumoniae*, AmiA, AliA, and AliB, all show the ability to bind oligopeptides and transport these molecules into the cell via the Ami ABC transporter (6). The lipoprotein p37 of *M. hyorhinis* is a thiamine binding protein and the gene encoding p37 is associated with a putative transporter.

This study was undertaken to test the hypothesis that LppB functions as the substrate binding protein of the GtsABC transporter to allow for active uptake of glycerol. *Mmy*SC is a restricted pathogen, so *Mcap* was chosen as the model organism because it has been reported to be transformable (92,106). First, transcriptional profiling was undertaken to understand the organization of the *gts* locus and determine if the four genes, *gtsA, gtsB, gtsC,* and *lppB* are transcribed as one polycistronic mRNA. Second, it was important to express and purify LppB to enable binding studies to be performed and for the production of antibodies for future experiments. This entailed the mutation of UGA tryptophan codons to UGG tryptophan codons for full-length protein expression in an *E. coli* host. Third, a strain of *Mcap* would be produced lacking the *lppB* gene to study the effects of mutation on glycerol uptake and hydrogen peroxide production.

Results

Transcriptional Profiling of the *gts* Locus

Initial sequence analysis of the *gts* region of *Mcap* suggests that several ORFs may be arranged in an operon (Fig. 5-2). The ORFs for *gtsA, gtsB,* and *gtsC* all overlap, and such an arrangement is usually indicative of operons in bacteria (16,189). The ORF of *lppB* is 113 nucleotides from the stop codon of *gtsC* and is in the same orientation as the other 3 ORFs (Fig. 5-2). Using the TransTermHP algorithm (102), a predicted rho-independent terminator was identified 10 nucleotides downstream of the stop codon of *lppB* (Fig. 5-3) (189). No other terminators were identified within the *gts* region.

To further characterize the RNA profile, reverse-transcriptase PCR was performed. First active transcription of each gene was confirmed. Primers were designed to amplify cDNA from *gtsA, gtsB, gtsC*, and *lppB* from *Mcap* cells grown in Hayflick medium (not supplemented with glycerol). If the *gts* locus is an operon it would be expected that all genes would be transcribed as a single transcript. Primers were designed to amplify a polycistronic mRNA spanning *gtsA/gtsB, gtsB/gtsC,* and *gtsC/lppB*. Transcripts of expected size (450 nt for AB, 300 nt for BC, 600 nt for CL, and 275 nt for UA) were again detected for each primer set (lanes AB, BC, CL in Fig. 5-4), as well as between *recD* and *gtsA* (lane UA, Fig. 5-4). No amplicons were seen in negative control lanes in which

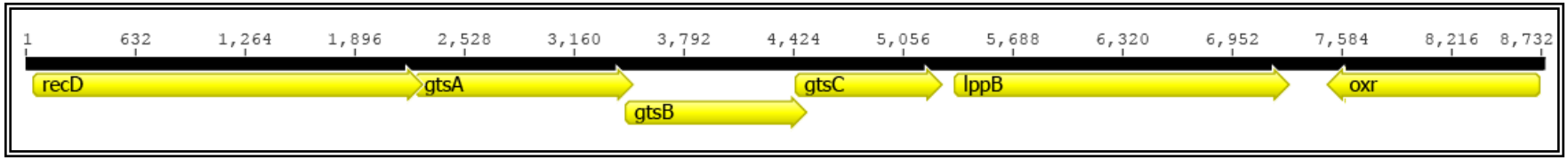

Figure 5-2. Putative Glycerol Transport Locus of *Mycoplasma capricolum* subsp. *capricolum*. *gtsA, gtsB*, and *gtsC* encode a putative glycerol ABC transporter, and *lppB* is the hypothesized glycerol binding protein. *recD* is a putative RecD/TraA helicase**,** and *oxr* is an NADH oxioreductase. Yellow arrows indicate ORFs, and are labeled according to the GenBank genome annotations. The ORFs for *gtsA, gtsB,* and *gtsC* overlap. The *recD* stop codon is 3 nt upstream of the *gtsA* start codon, and *lppB* begins 113 nt downstream of *gtsC*. The appearance of an overlap of *recD* and *gtsA* is due to the scale of the figure to encompass the entire region.

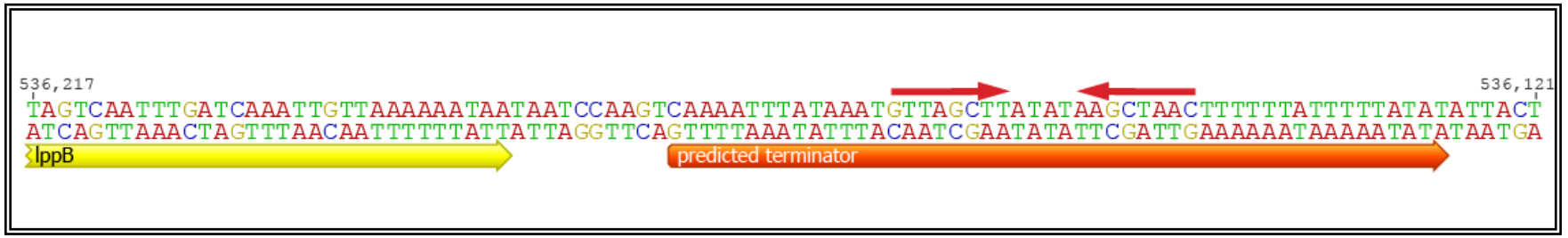

Figure 5-3. Predicted Rho-Independent Terminator in *gts* Locus. DNA sequence of the predicted rho-independent terminator identified with the TransTermHP algorithm. The yellow arrow represents the ORF of *lppB*, the orange arrow represents the predicted terminator. Red arrows above the DNA sequence show inverted repeats predicted to form the stemloop.

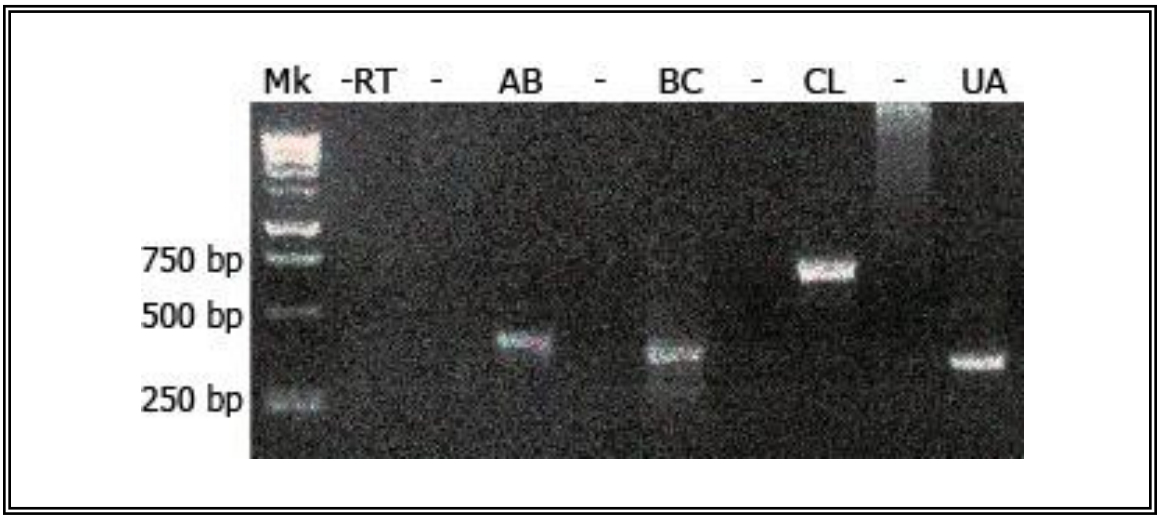

Figure 5-4. Transcript Analysis of *gts* Locus. Reverse-transcriptase PCR was used to show mRNA transcripts spanning the ORFs in the proposed operon of the *gts* locus. Lane MK is marker, lane –RT is no reverse transcriptase, lane - is no cDNA, lane AB is *gtsA/gtsB*, lane BC is *gtsB/C*, lane CL is *gtsC/lppB*, and lane UA is *recD/gtsA*.

reverse-transcriptase had been omitted, confirming that mRNA and not DNA contamination was being amplified.

Having shown transcription of the genes in this locus, efforts were made to map the 5' end of the transcripts of the genes in the *gts* locus. The approach used in this study was an RNase Protection Assay (RPA) to recognize the length of the mRNA from the AUG codon of the ORF being analyzed. Two RNA probes were designed to be complementary to the transcripts of *gtsA* or *lppB* beginning at the AUG codon and extending 500 nt upstream of the ORF. Three control reactions were included for each RPA; i) yeast RNA plus experimental probe with RNase, ii) yeast RNA plus experimental probe without RNase, and iii) 300-nt control RNA synthesized to be complementary to each probe, with RNase added. After hybridization with total RNA from *Mcap*, the appropriate samples were digested with an RNase A/T1 mixture, which selectively digests single-stranded RNA. RNA products resulting from each incubation were then separated by electrophoresis and RNA was transferred to a nylon membrane.

Detection revealed three major bands in the experimental lane for *gtsA*, one at ≈275 nt, ≈225 nt, and ≈190 nt (Fig. 5-5A) and a single band for *lppB* of approximately 350 nt (Fig. 5-5B). This raises the possibility that there are multiple initiation sites for *gtsA* or possibly post-transcriptional processing, but further study will be needed to determine the origin of each band. Interestingly, there is a single band found for *lppB* which runs at ≈350 nt. This is unexpected if *lppB* is a part of the *gts* operon as a band that is the same size as the undigested probe would be predicted. It is possible that *lppB* may be independently

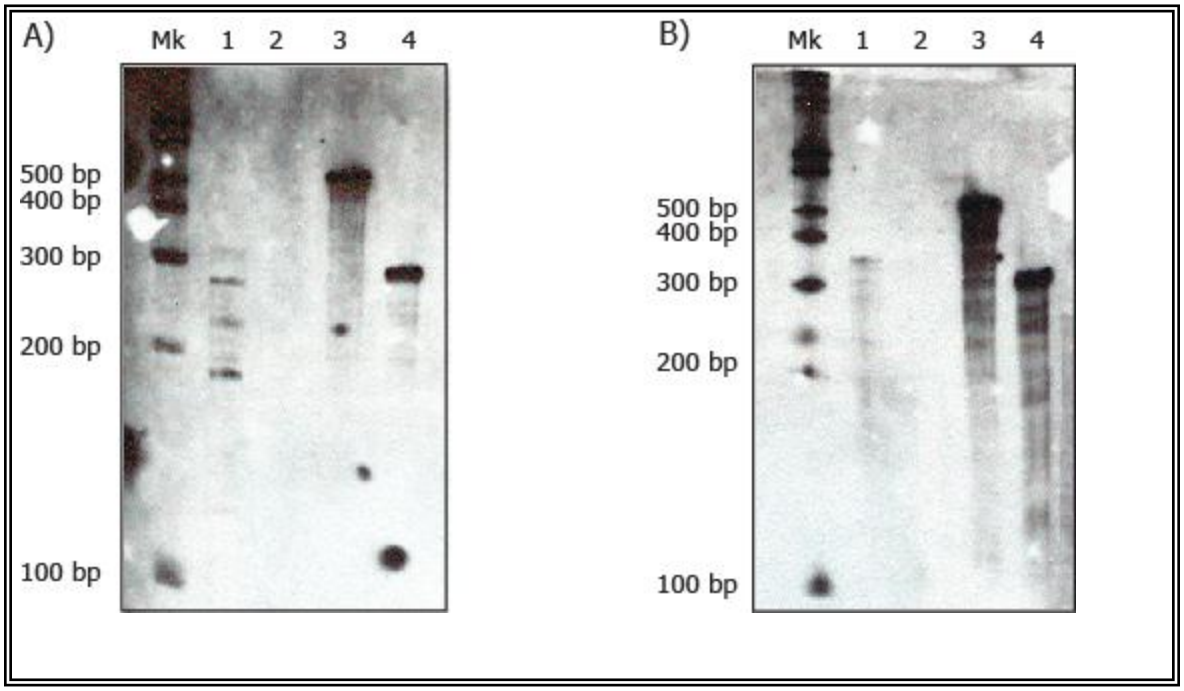

Figure 5-5. RNase Protection Assay Mapping 5' End of *gtsA* and *lppB.* A biotinylated probe was hybridized to total RNA from *Mcap.* After digestion, RNA species were separated and bands detected on membranes. A) Probe specific for *gtsA.* Bands were identified at 275, 225, and 190 bp. B) Probe specific for *lppB.* One band was identified at 350 bp. Lanes: Mk is RNA ladder, 1 is *Mcap* RNA, 2 is no target RNA control, 3 is no RNase control, 4 is positive control to known RNA transcript.

transcribed, or that there is also an internal initiation site that is responsible for transcription. The band is faint, and may be cross-reactivity of the biotinylated probe or possibly the streptavidin-HRP recognition molecule, but further study will be required to determine the origin of the band appearing in the *lppB* experimental lane. It will also be necessary to continue to characterize the transcription initiation sites of *gtsA* to determine if there are multiple sites upstream of the ORF using 5' primer-extension analysis.

Cloning of *lppB* for Protein Production

Analysis of the sequence of the *lppB* gene of *Mcap* revealed 8 UGA tryptophan codons, which in *E. coli* would be used to signal for translational stop. This necessitated the mutation of each UGA codon in *lppB* to UGG for the production of full-length protein from an *E. coli* over expression vector. In order to accomplish this, a PCR based multiple-mutation reaction (72) strategy was employed. Using the available sequence of *lppB* from the *Mcap* genome sequence, mutagenic primers were designed for each UGA codon. Although this method has been successfully utilized for *M. pneumoniae* genes (72), as well as by others in our laboratory (unpublished data), it was unsuccessful in the mutation of UGA codons in *lppB*. To overcome this obstacle, a synthetic gene was purchased in which all UGA codons were mutated to UGG codons (GenScript, Piscataway, NJ); beyond these changes the nucleotide sequence was confirmed to be unaltered. The synthetic *lppB* was cloned into the pUC57

vector (Fermentas). Oligonucleotide primers were designed with N-terminal NcoI and C-terminal XhoI (N-terminal BamHI and C-terminal HindIII for pMALC-2) restriction sites to amplify *lppB* without the lipoprotein signal sequence via PCR. Amplicons were then digested with the appropriate restriction enzymes, and cloned into the *E. coli* over expression vectors pET-20b (Novagen), pET-41a (Novagen), and pMALC-2 kindly provided by K. Wise. All plasmid inserts were then sequenced to ensure no mutations had occurred during PCR amplification and that the fusion proteins were in frame.

Over Expression of LppB-His_6

Once the *lppB* plasmids were verified, the pET-20b construct was transformed into the expression *E. coli* strain Rosetta (Invitrogen). To scale up production of protein, an auto-induction protocol was utilized (209). Two individual colonies of *E. coli* strain Rosetta with the pET-20b/*lppB* construct were inoculated into 25 ml of complex medium as described in Materials and Methods (pg. 37) and the cultures were incubated overnight. Bacteria from 1.5 ml of culture were pelleted via centrifugation and total protein samples boiled and separated on a 12% (w/v) SDS-PAGE gel. The protein was then transferred to nitrocellulose and probed with an α-His_6 tag monoclonal antibody (MP Biomedicals) that should recognize the LppB-His_6 fusion. As seen in Figure 5-6, a 72-kDa protein was detected in both induced lanes (lanes 2 and 4) as would be expected for LppB. To determine if the fusion protein was soluble, cells were

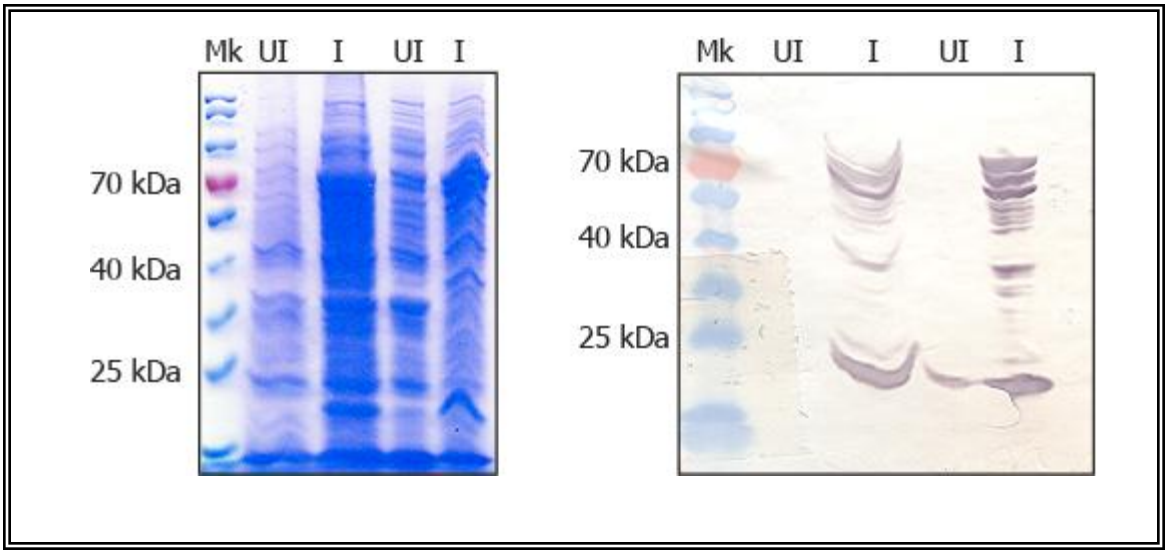

Figure 5-6. Induction of LppB-His$_6$ via Auto-induction. Two separate cultures were grown over night, pelleted via centrifugation, and total protein separated on a 12% polyacrylamide gel. Panel on left is gel stained with Coomassie Blue, panel on right is western blot. Protein was transferred and probed with an antibody specific for His$_6$-tag. As seen in the western blot, His$_6$-tagged protein was present in both induced lanes. The largest band is approximately 72 kDa, the predicted size of LppB; the lower bands are most likely degraded protein. Mk is protein ladder, UI is uninduced, I is induced.

lysed using a French press, and the cleared lysate was analyzed via western blotting. This revealed that LppB-His_6 was found in the low-speed spin pellet fraction indicating that the protein was in inclusion bodies or possibly embedded in membrane fractions (Fig. 5-6). The remaining two constructs were then analyzed for soluble protein expression as described for *lppB/*pET-20a, but LppB-His_6 was also localized to inclusion bodies despite the fusion to two different soluble binding partners, glutathione-S-transferase (GST) in pET-41a and MBP in pMAL C-2. To overcome this obstacle, GenScript used the synthetic gene made previously to over express soluble protein in the pCOLD II vector (Takara-Bio, Kyoto, Japan). The pCOLD*/lppB* vector was sequenced to ensure accuracy of the gene sequence, and no additional mutations were found. A total of 3.54 mg of protein that is >85% pure was received for future experiments.

Construction of *lppB* Mutant *Mcap* Strain

The definitive way to determine the role of LppB in glycerol uptake in *Mcap* was to delete the *lppB* gene and assess the glycerol metabolic phenotype of this mutant strain together with a complemented strain. As previously mentioned, there are few genetic tools available with which to manipulate mycoplasmas. Based on the availability of an *oriC* plasmid for *Mcap* (106), our strategy was to delete the *lppB* gene and replace it with the tetracycline resistance gene, *tetM*, via homologous recombination. This has previously been successful in *Mcap* for the inactivation of the *lppA* gene (92). pMCO3, the *oriC*

vector containing the *Mcap* origin of replication was used based on previous results showing the successful transformation and replication of this vector in *Mcap* (92,106). This plasmid is capable of replicating in *Mcap* cells, but has been shown to integrate after a number of passages (106). The construct consisted of the *tetM* gene driven by the spiralin promoter, which has been shown to be functional in multiple mycoplasma species including *Mcap* (38,106), flanked by 1 kb of DNA from the upstream and downstream regions of *lppB*, including the start and stop codons. Concurrently, a pMCO3-derived construct was assembled for complementation studies. This plasmid consisted of the full-length *lppB* gene, the spiralin promoter and the addition of the *aac-aphD* gentamicin resistance gene. This would allow for the *lppB* gene to be transformed into the *lppB* mutant strain with selection for tetracycline and gentamicin resistant colonies to attempt to reconstitute the wild-type phenotype.

Transformation of the knock-out construct into *Mcap* was performed by utilizing the electroporation protocol developed in our laboratory as described in Materials and Methods (pg. 46) (98). Each transformation was performed along with the original unmodified pMCO3 plasmid as a positive control. Despite numerous attempts and modifications (increasing and decreasing amount of plasmid used to transform, recovery time for electroporated cells, and changing tetracycline concentrations) to the protocol, transformation of the pMCO3/*lppB* and pMCO3/gentamicin-resistant complement construct into *Mcap* or *Mmy*LC GM12 wild-type was not successful, although the positive control transformation

worked on a regular basis on average yielding over 100 transformants per experiment.

While this research was in progress, an *lppB* transposon mutant, MCK 157, was identified in a transposon library generated by the group of Dr. J. Glass (J. Craig Venter Institute). PCR amplification of the *gts* locus and subsequent sequencing of the *lppB* gene confirmed the transposon insertion site, after amino acid 500 out of 628. It is unknown whether this insertion disrupts the function of LppB.

Discussion and Conclusions

Several studies have implicated glycerol metabolism as a virulence factor of mycoplasma (71,168). The *gts* locus in the *M. mycoides* cluster encodes a putative ABC transporter involved in glycerol uptake, and the presence of this locus has been correlated to pathogenicity in *Mmy*SC (228). A putative lipoprotein ORF, *lppB*, is associated with this locus in all cluster members for which sequences are available (52,228) as well as in several other mycoplasma species (55,203,227). This is significant in that gene order observed in these genomes is not very well conserved, so the association of *lppB* in multiple species suggests that it is a part of the operon. Our hypothesis is that *lppB* is a part of the *gts* locus which is transcribed as an operon, and that the protein LppB serves as the substrate binding protein of the glycerol uptake system in *Mcap*.

Initial sequence analysis and transcript mapping studies support the *gts* locus being structured as an operon. Reverse-transcriptase analysis showed the presence of mRNA that spanned each gene in the locus. The RPA for *gtsA* allowed the visualization of three bands of different sizes, one at 275 nt, one at 225 nt, and a third at 190 nt. This indicates that there may be multiple initiation sites for *gtsA*. The band that was identified in the reverse-transcriptase reaction (Fig. 5-4) between *gtsA* and *recD* may represent the promoter of *gtsA* and not the inclusion of *recD* in the *gts* operon. Now that potential initiation sites have been identified for *gtsA*, 5' primer extension can be used to map the exact nucleotide(s) at which transcription initiation begins. Of interest here are the RPA results obtained when looking for the 5' end of the mRNA for *lppB*. A band of approximately 350 nt was identified in lane 1 (Fig. 3-5B), yet the size of the probe is 500 nt (lane 4, Fig. 3-5B). It was expected that a band would be seen that was the same size as the probe if *lppB* was a part of the polycistronic message. This could be due to the presence of a promoter upstream of *lppB* which leads to low levels of expression of the single gene in addition to the polycistronic mRNA that is produced. It is also possible that *lppB* is exclusively transcribed as an independent mRNA, and is actually not part of the operon. Further transcriptional analysis will be necessary to determine if *lppB* is included in the *gts* operon, including fine scale promoter mapping using 5' primer extension.

The production of LppB protein proved to be very challenging. Mutation of all 8 UGA tryptophan codons to UGG for expression in *E. coli* was

unsuccessful, so a synthetic gene was purchased. The only mutations introduced into the *lppB* gene were the UGG codons so that the gene would be more conducive to expression in mycoplasma when it was required to complement *lppB* mutant strains. Once the synthetic gene was cloned into *E. coli* expression vectors, protein was produced. Unfortunately the over-expression vectors used lead to protein aggregation into inclusion bodies, as well as a high level of degradation. To overcome this problem the same company that synthesized *lppB* over expressed LppB and produced 3.54 mg of purified protein in addition to the plasmid constructs that were created. The pCOLD II vector from which the protein was produced was developed to efficiently express protein at colder temperatures using the promoter from the *cspA* gene (cold-shock protein A), when most protein synthesis is halted. This vector could have been more successful at LppB expression due to the colder temperatures which results in a slower rate of synthesis and protein folding. The next future step with the purified protein is to make antibodies for detection of LppB on the cell surface, as well as to look for protein expression via western analysis. Ultimately the LppB protein will be used to assay the ability of LppB to bind glycerol; this will be accomplished by binding LppB-His_6 to a nickel column and assaying the glycerol binding competition between unlabeled and radioactively labeled glycerol. This will test the hypothesis and ascertain the function of LppB on the cell surface.

Multiple attempts were made to create a deletion of *lppB* via homologous recombination in *Mcap*. While the positive control vector was successful in

transforming mycoplasma cells, yielding tetracycline resistant colonies, this was not seen for the experimental constructs. There could be several reasons for this; it could be the increased size of the constructs made transformation much less efficient, or that a restriction site for a previously unknown restriction enzyme in *Mcap* may have inadvertently been introduced into the vector. However an *lppB* transposon mutant was generated in the laboratory of a collaborator, and the insertion site has been mapped. The functionality of the mutant will be tested by comparing glycerol uptake and H_2O_2 production with the wild-type. It is possible that the truncated LppB will still retain some function as the insertion site is only 128 amino acids from the C-terminus. It is also possible that the transposon insertion will decrease the stability of LppB, leading to degradation.

The studies reported here suggest that *lppB* is co-transcribed with the previous gene *gtsC,* although further study is required to fully characterize the transcriptional profile of these genes. This also reinforces the hypothesis that LppB is associated with the putative glycerol transport system, most likely as the substrate binding protein. With protein currently in hand, the binding specificity of glycerol to LppB will be assayed and antibodies to LppB generated. The hydrogen peroxide producing capabilities of the transposon insertion *lppB Mcap* strain will also be evaluated as an initial gauge to detect LppB activity of the mutant strain. Complementation will also be attempted with the gentamicin resistant pMCO3/*lppB* construct to restore a wild-type phenotype, as complementation has not yet been used to confirm deletion effects in this locus. Together these studies will allow for the characterization of the role that LppB

plays on the cell surface of *Mcap*, and if successful will be the first report describing the function of a lipoprotein in the *M. mycoides* cluster.

Chapter 6 – Overall Conclusions and Significance

This body of work has furthered the understanding of the bacteria comprising the *M. mycoides* cluster. Despite the ability of these organisms to cause economically significant diseases in ruminants, little is known about host specificity and pathogenic mechanisms in these mycoplasma species. The identification and characterization of lipoproteins previously unknown has increased the knowledge of differences in the closely related *M. mycoides* cluster. Through genomic comparisons and protein characterization it will be possible to better diagnose, treat, and prevent disease caused by the *M. mycoides* cluster.

Comparisons of the five contingency loci in the four species included in this study have revealed several important facts. First, these loci locations appear to be highly conserved between *Mmy*SC, *Mcap*, *Mmy*LC and *Mmycapri*, suggesting that similar loci will be identified in the other two bacterial species in the cluster, *M*BG7 and *Mccp*. Being able to compare all of the putatively phase-variable lipoprotein genes will most likely prove beneficial to identifying proteins that may play important roles in host specificity and possibly in disease. Those that are more conserved between species that infect the same host, or cause the same type of disease, may give clues as to the protein function. Second, the putative phase-variable lipoprotein gene sequences have been found to be unique to each respective species and strain to some degree. Lipoprotein genes

were identified in each mycoplasma that were not present in the other members of the cluster examined, having the potential to aid in identification and differentiation of the species in this highly related group. Finally, analysis of putative promoter regions has revealed that many of the lipoprotein genes are preceded by a poly[TA] tract implying a potential to phase-vary by a conserved mechanism. The comparisons included in this research are important to our understanding of the *M. mycoides* cluster not only for the possibility to discover disease or host determinants, but also to the understanding of the evolution of this group of closely related bacteria.

The next step to this work is to begin to determine the function of the identified putative lipoproteins. By examining the role that these surface proteins play in the survival of mycoplasma within the host a clearer understanding of the pathogenicity and host specificity will be achieved. In the event that the lipoproteins encoded in these contingency loci do not play a role in disease progression it will be necessary to expand the search to uncover those genes that are involved, and the comparisons of the sequences in this study will serve as starting points in an expanded search.

Characterization of lipoproteins in these bacteria has not progressed much past immune recognition in experimental and naturally occurring infection. This body of work has laid the foundation to assess the function of the lipoprotein LppB on the cell surface of *Mcap.* If successful, this will be the first report of the use of a transposon insertion mutant and complementation construct to characterize a protein function in the *M. mycoides* cluster. This characterization

will not only be important in understanding a potential virulence mechanism, but will also help to further knowledge of nutrient acquisition and metabolism in this caprine pathogen; this information can likely be extended to other mycoplasma that contain the *gts* locus.

These data will also enable better differentiation of *Mmy*LC species from others of the *M. mycoides* cluster as well as to distinguish between strains and field isolates of this species. VlcH is the first lipoprotein to be identified and shown to undergo phase-variation in *Mmy*LC. The murine antibodies used in this study were utilized for strain differentiation and strain identification in the case of genome transplantation (107), and promise to be useful in epidemiological surveys.

The results reported herein collectively increase our knowledge about the bacteria in the *M. mycoides* cluster. Twenty-seven newly identified lipoproteins have been identified and compared, greatly enhancing the understanding of the relationship of these closely related bacteria. Ground work has been laid to improve the detection and diagnosis of infection, including the production of an antibody specific to VlcH in *Mmy*LC GM12. These results will be useful in the long run to improve diagnostic techniques and develop more effective treatments for infection with these ruminant pathogens.

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Vita

Tara R. Martin was born in Columbia, MO and grew up with the University of Missouri being the center of the community. She has two younger sisters, and most of her family is located in central Missouri. Throughout school science continued to be a fascinating subject of study, and Tara was always the student to which teachers were always saying “I will have to look that up!” After high school graduation it was fitting that she continued her education in Columbia, and the University would now be the center of her life.

While an undergraduate at Mizzou she chose to study biological sciences, and was an avid football fan. She was in Marching Mizzou for 5 years where she met many friends and her future husband. After graduation in 2001 she became engaged and married in 2003. That same month she joined the Molecular Microbiology and Immunology/Veterinary Pathobiology Graduate program at the University of MO.

Appendix 1 – Oligonucleotides Used for Contingency Locus Retrieval and Cloning

The following table contains all oligonucleotides used to perform the experiments as described in Materials and Methods.

Oligonucleotide Name	Oligonucleotide Sequence (5' to 3')	Use
TGM9-5-S1 (3-609)	GCAATTGCATGTACTGATAAGGTG	Southern Probe *Mmycapri* Gap
LCA-pep-F (4-009)	CGCGGATCCACTTTGACTATAAAATCAG	pMAL vector cloning of *vlcA*
LCA-pep-R (4-010)	CCCAAGCTTATTTTTCTAATTTTTCTTTTGA	pMAL vector cloning of *vlcA*
LCB-pep-F (4-011)	CGCGGATCCA GTGTGGTTGT AAGTAAAG	pMAL vector cloning of *vlcB*
LCB-pep-R (4-012)	CCCAAGCTTCTAGGCTTCAATTGTAAAAC	pMAL vector cloning of *vlcB*
LCC-pep-F (4-013)	CGCGGATCCGATGGTAAA ATTTTTTCAA	pMAL vector cloning of *vlcC*
LCC-pep-R (4-014)	CCCAAGCTTCTAATTAATTGTGTTGTGGTC	pMAL vector cloning of *vlcC*
TGM9-5-S2 (3-616)	GACAACAAGCTATACAAATAGCTA	*Mmy*LC, *Mmycapri* SgaT Southern probe
GM12-VmcAD-M13-1(3-583)	GTGATTACAGATAATTTTCAAATT	*Mmy*LC SgaT Region Amplification
GM12-VmcAD-Sp6-1(3-584)	ACTAATTTTTGCTATTAATGATCC	*Mmy*LC SgaT Region Amplification
Gm12-VmcEF-F3 (3-644)	GGTGGTGGAGATAGTGCTTCAGCT	*Mmy*LC GM648-13 Gap Region Amplification
Gm12-VmcEF-R3 (3-645)	GATACTGTTTTTCAATGAAACCGT	*Mmy*LC GM648-13 Gap Region Amplification
SC-s21-F (4-178)	GCAAGTGTTATTGTTCATGATGGA	*Mmy*LC s21 Region Amplification
SC-RuvB-R (4-179)	GCAATAATTAAAGGAGTTGTATT	*Mmy*LC s21 Region Amplification
MC-SGAT-F1 (2-290)	GGTGGTGGAGCTGGTATTATTGCT	*Mmy*LC GM648-13 SgaT Southern probe

Oligonucleotide Name	Oligonucleotide Sequence (5’ to 3’)	Use
d07-Hind-F (4-365)	CTTTAAAACAATCTGAAAAAGACT	*Mmy*LC GM12 LicA PCR
d07-Hind-R (4-366)	GGTAAGTAAATAAAAAATCTCTAA	*Mmy*LC GM12 LicA PCR
SGATR (5-120)	ATGATCCAGCTAAAACTATTTTTG	*Mmy*LC GM648-13 SgaT inverse PCR
SGAT-Mun1 (5-121)	AAACATAGCTGATTCTAAACAACA	*Mmy*LC GM648-13 SgaT inverse PCR
d07-F (4-367)	GATCAACTCAATCATTATGTTTAT	*Mmy*LC GM12 Southern probe
VlcA-F (5-049)	CCAAGCTTCTAATACGACTCACTATAGGGTGACAGTTCTATCGATTTCATCTAAGA	RT primers for *vlcA*
VlcA-R (5-050)	GAAAAAGAAATGCAGCAGATCAAC	RT primers for *vlcA*
VlcB-F (5-051)	CCAAGCTTCTAATACGACTCACTATAGGGTGACCAAATAGATCACCCGCATTAGCT	RT primers for *vlcB*
VlcB-R (5-052)	CCGTTGAGTGTGGTTGTAAGTAAA	RT primers for *vlcB*
VlcC-F (5-053)	CCAAGCTTCTAATACGACTCACTATAGGGTGATGTGTTGTGGTCTTCCATATCTAG	RT primers for *vlcC*
VlcC-R (5-054)	CAACTACTTCAAATAACAATTCAG	RT primers for *vlcC*
s21-F (5-055)	CCAAGCTTCTAATACGACTCACTATAGGGTGATTTGTATTTACGGTTTTGTTTTTG	RT primers for *rpsU*
s21-R (5-056)	TTGGAAGTTGAAAAAGTTAATGGC	RT primers for *rpsU*
GM12-d08-F1 (4-319)	GTCATATTTAAAAAATGCACCAAG	VlcH promoter amplification
GM12-d07-F (4-290)	CAATAAATCTAGCTTGATATTATG	VlcH promoter amplification
LppBkodn1kb (6-026)	CAGCAGCTTTTTTTAGTTTTTCTT	*lppB* knock-out construct
LppBkodn (6-029)	GTAATACGATTAAATTTTTCTCATCTCTTCTTTTCCT	*lppB* knock-out construct
tet-dn (6-030)	CATTATGCTAATTTAACACTAAGTTATTTTATTGAAC	*lppB* knock-out construct
LppBkoup1kb (6-031)	GAACTACCAAAAGAAAAAATTAAAC	*lppB* knock-out construct
LppBkoup (6-034)	TCATAAGAATATCTTGTTAAAAAATAATAATCCAAG	*lppB* knock-out construct

Oligonucleotide Name	Oligonucleotide Sequence (5' to 3')	Use
tet-up (6-035)	AGTATTCTTATAGAAGCATGCCTGCAGAATTAAAA	*lppB* knock-out construct
LppB-RNA-R (6-039)	GGTAAAGCTTTTATATTACCAGTT	RT primer for *lppB*
GtsC-RNA-F (6-040)	GCAAAAAGCATAGAATCAGAAAAAT	RT primer for *gtsC*
GtsC-RNA-R (6-041)	GAAAAGCTTTTAAAAAATTAGACC	RT primer for *gtsC*
GtsB-RNA-F (6-042)	TGTTGATTTAAATTATGCTCATAC	RT primer for *gtsB*
GtsB-RNA-R (6-043)	CATAAATAGTTCTATTAGCTTCAT	RT primer for *gtsB*
GtsA-RNA-F (6-044)	ATGATAAAAGCTATTATATAAGAC	RT primer for *gtsA*
GtsA-RNA-R (6-045)	GGACCTAGTAAAGAAATAATTTTA	RT primer for *gtsA*
mc455-RNA-F (6-046)	GTTATTGTTGTAGTGTTCATAAAAC	RT primer for *recD*
gt4001-F (8-073)	ATGAAT ATAGTTGAAAATGAAATATG	gentamicin pMCO3 construct
gt4001-R (8-074)	TATAAAAGGACTTATAAAGATTGA	gentamicin pMCO3 construct
spr-pro-R (8-075)	TCAAAGGATCTTATATTTCCTTTC	gentamicin pMCO3 construct
LppB-RPA-T7 (8-108)	TAATACGACTCACTATAGGAACTCCAATTGCTTATGTTACAAA	*lppB* RPA probe
GtsA-RPA (8-109)	GCATAACTTTTATTCTTTCATTTT	*gtsA* RPA probe
LppBkodnPST (6-142)	CATCTGCAGTTTTCTCATCTCTTCTTTTCCTATA	*lppB* RPA probe
LppB-RPA+T7 (8-542)	TAATACGACTCACTATAGGCTTGCTTCATGTTCTCTACCTAAA	*lppB* RPA positive control transcript production
LppB-RPA+control (8-543)	AACTCCAATTGCTTATGTTACAAA	*lppB* RPA positive control transcript production
GtsA-RPA+T7 (8-544)	TAATACGACTCACTATAGGCTTAAACTAATATCATCAAATTGC	*lppB* RPA positive control transcript production
GtsA-RPA+control (8-545)	TTTCTCCAGTATATGCTGATATTT	*lppB* RPA positive control transcript production