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An Aminopeptidase Acting as a Potential Factor in Host Adaptation of Mycoplasma Gallinarum

Xiufeng Wan

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AN AMINOPEPTIDASE ACTING AS A POTENTIAL FACTOR IN HOST
ADAPTATION OF *MYCOPLASMA GALLINARUM*

By
Xiufeng Wan

A Dissertation
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
in Veterinary Medical Science
in the College of Veterinary Medicine

Mississippi State, Mississippi

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2002

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ADAPTATION OF *MYCOPLASMA GALLINARUM*

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ADAPTATION OF *MYCOPLASMA GALLINARUM*

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Unlike most other host-specific mycoplasmas, *Mycoplasma gallinarum* exists as a commensal with a host range including most poultry as well as some mammals. This property of *M. gallinarum* may reflect unique mechanisms for its colonization and persistence in hosts. Whereas *M. gallinarum* shows leucine and arginine aminopeptidase activity, the genes encoding the enzymes had not been cloned and characterized. We identified an aminopeptidase gene (APN) by oligonucleotide hybridization to a genomic library of *M. gallinarum* in lambda ZAPII bacteriophage. Nucleotide sequence analysis of overlapping phage clones identified a 1,362 bp open reading frame (ORF) encoding a putative leucine aminopeptidase gene. Database searches indicate that this ORF has 68% nucleotide identity and 51% amino acid identity with the *M. salivarium* leucine aminopeptidase gene. The active sites of the leucine aminopeptidases in other eukaryotes and prokaryotes were conserved in the cloned aminopeptidase gene. Northern-blot hybridization analysis showed that this ORF is expressed as a 1.5 kb transcript. Southern-blot hybridization analysis demonstrated this gene was present as a single copy in *M. gallinarum*. Characterization of the leucine aminopeptidase demonstrated that it is a metallo-aminopeptidase

(EC 3.4.11.1) and is located in the cytoplasm with a weak interaction with the cell membrane. The subcellular location was further confirmed by immunoblotting with polyclonal anti-recombinant APN serum and *M. gallinarum* Triton-114 partitions. Immunoblotting results with sera from three chickens experimentally infected with *M. gallinarum* showed that there were very few proteins in *M. gallinarum* exposed to the host immune responses and that leucine aminopeptidase was not able to stimulate production of specific humoral antibody. Our results suggest that this leucine aminopeptidase play a role in nutrition supply for the host adaptation of *M. gallinarum* and that the enzyme was not strongly immunogenic.

DEDICATION

I would like to dedicate this research to my parents, Qizhi Wan and Jingxiang Yu, my wife Liping, and my son Hamilton.

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CHAPTER I
REVIEW OF LITERATURE

Mycoplasmas

Mycoplasmas belong to gram-positive bacteria but they differ from other gram-positive bacteria in lacking a cell wall (Fox et al. 1980; Woese et al. 1980). Genus I *Mycoplasma* and Genus II *Ureaplasma* constitute the family *Mycoplasmataceae*, which belongs to the Order I *Mycoplasmatales* in the class *Mollicutes*. According to the standards of International Committee on Systematic Bacteriology, Subcommittee on the Taxonomy of *Mollicutes* (1979), *Mollicutes* (molis, soft; cutis, skin, Lat.) is a class of organisms “lack of cell wall, typical colonial appearance, filterability through a membrane filter with a pore size 450 nm in diameter.” The *Mollicutes* include also Order II *Entomoplasmatales*, Order III *Acholeplasmatales*, Order IV *Anaeroplasmatales*, and some undefined taxonomic phytoplasma (Razin et al. 1998). More than 178 species of *Mollicutes* have been identified from humans, animals, insects, and plants since the first animal mycoplasma, *Mycoplasma mycoides*, was reported by Nocard and Roux in 1898 (Maniloff et al. 1992; Razin et al. 1998; Garnier et al. 2001). The first human mycoplasma was isolated from female genital organs in the 1930s (Dienes and Edsall 1937; Dienes 1940).

Physical properties

Mycoplasma cells vary in diameter from 0.2 to 0.5 μm . Their shape can be coccoid, coccobacilliform, bacilliform, filamentous, or ring form (Yoder 1991). Mycoplasma cells can

form a small, smooth, circular colony on culture plates. Some mycoplasmas may be motile on the surface of eukaryotic cells and show chemotactic behavior. For example, *M. pneumoniae* shows gliding motility, but the associated genes have yet to be identified (Razin et al. 1998). Mycoplasmas grow very slowly, and inhabitants of mammals and birds have an optimal growth temperature of 37 °C to 38 °C. Growth usually takes more than 3 days to form a visible colony.

The lack of a cell wall provides mycoplasmas the ability to resist beta-lactam antibiotics, such as penicillin and ampicillin, which prevent the formation of peptidoglycan, an essential element in the cell wall. Beta-lactam antibiotics, however, can be added in the media to eliminate other bacteria during preparation of mycoplasma cultures. However, the presence of inhibitory antibiotics in the cultures may affect the antigenicity of some mycoplasmas (Clark et al. 1985). Mycoplasmas are sensitive to antibiotics that inhibit protein synthesis, such as tetracyclines and erythromycins.

Genomic features

Mycoplasmas have a small genome, 580 to 1350 kb, and a low Mol%G+C, 23-40% (Razin et al. 1998; Woese et al. 1980). It has been proposed that mycoplasmas arose as a branch with a reduced genome and had a closely phylogenetical relation with gram positive bacteria *Bacillus* spp. and *Clostridium* spp. (Maniloff et al. 1992). The low Mol%G+C might result from mutation of GC to AT pairs by an AT-biased directional mutation pressure (AT pressure). Different GC content in different mycoplasmas may reflect the footprints of their evolution (Maniloff et al. 1992). In contrast to other bacteria, mycoplasmas utilize the UGA codon in addition to UGG to encode tryptophan in mRNA (Yamao et al. 1985). UGA serves as a stop codon in most other bacteria, such as *E. coli*. This may represent an example of AT pressure during evolution (Maniloff et al. 1992).

The genome sequence of *M. genitalium*, a cause of non-gonococcal urethritis in humans, was elucidated in 1995 (Fraser et al. 1995). Since then, the genome sequences of three other source-originated mycoplasmas, *M. pneumoniae*, *M. pulmonis*, and *U. ureaplasma*, have been completed (Himmelreich et al. 1996; Glass et al. 2000; Chambaud et al. 2001). The number of genes coding proteins varies from 484 in *M. genitalium* to 782 in *M. pulmonis*. These numbers are much smaller than corresponding gene numbers in other bacteria. For example, *Escherichia coli* K-12 has 4,289 protein-coding genes (Blattner et al. 1997). There are also fewer genes encoding tRNA in mycoplasma genomes. *Mycoplasma pulmonis* only has 30 structural RNA genes, whereas *E. coli* K-12 has 115 structural RNA genes. The reduced gene number reflects one feature of mycoplasmas: they lack the genes to synthesize a cell wall and many of the genes of major biosynthetic pathways for amino acid, fatty acid, and nucleic acid precursors (Himmelreich et al. 1997). On the other hand, there are some repetitive genomic sequences in mycoplasma genomes that can be classified as structural gene fragments, integrated viral sequences, and insertion-like (IS) elements. For instance, *M. pneumoniae* has multiple P1 operons; the *S. citri* genome contains the SpV1 virus sequence; and IS elements and transposons were reported to be distributed in different mycoplasma genomes, such as *M. hominis* and *M. hyopenumoniae*. The ongoing *Mollicutes* genome sequencing projects include *M. mycoides* sbsp. *Mycoides SC*, *M. hyopenumoniae*, *M. gallisepticum*, *M. penetrans*, *S. citri*, and *S. kunkelii*. The genomic data obtained by comparative genome analysis will provide a more comprehensive understanding of these unique microorganisms.

Infectious aspects

Mycoplasmas have been known as a widespread “ideal parasite” (Razin et al. 1998), generally causing little disease. However, they have been associated with diseases after the host is herded or crowded, like other infectious pathogens.

In humans, more than 16 mycoplasmas have been associated with various diseases (Tully 1993). Among them, *M. salivarium*, *M. orale*, *M. buccale*, *M. faucium*, *M. lipophilum*, *M. pneumoniae*, and *A. laidlawii* may colonize the oropharynx of humans. *Mycoplasma primatum*, *M. spermatophilum*, *M. penetrans*, and *U. Urealyticum* primarily colonize in the genitourinary tract of humans. *Mycoplasma hominis*, *M. genitalium*, and *M. fermentans* may colonize in both the oropharynx and genitourinary tract.

More than 20 species of mycoplasmas have been isolated from avian species (Stipkovits and Kempf 1996; Yoder 1991). The mycoplasma species from chickens and turkeys include *M. gallisepticum*, *M. synoviae*, *M. meleagridis*, *M. iowae*, *M. gallopavonis*, *M. cloacale*, *M. gallinarum*, *M. gallinaeaceum*, *M. pullorum*, *M. iners*, *A. equifetale*, *M. imitans*, and *Ureplasma gallorale*, and *Ureaplasma spp.* The species from ducks and geese include *M. anseris*, *M. anatis*, *A. axanthum* and *M. imitans*. *Mycoplasma glycyphilum*, *M. lipofacies*, and *A. laidlawii* have been reported from both birds and waterfowl. The pigeon source mycoplasmas include *M. columbinum*, *M. columbinasale*, and *M. columborale*. Among these mycoplasmas, *M. gallisepticum*, *M. synoviae*, *M. meleagridis* and *M. iowae* have been most significant to the poultry industry. *Mycoplasma gallisepticum* can cause chronic respiratory disease (CRD) in chickens and infectious sinusitis in turkeys. *Mycoplasma synoviae* generally causes a subclinical respiratory infection and occasionally results in an infectious synovitis. *Mycoplasma meleagridis* infection can cause an airsacculitis and also may lead to progeny development and growth problems, and *M.*

iowae is a problem in the hatchery because it reduces hatchability and increases embryonic mortality (Yoder 1991).

Mycoplasmas may also spread widely to other animals, insects, and plants. *Mycoplasma pulmonis* is an important pathogen in the respiratory and genital tracts of mice and rats, and *M. capri* and *M. mycoides* may cause pleuropneumonia in goats and the bovine. *Mycoplasma suis* may be the etiological agent of enzootic pneumonia of pigs (Taylor-Robinson 1975), *M. equirhinis* causes disease in horses (Allam and Lemcke 1975), and *M. hyosynoviae* and *M. hyorhinis* may cause arthritis in pigs (Nielsen et al. 2001; Furlong and Turner 1975). *Mycoplasma hyopneumoniae* is a frequent cause in pneumonia in piglets, and *M. mobile*, *M. phocarhinis*, and *M. phocacerebrale* were isolated from aquatic animals (Furlong and Turner 1975; Wilson 1976). However, rabbits are unusual in their lack of mycoplasma infection. This characteristic makes them particularly valuable for generating antisera for mycoplasmas (Maniloff et al. 1992). The wide prevalence of mycoplasmas also includes insects and plants (Bove 1981; Maniloff et al. 1992; Garnier et al. 2001).

Nutrition and host specificity

Because mycoplasmas do not encode many of the major genes for synthesis of macromolecule precursors, amino acids, lipids, and nucleotides, the survival of mycoplasmas depends on a nutrient rich environment. The growth environment is critical. A change of the environment may kill mycoplasmas or change their physical properties (Maniloff et al. 1992). Excessively rich media may inhibit growth (Razin et al. 1998).

Nutritional requirements and optimal growth media have not been defined for most mycoplasmas. Mycoplasma media development is done experimentally. Generally, a large number of precursors for macromolecule synthesis are required. The specific amino acid needs

vary for each mycoplasma, even for different strains of the same species of mycoplasma. Two strains of *A. laidlawii* show different amino acid requirements. *Spiroplasma melliferum* only needs asparagine, cysteine, and glutamine, and this species may represent the *Mollicute* with the fewest required amino acids (Moniloff et al. 1992). In the host, amino acids may be provided by proteolytic degradation of proteins (Clark et al. 1985).

The inorganic requirements are also difficult to define. A high concentration of ions may inhibit mycoplasma growth. Zn^{2+} , Mn^{2+} , Co^{2+} , and Fe^{2+} have been reported to prevent the growth of *M. mycoides* if the ionic concentration is higher than 1mM (Moniff et al. 1992). The inhibitory effect may vary for different media. Watanabe (1994) demonstrated that *M. orale* was more sensitive to $MnCl_2$ than was *M. salivarium*, and $MnCl_2$ had a higher inhibitory effect in media with 10% (vol/vol) horse serum than in media with 20% (vol/vol) horse serum.

Mycoplasmas are grouped as fermentative and non-fermentative, based on utilization of glucose. *Mycoplasma gallisepticum* is a typical fermentative mycoplasma, which employs glucose as a principle energy source. Different metabolic products may be derived from glucose, based on specific growth conditions. Lactate is produced under anaerobic conditions and acetate is produced under aerobic conditions. With *M. fermentans*, lactate will be produced in both anaerobic and aerobic conditions (Maniloff et al. 1992; Miles et al. 1991).

Non-fermentative mycoplasmas do not ferment sugar to produce acid. They may hydrolyze arginine or oxidize lactate, pyruvate, α -ketobutyrate, or ethanol as an energy source. *Mycoplasma hominis* and *M. gallinarum* are arginine-utilizing and non-fermentative mycoplasmas. Arginine hydrolyzation is accomplished through the arginine dihydrolase pathway. Most arginine-utilizing mycoplasmas utilize arginine as the only energy source (Maniloff et al. 1992). However, *M. gallinarum* was reported to replace arginine with organic substrates such as

ethanol, L-lactate, pyruvate, or 2-oxobutyrate as alternative energy sources. The organic acid catabolism does not interfere with arginine hydrolysis in *M. gallinarum* (Taylor et al. 1994).

The diverse habitats of various mycoplasmas reflect different nutrient requirements. This difference may underly the host or tissue-specific properties of most mycoplasmas. However, some mycoplasmas are exceptions. *Mycoplasma iowae*, an important mycoplasma for the poultry industry, has been isolated from apple seeds (Maniloff et al. 1992). Moreover, *M. gallinarum* and another *Mollicute*, *A. laidlawii*, have been reported in both mammalian and avian hosts (Stipkovits and Kempf 1996; Yoder 1991; Tully 1993; Maniloff 1992; Taylor-Robinson and Cherry 1972; Taylor-Robinson, Williams, and Haig 1968; Singh and Uppal 1987). *Acholeplasma laidlawii* was even reported in both plants and insects (Maniloff et al. 1992). *Mycoplasma fermentans* has been isolated not only from urogenital tracts, respiratory tracts, and joint fluids, but also in cell culture. Ozcan and Miles (1999) found that different strains of *M. fermentans* had different utilization patterns for nutrient components such as glucose, fructose, and N-acetylglucosamine, and that the patterns were associated with the colonization sites of the strains. They suggest the different nutrition utilization was due to insertion sequence-like elements in the *M. fermentans* genome. The underling mechanisms need to be evaluated.

Membrane structures

Like many other bacteria, the cell membrane of mycoplasmas possesses important functions besides providing a physical barrier. Because mycoplasmas must import a large number of nutrients from their extracellular environments, the membrane plays a very active role in metabolism. Mycoplasma membranes mainly are composed of phospholipid bilayer and various proteins, which consist up to 2/3 of the membrane mass (Razin, Yogev and Naot 1998).

There are more than 28 different *U. urealyticum* transporters, which represent 9 transporter families: “(1) an F-type ATPase; (2) two Amt ammonium transporters; (3) CopA, a copper-importing P-type ATPase, and PacL, a cation transport P-type ATPase; (4) a K⁺ channel from the voltage-gated ion channel (VIC) superfamily; (5) a Mg²⁺ MgtE transporter; (6) XasA a glutamate: GABA antiporter from the amino-acid-polyamine-organocation (APC) transporter superfamily; (7) two multidrug antimicrobial extrusion family transporters (MATE); (8) a *ptsH* element of a phosphoenolpyruvate-dependent sugar phosphotransferase transport system (PTS), which probably has a regulatory instead of transport function as the genome lacks the PTS EI component needed for sugar transport; and (9) a broad array of ABC transporters” (Glass et al. 2000). However, the transporters for bases, nucleotides, nickel, and urea have not been identified in *U. urealyticum*, and possibly they are in the hypothetical proteins whose functions have not been defined (Glass et al. 2000).

The gene numbers of lipoprotein in the complete genomes vary from 29 in *M. genitalium* to 54 in *M. pneumoniae*, 60 in *M. pulmonis*, and 19 in *U. urealyticum* (Himmelreich et al. 1996; Himmelreich et al. 1997; Chambaud et al. 2001; Glass et al. 2000). Some of these annotated proteins are putative lipoproteins. The prokaryotic signal peptides were present in mycoplasma membrane-bound proteins to direct the protein into the target location site after synthesis and processing (Yogev et al. 1991; Mader et al. 1991; Markham et al. 1993; Cleavinger et al. 1994; Cleavinger et al. 1995; Theiss et al. 1996; Voelker and Dybvig 1999; Flitman-Tene et al. 2000; Nishiguchi et al. 2001). Edman et al. (1999) compared the signal peptides from mycoplasma membrane protein with those from gram-positive bacteria and *E. coli*. They found mycoplasma signal peptides are different from the other two categories in many aspects: mycoplasma signal peptides have longer sequences than do any other bacterial signal peptides; mycoplasma signal peptides have their own unique periodicities of side chain hydrophobicity and volume and a

differing N terminal charge. Some differences with other bacteria signal peptidases were also found. The membrane proteins in *M. pneumoniae* were detected based on a signal peptide preceding a cysteine residue. However, details about processing and targeting of the membrane proteins are unknown. Currently, three categories of signal peptidases have been reported in prokaryotes, and they are lipoprotein diacylglycerol transferase, prolipoprotein signal peptidase, and phospholipids N-acyltransferase. The lipoprotein diacylglycerol transferase has been found in *M. genitalium*, *M. pneumoniae*, and *M. pulmonis*. The prolipoprotein signal peptidase was identified only in *M. pulmonis*. The phospholipids N-acyltransferase has not been identified in any of these three genome sequences (Himmelreich et al. 1997; Chambaud et al. 2001). Jan et al. (1995) showed that *M. gallisepticum* has an acylation mechanism similar to that in gram-negative bacteria, but different mycoplasmas probably have different mechanisms (Razin et al. 1998).

The fatty acids of membrane lipids, including phospholipids, glycolipids, and neutral lipids, are the primary sources of the hydrophobic core in mycoplasmal membranes. Like most other macromolecular synthesis precursors, mycoplasmas have a fastidious requirement for lipids. Different from other bacteria, mycoplasmas have a unique need for cholesterol. These membrane lipids have been associated with fusogenic properties of mycoplasmas. Because of the lack of a cell wall, mycoplasmal cell membranes contact directly with eukaryotic cell membranes. The membrane fusion leads to exchange of DNA and other biological materials between mycoplasmas and the eukaryotic cells, such as the importation of the nutrient requirements from the host cells and the exportation of DNA into the host cells (Rottem and Naot 1998; Razin et al. 1998). Dimitrov et al. (1993) reported that cell fusion of *M. fermentans* with T lymphocytes may require a proton gradient across the cell membrane. The membrane fusion may facilitate gene transfer between mycoplasmal cells and the host cells.

Molecular genetics

The bacterial *oriC* replication origin has been found in many *Mollicutes* genomes. It was reported that *oriC* origin was identified in *S. citri*, *M. hominis*, and *M. capricolum* and in the four completely sequenced mycoplasmas genomes, *M. genitalium*, *M. pneumonia*, *M. pulmonis*, and *U. urealyticum* (Chambaud et al 2001; Renaudin et al. 1995; Himmelreich et al. 1997; Glass et al. 2000; Sano and Miyata 1994; Hilbert et al. 1996). However, there exists a divergence between the replication origins in *Mollicutes* and those from bacteria. Not all of the components in bacterial *oriC* have been found in mycoplasma *oriC*, although generally *rnpA-rmpH-dnaA-dnaN-recF-gyrB* is highly conserved in mycoplasmas (Razin et al. 1998). The *gyrA* and *gyrB* genes were located elsewhere in the *M. pulmonis* genome and were not found in *M. hominis* and *M. capricolum*. In *M. genitalium*, AT and GC content skews symmetrically around the origin and terminus of replication (McLean et al. 1998).

Some mycoplasmas, such as *M. orale*, *M. hyorhina*, and *U. urealyticum*, have only a single DNA polymerase, whereas there are three DNA polymerases present in *S. siproplasma* and *A. laidlawii*. The DNA polymerases of *M. orale*, *M. hyorhina*, *M. genitalium*, *M. pneumonia*, and *M. pulmonis* lack 3'→5' proofreading function and impart a high rate mutation in these mycoplasmas (Barnes 1994; Razin et al. 1998).

The mycoplasmal RNA polymerases are similar to other bacteria polymerases in that they are encoded by conserved genes *rpoA* (α subunit), *rpoB* (β subunit), and *rpoC* (β' subunit). However, unlike other bacterial RNA polymerases, mycoplasmal RNA polymerases are not sensitive to rifamycin and streptolydigin. There is a single sigma factor in mycoplasmas, but at least 6 in *E. coli* and 18 in *B. subtilis* (Razin et al. 1998).

It was reported that *E. coli* transcription factors can recognize mycoplasma promoters that are similar to those in *E. coli* (Dhandayuthapani et al. 1998). The Shine-Dalgarno sequences

(AGGAGG) may be present in some mycoplasmal genes, whereas some mycoplasmal genes do not have this leader translation signal (Razin et al. 1998). The genome analysis using positional weight matrices (Boyle and Boyle, submitted) showed the annotated open reading frames in *M. pneumoniae* and *M. genitalium* do not have apparent Shine-Dalgarno sequences (Figure 1.1 A and B). However, in *U. ureaplasma* and *M. pulmonis*, the Shine-Dalgarno sequences are very apparent (Figure 1.1 C and D). Loechel et al. (1991) found another signal sequence (UUAACAACAU) was applied as a ribosome binding site in the *tuf* gene of *M. genitalium*. This sequence also worked well for *E. coli LacZ* gene. The rho-independent termination is usually employed to terminate the transcription in mycoplasmas. This termination class utilizes the step loop structure at the 3' of the gene sequence followed by a series of U residues and does not depend on the rho termination factor (Calcutt et al. 1999; Taschke and Herrmann 1986; Bove 1993). The elongation factors, *NusA* gene and *NusG* gene, have been identified in *M. genitalium* and *M. pneumoniae*. *NusA* decreases termination efficiency, and *NusG* increases termination efficiency in *E. coli* (Burns et al. 1998; Mah et al. 1999). Their roles in mycoplasmas are unknown.

Like other bacterial pathogens with small genomes, mycoplasmas have evolved to maintain genetic polymorphism so that they can evade the host immune response (Palmer 2002). As a result, a large portion of their genomes encoded lipoproteins, which play an important role in mycoplasma adhesion. These are also targets of the immune responses of the hosts.

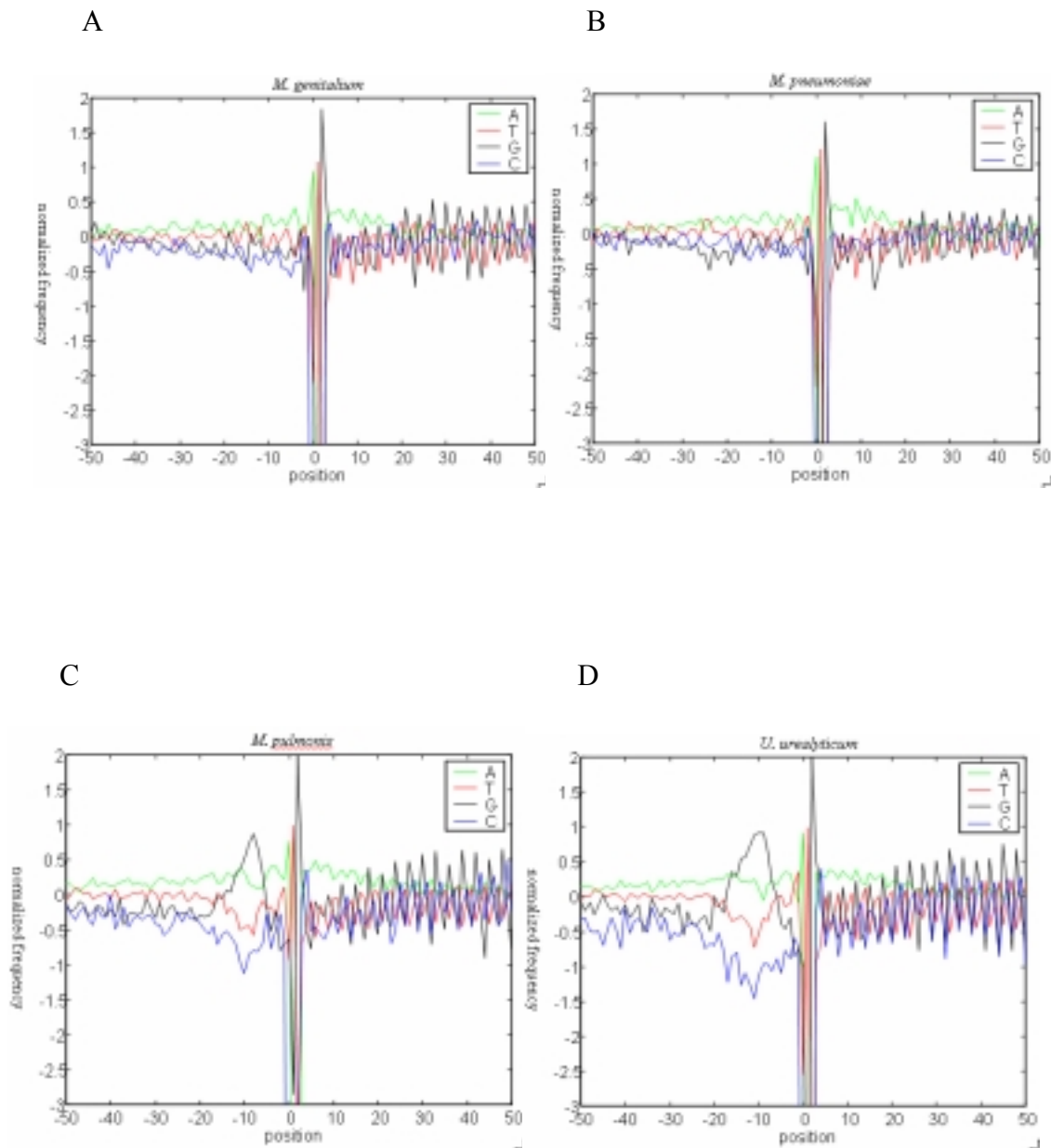


Figure 1.1 The positional weight matrices of mycoplasmal genomes. A. *M. genitalium*; B. *M. pneumoniae* genomes; C. *U. urealyticum*; and D. *M. pulmonis*. The genomes of *M. genitalium* and *M. pneumoniae* genomes do not have G rich regions at the SD region, whereas those of *M. pulmonis* and *U. urealyticum* do. These results suggest there might be different translation initiation mechanisms present in these two groups of mycoplasmas.

Pathogenesis and persistence in the hosts

The relations between mycoplasmas and associated hosts can be symbiosis or parasitism. Mycoplasmas generally exist as “ideal parasites” in chronic infections rather than as lethal pathogens in acute infections. However, parasitic and symbiotic existences are difficult to define confidently (Mims et al. 2001). Because of the knowledge gained about their pathogenesis, mycoplasmas have been implicated in various important human diseases, such as AIDS, the gulf war syndrome, chronic fatigue syndrome, Crohn’s disease, and various arthritides (Razin et al. 1998). *Mycoplasma fermentans*, *M. pirum*, *U. Urealyticum*, and *M. hominis* have been reported to be associated with HIV infections (Pollack et al. 1993; Cordova and Cunha 2000). As stated in the introductory section, mycoplasmas can also have different pathogenicities in different animals.

Factors involved in pathogenesis

Like many other bacterial pathogens, mycoplasmas enter hosts through epithelial cells of the body surfaces, especially the mucosal tissues of the respiratory or urinogenital tracts. Mycoplasmas can be transmitted among hosts both vertically or horizontally. Both pathways have been demonstrated for *Mycoplasma gallisepticum* infection (Yoder 1991). Kleven (1981) found leghorn chickens have a greater transmission potential during rather than after the first 4 weeks of infection. Lin and Kelven (1982a) found that egg transmission of *M. gallisepticum* occurred at a high rate the first 4 weeks postinfection. Grattard et al. (1995) demonstrated vertical transmission of *U. urealyticum* and *M. hominis* by isolating both mycoplasmas from women at delivery and from their neonates.

The pathogenesis of mycoplasmas may be affected by different factors. Different species of hosts, even different individuals, may show differing resistance to mycoplasma infection.

Turkeys are more susceptible to *M. gallisepticum* infection than are chickens. *Mycoplasma gallisepticum* may cause serious airsacculitis in turkey poults (Lin and Kelven 1982b), and different infection routes may result in differing pathogenicity. For *M. gallisepticum*, the intrasinus or intra-air sac inoculation may generate more serious lesions than the introocular, intranasal, or intratracheal routes (Yoder 1991). Also, different strains of mycoplasmas may show differing virulence within a species. Power and Jordan (1976) showed that *M. gallisepticum* S6 caused high mortality in chicks, turkey poults, and embryos, whereas two other strains of *M. gallisepticum* had much less virulence. In addition, co-infections with other viruses, bacteria, or parasites usually increased pathogenicity of *M. gallisepticum* infection. The clinical signs in chickens with *M. gallisepticum* are often serious when the chickens are co-infected with infectious bronchitis virus or avian influenza virus. Moreover, the pathogenicity may also be complicated by environmental conditions. Stress promotes infection by mycoplasmas, and this is a probable explanation for the observation that *M. gallisepticum* infection is more severe during cold winter. Mycoplasma infection rate depends also on how long it survives in the environment. Nagatomo et al. (2001) demonstrated that survival of *M. bovis genitalium*, *M. gallisepticum*, *M. bovirhinis*, and *M. gateae* depended on medium components and environmental temperature.

The pathogenesis of mycoplasma infections has been investigated extensively. As with other microorganisms, mycoplasmas are challenged by diverse environments. To survive in a potentially adverse environment, the bacteria must have mechanisms to compensate for challenges. Only those bacteria that can “enter, adhere to, and replicate” within the hosts would be maintained in the population (Razin et al. 1998). One challenge for the mycoplasmas *in vivo* is to avoid the host immune responses. As a result of selection pressures, mycoplasmas have developed a special tip structure organelle for adhesion and a series of strategies to avoid clearance by the host immune response. Studies *in vitro* suggested that much of the tissue damage

associated with mycoplasma infection results from innate and adaptive immune responses generated against the bacteria.

Studies show that mycoplasmas do not produce toxins, although metabolism by-products, such as hydrogen peroxide, superoxide radicals, and ammonia, may damage host cells (Megid et al. 2001; Avron and Gallily 1995; Stalheim and Gallagher 1977; Kannan and Baseman 2000b). Recently, the bacteriophage MAV1 was found to increase the virulence of *M. arthritis* to host cells after infecting this species of mycoplasma. Voelker and Dybvig (1999) identified a potential virulence factor in this virus.

Adhesion to host cells

Adhesion of mycoplasmas to host cells is a prerequisite for successful infection and colonization. Various adhesion-related lipoproteins and associated factors have been identified and characterized in different mycoplasmas. Loss or mutation of the corresponding genes encoding these cytoadhesins will remove or weaken the adhesion ability of these pathogens and thus their virulence. In addition to the cytoadhesins, adhesion of mycoplasmas requires the presence of corresponding receptors in host epithelial cells, such as sialoglycoconjugates and sulfated glycolipids (Razin et al. 1981; Kahane et al. 1982; Kahane 1983 & 1984; Zhang et al. 1994; Krivan et al. 1989). Receptors vary due to different adhesion circumstances. Even the same strain of mycoplasma may have different attachment mechanisms due to different host receptors (Gabridge et al. 1977). As a result, the presence of a flexible adhesion complex in mycoplasmas provides more opportunity to survive in hosts. The data relating to a large number of cytoadhesins and their accessory proteins identified so far have given strong evidence of this flexibility. The adhesion-associated proteins and adhesion mechanisms of several mycoplasmas are discussed in detail as below.

Mycoplasma pneumoniae. *Mycoplasma pneumoniae* is one of the mycoplasmas whose adherence mechanisms have been best characterized. This mycoplasma has specificity for various sialoligosaccharides of glycoproteins, which are found on host cells and erythrocytes (Gabridge and Taylor-Robinson 1979; Loomes et al. 1984; Roberts et al. 1989). This mycoplasma can also bind specifically to sulfated glycolipid, which can be inhibited by dextran sulfate (Krivan et al. 1989). Ultra-structural studies with tracheal tissue infected with *M. pneumoniae* showed that a specialized extension of the mycoplasma cell membrane, the attachment organelle, is found in close association with tracheal cells (Herrmann and Reiner, 1998). These organelle proteins are located in the *M. pneumoniae* Triton X-100-insoluble fraction or triton shell. Expressed on the surface of the *M. pneumoniae* attachment organelle are the products of two adhesin genes, P1 and P30 (Krause et al. 1982; Dallo et al. 1990; Krause 1996; Razin and Jacobs 1992). The P1 and P30 gene products are integral membrane proteins and are critical for adherence to respiratory epithelium cells. Avirulent mutant isolates of *M. pneumoniae* lacking the expression of P1 or P30 or both genes failed to adhere to host cells *in vitro* (Hedreyda and Krause 1995). Studies demonstrated that the P1 protein product functions as a bonafide adhesin by recognizing host cell glycoproteins (Dallo et al. 1988), and the P30 gene product was found to serve an accessory role required for the adherence properties of the P1 protein (Romero-Arroyo et al. 1999). Interestingly, both P1 and P30 genes show homology to eukaryotic cytoskeletal proteins, keratin, fibrinogen, myosin, and actin (Su et al. 1987; Dallo et al. 1996). These proteins have proline rich repeats (X-Pro-Pro-X) in the C terminus that might be involved in forming the spatial organization of tip structure of the organelle.

The *Mycoplasma pneumoniae* attachment organelle is a network of lipoproteins. Besides P1 and P30 mentioned above, HMW1, HMW2, HMW3, HMW4, P65, P40, P90, and proteins A, B, and C are also reported as components of the attachment organelle (Krause 1998; Baseman et

al. 1987; Franzoso et al. 1993). These proteins have been localized subcellularly by immunoelectron microscopy (Seto et al. 2001). Mutant characterization demonstrated these proteins were involved directly or indirectly in enhancing the adhesion of *M. pneumoniae* to all hosts. For instance, HMW1, located in filamentous extensions of the mycoplasma cells, is involved in P1 trafficking to the attachment organelle and in locating other components of the organelle (Hahn et al. 1998; Seto et al. 2001). HMW3 dominates the tip structure of the organelle as a polymer and enhances the attachment by increasing surface area. HMW3 forms an electron-dense core anchored to the terminal button with the functions of other proteins such as P30, P90, and P40 (Stevens and Krause 1992; Krause 1998). P65 is another cytoskeleton protein found in the organelle. However, the level of P65 was reportedly be related to the level of other organelle components, such as HMW1, HMW2, P1, P30, A, B, and C (Jordan et al. 2001). Recently, Layh-Schmitt et al. (2000) demonstrated that pyruvate dehydrogenase might also be a component of the attachment organelle. DnaK, one subunit of pyruvate dehydrogenase, might enhance the translocation of protein from the cytoplasm to the membrane.

Based on the *M. pneumoniae* organelle assembly model established by Seto et al. (2001), all of the components would be translocated in a specific order. HMW1 would be translocated first, followed by a group of proteins, and HMW3, P1, P30, P90, and P40. P65 would be translocated later.

Mycoplasma genitalium. The attachment mechanisms of *M. genitalium* and *M. pneumoniae* have also been investigated, because both are significant to human health. *Mycoplasma genitalium* infects and colonizes the urogenital tract, whereas *M. pneumoniae* localizes in the respiratory tract. The MgPa cytheadhesin of *M. genitalium* showed large homology with P1 in *M. pneumoniae* (Hu et al. 1987; Inamine et al. 1989; Mader et al. 1991; Opitz and Jacobs 1992). A proline-rich structure with no cysteine residues in the C terminus of MgPa was

conserved. Proline-rich organization indicates an inflexible structure, and cysteine-rich organization indicates a flexible structure. A proline-rich region is usually involved in the interaction of multiple proteins (William 1994). This suggests that this region has important functions during the interaction between cytoadhesins and their receptors. Interestingly, the other two cytoadhesins in *M. genitalium*, P32 and P69, not only were homologous with P30 and HMW3 in *M. pneumoniae*, but also conserved the operon-like organization of the latter. Moreover, the accessory proteins in *M. pneumoniae*, HMW1, HMW2, and HMW4, were also identified in *M. genitalium*.

Despite the similarity between MgPa and P1, epitope mapping experiments with different anti-MgPa monoclonal antibodies demonstrated different adherence sites. The functional domain of MgPa is apparently located in a region of the sequence that is not homologous with P1 in *M. pneumoniae* (Opitz and Jacobs 1992). These results suggest that mycoplasmas have adapted to different epithelial cells during evolution. The different A+T content in MgPa and P1 suggests these two genes might have evolved independently (Dallo et al. 1989). The associated similarity might result from a gene transfer during evolution (Reddy et al. 1995).

Mycoplasma gallisepticum. The adhesion of *M. gallisepticum* to glycoprotein on ciliated tracheal epithelial cells is necessary for the initiation of chronic respiratory disease in poultry (Glasgow and Hill 1980; Whithear 1996). Experiments to evaluate the adhesion of *M. gallisepticum* to glass suggested that an ionic bond rather than salt bridges functions in the attachment process (Kahane et al. 1979).

Similar to other mycoplasmas, a number of lipoproteins have been associated with this adhesion of *M. gallisepticum* to host cells. The cytoadhesin mgc1 cloned from a virulent field strain of *M. gallisepticum*, the S6 strain, showed homology to MgPa in *M. genitalium* (26.3%) and

to P1 in *M. pneumoniae* (28.7%) (Dohms et al. 1993; Keeler et al. 1996). The *mgc1* gene was identified as the second gene of a three gene operon. Subsequent studies identified the first gene of the same operon as a P30 homolog termed *mgc2*. This 0.9 kb open reading frame showed about 40.9% predicted amino acid homology with the P30 of *M. pneumoniae* and 31.4% with P32 of *M. genitalium* (Hnatow et al. 1998). An additional P30-like cytoadhesin, *pvpA*, was identified in *M. gallisepticum* with 54% and 52% homology to P30 of *M. pneumoniae* and P32 of *M. genitalium*, respectively. *pvpA* also shared 50% homology with P30 of *mgc2* and 49% to HMW3 of *M. pneumoniae*. Similar to some organelle components in *M. pneumoniae*, *pvpA* protein has a proline-rich carboxy-terminal region. Interestingly, the *pvpA* gene showed length variation in different strains of *M. gallisepticum* (Boguslavsky et al. 2000). *GapA*, another P1-like cytoadhesin in *M. gallisepticum*, is a trypsin-sensitive, surface-exposed protein, and the encoding gene exists as a single copy in *M. gallisepticum* (Goh et al. 1998). Papazisi et al. (2000) found that two proteins, p116 and p45, may also play an important role in the adherence process.

Additionally, *pMGA* cytoadhesins were found to function in the adhesion of *M. gallisepticum* (Markham et al. 1992). Studies suggested that there are between 33 and 79 copies of this gene in the virulent *M. gallisepticum* S6 and R strains, respectively (Baseggio et al. 1996). The currently ongoing *M. gallisepticum* genome sequencing project will unveil the structural organization of this cytoadhesin family.

Other mycoplasmas. A number of adherence associated proteins were also identified and characterized in other mycoplasmas. Some of them are homologous to the proteins described above. However, some are novel and do not show any homology with the currently identified cytoadhesins, such as P50 in *M. hominis* and P97 in *M. hypopneumoniae* (Feldmann et al. 1992; Zhang et al. 1995). Similar to *pMGA* in *M. gallisepticum*, there are also a number of gene repeats

in different mycoplasmas genomes, such as *vlp* family in *M. hyorhinitis*, *vsp* family in *M. bovis*, and *vsa* family in *M. pulmonis* (Rosengarten and Wise 1991; Behrens et al. 1994; Bhugra et al. 1995). As stated above, different numbers of cytoadhesins provide the opportunity for mycoplasmas to infect different hosts. The motility of mycoplasmas carries them to the target colonization sites, at which the attachment process begins.

Antigenic variation

Antigenic variation of surface antigens is one mechanism by which mycoplasmas evade phagocytosis from the host immune system (Razin et al. 1998). This phenomenon is also called phenotypic switching because this process will result in alteration of the microorganism's phenotype. Antigenic variation may result from random point mutations in associated genes. This variation is irreversible and inherited by the next generation. This phenomenon is also referred to as phase variation, and it may occur even without host immune responses and generate a large mutant pool. However, host phagocytes and shifting of the bacteria's living environments may provide selection pressure for this process. As a result, only the population with "preferred" mutations will survive. Another source of antigenic variation comes from regulated antigenic changes that respond to the extracellular signals, which might regulate the expression of a gene family in mycoplasmas and lead to alteration of the bacteria's antigenic character.

The changes in mycoplasma surface proteins are usually studied with monoclonal antibody reagents and colony blots of colonial mycoplasma populations. In these experiments, a given strain would appear heterogeneous for the expression of a particular protein, as detected by a monoclonal antibody reagent. Those colonies that lack expression of the protein will generally initiate expression of the protein with replating (Rosengarten and Yogev 1996). This has led to the proposal that such mechanisms are utilized by mycoplasmas to avoid elimination by the host

immune response (Citti and Rosengarten 1997). As stated above, mycoplasmas could have a different protein export system from other bacteria. So mycoplasmas might have an efficient and unique signal transport pathway by utilizing the minimal associated genes in their genomes. However, the minimal genomes of mycoplasmas have a limited number or no genes associated with this signal transport process (Razin et al. 1998). Thus, phase variation with random mutations may be a more frequent mechanism utilized by mycoplasmas.

Antigenic variation via regulation of gene family expression. Although they have a minimal genome size, many mycoplasmas have a number of gene repeats, such as *pMGA* gene family in *M. gallisepticum*, *vlp* family in *M. hyorhinitis*, *vsp* family in *M. bovis*, and *vsa* family in *M. pulmonis*. In *M. gallisepticum*, the *pMGA* gene family occupies up to 7.7% and 16% of the total genome size in strains F and R, respectively (Razin et al. 1998). Presence of these various gene copies allows the flexibility of turning on or off an individual gene copy during expression. In addition, the selection of a gene from the family can cause alternation of its length to provide further flexibility.

Although there are a large number of *pMAG* gene copies in *M. gallisepticum*, only a single copy of this gene will be expressed for an individual strain. For example, *M. gallisepticum* S6 strain expresses the *pMGA1.1* gene, whereas the *M. gallisepticum* R strain expresses the *pMGA1.2* gene. In RT-PCR experiments, a low level of mRNA for two additional *pMGA* genes (*pMGA1.4* and *pMGA1.8*) was also found in the *M. gallisepticum* S6 strain population. However, only the *pMGA1.1* gene was expressed at the protein level (Glew et al. 1995). These observations were confirmed further and extended by Milosevic et al. (2000). They examined *pMGA* gene expression in samples of virulent *M. gallisepticum* obtained from infected chickens throughout Europe and the United States. Their results showed that the samples expressed either the *pMGA*

1.2 or *pMGA* 1.9 gene, but not both. It is intriguing that in the samples that expressed only the *pMGA*1.2, analysis of the primary sequence revealed duplication or deletion of short sequences. Moreover, other deletions of sequence within the *pMGA*1.2 gene of some samples resulted in the loss of epitopes recognized by monoclonal antibodies. These studies suggest a mechanism by which *M. gallisepticum* strains can survive in the presence of host immunity, given the limited number of *pMGA* genes expressed. However, it is unclear if the *pMGA* variant proteins possess additional functions in the host adaptation of *M. gallisepticum*.

One example of a gene family in which surface proteins differ in amino acid sequence is the *vsp* gene family of *M. bovis*. At the gene level, the *vsp* genes show conservation in the 5' region encompassing the lipoprotein signal peptide sequence. However, the C-terminal region of the genes contain 1 to 4 regions or blocks of repeated sequences. The expansion or contraction of these regions can result in surface *vsp* proteins with different antigenic properties (Rosengarten et al 1994; Sachse et al. 2000).

The *vlp* family of *M. hyorhinis* shows variation both in size and expression. The *vlp* lipoproteins are encoded by a family of 7 genes. In the promoter region of these genes is a sequence of adenines that undergoes random length changes by the addition or deletion of a single base pair. Only those *vlp* genes with a total of 17 adenines in this region are transcribed, and silencing the gene correlates with the presence of an additional base pair. Similar to the *vsp* genes of *M. bovis*, the C-terminus of the *vlp* genes has several regions of repeated sequence (Yogev et al. 1991&1995). Examination of colony blots of *M. hyorhinis* isolates with *vlp*-specific monoclonal antibodies demonstrated that more than one *vlp* gene can be expressed at a time (Rosengarten and Wise 1991).

Similar to the *pMGA* family of *M. gallisepticum*, the number of *vsA* copies can vary for different *M. pulmonis* strains. Seven *vsA* genes were found in the *M. pulmonis* UAB 6510 strain,

and 11 of these genes were reported in strain KD735-15 (Bhugra et al. 1995; Shen et al. 2000). Shen et al. (2000) compared the *vsa* genes in *M. pulmonis* KD735-15 with those in strain CT. They found that *vsaB* and *vsaD* are absent in CT and *vsaG* and *vsaH* are absent in KD735-15. Like *M. gallisepticum*, only a single *vsa* gene is expressed for a given strain of *M. pulmonis* (Bhugra et al. 1995). The difference between active *vsa* genes and silent *vsa* genes is that the latter do not have a lipoprotein signal peptides. However, both genes conserved the *vsa* mature protein coding sequence and 3'-terminal repetitive elements (Simmons et al. 1996).

In addition to the variable expression of genes for a large gene family, mycoplasmas also have mechanisms to shield the products of single copy genes from the host immune response. Zhang and Wise (2001) found the expression of P120 masked the expression P56 in *M. hominis* and P120 was present only when the P56 protein was absent. These mechanisms would enhance the antigenic diversity of mycoplasma surface protein presented to the immune system.

Phase variation via random mutations. The mutations contributing to phase variation could occur at the promoter regions, the sequence preceding the mature protein or the inner sequence of the protein. The first case would down-regulate the expression, and the latter two would lead to phenotypic shifting in translational level. In addition, phase variation can occur through genomic rearrangement, which might result from gene conversion, DNA inversion, gene duplication or homologous recombination, or movements of a transposon within the chromosome (Razin et al. 1998; Lysnyansky et al. 1996).

At 21 bases upstream of the -35 box of *pMGA* in *M. gallisepticum*, there are a series of GAA tri-nucleotide repeats (Markham et al. 1993). Previous studies suggest that the exact number (12) of GAA repeats is required for proper positioning of the RNA polymerase and *pMGA* gene expression (Baseggio et al. 1996; Glew et al. 1998&2000; Liu et al. 2000). More or

less than this number blocks expression of the *pMGA* gene. In the *vlp* promoter of *M. hyorhina*, there is a poly (A) track between the -10 box and -35 box. The alteration of this poly (A) site with random insertion or deletion also led to an on and off switching of transcription (Yogev et al. 1991; Citti and Wise 1995).

There is a poly(A) site located between the signal peptide and mature *vaa* gene in *M. hominis*. Insertion or deletion of a single nucleotide at this site could lead to a translational frameshift, which would result in a UAG stop codon right after the poly(A) tract and before the mature *vaa* (Zhang and Wise 1997). Thus mutation of this poly(A) would turn on or off the *vaa* expression at the translational level. This phase variation mechanism is employed also in the P78 gene, a surface protein in *M. fermentans*. Mutation of poly(A) located before the mature protein resulted in the selective expression of this gene (Theiss and Wise 1997). Phase variation can also occur in “hot spots” within the structure genes. The insertion or deletion of a single adenine into a normal stretch of 7 adenines within P1 of *M. pneumoniae* truncates P1 translation products (Su et al. 1989).

The phase variation of *vspA* can occur by genomic rearrangement. This process can silence the *vspA* gene. Sequence analysis found an insertion of about 800 bp was located 70 bp upstream of the initiation codon of *vspA* (Razin et al. 1998). A gene family adjacent to *vspA* has significant homology to bacterial IS elements, suggesting a possible involvement of regulatory proteins with these elements in phase variation. Lysnyansky et al. (2001) identified gene inversion as one possible mechanism of phase variation in the *vsp* genes of *M. bovis*. Their studies showed that the two “cassettes” located upstream of the gene were active in phase variation of *vsp* in *M. bovis*. The first cassette is located at a 71-bp region upstream of the ATG initiation codon. This cassette has 98% homology among all *vsp* genes. The second cassette has a size of 50 to 180 bp and is located upstream of the first cassette. When the second cassette was

juxtaposed to a silent *vsp* gene, this gene was found to be expressed. This result demonstrated the active role of the second cassette in regulation of gene expression.

Reiterated sequences in other bacterial species were shown to be associated with ligand binding and generation of antigenic variation (Razin et al. 1998). These repetitive sequences are present in mycoplasmas, such as P30 and P1 in *M. pneumoniae*, *vlp* in *M. hyorhinae*, and *vsp* in *M. bovis*, and they may generate another mechanism of antigenic variation by size variation.

As stated above, antigenic variation provides mycoplasmas a good shield to escape host immune responses. However, mycoplasmas might employ mechanisms other than antigenic variation to survive in a specific host. Jensen et al. (1998) investigated antigen variation among strains of *M. hominis* isolated from one patient and those from different patients. Their results showed that there was almost no antigenic variation or genomic variation among isolates from the same patient. However, isolates from different patients exhibited a high level of antigenic and genomic variation. These results suggest mycoplasmas may utilize other mechanisms to avoid host immune responses once it establishes infection in the hosts. Why did *M. hominis* not undergo antigenic variation within the same patients? One possible reason may be the disadvantage of antigen variation by random mutation, which may generate many dead cells. This phenomenon was different from other reports of antigenic variation, which usually occurs in infected individuals at the population level (Mims et al. 2001).

Interactions with the host immune system

A successful mycoplasma infection has to overcome various challenges, including adherence, colonization, and transmission in the host population. First, mucus secreted by goblet cells will provide a physical barrier against the mycoplasmas infection. Both nonspecific and specific immune responses from the host are encountered by those mycoplasmas passing through

mucus. Secretion IgA can bind mycoplasma cells and prohibit them from binding to mucosal cells and thus from entering the host (Mims et al. 2001). Cell-surface lipoproteins of mycoplasmas are the primary targets of humoral immunity (Chambaud et al. 1999).

Infection by mycoplasmas stimulates both systemic and local immune responses. However, some mycoplasmas may mimic molecules of the host and avoid recognition by elements of the immune system. Furthermore, some mycoplasmas have evolved some special strategies to induce immunosuppression.

The following sections review current findings with the interaction of mycoplasmas and their hosts: 1. how mycoplasmas activate the host immune responses; 2. how mycoplasmas result in immunosuppression of the hosts; and 3. how mycoplasmas mimic host molecules to avoid recognition by the host.

Phagocytosis of mycoplasmas. The non-specific immune response that mycoplasmas face includes phagocytosis from macrophages and polymorphonuclear leucocytes of the respiratory and genitourinary mucosae (Marshall et al. 1995). These phagocytic cells set up the first line of host defense.

The first step for phagocytosis is the attachment of mycoplasmas to phagocytes. Cytadhesins, sialoglyco-conjugated proteins, or lipids will take part directly in the interaction between mycoplasmas and phagocytes. The morphology of mycoplasmas affects the attachment. Filamentous mycoplasmas are difficult to engulf, although this effect may be removed by opsonisation. A highly hydrophobic membrane may facilitate the engulfment of mycoplasmas (Marshall et al. 1995). However, this process may be inhibited by antiphagocytic proteins or capsular material. The capsule of *M. dispar* was found to prohibit the activation of bovine alveolar macrophages and phagocytosis (Almeida et al. 1992). Treatment with trypsin will

weaken this inhibitory effect (Jones et al. 1972). Other inhibitory factors may come from physical barriers, such as binding of the immunoglobulin Fc region to mycoplasma cells (Marshall et al. 1995).

Following attachment, mycoplasmas cells will be engulfed by invagination of the phagocyte membrane. This is followed by a respiratory burst and fusion of the engulfed cells with lysosomes to create phagosomes, which destroys the mycoplasma (Marshall et al. 1995). Mycoplasmas can avoid phagocytosis and killing by mycoplasmal proteases, lipases, and phospholipases. The oxygen radical generation affects the integration and metabolism of phagocytes. Moreover, ammonia produced by mycoplasmas may damage the lysosome or phagosome.

However, some mycoplasmas may survive after being engulfed by phagocytes. Thus, the opsonization with both specific antibody and complement components results in efficient phagocytosis and destruction of mycoplasmas. *Mycoplasma fermentans* cells were found in the peripheral mononuclear cells of AIDS patients (Lo et al. 1989 a&b). Complement-mediated phagocytosis provides more chance for mycoplasmas to survive than does antibody-mediated phagocytosis (Webster et al. 1988; Marshall et al. 1995). However, the mechanism by which the intracellular mycoplasmas escape the phagocyte bactericidal effect is not understood well.

Different types of phagocytes use different mechanisms to kill mycoplasmas. Polymorphonuclear leucocytes and mononuclear phagocytes are employed efficiently via antibody mediation, whereas phagocytes use direct mycoplasmacidal activity. As a result, different strains of mycoplasmas may be eliminated by different mechanisms because of different features of mycoplasmal cells (Davidson et al. 1988; Lai et al. 1987; Marshall et al. 1995).

Activation of host immune responses. Infection by mycoplasmas stimulates the release of proinflammatory cytokines from phagocytes. These cytokines play an important role in interactions of mycoplasmas with host immune systems. The immunological responses of hosts vary based on the associated antigens of various mycoplasmas. The cytokines detected in mycoplasma infection are IL-1, IL-2, IL4, IL8, TNF- α , IFN- α , IFN- γ , and granulocyte-monocyte colonystimulating factor (GM-CSF) (Razin et al. 1998). These proinflammatory cytokines activate B cells, T cells, NK cells, monocytes, and polymorphonuclear cells. They also upregulate adhesion molecule expression on the surface of endothelial cells and neutrophils surface. Inflammation results as chemokines attract leukocytes (Ross et al. 1992).

NK cells may be activated directly by mycoplasmas or indirectly by different cytokines secreted by macrophages in response to the mycoplasma infection, such as TNF- α , IFN- α , IFN- γ , IL-1, and IL-2. Although there are many reports of NK activation *in vivo* by different mycoplasma species, it is not clear whether they are activated directly by mycoplasma or indirectly by proinflammatory cytokines *in vivo* (Razin et al. 1998). The expression of MHC class I and class II on macrophages can be upregulated by proinflammatory cytokines or by mycoplasma antigens. The *M. arthritidis* mitogen (MAM) was reported to directly activate NK cells (D'Orazio et al 1996). Binding of the N-terminus of MAM can activate MHC class II, whereas the C-terminus of MAM mediates its recognition by T cell receptors (Langlois et al. 2000). The activation of MHC by *M. arginini* depends on macrophage-derived TNF- α , but not IFN- γ , IL-4, and GM-CSF (Stuart 1993).

As occurs with other infectious pathogens, T and B cells are activated both by mycoplasma mitogens and cytokines released by macrophages. After activation, B cells proliferate, differentiate into antibody secreting plasma cells, and then generate specific antibodies. T cell activation requires antigen presenting cells to present mycoplasma derived

antigen to the T-cell receptor in the context of MHC. Activated T lymphocytes secrete various cytokines, including both upregulating and downregulating cytokines. Upregulating cytokines, such as IL-4, IL-6, and TNF- α , may activate memory B cells to differentiate into plasma cells and secrete specific antibody. *Mycoplasma fermentans*-infected macrophages can induce T-cell apoptosis, which might be responsible for the persistent infection of this mycoplasma (Jendro et al. 2000).

Immunosuppression. Arginine metabolism through the arginine dihydrolase pathway is an energy yielding pathway for non-fermentative and some fermentative mycoplasmas (Miles 1992). Interestingly, arginine depletion was found to suppress the activation of lymphocytes *in vitro*, although this effect has not been verified *in vivo* (Razin et al. 1998). The temporary suppression of lymphocytes caused by *M. arthritidis*, an arginine-utilizing mycoplasma, might reflect the effects of arginine depletion *in vivo*.

Cole et al. (1993) demonstrated that the superantigen MAM may also be involved in suppressing T-cell mediated immunity and activating B-cell immunity. Splenocytes from mice injected with MAM showed decreased interleukin-2 (IL-2) production but a marked increase of IL-4 and IL-6 production. This suggested that mycoplasmas may directly suppress T-cell cytokine production. *Mycoplasma pneumoniae*, *M. bovis*, and *M. pulmonis* can also suppress host lymphocytes directly. The former two mycoplasmas can affect the T-cell mediated immune responses, and *M. pulmonis* is able to decrease humoral immunity (Thomas et al. 1990; Jacobs et al. 1988; Aguila et al. 1988; Razin et al. 1998). *Mycoplasma hyorhinis* can also suppress both cell mediated and humoral immunity and the production of IL-2 (Teh et al. 1988).

Mycoplasma fermentans and *M. penetrans* interfered with host immune responses by killing the lymphoid cells. *Mycoplasma fermentans* (incognitus strain) exhibits an ability to fuse

with T lymphocytes and induce apoptosis (Dimitrov et al. 1993), and *M. penetrans* can penetrate human CD4⁺ lymphocytes and monocytes and kill these cells (Lo et al. 1993; Sasaki et al. 1995).

Mycoplasmas also induce the production of downregulating cytokines, such as IL-10 and IL-13, both of which can prevent inflammation. IL10 is secreted by the Th₂ subset of CD4⁺ cells and macrophages. IL-10 can inhibit IFN γ secretion and mononuclear cell inflammation. IL-13 is secreted by the Th₂ subset of CD4⁺ cells, CD8⁺ T cells, neutrophils, and non-immune cells and tissues. IL-13 can inhibit mononuclear phagocyte activation and the proliferation and differentiation of B cells. Under normal situations, both cytokines have a role in regulating immune responses. However, some pathogens can take advantage of this mechanism and employ the downregulation of cytokines in suppressing immune responses so that they can escape the host immune responses (Mims et al. 2001).

Several mycoplasmas, such as *M. pneumoniae*, *M. arthritis*, *M. fermentans*, *M. hyorhinitis*, and *M. hypopneumoniae*, exhibit the capacity to upregulate IL-10 secretion (Kita et al. 1992; Pietsch et al. 1994; Mu et al. 2001; Thanawongnuwech et al. 2001; Jayagopala et al. 2000; Matsumoto et al. 1998). IL-10 mRNA levels were increased in mice that were intranasally inoculated with *M. pneumoniae* (Pietsch et al. 1994). Mu et al. (2001) showed that the levels of IL-2, INF γ , IL-12, and TNF α were decreased, whereas those of IL-4, IL-6, and IL-10 were elevated in mice injected with *M. arthritis* MAA superantigen. The *M. fermentans* complement-activating protein M161Ag was shown to increase IL-10 along with IL-1 β , TNF γ , IL-6, and IL-12 in human peripheral blood monocytes (Matsumoto et al. 1998). In *M. hyorhinitis* infected pigs, there was also an increase of expression of IL-6, IL-10, and TNF- α (Jayagopala et al. 2000). IL-10 is found also along with IL-2 and IL-6 in an adult infected with *M. pneumoniae* (Mizukane et al. 2002). Zurita-Salinas et al. (1996) showed IL-13 was generated in cultured human fibroblasts contaminated with mycoplasmas.

Different antigens show different abilities to induce anti-inflammatory cytokines. Rink et al. (1996) compared the induction of cytokines from *M. arthritidis*-derived superantigen (MAS) and LPS. They found MAS showed a significantly decreased capacity to induce the anti-inflammatory cytokine IL-10 and IL-1 α , although both MAS and LPS have the same ability to induce proinflammatory cytokines IL-1, IL-6, and IL-8 compared to LPS. Interestingly, *M. fermentans*-derived lipid inhibited MHC II expression without mediation by IL-6, IL-10, TNF, transforming growth factor-beta, type I interferon, prostaglandins, or nitric oxide (Frisch et al. 1996).

Evasion of host immune responses. Mycoplasma components may mimic the antigenic character of host molecules. Activation of both T and B cells by such mycoplasmas components, which exhibit similar antigenic character to the host, may cause autoimmune manifestations in the host. For instance, the T cells can be stimulated as non-specific due to nonfunctional down-regulating systems, such as down-regulating cytokines induction. Bar et al. (2000) suggested that the pathogenesis of thrombotic thrombocytopenic purpura resulted from the cross-reaction between mycoplasma proteases and host proteases.

Aminopeptidases in prokaryotes

Peptidases catalyze peptide degradation. Those peptidases that cleave within the peptides are endopeptidases, whereas exopeptidases function on the N-terminus or C-terminus of peptides. Endopeptidases can be dipeptidases or tri-peptidases according to their role in cleavage of di-peptides or tri-peptides, respectively. Exopeptidases may be classified as aminopeptidases or carboxypeptidases, respectively, based on their functions on the N-terminus or C-terminus (Gonzales and Robert-Baudouy 1996).

Aminopeptidases are distributed widely among both prokaryotes and eukaryotes. They have been investigated extensively, especially recently because of their multifunctional roles in metabolism, pathogenicity, and control of cellular functions (Gonzales and Robert-Baudouy 1996; Neidhardt 1996; Riemann et al. 1999). Aminopeptidases in eukaryotes are mainly extracellular, but those in prokaryotes are not secreted into the extracellular environment. Gonzales and Robert-Baudouy (1996) stated that only 16% of 102 selected prokaryotic aminopeptidases were extracellular, while the others are either cytoplasmic or membrane-bound. The following review will refer to the aminopeptidases in prokaryotes, except for special comments.

Properties of aminopeptidases

Aminopeptidases can be classified into metallo-aminopeptidases, cysteine aminopeptidases, and serine aminopeptidases based on their inhibitors (Gonzales and Robert-Baudouy 1996). Metallo-aminopeptidases may be inhibited by chelating agents such as EDTA and 1,10-phenanthroline. Cysteine aminopeptidase are inhibited by “ Hg^{2+} , iodoacetamide, *N*-ethylmaleimide, *p*-chloro-mercuribenzoate”, and serine aminopeptidases are sensitive to “phenylmethylsulfonyl fluoride and isopropyl fluorophosphate (Gonzales and Robert-Baudouy 1996).” Divalent metallic cations are required to enhance the functions of metallo-aminopeptidases. Zn^{2+} is the most commonly associated cation. The number of Zn^{2+} ions needed per molecule varies according to different aminopeptidases. Bovine lens leucine aminopeptidase (bLAP; EC 3.4.11.1) requires two Zn^{2+} ions, although the second Zn^{2+} can be exchanged with other divalent cations, such as Mg^{2+} (Kim and Lipscomb 1993). Other cations, such as Mn^{2+} , can also be involved also in enhancing and restoring some aminopeptidase enzyme activities. Some metallo-aminopeptidases can be activated with various divalent cations, and some can be inhibited by various cations. Aminopeptidase B, one of the four cysteinylglycinases of *E. coli* K-

12, was activated by various divalent cations such as Ni^{2+} , Mn^{2+} , Co^{2+} , and Cd^{2+} (Suzuki et al. 2001). Direct ionic interaction with an active metal cation may lead to loss of function of an aminopeptidase (Green et al. 2000). Cu^{2+} , Hg^{2+} , and Cd^{2+} inhibited peptidases within *L. reuteri* CRL 1098. These required cations may help form and stabilize an optimal three dimensional structure for the activity of the associated enzyme. The functions of dipeptidases or tri-peptidases can be restored by Co^{2+} or Zn^{2+} in 10 mM potassium phosphate buffer (pH 7.0), whereas the function of aminopetidases can be restored by Mn^{2+} in 10 mM potassium phosphate buffer (pH 6.0) (Rollan and Valdez 2001).

There are several different models of inhibition and activation of metallo-aminopeptidases with metal ions. The first model proposed that the metal ions bind directly with substrate at active sites (King et al. 1989; Mock and Liu 1995). King et al. (1989) demonstrated there was a bidentate ligand between the Mn^{2+} and the substrate. They suggested this was the reason why porcine kidney prolidase prefers Mn^{2+} rather than other metal ions. Mock and Liu (1995) suggested the binding of two metal ions to the substrates within the active sites stabilized the three dimensional structure of substrate-enzyme intermediate. In contrast, a second model proposed that direct binding of metal ions to the substrate is not necessary (Wilce et al. 1998). In this model, the functions of metal ions would increase the acidity of the water bond for a proline-specific aminopeptidase in *E. coli*. Lowther et al. (1999a) proposed two mechanisms may be present in the catalysis of methionine aminopeptidase in *E. coli*. They demonstrated that the tetrahedral intermediate was stabilized by interaction of Glu204 with the metal center or by chelation of the trigonal bipyramidal cobalt ion (Co1) and potential hydrogen binding with His178. In the same year, Lowther et al (1999b) confirmed the second proposed interpretation and included His79 into this interaction as the catalytic mechanism for methionine aminopeptidase in *E. coli*.

Metallo-aminopeptidases can be sub-classified into three families (Gonzales and Robert-Baudouy 1996). One family has the conserved motif HExxH. Zn^{2+} is also associated with this family. The members of this family include PepN aminopeptidases of *E. coli* and other lactic acid bacteria. Membranes of the second family have very strong thermostability and can be inhibited by bestatin. Bovine lens leucine aminopeptidase belongs to this family. The third family includes those enzymes employing Co^{2+} as the cofactor, such as aminopeptidase-A of *Lactococcus lactis* ssp. *lactis*.

Both cysteine aminopeptidases and serine aminopeptidases do not utilize metallic ions as cofactors. As the name states, they require an active cysteine or serine residue in the active site. *PepC* in *L. lactis* ssp. *cremoris* AM2 is a cysteine aminopeptidase gene (Chapot-Char et al. 1993). The catalytic mechanisms are similar for both cysteine and serine aminopeptidases. Both utilize “nucleophilic attack” to specific sites of the substrate groups, such as the sulphur of the sulphhydryl group for cysteine aminopeptidase and the oxygen of the hydroxyl group for serine aminopeptidases (Gonzales and Robert-Baudouy 1996).

Different aminopeptidases have different pH and temperature optimums. Usually, pH values vary from 6 to 9. The temperature for enzyme activity is similar to the living temperature of the prokaryotic cell growth. The aminopeptidases from *E. coli* show maximum catalytic activity at 37 °C. The optimal temperature is 75 °C for an intracellular aminopeptidase from the thermophilic *Sulfolobus solfataricus* (Hanner et al. 1990).

Some aminopeptidases show very broad substrate specificity. They can digest various kinds of amino acids from the N terminus of the peptides. However, some aminopeptidases can catalyze the cleavage of only one type of amino acid from the N terminus of the peptides. An aminopeptidase from *Sphingomonas capsulate* released glycine, alanine, leucine, proline, and glutamate from different N-terminal amino acids, and the reactions with glycine and alanine had

the highest efficiency (Byun et al.2001). In addition to the N-terminal amino acid, residues within the substrate play a role in the substrate specificity of aminopeptidase (Ben-Bassat et al. 1987; Yoshimota et al. 1994). Yoshimota et al. (1994) synthesized 150 peptides with different amino acids at 4 specific subsites to test their relationship with enzyme activity of aminopeptidase P from *E. coli*. Their results showed that the amino acid at the second subsite affected the digestion of the N terminal amino acid.

Some aminopeptidases are monomers, but others may consist of two or more peptides. Gonzales and Robert-Baudouy (1996) recorded that 47% of their selected 102 aminopeptidases were monomers, most of which were secreted enzymes. They also found that most of the other 53% were paired as 2, 4, or 6 peptides. The quaternary structures of these enzymes have the hydrophobic regions inside and hydrophilic regions outside the molecule, giving the structure more stability. The leucine aminopeptidase from *M. salivarium* was suggested to consist of two molecules of 50 and 46 kDa (Shibata and Watanabe 1987).

To date, a number of structures of aminopeptidases have been resolved. There are 19 aminopeptidases entries in the Protein Data Bank (<http://www.pdb.org>). These structures reflect the underlying catalytic mechanisms for each specific enzyme. Wilce et al. (1998) solved the structure of a proline-specific aminopeptidase in *E. coli*. This enzyme catalyzes the cleavage of proline from the N-terminus of the substrate. Their results indicated that this enzyme was a monomer that folds into two domains under physiological conditions. At the active site, the C-terminal domain and a Mn^{2+} forms a center, with which a hydroxide ion acts on the Xaa-Pro with a nucleophilic attack. Combining the results from three inhibitors, they proposed the second model described above for the interaction between ions and substrates. Methionine aminopeptidase in *E. coli* is another metallo-aminopeptidase that functions in the cleavage of N-terminal methionine (Lowther et al. 1998). The resolution of its three dimensional structure

reflects its catalytic mechanisms. Binding of substrate to the enzyme involves interaction with a metal cofactor. This structure was stabilized by chelation of Co1 and potential hydrogen binding with His178 and His79 (Lowther et al. 1999a&1999b). However, inhibition occurred when the Co1 was still bound to His178, which was similar in many aspects to the model proposed by Wilce et al. (1998).

As stated above, some aminopeptidases can be secreted into the extracellular environment. They can also be membrane associated enzymes. The membrane associated proteins can be classified into two categories: integral membrane proteins and weakly bound internal membrane proteins. Similar to other membrane bound proteins, these enzymes usually have an apparent hydrophobic spanning region within their sequences. Aminopeptidases can also be soluble proteins present in the cytoplasm or in the periplasm of gram-negative bacteria. Periplasmic aminopeptidases generally have a signal peptide at the N terminus of the sequence, which plays a role in the protein targeting process (Gonzales and Robert-Baudouy 1996).

The leucine aminopeptidase of *M. salivarium* was shown to be a membrane-bound enzyme. The protein was reported with an apparent hydrophobic region in its C-terminus (Shibata and Watanabe 1987; Shibata et al. 1995), although they indicated this enzyme was a putative cytoplasmic protein when they submitted their sequence into GenBank (accession number P47707). Jensch and Fricke (1997) applied different subcellular location methods and identified the subcellular locations of alanyl aminopeptidase (AAP) and leucyl aminopeptidase (LAP) from *Pseudomonas aeruginosa*. Their results demonstrated LAP was cytosolic but located near the cytoplasmic membrane and that AAP was a periplasmic enzyme that had hydrophobic interactions with the cytoplasmic membrane. There are numerous aminopeptidases with similar features to LAP. They interact with the cytoplasmic membrane, especially the internal side. They usually display enzyme activity in both soluble fractions and in the membrane associated fraction.

Because the interaction between aminopeptidases and membranes is very weak, these aminopeptidases can be separated easily from membranes even without detergents (Gonzales and Robert-Baudouy 1996). Some typical examples are listed by Gonzales and Robert-Baudouy (1996): *E. coli* N-aminopeptidase (Murgier et al. 1977); the aminopeptidase from *A. calcoaceticus* (Ludewig et al. 1987); and I-aminopeptidase from *B. stearrowthermophilus* (Yoshimoto and Tsuru 1982). Generally, cytoplasmic aminopeptidases do not have a leader sequence (Lazdunski 1989). The aminopeptidase N of *L. lactis* is a cytoplasmic aminopeptidase (van Alen-Boerrigter et al. 1991). However, this enzyme may also be associated with the membrane (Gonzales and Robert-Baudouy 1996).

Functions of aminopeptidases

The basic function of aminopeptidases in prokaryotes is to digest the amino acids from extracellular peptides, which can then be used in biochemical metabolism of prokaryotes. *Lactococcus lactis* is an important bacterium in the dairy fermentation processes. During this process, lactose is fermented into lactic acid. Short peptides and free amino acids are released from milk caseins by proteolytic activity. These free amino acids are utilized by *L. lactis* as nutrients (Tan et al. 1993; Morales et al. 2001). The enzymes involved include various aminopeptidases and other peptidases. McDonnell et al. (1999) found two different cytoplasmic aminopeptidases were involved in releasing proline from Tyr-Pro-Phe-Pro-Gly. This result suggested a mechanism utilizing a combination of different aminopeptidases in the breakdown of proline-containing peptides.

Aminopeptidases can be involved also in the degradation of intracellular peptides. The degradation by intracellular peptides can be important during starvation (Goldberg and Dice

1974). *Escherichia coli* and *S. typhimurium* with aminopeptidase activity will be more stable than those strains with fewer or no corresponding aminopeptidases during carbon starvation (Reeve et al. 1984). Sequential digestion with different specific aminopeptidases may be involved in this degradation process (Miller and Green 1983). This result suggests an important role of internal peptides for survival of bacteria during starvation.

Aminopeptidases also function in protein maturation. During protein synthesis, the N-formyl-methionine terminus is formed for nascent polypeptides. The maturation of the polypeptides involves the initial removal of the N-formyl group. The removal of the methionine residue requires methionine aminopeptidase. Solbiati et al. (1999) demonstrated that removal of the N-formyl group must be completed before the further removal of the methionine residue. Their results showed methionine aminopeptidase from *S. typhimurium* is inactive on an N-formyl-methionyl peptide.

Aminopeptidases were also reported with other functions in prokaryotes (Gonzales and Robert-Baudouy 1996). The aminopeptidase encoded by *xerB* in *E. coli*, which is homologous to *pepA* in *S. typhimurium*, has been found to be involved in the stability of high copy number of ColE1 plasmids (Stirling et al. 1989). D-aminopeptidase, which digests D-form amino acids, has been demonstrated to take part in the synthesis and degradation of peptidoglycan (Asano et al. 1992). Aminopeptidase N from *E. coli* and two other aminopeptidases from *S. typhimurium*, aminopeptidase N and aminopeptidase A, were also reported as enzymes hydrolyzing and activating albomycin (Braun et al. 1983).

The functions of aminopetidases from eukaryotes have been found to be much broader than those from prokaryotes. In addition to peptide degradation and amino acid scavenging, they can cleave peptide mediators, which will lead to activation or inactivation of function. In addition,

they can function in “adhesion or signal transduction” (Riemann et al. 1999). Moreover, aminopeptidases located on the cell surface might be important in the “control growth and differentiation of many cellular systems by modulating the activity of peptide factors, as well as by regulating their access to adjacent cells” (Riemann et al. 1999; Shipp and Look 1993). CD13, aminopeptidase N, is an example of these multifunctional aminopeptidases.

Aminopeptidases in mycoplasmas

Aminopeptidase activities of mycoplasmas have been studied widely since the 1970s. Because arginine is used as an energy source, arginine aminopeptidase activity was investigated for almost all arginine-utilizing mycoplasmas. The current reports about mycoplasma aminopeptidase activity are reviewed.

Choules and Gray (1971) discovered the aminopeptidase activity associated with Leu, Ser, and Tyr from *A. laidlawii* was the cause of protein degradation during *A. laidlawii* cell membrane preparation. This study first proposed the importance of aminopeptidases in mycoplasmas.

Since then, different aminopeptidase activities have been investigated in various mycoplasma strains. Neill and Ball (1980) investigated the aminopeptidase activities in *A. laidlawii*, *M. bovirhinis*, *M. bovis*, and *M. dispar*, all of which cause calf pneumonia. Their results showed that the ability of these four mycoplasmas to degrade the same substrates decreased in order *A. laidlawii* > *M. bovirhinis* > *M. bovis* and *M. dispar*. Interestingly, their pathogenicity increased in this order. Their results suggest that more aminopeptidase activity might result in less pathogenicity. Among the 19 substrates tested, *A. laidlawii* showed a relative high activity associated with Ala, Val, Asp, Leu, Phe, Arg, Tyr, and Trp. *Mycoplasma bovirhinis* showed a relative high activity for Ala, Leu, Lys, Arg, and His-substrate. *Mycoplasma dispar* and *M. bovis* showed low activity for these substrates.

Ball et al. (1982) compared the aminopeptidase activity of 11 arginine-utilizing mycoplasmas with 22 species of mycoplasmas not utilizing arginine on 20 different aminoacyl β -naphthylamide substrates. Their results demonstrated that arginine-utilizing mycoplasmas can be classified into two groups: group A with a high arginine aminopeptidase activity and group B with a low arginine aminopeptidase activity. Their results suggest arginine aminopeptidase activity was relatively higher in arginine-utilizing mycoplasmas than in mycoplasmas not utilizing arginine. These results caused them to test arginine aminopeptidase activity in 23 more species of arginine-utilizing mycoplasmas (Ball et al. 1985). They separated them into three groups according to the enzyme activity. The first group showed high enzyme activity for all strains, including *M. gallinarum*, *M. iners*, *M. moatsii*, *M. opalescens*, and two unidentified canine isolates. The second group, including *M. alvi*, *M. buccale*, *M. capricolum*, *M. columbinasale*, *M. columbinum*, *M. equirhinis*, *M. iowae*, *M. lipophilum*, *M. meleagridis*, *M. orale*, *M. primatum*, *M. putrefaciens*, *M. sualvi*, and *M. subdolum*, showed low activity. In the third group, the arginine aminopeptidase activity varied greatly for different strains of the same species of mycoplasmas. This group included *M. argininim*, *M. gateae*, *M. maculosum*, and *M. spumans*.

Shibata and Watanabe (1989) incubated bradykinin with different mycoplasma cells, *M. pneumoniae*, *M. genitalium*, *M. fermentans*, *M. hominis*, *M. salivarium*, *M. orale*, *U. urealyticum* and *A. laidlawii*, at 37 °C for 1 hour and then injected the mixture into rabbits. The results indicated mycoplasmas were able to inactivate the vascular permeability-increasing activity of bradykinin. They also suggested that arginine aminopeptidase and carboxypeptidase were responsible for this activity.

Mycoplasma salivarium and *M. orale*, important pathogens in periodontal disease, show Leu and Arg aminopeptidase activity. *Mycoplasma salivarium* has a higher Leu aminopeptidase

activity than did *M. orale* (Watanabe et al. 1985; Watanabe et al. 1984). The Leu aminopeptidase was purified and characterized (Shibata and Watanabe 1987; Shibata et al. 1995). This enzyme is a membrane-bound enzyme composed of 50 kDa and 46 kDa domains. It is a metallo-aminopeptidase showing both Leu and Arg aminopeptidase activity. Mn^{2+} and Zn^{2+} enhanced the enzyme activity to 9 and 2 fold, respectively. An aminopeptidase *My* gene of *M. salivarium* was cloned and sequenced. The *My* gene encoded a 58 kDa protein and conserved the active sites of bovine leucine aminopeptidase (Burley et al. 1990; Shibata et al. 1995). This protein has an apparent hydrophobic region that suggests it is membrane-associated. However, leader peptide was identified using SignalP (Nielsen et al. 1996; <http://www.cbs.dtu.dk/services/SignalP-2.0/>). The reason might be that SignalP looks for bacterial signal peptidases that have features different from the signal peptides from mycoplasmas (Edman et al. 1999).

Including the putative aminopeptidase genes from the complete genomes of *M. genitalium*, *M. pneumoniae*, *M. pulmonis*, *U. urealyticum*, there are more than 37 various aminopeptidase and peptidases protein sequences in GenBank. There are 7, 9, 10, and 7 aminopeptidases in *M. genitalium*, *M. pneumoniae*, *M. pulmonis*, and *U. urealyticum*, respectively (Table 3.2). The specificity of these aminopeptidases varies. However, none of these genes have been characterized. To date, the *My* gene from *M. salivarium* is the only gene characterized from mycoplasmas.

***Mycoplasmas gallinarum* as a model to study host adaptation**

General features of *M. gallinarum*

M. gallinarum belongs to class *Mollicutes*, order I *Mycoplasmatales*, family I *Mycoplasmataceae*, Genus I *Mycoplasma* (Razin et al. 1998). It has a genome size of 727 kb and 26.5-28 Mol%G+C (Askaa et al. 1973; Maniloff et al. 1992).

The antigenic and biochemical features of *M. gallinarum* are relatively homologous. However, different strains of this species have been reported that demonstrate some DNA polymorphisms. Seven field strains of *M. gallinarum* were compared with the type strain PG 16 for genotypic heterogeneity using restriction endonuclease analysis of genomic DNA with *EcoRI*, *HindIII*, *BamHI*, *PstI*, and *XhoI* (Dov□et al. 1991). Based on the result of *BamHI* digestion, they found three different genotypes among the field strains from PG16. This might reflect the molecular basis for the wide distribution of *M. gallinarum* through different hosts, although all seven field strains involved in this research were isolated from different chicken tissues.

Previous experiments showed an antigenic difference between the type strain *M. gallinarum* (PG16) and *M. gallisepticum* (S6). The immuno-diffusion and immuno-electrophoretic patterns between PG16 and the anti-sera of S6 showed two precipitin lines, while six precipitin lines exist for S6. The antisera of PG16 had two precipitin lines for S6, whereas there were five lines for PG16. The similarities were suggested to be group specific, and the differences were suggested to be species specific (Ahuja and Chandiramani 1976).

Mycoplasma gallinarum is a non-fermentative and arginine-utilizing mycoplasma (Aluotto et al. 1970). Its growth requires 13 amino acids, including Ala, Arg, Asp, Cys, Glu, Gly, His, Ilu, Leu, Met, Phe, Thr, and Tyr (Lund and Shorb 1966). As with other non-fermentative

mycoplasmas, arginine can be utilized by *M. gallinarum* as an energy source via the arginine dihydrolase pathway. *Mycoplasma gallinarum* also shows a strong arginine aminopeptidase activity and relatively low leucine aminopeptidase activity (Ball et al. 1982). The activity of aminopeptidase may play a role in supplying the required nutrients. However, arginine is not the single energy source for *M. gallinarum*. Taylor et al. (1994) found this species can oxidize organic acid as alternative energy sources. These two processes can coexist without a prohibitory effect on each other.

There are very few reports concerning the molecular genetics of *M. gallinarum*. Currently, only the 16s rRNA gene has been sequenced (Genbank access number: L24105). It was reported that cross reaction with EF-TU protein of *M. synoviae* occurs, suggesting the presence of this protein in *M. gallinarum* (Benčina et al. 1999).

Pathogenesis of *M. gallinarum*

Mycoplasma gallinarum has been isolated from various hosts, including poultry, pig, cattle, and sheep (Singh and Uppal 1987; Taylor-Robinson and Dinter 1968; Poveda, et al. 1990). This mycoplasma is also one of the mycoplasmas most frequently isolated from poultry (Shah-Majid and Nihayah 1987; Kleven, Eidson, and Fletcher 1978). Generally, *M. gallinarum* does not cause any signs or lesions among infected hosts (Power and Jordan 1976; Varley and Jordan 1978a&b). The infection of hens with *M. gallinarum* was shown to have no effect on hatchability (Shah-Majid 1994).

This species of mycoplasma has been demonstrated to be much less pathogenic than other mycoplasmas of poultry, including *M. gallisepticum*, *M. synoviae*, *M. iowae*, and *M. meleagridis* (Kleven 1998; Power and Jordan 1976; Varley and Jordan 1978a&b). Transmission and scanning electron microscopy showed differences that may explain the difference in the

pathogenesis of *M. gallinarum* and *M. gallisepticum* (Abu-Zahr and Butler 1976). They showed that *M. gallisepticum* directly attached to the epithelial cells. It colonized the cells first and then the lamina propria. In contrast, *M. gallinarum* did not develop a close association with the epithelium cells and mycoplasma cells appeared first in the lamina propria.

M. gallinarum inhibited the replication of goose parvovirus strain "B" in cell culture. However, *A. axanthum* does not have this effect. Continuous supplementation of arginine into the *M. gallinarum* and virus culture can remove the inhibitory effects (Kisary and Stipkovits 1975).

Inoculation of chickens with *M. gallinarum* may cause airsacculitis when combined with infection by infectious bronchitis virus or vaccination for Newcastle disease or infectious bronchitis (Kleven et al. 1978; Kleven 1998). Shah-Majid (1996) investigated the effects on the tracheal epithelium by coinfection of *M. gallinarum* and Newcastle disease virus (F strain). They found many non-ciliated tracheal epithelium cells during 1 to 3 days after the infection. These cells regained the cilia by 1 week after the infection.

The presence of *M. gallinarum* in different tissues was reported to depend on the host species infected. In a series of laboratory experiments, *M. gallinarum* was only recovered from the infra-orbital sinus and trachea of infected chicks (Varley and Jordan 1978a). However, *M. gallinarum* can be recovered from different tissues of experimentally infected turkey poults, including the infra-orbital sinus, trachea, left lung, left abdominal air sac, clavicular air sac, heart blood, and muscle, caecal tonsil, bursa, spleen, liver, gonad, kidney, yolk, and right or left hocks. None was recovered from pericardium, foot pads, conjunctiva or brain (Varley and Jordan 1978b).

The immunological responses of poultry were investigated in early research. Based on the results of rapid serum agglutination tests and haemagglutination inhibition tests, in birds where *M. gallinarum* was recovered, 70% were serologically positive (Varley and Jordan 1978a;

Varley and Jordan 1978b). These results were confirmed further by Benčina and Bradbury (1991a&1992) using immunofluorescein and immunoperoxidase techniques. Antibody responses were investigated among chickens coinfecting with *M. gallinarum*, *M. gallisepticum*, and *M. synoviae* (Benčina et al. 1991b). They found that mycoplasma specific humoral IgG and IgM levels were very low or non-detectable, whereas IgG and IgM were detected with a high frequency (over 50%) in the respiratory mucus and in the Harderian gland. IgA was relatively undetectable for all samples.

***Mycoplasma gallinarum* serving as a model for mycoplasmas host adaptation investigation**

The wide distribution of *M. gallinarum* among different species of hosts and its persistent colonization demonstrates the presence of a unique host adaptation mechanism in this mycoplasma. The low pathogenesis of *M. gallinarum* and the weak immunological host responses suggest a unique mechanism to escape host immune responses. Thus, *M. gallinarum* serves as a good model to investigate the host adaptation mechanisms between mycoplasmas and eukaryotes cells.

Hypothesis and objectives

The hypothesis tested in this study is that some membrane associated proteins of *M. gallinarum* play an important role in the host adaptation of this species.

The objectives of this study were to: 1). identify and clone the aminopeptidase gene from *M. gallinarum*; 2). generate the recombinant protein for this enzyme and polyclonal antisera against this recombinant protein; 3). determine the subcellular location and the function of this enzyme in host adaptation of *M. gallinarum*; 4). investigate the specific humoral response to leucine aminopeptidase of *M. gallinarum* in natural infection of *M. gallinarum*.

CHAPTER II

DETECTION OF CYTADHESIN GENES IN *MYCOPLASMA GALLINARUM*

Abstract

P1-like and P30-like probes were designed from previously identified cytoadhesins to examine the presence of the P1 or P30 like genes in *M. gallinarum* genomic DNA by Southern-blot hybridization analysis. The 28 bp P30-like probe and the 29 bp P1-like probe were not able to bind with *Eco* RI digested *M. gallinarum* genomic DNA. However, during Southern hybridization analysis, the 18 bp P30-like probe showed a 3-kb band in *Eco* RI digested *M. gallinarum* genomic DNA. The 3-kb DNA fragment was then purified and ligated into the plasmid *pBlueScript-KS(-)* to construct a genomic plasmid library. The 18 bp P30-like probe was utilized to screen this library, and three positive colonies were obtained. Sequence analysis of the 2,274 bp fragment demonstrated that the cloned sequence was composed of a partial aminopeptidase gene candidate and a partial glutamyl-tRNA synthetase gene candidate. Sequence alignments showed this partial nucleotide sequence had a 54% nucleotide identity and 68% amino acid identity to a leucine aminopeptidase gene from *M. salivarium*. Further analysis showed a homology of 42% between the cloned nucleotide sequence and P30 gene from *M. pneumoniae*, which might be the reason why library screening using P30 gene resulted in an aminopeptidase-like gene. Our results suggest that there is no P1-like and P30-like cytoadhesins present in *M. gallinarum*, and that the adhesion of *M. gallinarum* to its hosts may involve other cytoadhesins or components in their membrane proteins.

Introduction

The class *Mollicutes* constitutes a special subgroup of gram-positive bacteria with minimal genomes and no cell wall. Within this class, mycoplasmas, members of the family *Mycoplasmataceae*, have been most widely studied by scientists because of the mycoplasma association with human and animal health. Mycoplasmas are spread widely among various hosts, such as plants, animals, and arthropods (Maniloff et al. 1992). In general, mycoplasmas virulent for humans and animals have evolved mechanisms of host adaptation to persist in the presence of an immune response, and therefore induce chronic inflammatory-type infections of the respiratory and urogenital tracts and, in some cases, induce arthritis-like lesions in joints (Baseman et al. 1996; Citti and Rosengarten 1997). Therefore, mycoplasmas should have some strategies to avoid clearance from the motile mucus and shield from innate and acquired immune responses. Generally, mycoplasmas exist as “ideal parasites” (Razin et al. 1998). The physical association with the host epithelial cells is required not only to avoid the surface-dependent motility but also to acquire nutrients.

Mycoplasma are able to attach to various host cells *in vitro*, such as erythrocytes, spermatozoa, fibroblasts, lung cells, skin cells, gill arches and rakers, and tracheal organ cultures (Stadtlander and Kirchhoff 1995; DeBey and Ross 1994; Zielinski and Ross 1993; Washburn et al. 1993; Gabridge 1984; Watanabe et al. 1990; Cassell et al. 1981). Different cytoadhesins, membrane-associated proteins of mycoplasmas, have been identified to function in these attachments to the glycoprotein or glycolipid receptors on the eukaryote epithelial cell surfaces and are associated with the interaction between mycoplasma cells and their hosts.

P1 cytoadhesin is an important virulence factor in *M. pneumoniae*, and it mediates the attachments of *M. pneumoniae* to host cells (Krause 1996). The P30 cytoadhesin was identified also as another cytoadhesin functioning in adhesion of *M. pneumoniae* to host cells (Dallo et al. 1990; Romero-Arroyo et al. 1999). Both P1 and P30 constitute the attachment organelle in *M.*

pneumoniae along with other accessory proteins. A loss of P1 or P30 may remove the capability of *M. pneumoniae* cells to attach to epithelial cells and thus decrease the virulence of this mycoplasma (Baseman et al. 1982; Krause and Baseman 1983; Krause 1996). P1-like cytoadhesins also have been identified in many other mycoplasma species, such as *M. genitalium*, *M. gallisepticum*, *M. pirum*, and *M. synoviae* (Dallo et al. 1989; Morsy et al 1993; Tham et al. 1994). In addition to *M. pneumoniae*, P30-like cytoadhesins were also identified in different mycoplasmas, including *M. agalactiae*, *M. gallisepticum*, and *M. genitalium* (Fleury et al. 2001; Boguslavsky et al. 2000; Hnatow et al. 1998; Reddy et al. 1995).

Antigenic variation was reported to be an important strategy employed by mycoplasmas to avoid elimination by host immune responses. Besides phenotype shifting by changing the expression products, mycoplasmas can also turn on or off the expression of the adhesion-associated genes by mutating nucleotides in the promoter region or by gene rearrangement (Theiss and Wise 1997; Razin et al. 1998).

Unlike most other host-specific mycoplasmas, *M. gallinarum* has been identified in a wide range of vertebrate hosts, including poultry, cattle, pigs, and sheep (Singh and Uppal 1987; Taylor-Robinson et al. 1968; Taylor-Robinson and Dinter 1968; Wang et al. 1990). The colonization of the respiratory tract of poultry by *M. gallinarum* alone does not result in pathologic changes, although co-infection with respiratory viruses was reported to induce clinical signs (Kleven et al. 1978; Shah-Majid 1996). Furthermore, *M. gallinarum* has not been found to induce strong antibody responses (Benčina and Bradbury 1992). Therefore, *M. gallinarum* may serve as a good model for basic studies in host adaptation.

Currently there are no reports concerning the mechanism of *M. gallinarum* adhesion and its association with the prevalence of this mycoplasma in various hosts. The goal of this project is to identify *M. gallinarum* cytoadhesins, such as P1-like and P30-like genes, which may be critical in host adaptation of this mycoplasma in various hosts.

Materials and methods

Mycoplasma strain and culture conditions

The *M. gallinarum* used in these studies (kindly provided by S. H. Kleven, University of Georgia, Athens, GA) was filter cloned three times and then used as an inoculum for a 400 ml culture in Frey Broth incubated 24 hrs at 37 °C. Aliquots of the culture were stored at minus 80 °C for use throughout the study (Ellakany et al. 1997).

Nucleic acid preparation

Genomic DNA was isolated from overnight broth cultures of *M. gallinarum* using the QIAGEN genomic DNA isolation kit (QIAGEN, Inc., Valencia, CA). Plasmids were extracted from 24 hrs cultures of *E. coli* using the QIAGEN miniprep plasmid extraction kit (QIAGEN, Inc., Valencia, CA).

Hybridization probes preparation and Southern-blot hybridization

To identify whether P1 or P30 genes are present in *M. gallinarum*, Southern-blot hybridization analysis was conducted with *M. gallinarum* genomic DNA and P1 or P30 probes designed from mycoplasma P1 or P30-like homologs.

The P1 degenerate oligonucleotide 5'-GCNGTNGGNKSNGTNTTY-3' was synthesized complementary to the conserved regions identified in amino acid sequence alignments from the P1 gene from *M. pneumoniae* (Su et al. 1987), MgPa gene from *M. genitalium* (Inamine et al. 1989), mgc1 from *M. gallisepticum* (Keeler et al. 1996), and P1-like gene from *M. pirum* (Tham et al. 1994). The P30 degenerate oligonucleotide 5'-CTNGGNCTNGSNATHGGN-3' was synthesized according to conserved regions identified in amino acid sequence alignments of the

P30 gene from *M. pneumoniae* (Dallo et al. 1990), P32 gene from *M. genitalium* (Reddy et al. 1995), and Mgc2 from *M. gallisepticum* (Hnatow et al. 1998). In these two probes, K=G+T, S=G+C, H=A+T+C, Y=C+T, and N = all four nucleotides used in the synthesis. The P30 probe was increased to 28 bp to increase the specificity, 5'-WTNGGNWTNGGNATHGGNWT NCCNATHG-3', where W=A+T, N=A+T+C+G, H=A+T+C. These probes were synthesized by Genosys, Woodlands, TX.

The oligonucleotide probes were fluorescein-labeled using the enhanced chemiluminescence (ECL) 3'-oligolabelling systems according to the manufacturer's protocol (Amersham, Arlington Heights, IL). The probes were fluorescein-labeled and made by incubating at 37 °C for 90 mins a mixture of 100 pMol oligonucleotide, 10 µl fluorescein-11-dUPT, 16 µl cacodylate buffer, and 16 µl terminal deoxynucleotidyl transferase.

For Southern-blot hybridization, 5 µg *EcoRI* digested *M. gallinarum* genomic DNA was loaded into 0.8% agarose gel and blotted onto nylon membranes (Amersham, Arlington Heights, IL) by capillary transfer after electrophoresis at 30 volts for 17 hrs. The nylon membranes were baked at 80 °C for 2 hrs. The membranes were first prehybridized at 42 °C for 4 hrs, and then 10 ng labeled probe was added to the hybridization solution (Amersham, Arlington Heights, IL) and hybridized for 16 hrs at 42 °C. Blots were then washed twice with a low stringency buffer (5×SSC, 0.1% SDS) for 5 mins at room temperature. Finally, the blots were washed twice with a high stringency buffer (1×SSC, 0.1%SDS) for 15 mins at 42 °C. Afterwards, the blots were treated with chemiluminescence substrate. The chemiluminescent detection was performed with ECL detection reagents and exposed to X-ray film for various time periods.

Genomic plasmid library construction and screening

Restriction fragments in the 3 kb size range were gel-isolated from *EcoRI* digested *M. gallinarum* genomic DNA and ligated into *pBlueScript* PBS-KS (-) plasmid vector (Stratagene, Co., La Jolla, CA). For the ligation reaction, a mixture of 60 ng isolated DNA and 20 ng *E. coli* predigested *pBlueScript* PBS-KS(-) vector was incubated at room temperature for 5 mins with Fast-link ligase (Epicenter, Madison, WI). After heat-inactivation of the ligase, the ligation reaction products were then transformed into *E. coli* strain XL1-Blue (Stratagene, Co., La Jolla, CA) and spread at a density of 100 antibiotic-resistant colonies per plates. Colony lifts were prepared according to the protocol adapted from Sambrook et al. (1989). Briefly, duplicate nylon membranes were placed on the plates for 1 min and then moved to 10%SDS to lyse the cells. The lifts were denatured and neutralized for 5 mins each and then incubated on 2×SSC buffer for another 5 mins. After drying on filter paper, the lifts were baked at 80 °C for 2 hrs. The colony lifts were then hybridized with the P1 and P30 oligonucleotide probes by the methodology described for genomic Southern blot hybridization.

Nucleotide sequencing and sequence analysis

The inserts from library clones were sequenced by dye terminator cycle sequencing using the Big-Dye terminator reaction (Applied Biosystems, Foster City, CA) and *pBlueScript* PBS-KS(-) phagemid promoter primers or internal primers T7.1.854 (Stratagene, Co., La Jolla, CA). Sequencing reaction products were analyzed on an ABI Prism 310 Genetic Analyzer (Perkin-Elmer Corp., San Fernando, CA). Nucleotide sequence data were analyzed with MacDNASIS Pro V3.1 software (Hitachi Software Engineering America, Ltd., San Francisco, CA) and with the GenBank database (National Center for Biotechnology Information). Alignments of predicted amino acid sequence were conducted with the Clustal W version 1.81 program (Thompson et al. 1994).

Results

Southern-blot hybridization analysis

Hybridization results are shown in Figure 2.1. The results were negative for the hybridization of *M. gallinarum* genomic DNA with the P1-like probe. There was a significant hybridization signal at 3 kb with the 18bp P30-like probe. However, there was no hybridization signal with 28bp P30-like probe. Interestingly, the results for two P30-like probes were different for *M. gallisepticum* genomic DNA.

Genomic library screening, nucleotide sequencing, and sequence analysis

By screening the genomic plasmid library, three positive clones were identified and characterized by nucleotide sequencing. Database searches with the nucleotide sequence data obtained from one colony showed a homology with the leucine aminopeptidase gene from *M. salivarium*. Additional sequence of the insert was obtained with internal primers (Table 2.1). This sequence contained a partial aminopeptidase-like gene and a partial glutamyl-tRNA synthetase-like gene (Figure 2.2). The partial nucleotide sequence from this aminopeptidase-like gene showed a homology of 54% identity with the nucleotide sequence of the leucine aminopeptidase gene from *M. salivarium*. The amino acid sequence derived from the aminopeptidase nucleotide sequence had a 68% identity with the protein sequence of leucine aminopeptidase from *M. salivarium* (Figure 2.3).

Discussion

A large number of lipoproteins, despite their small genome sizes, are encoded in mycoplasma genomes (Chambaud et al. 2001). These lipoproteins have been reported as the primary targets of the immune responses (Chambaud et al. 1999; Kaufmann et al. 1999). A special formation of these lipoproteins, attachment organelles of mycoplasmas, would generate a close, physical association with the eukaryotic epithelial cells (Krause 1996). Various adhesion associated proteins have been characterized in these organelles in a number of mycoplasmas, including P30-like and P1-like cytoadhesins (Dallo et al. 1989; Morsy et al. 1993; Tham et al. 1994; Fleury et al. 2001; Boguslavsky et al. 2000; Hnatow et al. 1998; Reddy et al. 1995). Additional studies characterizing the adhesion of mycoplasmas showed that different gene families were employed by mycoplasmas as a strategy to face challenges from host immune responses, such as the *pMGA* gene family in *M. gallisepticum*, *vaa* gene family in *M. hominis*, *vlp* gene family in *M. hyorhina*, and *vsp* gene family in *M. bovis* (Markham et al. 1993; Zhang and Wise 1996; Rosengarten and Wise 1991; Beier et al. 1998). It is very intriguing that, for some gene families, only a single gene copy was shown to be expressed in the associated mycoplasma strain (Bhugra et al. 1995; Glew et al. 1995; Milosevic et al. 2000). There have also been more adhesion associated genes identified that were not homologous with other cytoadhesins, such as P97 in *M. hypopneumoniae* (Zhang et al. 1995).

Our results showed a positive signal in Southern-blot results with the P30-like 18 bp probe and no signal with the P30-like 28 bp probe. However, sequencing results demonstrated that the insert recognized with the P30-like probe was an aminopeptidase-like gene. The conserved region of the P30 protein sequence, 5'-LGLGIG-3', which was used to design the P30-like probe, was not identified in the amino acid sequence predicted from the cloned aminopeptidase gene. The cloned aminopeptidase gene fragment only had a 42% nucleotide identity with the *M. pneumoniae* P30 gene nucleotide sequence (Figure 2.3). The P30-like 28 bp

did not give a positive result for Southern hybridization analysis, suggesting that the P30-like gene might not be present in *M. gallinarum*. The hybridization results of P1 suggested that a P1-like gene is not encoded in *M. gallinarum*. Thus, *M. gallinarum* cytoadhesins may have diverged from the P1 and P30-like genes, or alternatively, other membrane components may be involved in interaction with host cells.

The glycolipids of *A. laidlawii* PG8 demonstrated a capability to bind to human lymphoid cells (Toujima et al. 2000). Similarly, *Mycoplasma gallinarum* may also have some other unique membrane components other than cytoadhesin associated components involved in adhesion. More experiments are required to explore the membrane adhesion of *M. gallinarum*.

In prokaryotes, aminopeptidases have been associated with the digestion of intracellular and extracellular peptides, which may be significant in the nutritional supply to prokaryotes (Tan et al. 1993; Goldberg and Dice 1974). These functions of aminopeptidases may be especially important to the persistence of parasites, such as mycoplasmas, which are not able to biosynthesize amino acids and the precursors for other macromolecules. In eukaryotes, aminopeptidases were associated with cell adhesion and signal transduction (Riemann et al. 1999).

Mycoplasma gallinarum is a commensal in poultry, cattle, sheep, and pigs (Singh and Uppal 1987; Taylor-Robinson et al. 1968; Taylor-Robinson and Dinter. 1968; Wang et al. 1990). This species of mycoplasma is non-fermentative and requires most of the amino acids for their growth. Interestingly, a higher aminopeptidase activity was reported in bovine mycoplasmas with a lower pathogenesis (Neill and Ball 1980). It was shown that *M. gallinarum* had both leucine and arginine aminopeptidases (Ball et al. 1982). Currently, these genes and their functions have not been characterized. It is unclear if these aminopeptidases are associated with the non-host specific features and the low pathogenesis of *M. gallinarum*.

The sequences identified in this study showed a higher homology with the leucine aminopeptidase from *M. salivarium*. Cloning of the complete open reading frame of this gene and

functional characterization will be helpful in unveiling the details about the roles of this gene in host adaptation of *M. gallinarum*. It may also provide some basis for the function of aminopeptidases in mycoplasma host adaptation.

Table 2.1 Internal sequencing primers used in chapter II

Name	Oligonucleotides
T7.1.854	5'-ACAAATGAAGGAAAATGAGG-3'
T7.1.1332	5'-TTAGCCGTCTTTTCATTTTT-3'
T3.1.1045	5'-TTTATTATGCCTATTCGTT-3'
T3.1.605	5'-TTTCTTGCTTTATTAGGTTG-3'
T3.1.242	5'-ATGCCTAAAAATGTTGAATA-3'

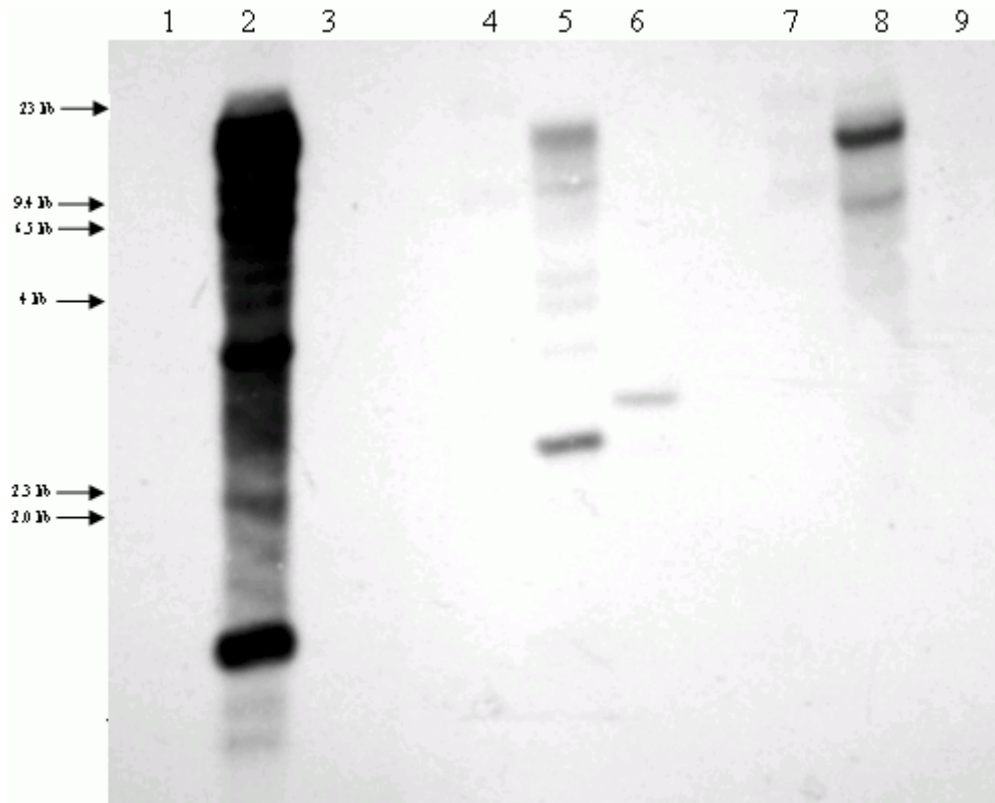


Figure 2.1 The Southern-blot hybridization results using cytoadhesin probes. Lanes 1, 4 and 7 were loaded with 2 μ g lambda DNA digested with *Hind* III; Lanes 2, 5 and 8 were loaded with 5 μ g *M. gallisepticum* F strain genomic DNA digested with *Eco* RI; Lanes 3, 6 and 9 were loaded with 5 μ g *M. gallinarum* genomic DNA digested with *Eco*RI. Lanes 1, 2, and 3 were hybridized with P1-like Probe; Lanes 4, 5, and 6 were hybridized with P30-like 18 bp probe; Lanes 7, 8, 9 were hybridized with P30-like 28 bp probe.

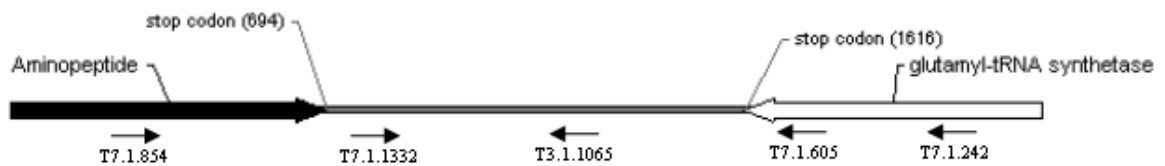


Figure 2.2 Gene organization of the cloned 2,274 bp sequence from *M. gallinarum*. BLAST showed two ORFs: one is aminopeptidase gene and the other one is glutamyl-tRNA synthetase. Internal sequencing primers were labeled in the top of this figure.

CHAPTER III

CLONING, SEQUENCING, AND CHARACTERIZATION OF AN AMINOPEPTIDASE

GENE FROM *MYCOPLASMA GALLINARUM*

Abstract

Unlike most other host-specific mycoplasmas, *Mycoplasma gallinarum* is a commensal with a host range including most poultry as well as some mammals. This property of *M. gallinarum* may reflect unique mechanisms for its colonization and persistence in hosts. The aminopeptidases have been suggested to play a potential role in the host colonization by supplying nutrition from peptide degradation and amino acid scavenging. Whereas *M. gallinarum* shows leucine and arginine aminopeptidase activity, the genes encoding the enzymes had not been cloned and characterized. We identified an aminopeptidase gene by oligonucleotide hybridization to a genomic library of *M. gallinarum* in lambda ZAPII bacteriophage. Nucleotide sequence analysis of overlapping phage clones identified a 1,362 bp open reading frame (ORF) encoding a putative leucine aminopeptidase gene. Database searches indicate that this ORF has 68% nucleotide identity and 51% amino acid identity with the *M. salivarium* leucine aminopeptidase gene. The active sites of the leucine aminopeptidases in other eukaryotes and prokaryotes were conserved in the cloned aminopeptidase gene. Northern-blot hybridization analysis showed that this ORF is expressed on a 1.5 kb transcript. Southern-blot hybridization analysis demonstrated this gene was present as a single copy in *M. gallinarum*. Characterization of the leucine aminopeptidase demonstrated that leucine aminopeptidase is a metallo-aminopeptidase (EC 3.4.11.1) and is located in cytoplasm with a weak interaction with the cell membrane. These features suggest a potential role of this leucine aminopeptidase in the nutritional supply of *M. gallinarum*.

Introduction

Mycoplasma gallinarum has been identified as a commensal in a wide range of vertebrate hosts, including poultry, cattle, pigs, and sheep (Shah-Majid 1996; Singh and Uppal 1987; Taylor-Robinson et al. 1968; Taylor-Robinson and Dinter 1968; Wang et al. 1990). *M. gallinarum* was reported as one of the most frequently isolated mycoplasmas from poultry (Shah-Majid and Nihayah 1987; Kleven et al. 1978). Generally, colonization of the respiratory tract of poultry by *M. gallinarum* does not result in pathologic changes and does not show clinical signs. *In vitro* studies by Taylor-Robinson and Cherry (1972) suggest that *M. gallinarum* does not cause reduction of tracheal epithelial cell ciliary activity in tracheal organ culture. However, *M. gallinarum* was reported to induce a temporary airsacculitis when chickens were infected by aerosol or air-sac inoculation in combination with a field strain of infectious bronchitis virus (IBV) or with vaccination for IB and Newcastle disease (Kleven et al. 1978). During an *In vitro* mixed infection with Newcastle disease virus, *M. gallinarum* may temporarily destroy the cilia on the epithelial surface of the tracheal organ culture, whereas the microvilli were not affected by this infection (Shah-Majid 1996). Cilia were repaired after about one week.

Mycoplasma gallinarum does not induce a strong antibody response, and the humoral IgG and IgM levels against the organism in infected individuals were reported to be almost undetected (Varley and Jordan 1978a; Varley and Jordan 1978b; Bencina et al. 1991a; Bencina et al. 1991b; Bencina et al. 1992). The capability to persist in various hosts and to escape the host immune responses suggested that some unique host adaptation mechanisms might be present in this mycoplasma.

As a comparison, *M. gallisepticum* is a pathogenic mycoplasma that causes chronic respiratory diseases in chickens (Yoder 1991). *M. gallinarum* was reported to prevent the reduction in ciliary activity by pathogenic *M. gallisepticum* in a mixed infection without

reduction in the viability of *M. gallisepticum* (Taylor-Robinson and Cherry 1972). If the epithelial cell exfoliation observed in respiratory infections with *M. gallisepticum* results from local inflammatory-type responses, it is possible that the presence of *M. gallinarum* may abrogate the pathological consequences induced by *M. gallisepticum* by suppressing local innate responses. The results from transmission and scanning electron microscopy showed the difference between the pathogenic mechanisms of *M. gallinarum* and *M. gallisepticum* (Abu-Zahr and Butler 1976). They showed the pathogenesis of *M. gallisepticum* came directly from its attachment to the epithelial cells. It colonized the cells first and then the lamina propria. In contrast, the pathogenesis of *M. gallinarum* may be related to factors other than the adhesion. They found *M. gallinarum* did not develop a close association with the epithelial cells because mycoplasma cells appeared first in the lamina propria, which are different from most other mycoplasmas (Krause 1996; Razin et al. 1998).

The aminopeptidase and carboxypeptidase activity of several human mycoplasmas, including some pathogens, has been demonstrated to inactivate the inflammatory mediator bradykinin (Shibata and Watanabe 1989). In other prokaryotes, proteases have been shown to down regulate immune responses by the degradation of phagocyte LPS receptor CD14 and TNF•• (Calkins et al. 1998; Sugawara et al. 2000). Moreover, other studies have demonstrated that bacterial proteases possess the ability to suppress important neutrophil functions, such as the oxidative burst, in response to stimulation with phorbol esters (Kadowaki et al. 1994; Nakayama et al. 1995; Yoneda et al. 1990).

Peptide degradation and amino acid scavenging by aminopeptidases are reported to be important in the nutritional supply in prokaryotes (Tan et al. 1993; Morales et al. 2001). Because mycoplasmas have been shown to lack the major genes to synthesize most macromolecules precursors, such as amino acids, lipids, and nucleic acid precursors, the aminopeptidase activity

may play a more important role in the survival of mycoplasmas in their hosts (Maniloff et al. 1992; Razin et al. 1998). However, the activity of aminopeptidases from mycoplasmas may be deleterious to the epithelial cells because they produce ammonia and hydrogen peroxide in the process of oxidizing the released amino acids (Cole et al. 1968; Abu-Zahr and Butler 1976).

Previous studies have indicated that *M. gallinarum* possesses leucine and arginine aminopeptidase activity (Ball et al. 1985). The functions of these aminopeptidases in the host adaptation of *M. gallinarum* are unknown. From our previous work, a partial aminopeptidase gene candidate was identified from *M. gallinarum* (Chapter II). Herein we clone, sequence, and characterize the complete open reading frame of this aminopeptidase gene candidate.

Our results showed that the cloned aminopeptidase enzyme was a leucine aminopeptidase (EC 3.4.11.1), and that this enzyme was located in the cytoplasm having a weak interaction with the cell membrane, or on the inner side of the membrane portion of *M. gallinarum* cells. These results suggested the cloned leucine aminopeptidase would at least play an important role in the nutrition supply for *M. gallinarum*.

Materials and methods

Mycoplasma strain and culture conditions

The *M. gallinarum* used in these studies (kindly provided by S. H. Kleven, University of Georgia) was filter cloned three times and then used as an inoculum for a 400 ml culture in Frey Broth incubated overnight at 37 °C. Aliquots of the culture were stored at -80 °C for use throughout the study (Ellakany et al. 1997).

Aminopeptidase activity assay and subcellular location of aminopeptidase

The method for membrane protein purification was adapted from the methods described previously (Neill and Ball 1980; Shibata and Watanabe 1987). Briefly, *M. gallinarum* cells were harvested by centrifuging a 1 liter 24 hrs culture at 40,000×g for 30 min and then washing twice with 0.15 M NaCl. The final pellets were diluted with 5 ml 0.15 M saline solution or 0.1 M BBS with 0.15 M saline (pH 8.0). The suspended pellets were sonicated 10 times on ice at the maximum setting for 15 sec with 30 sec intervals (Microson Ultrasonic Cell Distruptor, Model XL2000). The broken cells were then centrifuged at 1000×g for 10 mins, and the supernatants were collected as the source of total proteins of *M. gallinarum*. Protein concentration was determined using the Pierce bicinchoninic acid assay kit (Pierce Chemical Co., Rockford, IL).

Leucine aminopeptidase activity was assayed with the Sigma LAP diagnostic kit according to the manual's procedure (Procedure NO. 251, Sigma Diagnostics, St. Louis, MO). The arginine aminopeptidase activity assay procedure was the same as the leucine aminopeptidase assay except the LAP substrate was replaced with L-arginine-β-naphthylamide substrate (Sigma Chemical Co., St. Louis, MO). The substrate degrading products were quantified by absorbance at 600 nm and activity per mg of protein calculated.

To test the effects of metal ions on leucine and arginine aminopeptidase activity, the enzyme-substrate solution was adjusted with a final concentration of 1mM Zn²⁺, Mn²⁺, K⁺, or Mg²⁺ before the aminopeptidase activity assay. To test the inhibitory effect of tetrasodium ethylenediaminetetraacetate (EDTA) on the aminopeptidase activities, the total cell membrane was incubated with the final concentration of 1 mM EDTA at 37 °C for 15 mins before aminopeptidase activity assay. The experiments were repeated three times and conducted with an ANOVA analysis.

The above prepared sonicated cells were centrifuged at 114,000×g at 4 °C for 2 hrs to separate cell membrane proteins from cytoplasmic proteins (Pollack 1995&1998). The supernatants were filtered through 0.2 µm pore filters to yield the cytoplasmic protein fraction. The cell pellets were suspended with an equal amount of the supernatants of 0.1 M BBS with 0.15 M NaCl saline and then centrifuged at 2,000×g at 4 °C for 3 mins to collect the supernatants as membrane protein fraction. Protein concentrations were determined (Pierce Chemical Co., Rockford, IL) and the aminopetidase activity was assayed for both fractions.

Nucleic acid preparation

Genomic DNA was isolated from overnight broth cultures of *M. gallinarum* using the QIAGEN genomic DNA isolation kit (QIAGEN, Inc., Valencia, CA). Plasmids were extracted from 24 hrs cultures of component cells using the QIAGEN miniprep plasmid extraction kit (QIAGEN, Inc., Valencia, CA).

Total RNA was prepared from overnight cultures of *M. gallinarum* and *M. gallisepticum* using Trizol (GibcoBRL, Invitrogen Co., Carlsbad, CA). To remove genomic DNA, total RNA was treated with RNAase free DNAase I (2 units/µg RNA) at 37 °C for 1 hr, and then the DNAase I enzyme was removed by incubation with a protein-binding resin (Ambion, Inc. Austin, TX).

Recombinant genomic library construction and library screening

A recombinant bacteriophage lambda library was prepared as described by Minion et al. (1995). Briefly, 200 µg genomic DNA were partially digested with 52 unit *Tsp* 509 I in a final volume of 1 ml at 65 °C for 1 hr (New England Biolabs, Inc., Beverly, MA), purified by extraction with phenol/chloroform (1:1), and precipitated with ethanol. The purified digested

DNA was size-fractionated by centrifugation at $11,300 \times g$ for 16 hrs on sucrose gradients (Luthe 1983). The fragment sizes of the 64 fractions collected from the gradient were determined by gel electrophoresis. The gradient fractions containing DNA fragments in the 4-8 kb range were combined and purified by phenol/chloroform (1:1) extraction and ethanol precipitation. The purified DNA fragments (0.3 μg) were ligated into the *EcoRI* site of 1 μg λ ZAPII bacteriophage I vector by Fast-link ligase at 13 °C for 16 hrs (Epicenter, Madison, WI). After denaturing the ligase, a 3 μl ligation product was mixed with Gigapack Gold III packaging extracts (Stratagene, Co., La Jolla, CA), incubated at room temperature for 1 hr and 45 mins, and then mixed with 500 μl SM buffer. The packaged phage library was stored at 4 °C.

For library plating, *E.coli* XL1-Blue cells were collected from 10 ml fresh XL1-Blue cell culture (OD_{600nm} 0.8) by centrifuging at $837 \times g$ for 5 mins. The cell pellets were then resuspended with 10 ml of 10mM MgSO₄. The amount of 200 μl suspended XL1-Blue cells was mixed with 30,000 plaque form unit (pfu) packaged phages and 10 ml top agrose, and the mixture was poured onto 2XYT agar plates. The plates were incubated at 37 °C overnight. The recombinant phage library was screened according to a protocol adapted from Sambrook et al. (1989). Briefly, duplicate nylon membranes were put on the plates for 1 min, and then moved onto the filter paper soaked with 10% SDS to lyse the cells. Afterwards, the lifts were put onto the filter paper soaked with denaturation buffer for 2 mins and then transferred onto the filter paper soaked with neutralization buffer for 5 mins. After drying on filter paper, the lifts were baked at 80 °C for 2 hrs.

The oligonucleotide T7.1.854 (Table 2.1) was fluorescein-labeled using the enhanced chemiluminescence (ECL) 3'-oligolabelling system according to the manufacturer's protocol (Amersham, Arlington Heights, IL). The fluorescein-labeled probes were made by incubating at 37 °C for 90 mins the mixture of 100 pMol T7.1.854, 10 μl fluorescein-11-dUPT, 16 μl

cacodylate buffer, and 16 µl terminal deoxynucleotidyl transferase. The labeled T7.1.854 probe was used to screen the library for the aminopeptidase gene.

Before the hybridization, the lifts were incubated with 2×SSC for 10 mins, and the cell debris on the lifts was removed gently with Kimwipes (Kimberly-Clark Inc. Roswell, GA). Then the blots were prehybridized at 42 °C for 4 hrs. The 10 ng labeled probe was added into the hybridization solution and incubated for 16 hrs at 42 °C. Then the blots were washed twice with a low stringency buffer (5×SSC, 0.1% SDS) for 5 mins at room temperature. Finally, the blots were washed twice with a high stringency buffer (1×SSC, 0.1% SDS) for 15 mins at 42 °C. After hybridization, the blots were treated with chemiluminescence substrate. The chemiluminescent detection was performed with ECL detection reagents and exposed to X-ray film for various time periods.

Nucleotide sequencing and sequence analysis

The inserts from library clones were sequenced by dye terminator cycle sequencing using the Big-Dye terminator reaction (Applied Biosystems, Foster City, CA) and *p*Bluescript phagemid promoter primers (Stratagene, Co., La Jolla, CA) or internal primers (Genosys, The Woodlands, TX) listed in Table 3.1. Sequencing reaction products were analyzed on an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). Nucleotide sequence data were analyzed with MacDNASIS Pro V3.1 software (Hitachi Software Engineering America, Ltd., San Francisco, CA) and with the GenBank database (National Center for Biotechnology Information). Amino acid sequence prediction was based on mycoplasma codon usage with UGA encoding tryptophan (Inamine et al. 1990). Alignments of predicted amino acid sequence were conducted with the Clustal W version 1.81 program (Higgins and Sharp 1988; Thompson et al. 1994).

PCR and RT-PCR

Primers APN-F and APN-R (Table 3.1) were used to amplify the leucine aminopeptidase open reading frame from *M. gallinarum* genomic DNA. In the PCR reaction, 20 pmol APN-F, 20 pmol APN-R, and 50 ng *M. gallinarum* genomic DNA were used in a 50 µl volume. The PCR reaction was conducted at 94 °C for 10 mins, 30 cycles of 94 °C for 30 secs, 55°C for 30 secs, and 72 °C for 3 mins, and finally 72 °C for 5 mins.

The cDNA was synthesized using 2 µg total RNA as template, 1 pmol random hexamer primers and 200 units SuperScript II reverse transcriptase (Invitrogen Co., Carlsbad, CA) in a volume of 19 µl. The primers used in the amplification of cDNA were APN-F and APN-R as described above.

Identification of 3-terminus of mRNA

Total RNA was polyadenylated with poly (A) polymerase (Ambion, Inc. Austin, TX) prior to reverse transcription. Total cDNA was then synthesized from polyadenylated RNA primed with an Oligo(dT)₁₇ primer-adaptor 5'-ATAGACTCGAGTCGACATCGA-dT₁₇-3' (Frohman et al. 1988) and SuperScript II reverse transcriptase (Invitrogen Co., Carlsbad, CA). The first round PCR was conducted as described above with a single primer, APN-F, for 30 cycles with the conditions described above (Rother 1992). The products of the first round PCR were subjected to an additional 30 cycles of amplification with an internal gene specific primer 1220S (5'-TTTAGTTTTAGCAGATGGAT-3') and the adaptor-primer 5'-ATAGACTCGAGTCGACATCG-3' (Frohman et al. 1988; Rother 1992).

Northern-blot hybridization and Southern-blot hybridization analysis

The PCR amplification products covering the entire aminopeptidase open reading frame was labeled with biotin using BrightStar™ Psoralen-Biotin nonisotopic labeling kit (Ambion, Inc. Austin, TX). The labeled probe was used both in Northern-blot and Southern-blot hybridization analysis.

To confirm the transcription of the leucine aminopeptidase gene and determine the size of this transcript, Northern blots were hybridized with the biotin-labeled APN PCR product according to the manufacturer's instructions (Ambion, Inc. Austin, TX). The amount of 5 µg glyoxal-treated total RNA was loaded onto 1% agarose (Ambion, Inc. Austin, TX), and the gel was electrophoresed at 135 volts for 115 mins. The RNA was blotted to the nylon membrane by capillary transfer. Afterwards, the blots were baked at 80 °C for 2 hrs.

During hybridization, the blots were prehybridized with the hybridization buffer (Ambion, Inc. Austin, TX) at 42 °C for 3 hrs, and a final concentration of 0.1 pM of biotin labeled APN probe was added into the hybridization solution to hybridize at 42 °C for 16 hrs. The blots were then washed twice with a low stringency buffer (Ambion, Inc. Austin, TX) at room temperature for 10 mins, and then washed twice with a high stringency buffer (Ambion, Inc. Austin, TX) at 55 °C for 15 mins. The blots were detected with BrightStar™ BioDetect™ non-isotopic detection kit (Ambion, Inc., Austin, TX). *M. gallisepticum* lacks aminopeptidase activity (Ball et al. 1985) and was used as a negative control.

To quantify the copy number of the leucine aminopeptidase gene present in the genome, 2 µg *M. gallinarum* genomic DNA was digested with *Bsa* AI, *Cla* I, *Dde* I, *Eae* I, *Eco* RI, *Hind* III, *Nco* I, *Pst* I, and *Xmn* I overnight at 37 °C and evaluated by Southern blot. The reaction products were separated by 0.8% agarose gel electrophoresis at 30 volts 17 hrs and then blotted to nylon membranes by capillary transfer. The blots were baked and hybridized similarly to

Northern Blotting hybridization analysis. *Hind* III digested *M. gallisepticum* genomic DNA was used as a negative control.

Three dimensional structure prediction

Three dimensional (3D) modeling templates of the leucine aminopeptidase gene were obtained with SWISS-MODEL (<http://www.expasy.ch/swissmod/>) Blast by searching the ExNRL-3D database with the target sequence. A putative 3D structure of the aminopeptidase was predicted with the Swiss-Model First Approach Model. The resulting structure was analyzed with Swiss-Pdbviewer version 3.7 beta (Schwede et al. 2000).

Phylogenetic relation and hydrophobicity analysis

To investigate the phylogenetic relationships between the cloned aminopeptidase and other peptidases from mycoplasmas, phylogenetic trees were generated by Clustal W version 1.81 (<http://www.ebi.ac.uk/clustalw/>) based on the nucleotide sequence identities (Thompson et al. 1994; Higgins and Sharp 1988). The tree was drawn with TreeViewer. Protein sequences of 37 mycoplasmas peptidases and nine eukaryote leucine aminopeptidases were obtained from the Genbank database (Table 3.2). To analyze the subcellular location, hydrophobic features of leucine aminopeptidases in mycoplasmas were plotted by ProtScale (<http://www.expasy.ch/cgi-bin/protscale.pl>) (Kyte and Doolittle 1982).

Results

APN activity assay

The total proteins from *M. gallinarum* showed both leucine and arginine aminopeptidase activity. Arginine aminopeptidase activity was stronger than leucine aminopeptidase activity

(Table 3.3). These results are similar to previous reports (Ball et al. 1985). The addition of K^+ , Mg^{2+} , Mn^{2+} , or Zn^{2+} , did not significantly affect the arginine aminopeptidase activity. However, leucine aminopeptidase activity was enhanced most effectively by Mn^{2+} , which was followed by Mg^{2+} and K^+ . Zn^{2+} could not serve as a cofactor for leucine aminopeptidase of *M. gallinarum*. EDTA almost completely inhibited both leucine and arginine aminopeptidase activity and addition of Mn^{2+} restored the activities (Gonzales and Robert-Baudouy 1996).

Subcellular location of aminopeptidase

The arginine and leucine aminopeptidase activities were detected in both membrane and cytoplasmic fractions of *M. gallinarum*. The ratio of arginine aminopeptidase activity in the membrane fraction versus that in the cytoplasmic fraction was 1.77, and that ratio for leucine aminopeptidase activity was about 1.22. These results suggested that these enzymes might be a cytoplasmic having a weak interaction with the membrane (Scopes 1994). The enzyme activity shown in the membrane fraction could result from the enzymes which are adhering to the membrane.

Phage library screening and sequence analysis

The unamplified library had a titer of 6×10^6 pfu/ml, with less than 1% non-recombinants and an average insert size of about 5 kb. Six overlapping phage clones were identified and characterized by restriction endonuclease digestion. Comparison of restriction patterns of the phage clones suggests that a 3.8 kb region of the *M. gallinarum* chromosome had been cloned.

The clones were then sequenced with the internal primers listed in Table 3.1. A 3,865-bp fragment was obtained that contained two potential open reading frames (ORF) (Figure 3.1). Database searches indicated that the 1,362-bp ORF1 sequence had 68% nucleotide identity with

the *M. salivarium* aminopeptidase gene and that the 1.1 kb ORF2 sequence had 68% nucleotide identity with glutamyl tRNA synthetase gene of *M. pulmonis*. Alignment of the predicted amino acid sequence derived from ORF1 with the aminopeptidase protein of *M. salivarium* showed an amino acid identity of 51% between the two sequences (Figure 3.2).

The putative promoter region and Shine-Dalgarno sequence are shown in Figure 3.3. There are four TGA codons in this gene (Figure 3.3), which encode tryptophans in mycoplasmas while serving as stop codons in most eubacteria (Inamine et al. 1990; Yamao et al. 1985). A 30 bp inverted repeat at the 3' terminus of this gene (Figure 3.3) has the potential to form a stem loop structure for rho-independent termination of transcription. According to the methods of SantaLucia (1998), the stem loop was formed as in Figure 3.4.

The amino acid sequence showed the six active residues identified in other leucine aminopeptidases from both eukaryotes and prokaryotes have been conserved in this aminopeptidase gene candidate at position Lys 226, Asp 231, Asp 248, Asp 307, Glu 309, and Arg 311 (Figure 3.2) (Burley et al. 1990; Kim and Lipscomb 1993; Strater and Lipscomb 1995a; Strater and Lipscomb 1995b). The active sites were also labeled in the putative 3D structure of the leucine aminopeptidase in Figure 3.5.

Northern-blot hybridization analysis

To confirm gene expression and the size of the transcripts, mRNA was hybridized with the biotin-labeled primer that covered the whole *M. gallinarum* leucine aminopeptidase (ORF1). The Northern-blot showed the leucine aminopeptidase of *M. gallinarum* was expressed as a 1.5 kb transcript (Figure 3.6).

3' termination site determination and characterization of APN ORF

The 3' poly(A) tailing sequences showed the transcript terminated at poly(T) tails after the 3' of APN gene. The stem loop at the termination site of the leucine aminopeptidase gene transcript was shown in Figure 3.4 using DNA structural determination methods described previously (SantaLucia 1998).

Southern blot hybridization analysis

To quantify the copy number of aminopeptidase genes encoded in *M. gallinarum*, its genomic DNA was digested with *Bsa* AI, *Dde* I, *Ear* I, *Eco* RI, *Hind* III, and *Xmn* I, and hybridized with APN biotin-labeled probe. The results are shown in Figure 3.7 A. *Hind* III digested *M. gallisepticum* genomic DNA was used as a negative control.

Hybridization to blots of *Bsa* AI and *Ear* I digests resulted in 2.9 kb and 6 kb bands, respectively. *Dde* I digest generated three DNA fragments at molecular weights of 2.5 kb, 1.1 kb, and 1.4kb. *Eco* RI resulted in two bands, 2.4 kb and 2.8 kb. *Hind* III showed two bands, 2.2 kb and 1.9 kb. *Xmn* I showed four bands: a strong band at 1.8 kb and weak bands 2.5 kb, 0.9 kb, and 0.6 kb. The DNA was digested with different units of *Xmn* I to determine if the banding patterns were consistent (Figure 3.7 C)

Three dimensional structure prediction

The published X-ray crystallography 3D structures of LAN, LAP, LAM, LCP, and BPN were used as templates (Burley et al. 1990; Kim and Lipscomb 1993; Strater and Lipscomb 1995a; Strater and Lipscomb 1995b). The amino acid sequence homology between *M. gallinarum* leucine aminopeptidase and the templates are 38.3%. The sequence and secondary structure alignments of *M. gallinarum* leucine aminopeptidase with LAP are shown in Figure 3.8. Figure

3.5 is the predicted structure of the leucine aminopeptidase of *M. gallinarum* and showed four α -helixes and a β -sheet formed the active sites of the enzyme.

Phylogenetic relation and hydrophobicity analysis

The phylogenetic relationship between the aminopeptidase from *M. gallinarum* and other peptidases (Figure 3.9 A) showed the leucine aminopeptidases are present as a different family from the methionine aminopeptidase (pepM), proline aminopeptidase (pepP), proline iminopeptidase (PIP), oligoendopeptidase F (pepF), signal peptidase II (singalPII), and o-sialoglycoprotein endopeptidase (ISGendoP). Only the proline dipeptidase from *M. pulmonis* is closer to the family of leucine aminopeptidases. The cloned aminopeptidase could be a member of the aminopeptidase family M17, which is a family of cytoplasmic proteins. They release an N-terminal amino acid from Xaa-Xbb-, where Xaa- is preferably Leu and can also be Pro, and Xbb- may be Pro. The aminopeptidase of *M. gallinarum* may have similar features with the leucine aminopeptidases (EC 3.4.11.1). Among the seven annotated leucine aminopeptidases, the cloned aminopeptidase (MGN_APN) and the leucine aminopeptidase from *M. salivarium* (MSA_LAP) might diverge from a closer ancestor than other leucine aminopeptidases. The leucine aminopeptidases from mycoplasmas represent a different family of leucine aminopeptidase from other prokaryotes (Figure 3.9 B). However, the leucine aminopeptidases from *M. pneumoniae*, *M. genitalium*, and *U. urealyticum* showed a closer relationship to the aminopeptidases of other prokaryotes. The alignments of these seven leucine aminopeptidases were shown in Figure 3.10. The reported six active aminopeptidases identified in other leucine aminopeptidases were conserved in all the leucine aminopeptidases from mycoplasmas (Burley et al. 1990; Kim and Lipscomb 1993; Strater and Lipscomb 1995a; Strater and Lipscomb 1995b).

Hydrophobicity analysis showed that the leucine aminopeptidase of *M. gallinarum* does not have a strong hydrophobic region suggestive of a transmembrane region (Figure 3.11).

Discussion

We have identified a leucine aminopeptidase of *M. gallinarum*. The probe that hybridized with the aminopeptidase gene fragment in our plasmid library was designed from P30 cytoadhesin of *M. genitalium* and *M. pneumoniae*. We obtained a short sequence from this plasmid library, which showed homology with the leucine aminopeptidase gene of *M. salivarium*. By screening a *M. gallinarum* genomic library constructed in λ ZAP Express with oligonucleotides T7.1.854, we obtained a full-length leucine aminopeptidase of *M. gallinarum*. This is the second gene in *M. gallinarum* described after the 16s rRNA gene was published in 1993 (Boyle and Morrow 1993).

Within the 1462 bp ORF1, there are four TGA codons. The presence of these codons within the ORF demonstrated that UGA codons encode Trp in *M. gallinarum*, which is a common characteristic of mycoplasma genes (Inamine et al. 1990; Yamao et al. 1985). This property is unique to mycoplasmas and possibly results from AT pressure (Jukes et al. 1987). This feature causes difficulty in expressing mycoplasma genes in eubacteria systems (Minion 1998).

The sequence alignments showed that ORF1 encodes a leucine aminopeptidase. The 3' Poly(A) tailing reaction confirmed the termination site of the transcript encoding this leucine aminopeptidase gene. The potential stem loop in the 3' terminal indicates a rho-independent termination character of this transcript (Calcutt et al. 1999; Taschke and Herrmann 1986; Bove 1993). The promoter sequences and ribosomal binding sites are similar to those described in previous reports of mycoplasma genes (Bove 1993; Shibata et al. 1995). The presence of putative SD sequence suggests that *M. gallinarum* might use a leader translation initiation (Figure 1.1; Boyle and Boyle, submitted). Analysis of the predicted amino acid sequence suggested that the

active sites identified in other leucine aminopeptidases are conserved in *M. gallinarum* leucine aminopeptidase (Burley et al. 1990; Kim and Lipscomb 1993; Strater and Lipscomb 1995a; Strater and Lipscomb 1995b).

The Northern blotting results suggested that the leucine aminopeptidase gene of *M. gallinarum* is expressed as a monocistronic message of 1.5 kb. According to Southern blot hybridization results, there is a single copy of leucine aminopeptidase in *M. gallinarum*. *M. gallinarum* genomic DNA was digested with *Bsa* AI, *Dde* I, *Ear* I, *Eco* RI, *Hind* III, and *Xmn* I, and hybridized with a biotin-labeled APN probe. In the restriction map of Figure 3.1, *Bsa* AI flanks the ORF1 and produces a fragment of 2,833 bp. There are three *Dde* I restriction sites at 356, 1708, and 2,863, thus the complete digestion will release two signals of 1,352 bp and 1,155 bp. The single *Ear* I site is present at position 3657. There is an *Eco* RI site at position 1126 so that the digestion will divide this gene into two fragments. There are two *Hind* III sites at 1203 and 3405, thus digestion will divide ORF1 at position 1203 into two fragments, one of which would be 2,202 bp. There are three *Xmn* I sites at positions 184, 435, and 2871, respectively, each of which flanks ORF1. The Southern-blot hybridization results we have obtained are consistent with the restriction maps (Figure 3.7 A). However, *Dde* I digestion showed three bands at molecular weight at about 2.5 kb, 1.1 kb, and 1.4kb, which might be the result of incomplete digestion. The several bands that results from *Xmn* I digestion instead of an expected single band are probably a result of the star activity of *Xmn* I (Promega, Madison, WI). Southern hybridization analysis with different genomic DNA/enzyme ratios confirmed this possibility (Figure 3.7 B). The lower the genomic DNA/enzyme ratio, the more completion of *Xmn* I digestion. Southern-blot with three enzymes flanking the aminopeptidase gene, *Cla* I, *Nco* I, and *Pst* I, showed a single band, which also confirmed the single copy of aminopeptidase gene present in *M. gallinarum* (Figure 3.7 C).

Arginine aminopeptidases cleave the arginine amino acid from the N terminus of peptides. Our results demonstrated that arginine aminopeptidase activity was not affected by the metallic ions, K^+ , Mg^{2+} , Mn^{2+} , or Zn^{2+} . But it was inhibited by EDTA and this activity was resumed by Mn^{2+} , suggesting that arginine aminopeptidase is a metallo-aminopeptidase. Leucine aminopeptidases mainly cleave N terminal leucyl substrates, and they can cleave most other peptides (Burley et al. 1990). Our results demonstrated the leucine aminopeptidase of *M. gallinarum* is a metallo-aminopeptidase. EDTA can inhibit the activity of both leucine and arginine aminopeptidase activity. Mn^{2+} not only enhanced leucine aminopeptidase activity but also restored aminopeptidase activity inhibited by EDTA. Our results implied arginine and leucine aminopeptidase activities may result from different enzymes of *M. gallinarum*.

The putative leucine aminopeptidase predicted by the cloned gene potentially belongs to the leucine aminopeptidase family (EC 3.4.11.1). According to the enzyme nomenclature from the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB), the enzymes in this family “release an N-terminal amino acid, Xaa-Xbb-. Xaa- is preferably be Leu, and can also be Pro although not Arg or Lys. Xbb- may be Pro (<http://www.chem.qmul.ac.uk/iubmb/enzyme/>).” However, because this enzyme was not activated by Zn^{2+} and even lost some activity because of Zn^{2+} , it may be more similar to leucine aminopeptidase in *E. coli* than other typical leucine aminopeptidases, such as bovine leucine aminopeptidase. Leucine aminopeptidase (Genbank access No: P11648) was reported to be activated by Mn^{2+} but inhibited by Zn^{2+} and EDTA, and this enzyme was assumed to be involved in the processing and regular turnover of intracellular proteins as well as in catalyzing the removal of unsubstituted N-terminal amino acids from various peptides (Gonzales and Robert-Baudouy 1996). In contrast, bovine leucine aminopeptidase (Genbank access No: P00727) can be activated by Zn^{2+} as well as Mn^{2+} and Mg^{2+} (Burley et al. 1990).

The phylogenetic relationship and hydrophobic plot comparison showed the leucine aminopeptidase characterized in this study was related closely to the leucine aminopeptidase in *M. salivarium*. Shibata et al. (1995) showed this enzyme is a membrane associated enzyme. However, more recently, they annotated it as a possible cytoplasmic protein (Genbank access No: P47707). Based on our results, the leucine aminopeptidase of *M. gallinarum* is a cytoplasmic protein having a weak interaction with the cell membrane.

Because mycoplasmas lack many of the genes to synthesize most macromolecule precursors, such as amino acids, lipids, and nucleic acid precursors, the survival of mycoplasmas depends on the nutrient environment (Maniloff et al. 1992; Razin et al. 1998). Thus, mycoplasmas require a “fastidious” environment (Maniloff et al. 1992). *Mycoplasma gallinarum* is one of the most fastidious mycoplasmas and requires 13 amino acids for its growth, including leucine, arginine, alanine, asparagines, cystine, glutamic acid, glycine, histidine, isoleucine, methionine, phenylalanine, threonine, and tyrosine. The leucine aminopeptidase might play an important role in peptide degradation and amino acid scavenging in *M. gallinarum* (Tan et al. 1993; Morales et al. 2001). The possible association of *M. gallinarum* aminopeptidase with the cell membrane could result in the close proximity of this enzyme with membrane transport systems. Peptides transported across the membrane could be cleaved by the enzyme to provide a source for leucine as well as to render peptides susceptible to further degradation (Gonzales and Robert-Baudouy 1996).

The lack of transmembrane regions in the hydrophobicity plot of the cloned aminopeptidase suggested that this enzyme is a cytoplasmic protein having a weak interaction with the cell membrane. Alternatively, the several weak hydrophobic regions predicted are possibly involved the interaction of this enzyme with other membrane proteins by noncovalent forces, which could be membrane transport proteins (Alberts et al. 1994).

Through comparison of the aminopeptidase activities between *A. laidlawii*, *M. bovirhinis*, *M. bovis*, and *M. dispar*, Neill and Ball (1980) found the pathogenicity of mycoplasmas decreased as the associated aminopeptidase activity increased. *A. laidlawii* is another non-host-specific strain, which was reported in humans, birds, cattle, and even plants and insects (Tully 1993; Maniloff 1992; Stipkovitis and Kempf 1996; Yoder 1991). *A. laidlawii* had the strongest aminopeptidase activity among these four investigated mycoplasmas. On the other hand, the depletion and oxidation of amino acids by mycoplasmas may generate ammonia and hydrogen peroxide, which could induce cytopathology (Cole et al. 1968; Abu-Zahr and Butler 1976). The role that the leucine aminopeptidase may play in host-pathogen interaction of mycoplasmas is unknown. *M. gallinarum* is also a non-host specific mycoplasma and is relatively nonpathogenic and does not produce clinical signs in its hosts (Yoder 1991). Whether the leucine aminopeptidase activity has a role in nonpathogenesis is unknown.

Other studies demonstrated that mycoplasmal aminopeptidase and carboxypeptidase activity can inactivate the inflammatory mediator bradykinin (Shibata and Watanabe 1989). Similarly, peptidases from the other prokaryotes were reported to down regulate immune responses by degradation of the phagocyte LPS receptor CD14 and TNF α , and even to suppress neutrophil functions, such as oxidative burst (Calkins et al. 1998; Sugawara et al. 2000; Kadowaki et al. 1994; Nakayama et al. 1995; Yoneda et al. 1990). It is unknown whether the cytoplasmic leucine aminopeptidase in *M. gallinaurum* is involved in the suppression of host immune responses.

Table 3.1 Internal sequencing primers used in chapter III

Name	Oligonucleotides
71S	5'-TTTTGTTGATTTTCATTTAATCAG-3'
187S	5'-TTTTTTCTAATGCGATATTCAATC-3'
341S	5'-TTCTTACTATCTTACCTCAG-3'
30S	5'-TTAACTTATCATCATCGTCAATG-3'
190S	5'-GATGCTCAAGTCCACTTTTTTCATTAC-3'
307	5'-ATTGTTGTCTCTTTTTGTCA-3'
652S	5'-TATTTAATTCTGATGAAGATAAAGAAG-3'
868S	5'-TGTCTGTAAATAAGGGTTCA-3'
1220S	5'-TTTAGTTTTAGCAGATGGAT-3'
1708	5'-TTTGCCGCTAATGAGACCTT-3'
APN-F	5'-AATAAATTGGAATTTTTGACAAAAAGA-3'
APN-R	5'-TTTTTCATTTAAT AAAGCAAATTCAAC-3'

Table 3.2 Peptidases in phylogenetic tree analysis

Name	Sources	Specificity	Cofactor	Subcellular location	Locus	Reference
AMPA_ECOLI	<i>E. coli</i>	Leu, Met	Mn ²⁺	cytoplasm	P11648	McCulloch et al. 1994
AMPA_PSEAE	<i>Pseudomonas aeruginosa</i>	Leu	Mn ²⁺	cytoplasm	O68822	Woolwine and Wozniak 1999
AMPA_PICPR	<i>Rickettsia prowazekii</i>	Leu	Zn ²⁺ , Mn ²⁺	cytoplasm	P27888	Wood et al. 1993
PPUT_LAP	<i>P. putida</i>	Leu	unknown	unknown	CAA09054.1	Sonke et al. 1998
VPRO_LAP	<i>Vibrio proteolyticus</i>	Leu	Zn ²⁺ ?	extracellular	AAA21940.1	Guenet et al. 1992
APX_STRGR	<i>Sireptomyces griseus</i>	Leu	Zn ²⁺	extracellular	P80561	Ben-Meir et al. 1993
SENT_LAP	<i>Salmonella enterica</i> subsp. <i>enterica</i> serovar Typhi	Leu	unknown	unknown	CAD02739.1	Parkhill et al. 2001
ALTE_METAIL	<i>Alteromonas</i> sp. O-7	unknown	Metal	unknown	BAB79616.1	Miyamoto et al. 2002
APE2_SULSO	<i>Sulfolobus solfataricus</i>	Leu	Zn ²⁺	cytoplasm	P95928	Condo et al. 1998
MPN_LAP	<i>M. pneumoniae</i>	Leu	unknown	cytoplasm	NP_110261.1	Himmelreich et al. 1996
MPN_PIP	<i>M. pneumoniae</i>	Pro	unknown	cytoplasm	P75092	Himmelreich et al. 1996
MPN_pepP	<i>M. pneumoniae</i>	Xaa-Pro	Zn ²⁺ , Mn ²⁺	unknown	P75313	Himmelreich et al. 1996
MPN_pepM	<i>M. pneumoniae</i>		Mn ²⁺	unknown	S62822	Himmelreich et al. 1996
MPN_signalPI	<i>M. pneumoniae</i>	unknown	unknown	unknown	NP_109981	Himmelreich et al. 1996
MPN_dipepP	<i>M. pneumoniae</i>	unknown	unknown	unknown	NP_110158	Himmelreich et al. 1996
MPN_ISGendoP	<i>M. pneumoniae</i>	unknown	unknown	unknown	AAB95743	Himmelreich et al. 1996
MPN_pepF	<i>M. pneumoniae</i>	unknown	unknown	unknown	NP_109885	Himmelreich et al. 1996
MGE_LAP	<i>M. genitalium</i>	Leu	Mn ²⁺	cytoplasm	P47631	Fraser et al. 1995
MGE_PIP	<i>M. genitalium</i>	Pro	unknown	cytoplasm	P47266	Fraser et al. 1995
MGE_pepM	<i>M. genitalium</i>		Co ²⁺	unknown	P47418	Fraser et al. 1995
MGE_pepP	<i>M. genitalium</i>	proline	Zn ²⁺	unknown	P47566	Fraser et al. 1995
MGE_ISGendoP	<i>M. genitalium</i>	unknown	unknown	unknown	AAC71262	Fraser et al. 1995
MGE_pepF	<i>M. genitalium</i>	unknown	unknown	unknown	NP_072846	Fraser et al. 1995
MGE_lsp	<i>M. genitalium</i>	unknown	unknown	unknown	AAC71428	Fraser et al. 1995
UUR_LAP	<i>U. urealyticum</i>	Leu	unknown	cytoplasm	AAF30919.1	Glass et al. 2000
UUR_pepM	<i>U. urealyticum</i>	Met	unknown	unknown	NP_078086.1	Glass et al. 2000
UUR_pepP	<i>U. urealyticum</i>	Pro	unknown	unknown	NP_078370.1	Glass et al. 2000
UUR_singalPII	<i>U. urealyticum</i>	unknown	unknown	unknown	NP_078148	Glass et al. 2000
UUR_metalPZn	<i>U. urealyticum</i>	unknown	Metal	unknown	NP_077936	Glass et al. 2000
UUR_pepF1	<i>U. urealyticum</i>	unknown	unknown	unknown	NP_077895	Glass et al. 2000
UUR_pepF2	<i>U. urealyticum</i>	unknown	unknown	unknown	NP_078148	Glass et al. 2000
MPU_LAP1	<i>M. pulmonis</i>	Leu	unknown	cytoplasmic	NP_326206.1	Chambaud et al. 2001
MPU_LAP2	<i>M. pulmonis</i>				NP_326557.1	Chambaud et al. 2001
MPU_pepM	<i>M. pulmonis</i>	Met	unknown	unknown	NP_326397.1	Chambaud et al. 2001
MPU_pepP	<i>M. pulmonis</i>	Pro	Metal	unknown	NP_326319.1	Chambaud et al. 2001
MPU_APN	<i>M. pulmonis</i>	unknown	unknown	unknown	NP_326561.1	Chambaud et al. 2001
MPU_ISGendoP	<i>M. pulmonis</i>	unknown	unknown	unknown	NP_325949	Chambaud et al. 2001
MPU_dipepP	<i>M. pulmonis</i>	unknown	unknown	unknown	NP_326107	Chambaud et al. 2001
MPU_pepF	<i>M. pulmonis</i>	unknown	unknown	unknown	NP_3261	Chambaud et al. 2001
MPU_signalPI	<i>M. pulmonis</i>	unknown	unknown	unknown	NP_326461	Chambaud et al. 2001
MPU_signalPII	<i>M. pulmonis</i>	unknown	unknown	unknown	NP_326499	Chambaud et al. 2001
MSA_LAP	<i>M. salinarum</i>	Leu	Mn ²⁺	cytoplasmic ^a	P47707	Shibata et al. 1995
MGN_APN	<i>M. gallinarum</i>	Leu	Mn ²⁺	cytoplasmic	AAL86922.1	
MCA_pepM	<i>M. capricolum</i>	Met	unknown	unknown	Q59509	Bork et al. 1995
MGS_pepM	<i>M. gallisepticum</i>	Met	unknown	unknown	AAB95408.1	Skamrov et al. 2000.

a. Shibata demonstrated MSA_LAP is a membrane protein (Shibata et al. 1995). However, they described it as a possible cytoplasmic protein when they submitted the sequence into Genbank (Locus: P47707).

Table 3.3 Effects of metallic cofactors on aminopeptidase activity of *M. gallinarum*

Metal name	APN Activity (OD _{600nm} Unit/mg protein) ^{a,b}	
	Leu substrate	Arg substrate
No metal ion	0.43 ± 0.03 ^{c,d}	11.9 ± 0.03 ^{g,h}
K ⁺	0.94 ± 0.06 ^{c,e}	12.17 ± 0.32 ^g
Mg ²⁺	1.95 ± 0.06 ^{c,e,f}	12.29 ± 0.14 ^g
Mn ²⁺	2.38 ± 0.06 ^{c,e,f}	12.31 ± 0.38 ^g
Zn ²⁺	0.39 ± 0.05 ^d	11.61 ± 0.15 ^h

- It is calculated by dividing the OD_{600nm} value by the test protein concentration.
- The values in table are the activity mean values from three experiments ± standard error.
- Significant difference for leucine aminopeptidase activity between the no metal ion control group and the group treated with K⁺, Mg²⁺, or Mn²⁺.
- No significant difference for leucine aminopeptidase activity between Zn²⁺ treated group and the no metal control group.
- Significant difference for leucine aminopeptidase activity between the group treated with K⁺ and the group treated with Mg²⁺ or Mn²⁺.
- Significant difference for leucine aminopeptidase activity between the group treated with Mg²⁺ and the group treated with Mn²⁺.
- No significant difference for arginine aminopeptidase activity between the no metal ion control group and the group treated with K⁺, Mg²⁺, or Mn²⁺.
- No significant difference for arginine aminopeptidase activity between Zn²⁺ treated group and the no metal control group.

Table 3.4 Effects of EDTA on aminopeptidase activity of *M. gallinarum* lysed cells

Inhibitor name	APN Activity (OD _{600nm} Unit/mg protein) ^{a,b}	
	Leu substrate	Arg substrate
No metal ion	0.40 ± 0.01 ^c	7.23 ± 0.12 ^e
EDTA	0.00 ± 0.00 ^c	0.00 ± 0.00 ^e
Mn ²⁺	1.26 ± 0.09 ^{c,d}	6.90 ± 0.28 ^{e,f}
EDTA+ Mn ²⁺	0.65 ± 0.07 ^{c,d}	7.05 ± 0.24 ^{e,f}

- It is calculated by dividing the OD_{600nm} value by the test protein concentration.
- The values in table are the activity mean values from three experiments ± standard error.
- Significant difference for leucine aminopeptidase activity between EDTA treated group and the other three groups.
- Significant difference for leucine aminopeptidase activity between Mn²⁺ treated group and EDTA+Mn²⁺ treated group.
- Significant difference for arginine aminopeptidase activity between EDTA treated group and the other three groups.
- No significant difference for arginine aminopeptidase activity between EDTA treated group and the other three groups.

Table 3.5 Aminopeptidase activity of *M. gallinarum* lysed cells

Arg APN activity		Leu APN activity	
(OD _{600nm} Unit/mg protein) ^{a,b}		(OD _{600nm} Unit/mg protein) ^{a,b}	
Membrane fraction	Cytoplasmic fraction	Membrane fraction	Cytoplasmic fraction
2.53	1.36	3.65	0.96

- It is calculated by dividing the OD_{600nm} value by the test protein concentration.
- The listed values are from the group with the maximum enzyme activity in cytoplasmic fraction among the four separated experiments.

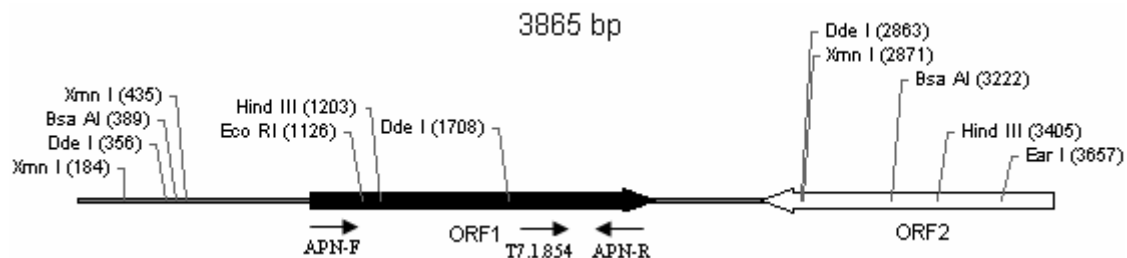


Figure 3.1 Restriction map of the cloned 3,865 bp sequence. Database searches indicate that the 1,362-base ORF1 sequence has 68% nucleotide identity with the *M. salivarium* leucine aminopeptidase gene, whereas the 1.1 kb ORF2 sequence has 68% nucleotide identity with glutamyl tRNA synthetase gene of *M. pulmonis*. Alignments of the predicted amino acid sequence, which was derived from ORF1, with the aminopeptidase protein of *M. salivarium* showed an amino acid identity of 51% between the two sequences.

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MSA_LAP      MELIKEIETSRNSSVLLKAI FEGDDAP TLLVKKQSQITEYLKENVA YVYLGGKSEFGYKD 60
MGN_APN      MNKL-EFLT KRDNFLVAVDYNL PFEWISKNGAISENLSKNEAYITIKEIK--NYDE 57
*:. :.*: *. :...** *: .: . : **:. *: * :.* ** : : . . .*.:

MSA_LAP      AYEFARDLAENCARSYQLDLTTFVTEK-LCIKGVVDAFTKGINFSAFYQYNLKFTFKRVN 119
MGN_APN      LVEFIKKNILNSQRAFQIDLNSFKVNLNINYEDILKAFISSIVFYQAKLFNKK--KEKSK 115
** .: . * . *:.*:**.* . : : : : ** . . * * : : * * : : : :

MSA_LAP      ENSLSFYLENI SQDVLNVFKKALILVDAQNFARNLGVTPPNELNSEQLAEI IRKDFKKYH 179
MGN_APN      KTNFSLLFN--SDEDKEVFNKFNLIAENRNKIRNLQIMPENYCNSVELANYIVEDFNLK 173
:..*: : : . * : : : **:* : : : : * * ** : * * * : ** : * : ** : :

MSA_LAP      NLKVKVLERKQIELLGMDDL LLSVNKGSVYEP RVVIEEYKGNPSSQEKTVLVGKGITFDSG 239
MGN_APN      NLKVTVL DKNMIEK LKMG LILSVNKGSTHEPRVVI EYNGNKSKDKIVFVVGKGITFDTG 233
****.* : : : * * * . : . : ** : : : : : : : : : : : : : : : : : : : :

MSA_LAP      GYSLKPKPFMLGMKYDMSGSAI VAAVMKAI AQLKPNKNVSAIMCITDNRINGDASLPDSV 299
MGN_APN      GINTKG-YHMSGMKYDMSGSAI VAYAVKTLAELKANVNVAAIMMITDNRQGDASLPENV 292
* . * . . * ***** . : : : * : * * : * * * * * * : * * * * :

MSA_LAP      YTSMSGKTVEVNNTDAEGR LVLADRL YYGATKLNATRLIDTATLTGTMLTALGQTYSGIY 359
MGN_APN      YESMSGLSVEVTD TDAEGR LVLADGL YYGATK LKATLFDVATLTG SVLTALGHTYSGIW 352
* **** : *** : ***** ***** : * * : * * * * : : * * * * * :

MSA_LAP      ATSCKIWHQFEDA AKIAHEKVWRMPLHEDFNKTN KESLVADLNNYSNNEKSDCNTAAMFL 419
MGN_APN      STNEGKWGLFEKAGQISHEKIWRMPLHDDFHQFNLASEVADLNNYNNSEKSDSNTAAMFL 412
:* . * ** . : * : * : * : * : * : * : * * * * * * . * . * * * * *

MSA_LAP      KEFTNNVPYIHC DVAGTADKKGMGLGILVSTFVEFGKSQQR
MGN_APN      KQFTNNVDYIHC DVAGTADVGGKPGV LIDTLVEFALLNEK
* : * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

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Figure 3.2 Alignment of ORF1 with *M. salivarium* leucine aminopeptidase gene. The alignment of amino acid sequence derived from *M. gallinarum* ORF1 with the leucine aminopeptidase gene of *M. salivarium* (Shibata et al. 1995) showed a 51% identity. The active sites of leucine aminopeptidases (Burley et al. 1990; Kim and Lipscomb 1993; Strater and Lipscomb 1995a; Strater and Lipscomb 1995b) were predicted in this aminopeptidase candidate and are designated as bold. In the consensus line, “*” designates identical amino acids, and “:” designates conserved amino acids, and dashes indicate gaps introduced to maximize homology.

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1 TGCTTTGTAGACAGTTTAAAGCATAAATCATAATTAGCAATCTGTAAATGCTTTAAATTTATATAGTGTGGTGGATTTCATTTAATCAGTCAAATCTTTTAGTTTATAAATGTC
121 TTTTGATATGTTGAATAGCTAAAGCAAATTTTTCATAGGCTTTATTTAAATGGTAGATAAAGAATTTTCTAATGCGATATCAATCAAATTTTATTTAAATAAAAAACATTTAATA
241 TTATTTTAAATCAACTATAAACACATCTAAATCTCGTAAATTTTGGTTTAAAAAGATATATTTTGGTGTATTTTGTAAATAAATATTTTCTTACTATCTTACCTCAG
361 GCAAAAAGTAAATTTTAAAAATAAATCACGTGAATAATTTCTCCGTTGACATTTATATGGGAATATGAAAACTTCATTTGAATTTTGTAGTAATGCACTAACTGCAATAA
481 TGTTTGGCAGTAAATTAATCATATTTATATAAAAAATTTAATAAAAAATTAATTTAAGTTTAACTTATCATCATCGTCAATGAAAAAAGATTTCTCCTTTAGCTTGAGAA
601 ATTAATAAATCAGGATTAACCCGATTCCTTTTCTGAAATAGTCACTTAAATTTAAACTTTAATCTTGAATCTTTTGTAGATAAATCAATTAATTTTCAAAAGTATATCATTT
721 GAATAATCATCAATCAAAAATTTCAATTTTATATTTGCTCTTAAGTTTCAATAATGTTTAAAGTAAAAAATTTAATTTATTAATTTATGCAAGGAATTAAGATGCTCAGTCC

-35 -10 +1
841 ACTTTTTTCATTAACATCTTTATATATTTGCTTTATATTTTATATAATTTAAAAATTAAGGAGATATGAATAAATGGAAATTTTGCACAAAAAGAGACAA
SD M N K L E F L T K R D N
961 CAATTTTTTATAGTTCAGTCGATTATAAATTTACCAATTTGAATGAAATTTCTAAAAAATGGCGCAATAAGTGAATTTATCTAAAAAGGAGCATATAACTATAAAGAAAT
N F L L V A V D Y N N L P P E W I S K K N G A I S E N L S K N E A Y I T I K E I
1081 TAAAAATATGATGAATTAGTAGAATTTATCAAGAAAAATTTTGAATTTCTCAAGAGCTTTTCAAATGATTTAAATCTTTTAAAAACGTATTGAATATAAATTTAGAGATATTTT
K N Y D E L V E F I K K N I L N S Q R A F Q I D L N S F K N V L N I N Y E D I L
1201 AAAAGCTTTTATTTCTTCAATCGTTTTTATCAAGCAAATTTGTTTAAACAGAAAAAGAAAAAGTAAAAAACTAATTTTCTTTATTTAATCTGTGAGATAAAGAAGTTTT
K A F I Y Q A K L F N K K G L I L S V N K G S T H E P R V I T T G V A T C A A
1321 TAATAAATTTAATTTAATCGCAGAAAAATAGAAAATAAATTTAGAACTTGCATAAATGCTGAAAATTTACTGCAATTCAGTAGAGTTAGCTAATTTATTTGTTGAAGATTTTCTTAATCT
N K F N L I A E N R N K I R N L Q I M P E N Y C N S V E L A N Y I V E D F S N L
1441 AAAAAATTTAAAGTCACCGTTTGTAGATAAATAATGATAGAAAAATTAAGAGTGGTTTAATTTTGTCTGTAATAAGGGTCAACACACGAACCAAGAGTGGTTTGTGGAATACAA
K N L K L V L D K N M I E K L K M K G L I L S V N K G S T H E P R V I T T G V A T C A A
1561 TGAACAACAGAAATCAAAGATAAATCGTCTTTGTTGAAAAAGGAATTTACTTTTGTAGTACAGTGGAAATTAATAAAAAAGGCTATCATATGTCAGGAATGAAATATGATATGTCAGGTTT
G N K K S K D K I V F V G K G I T F D T G G I N T K G Y H M S G M K Y D M S G S
1681 TGCCATTGGCTTATGCTGTAAAAACTTAGCCGAATTAAGCAAATGTTAATGTTCTGCAATTTATGATGATAACTGATAATCGTCAAGACGGAGATGCTTATGCCCCGAAATGT
A I V A Y A V K T L A E L K A N K V N V A A I M M I T D N R Q D G D A S L P E N V
1801 TTATGAATCTATGTCAGGTTGAGTGTGAAGTAACTGATACCGATGCTGAAGGACGTTTAGTTTGTAGCAGATGGATTATATATGGAGCTACTAAATTAAGCAACTACATATTTGA
Y E S M S G L S V E V T D T D A E G R L V L A D G L Y Y G A T K L K A T T L F D
1921 TGTGCAACTTAAACAGGATCAGTTTAAACAGCACTAGGACATACTTATCTGGAATTTGATCAACAAATGAAGAAAAATGAGGACTTTTCAAAAAAGCAGGCAAAATGATCATGAGAA
V A T L T G S V L T A L G H T Y S G I W S T N E G K W G L F E K A G Q I S H E K
2041 AATTTGAAGAATGCCATTACATGATTTTCAATTAATTTGGCTTCTGAAGTTCGCTGATTTAAATAAATAAATAGTAAAAATCAGATTTCAATTAATGCTGCAATTTCTTT
I W R M P L H D D F H Q F N L A S E V A D L N N Y N N S E K S D S N T A A M F L
2161 AAAACAATTTACTAACAATGTAGATTATATTCATTTGATGTTGCCCCGAAACCGTGTGCGTGGAAAACTCAAGGTGTTTAAATGATACATAGTTGAATTTGCTTTTATAAATGA
K Q F T N N V D Y I H C D V A G T A D V G G K P Q G V L I D T L V E F A L L N E
potential stem loop
2281 AAAATAGAACCAAGAAAGTTCTTATTTTTTAAATCTTTCAATTTGAAATTTCAAATTTGCCAATTAAGGTCCTATTAGCGGCAATAACTCATATTTAAATGTTTGGCTTATATCAGTTA
K *
2401 TTTCTACATCATGAAGTAACTAATTAAGACAAATTTTGAATTTTACAGAGATATAAGAATTAAGCCGCTTTTCAATTTTTTCAAATTAAGGTTGTATCACCAGCAAAAAATTTAA
2521 TTTGCTTTTCATTAATTTCAATTTCTGTTAGCAATTCCTTTTGAAGGTTCTAAAAATTTGATATGTTAAATTTCAATTTGATTTGAGTTATCAGTAAAAATTTCAAATTTCACTTT
2641 CAGCCTCAAATTTCAATAATATTTTTTTCAGTATCTACAGAAATGATCTGCAATTTGATTTAAATTTTATTTTCAATAATTTCAATCTTTCTTTAATTTACTTTTTTCCATAAAGAAAAATTTGC
2761 TTTTGCAGTTTCAAGCCTATGTGAGACAAATGTTGTTGGCTAAACGAATAGGCATAAATAAATTTTGGCCCTTTTTCTCAGTGATTTTGCAGTAAATTAATTTGCTTTTTGAAATGTTTC
2881 AACATAAATTAATTTGTTTATTTCTCTCTAAAGATTTTCAACACTTCTAATTTCAATTTGATAAATTTGCGCTTAACTTTGATTTATAGTTTAAATAAATTTCAATTTTCTTT
3001 TAATTTGTTGAATTTGATAAACACTTTCTTTAAAGTATCAACAAATAAATTTTCAACATCATCTCAAGATTTAACAATTTAATAAATCACTAGTGGAAATTTTTTCAAAATTTG
3121 TTTTGAGAAATCATGCAATTTTGAATATCAAATTTAGAAAGACTTTTACTTAACTTTTGGGATCAAAAGCAATTAATTTTCTTTTGAACATTTTTCACGTTGTTGACTATCTGT
3241 TCAACCTAAATAAGCAAGAAAAATAAAAATAGCTTTGAGGAATATAACCTTCAATTTCTATAATCTTCAATAAATTTTAAACTAGTATCTCTTTTGAAGATTTTGGCCCTCCATTT
3361 TGTAATTAATGTCATATGCGCAATTTCTGACTTTCTCAATTTAAAGCTTGAATAATGCAAGTGTGTTAGGAGTATAGTTATATGTTCTTCACTCTTAAATCATGACTATTTCCAT
3481 ATCATGATCATCAACACTACTGCGAAATATATGATAGGATAACCATCTTTTTTGAATTTACTCAATCTCAATATCATTTGATGAAAACTAATTTTCCCTCAACAAATATCATTTCA
3601 TTTATATTTCAACATTTTATAGCAATTTGCTTAAACGAACTGAAATATTTCTTGGCATCATCTCTTTTGGTTTTTCAAATTCAGAAATTTTCAATCAATTTTATCATATCTAAAGATGAAAT
3721 ACCTTTTGGCTTCTGCTTCAAGATGTTGTTTCTCAATTTCTCAGAAGTATCATATGCTTTATAAGTAAACCTTTGCTTAAAGTTTCAATTTGATGATTTGTTGATATCTATCTAATTTTTC
3841 GCCTTTGCTATATTTTCCATATTTT

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Figure 3.3 Nucleotide and deduced amino acid sequences of the aminopeptidase gene cloned from *M. gallinarum*. The marked SD denoted the putative Shine-Dalgarno sequence. The potential stem loop at the end of the APN gene suggests a rho-independent termination signal of transcription. The putative promoter regions are shown upstream of the APN gene. The four TGA used to encode Trp were denoted in bold letters in both nucleotide and amino acid sequences. The Genebank access number is AY080895.



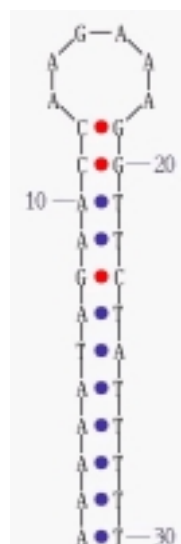


Figure 3.4 The potential loop at 3' terminus of the leucine aminopeptidase identified gene in *M. gallinarum*. This loop was made according to the methods described by SantaLucia (1998).

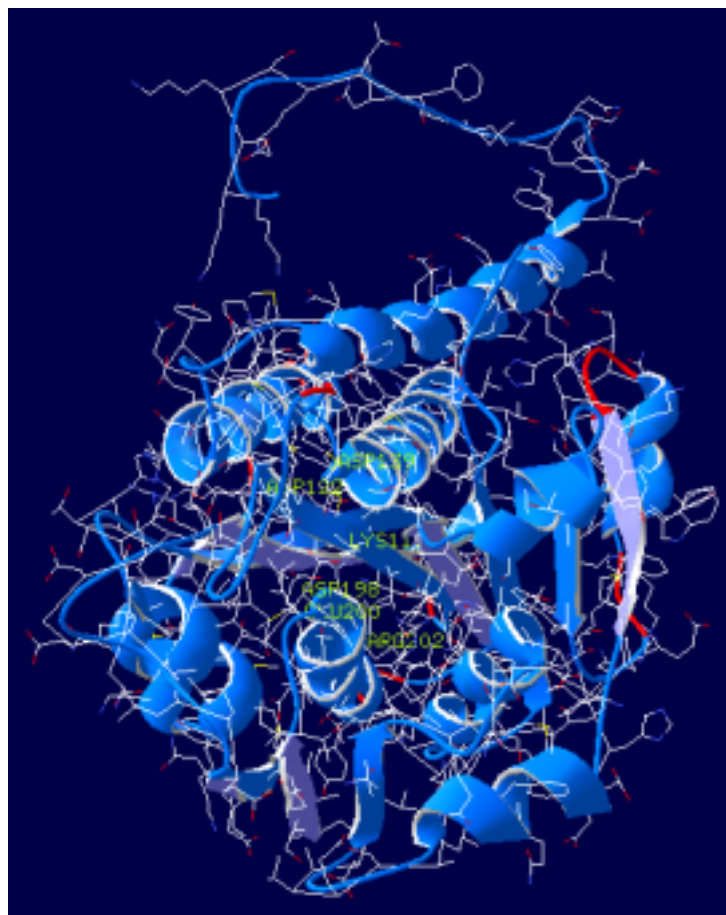


Figure 3.5 The predicted 3D structure of the leucine aminopeptidase identified in *M. gallinarum*. This structure covered the length of the protein from amino acid 110 to 433. The 3-D modeling templates of the leucine aminopeptidase gene were obtained with SWISS-MODEL (<http://www.expasy.ch/swissmod/>) Blast by searching the ExNRL-3D database with the sequence, and the structure was predicted with the Swiss-Model First Approach Model and analyzed with Swiss-Pdbviewer version 3.7 beta (Schwede et al. 2000). The six active sites reported in other leucine aminopeptidases (Burley et al. 1990; Kim and Lipscomb 1993; Strater and Lipscomb 1995a; Strater and Lipscomb 1995b) were conserved in this structure. They are labeled as Lys 117, Asp 122, Asp 139, Asp 198, Glu 200, and Arg 202, representing the amino acid Lys 226, Asp 231, Asp 248, Asp 307, Glu 309, and Arg 311 in the original sequence.

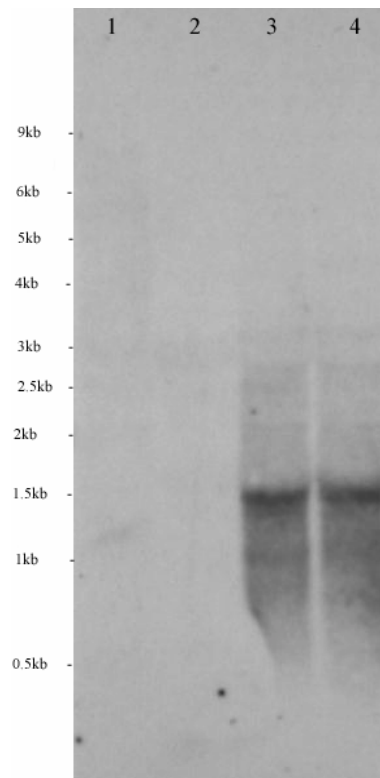


Figure 3.6 The results of Northern-blot hybridization analysis. Lane 1, RNA molecular marker; lane 2. *M. gallisepticum* total RNA; lane 3-4, *M. gallinarum* total RNA. The blot was hybridized with APN biotin-labeled probe which was amplified by PCR from *M. gallinarum* genomic DNA and covered ORF1.

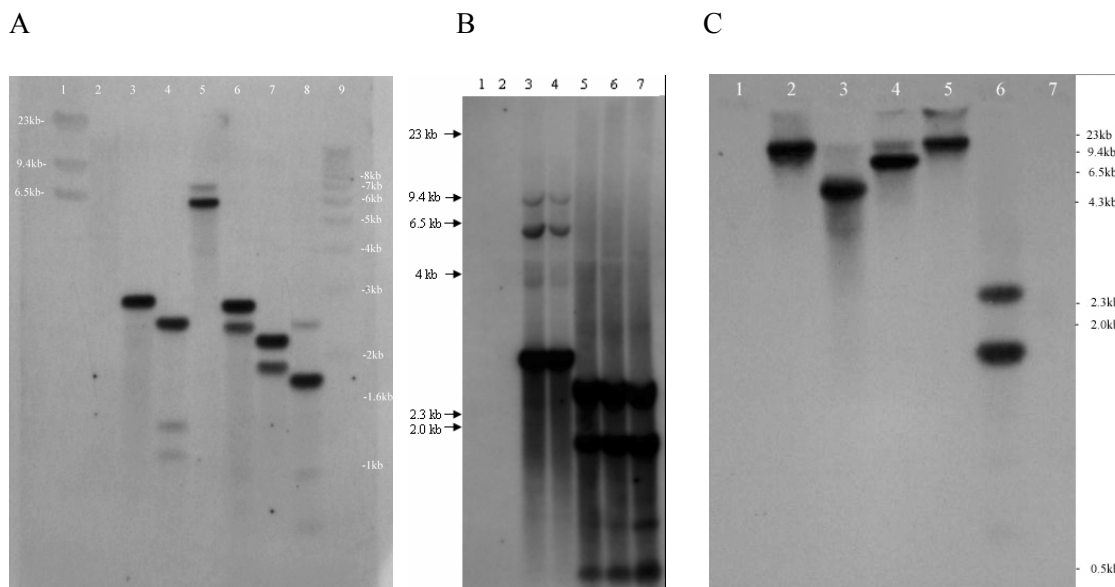


Figure 3.7 The results of Southern-blot hybridization analysis. The blot was hybridized with APN biotin-labeled probe that was amplified by PCR from *M. gallinarum* genomic DNA and covered ORF1. A. Lane 1, lambda DNA digested with *Hind* III; Lane 2, 2 µg *M. gallisepticum* F strain genomic DNA digested with *Hind* III; Lane 3-8, 2 µg *M. gallinarum* genomic DNA digested with 10 units of *Bsa* AI, *Dde* I, *Ear* I, *Eco* RI, *Hind* III, and *Xmn* I; Lane 9, 1kb DNA ladder. B. Southern-blot results from partial digested *M. gallinarum* genomic DNA with *Xmn* I. Lane 1, lambda DNA digested with *Hind* III; lane 2, 2 µg *M. gallisepticum* F strain genomic DNA digested with *Hind* III; lane 3-4, 2 µg *M. gallinarum* genomic DNA digested with 10 and 20 units of *Bsa*I, respectively; Lane 5-7, 2 µg *M. gallinarum* genomic DNA digested with 2, 5, and 10 units *Xmn*I, respectively. C. Lane 1, 2 µg *M. gallisepticum* F strain genomic DNA digested with *Hind* III; Lane 2-6, 2 µg *M. gallinarum* genomic DNA digested with 10 units of *Cla* I, *Ear* I, *Nco* I, *Pst* I, and *Xmn* I; Lane 7, lambda DNA digested with *Hind* III.

```

MGN_APN 110                               KKEKSCK TNFSLLFNSD
LAP      101 QDLEIPSVEV DPCGDAQAAA EGAVLGLYEY D--DLKQKRK VVVS AKLHGS
                                     . *   *   . . .

MGN_APN
LAP      hh  sssss s   hhhhh hhhhhh                               sssss

MGN_APN 117 EDKEVFNKFN LIAENRNKIR NLQIMPENYC NSVELANYIV EDFSNLKN-L
LAP      149 EDQEAWQRGV LFASGQNLAR RLMETPANEM TPTKFAEIVE ENLKSASIKT
          * * *   . * * * * * * * *   . . * . . * . . .

MGN_APN
LAP      hhhhhhhh hhhhhhhhhh hhhh          hhhhhhhhhh hhhh
          hhhhhhhh hhhhhhhhhh hhhh          hhhhhhhhhh hhhhhh ss

MGN_APN 176 KVTVLDKNMI EKLKMG LILS VNKGSTHEPR VVIVEYNGNK K-SKD KIVFV
LAP      199 DVFIRPKSWI EEQEMGSFLS VAKGSEPPV FLEIHYK GSP NASEPPLV FV
          * . * . * *   ** . ** * *** *   . . * . * . *   . ***

MGN_APN
LAP      sss hhhh hh   hhhhh hhh   s ssssssss          ssss
          sssss hhhh h   hhhhhh hhh   s ssssssss          ssss

MGN_APN 225 GKGITFDTGG INTKGYHM-S GMKYDMSGSA IVAYAVKTLA ELKANVNVA
LAP      249 GKGITFDSGG ISIKAAANMD LMRADMGGAA TICS AIVSAA KLDLPINIVG
          ***** . ** * . *   * . ** . * *   . * . . *   . * . .

MGN_APN
LCP      sssss          hhhhh hhhhhhhhhh hh   sssss
          sssss sss sss   h hh   hhhh hhhhhhhhhh hh   sssss

MGN_APN 274 IMMITDNRQD GDASLPENVY ESMSGLSVEV TDTDAEGR LV LADGLY GAT
LAP      299 LAPLCE NMP S GKANKPGDVV RARNGKTIQV DNTDAEGR LI LADALCYAHT
          . . . *   * * . * *   . . * . . *   . ***** . *** . * . *

MGN_APN
LCP      sssss          sss ss   sssss          hhhh hhhhhhhh
          sssss          sss ss   sssss          hhhh hhhhhhhh

MGN_APN 324 KLKATTLFDV ATLTG SVLTA LGHTYSGIWS TNEGK-WGLF EKAGQISHEK
LAP      349 -FNPKVI INA ATLTGAMDIA LGSGATGVFT NSSWLWNKLF EAS IETG-DR
          . . . . . ***** . * **   . * . .   .   ** * . . . . .

MGN_APN
LAP      sssss s   hhhhh          sssss          hh hhhhh s
          sssss s   hhhhh          sssss s hhhhhhhh hhhhhh s

MGN_APN 373 IWRMPLHDDF HQFN LASEVA DLNNYN NSEK SDSNTAAMFL KQFTNNVDYI
LAP      397 VWRMPLFEHY TRQVIDCQLA DVNNIGKYRS AGACTAA AFL KEFVTHPKWA
          . ***** . . . . . * . **   . . . *** ** * . *   .

MGN_APN
LAP      ssss   hh hhhhh          sss          hhhhhhhh h   sss
          ssss   hh hhhhh          sss          hhhhhhhh h   ss

MGN_APN 423 HCDVAGTADV G
LAP      447 HLDIAGVMTN KDEV P YLRKG MAGRPTRTLI EFLFRFSQ
          * * . **

MGN_APN
LAP      ssss
          ssss sss          s ss   hhhhh hhhhhhhh
    
```

Figure 3.8 Alignment of the leucine aminopeptidase of *M. gallinarum* and the template LAP obtained from the ExNRL-3D database (Burley et al. 1990; Kim and Lipscomb 1993; Strater and Lipscomb 1995a; Strater and Lipscomb 1995b). MGN_LAP represents the cloned aminopeptidase gene from *M. gallinarum*. LAP is an abbreviation for bovine lens leucine aminopeptidase. There is a 38.3% homology between MGN_LAP and LAP. * designates the identical amino acids; conservative amino acid are designed by “.”. Dashes indicate gaps introduced to maximize homology. The letter of s denotes sheet and h represents helix.

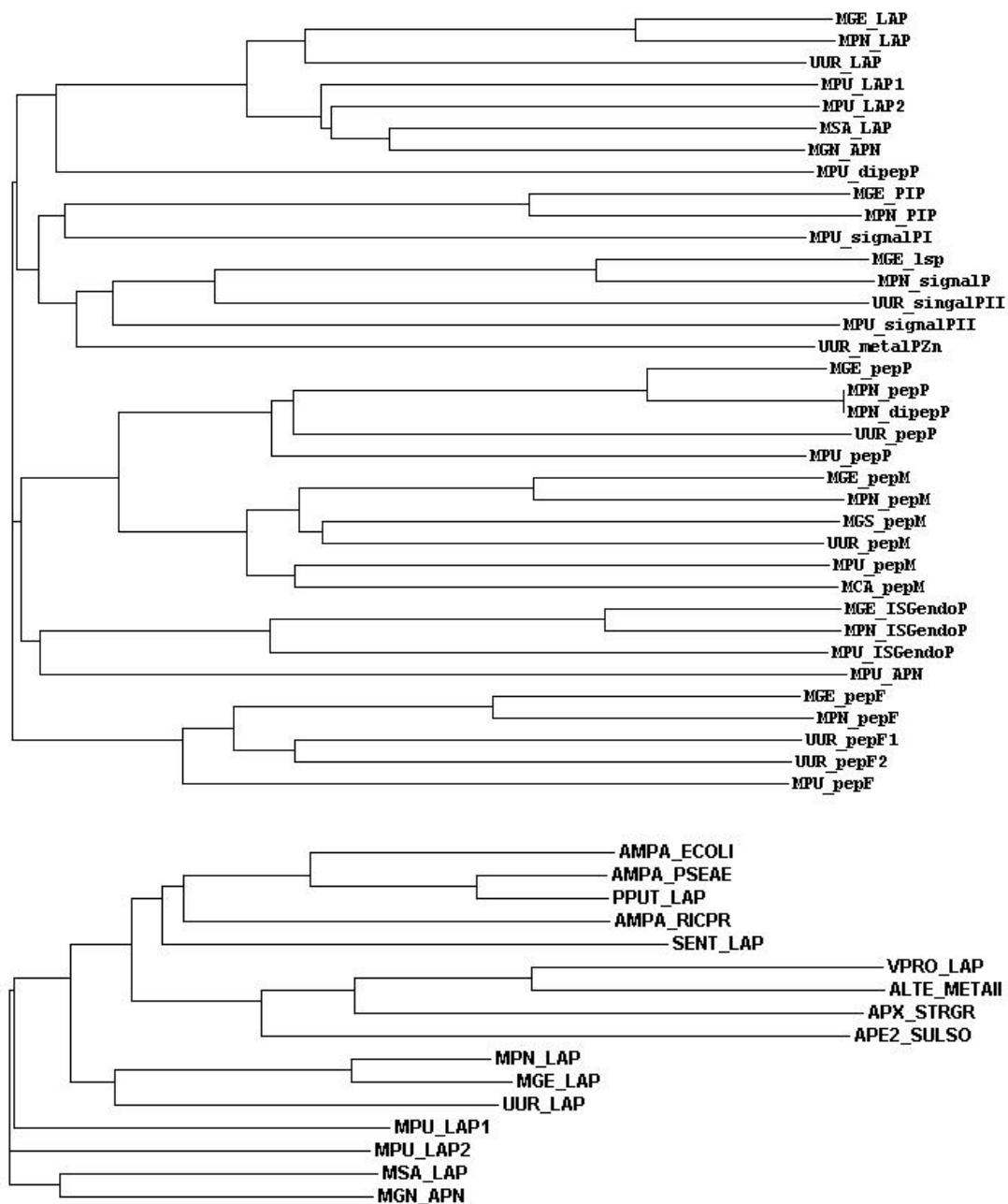


Figure 3.9 Phylogenetic relationships of leucine aminopeptidases. A. Phylogenetic relations for 37 peptidases from mycoplasmas. B. Phylogenetic relations for 7 leucine aminopeptidases from mycoplasmas and 9 leucine aminopeptidases from other prokaryotes. The phylogenetic trees were predicted with the Clustal W version 1.81 (<http://www.ebi.ac.uk/clustalw/>) (Thompson et al. 1994; Higgins and Sharp 1988). The sources of the abbreviation were shown in Table 3.2.

```

MSA_LAP      --MELIKEIETSRNSSVLLKAI FEGDDAP TLLVKKQSQITEYLKENVAVVYLGKKSEFGY 58
MGN_APN      --MNKL-EFLTKRDNPLLVAVDYNL PFEWISKNGAISENLKNEAYITIKK--NY 55
MPU_LAP2     --MLLEKQTRERNEMLLKAVFDS EAKIEGLIKTKNVI TE FVEKNEAYVFLGKKEDFSR 57
MPU_LAP1     MKGKKVIKVSNEKEFPISLKLIFKQENMNDPLIQEDHQITEINSENTLYYFVKDKSKYDI 60
MPN_LAP      -----MKLN--KLF--DSKT VVFKKSDKYGKSDCKTKCVEG-QIEFG--VKIPTDFP- 46
MGE_LAP      -----MRIN--KPFSDSNTVVFVSSKTYG---VKEEAHNPVVEFG--VVLPTDFP- 45
UUR_LAP      -----MTLNEKKEFVVELKAVEKNHN-LKGFEE NTTEHV VFGGLYQKQYLILTPDFDS 53
              :           :           .           :           .

MSA_LAP      KDAYEFARDLAENCARSYQLDLTTFVTEK-LCIKGVVDAFTKGINFSAFQYYNLKTPFKR 117
MGN_APN      DELVEFIKKNILNSQRAFQIDLNSFKVNLNINYEDILKAFISSIVFYQAKLFNKK--KEK 113
MPU_LAP2     EVWNSFLDSFIPSMKRSYQLDLILSFETPS-YKLEDIIYDLYIKYDIKADLFSHRKKESD 116
MPU_LAP1     KPFKKAASKIVENQRNRLNIDISSFSHNS-IALESIVKIFVEKYEFKHANLFKFKDKDDK 119
MPN_LAP      -AFNRALVTFLLKTQKNQNLVDLDSFVELYKAEN--LCYKTALKVVASITFCETPPTMK 103
MGE_LAP      -AFNRALVQFLKRKTKLNLINLDSLIELYKKNENSGCFHTAIKTVITSVTFCETPPTMK 104
UUR_LAP      NELSRSLISFLEKSPKSVSVDLNSFLSLVPEPSR---HASLLNVVISALEYVEVTPPSLK 109
              .           .           .           .           .

MSA_LAP      VNENSLSFYLENISQDVLNVFKKALILVDAQNFARNLGVTPPNELNSEQLAEIIRKDFKK 177
MGN_APN      SKKTNPFLFN--SDEDKEVFNFKNLIAENRNKIRNLQIMPENYCNSVELANIYVEDFSN 171
MPU_LAP2     ENKVS LTIWNN-ANRCFAEYVEKAKLISLNVNFARDLQTTPPNI CNSEWMAKKVEEKAKS 175
MPU_LAP1     QYSINLYVSDKQKILVLETLLEKTLILTNALNTVRKIQIYPPNICNSEWLDADFVYEDLKK 179
MPN_LAP      TEPQKNVEVAVQCDSEHTSLIKEYEVVGNVNMARQLQDTPSDQLYPEEFVKRFEKAATG 163
MGE_LAP      TKPEKNVEVAVQCAVEYHNLVKEYETVGEYVNLARELQDTPSDLLYSEVVFVKHFEKAASK 164
UUR_LAP      SKIETKNIHNLVVDQKYHDLINKLHVIAQSQTITRRLQDTPANLMTPGDFEERI KELFKD 169
              .           .           .           .           .

MSA_LAP      YHNLKVKVLERKQIELLGMDDL LLSVNGKGSV--YEP RVVIEYKGNPSSQEKTVLVGKGI 234
MGN_APN      LKNLKVTVLDKNMIEKLMGLILSVNGKST--HEPRVIVVEYNGNKKSKDKIVFVGKGI 228
MPU_LAP2     FENVEVKVLDKKAIEKHGMNLLSVNGKSM--FEPRVVLSYNGDSSSKERTSLIGKGI 232
MPU_LAP1     IDSLKINIYNSQI EDLGMNLF LSVNGKSA--YEPRLVSIYNGDPSSEKTVLVGKGI 236
MPN_LAP      LG-VKITVLKQADL I KKKMGLLLGVNKGSE--REARLLVSIYNNKKSSETLALVKGKI 219
MGE_LAP      LP-VKIKVLKQSDL I KKKMGLLLGVNQGSE--REARLLVSIYQANKNSKEKLAFVKGKI 220
UUR_LAP      LPEVKVSVLYRKDLEAKGMNAHVGVGKAASVDKAQPRLVVVEYNNNPETNEKYAFVKGKV 229
              .           .           .           .           .

MSA_LAP      TFDGGYSLKPPKFMGLMKYDMSGSAI VAAVMKAI AQLKPNKNVSAIMCITDNRINGDAS 294
MGN_APN      TFDTGGINTKG-YHMSGMKYDMSGSAI VAYAVKTLAELKANVVAAIMITDNRQDGDAS 287
MPU_LAP2     TFDGGYSLKPAKSMVGMKYDMSGAAI VS AVV FALSALGVKRNVS AVLAI TDNRVNGDAN 292
MPU_LAP1     TFDGGYNIKT-SHMGGMKFDMSGAAI AAYALKAMAQLRAKSNFAALMLITDNRNLSVAN 295
MPN_LAP      TYDSGGMNIKTGDYMRGMKYDMSGAAI VCSTVLALAKNVKTNVVAAL TENLPGPHAQ 279
MGE_LAP      TYDSGGMNIKTGDYMRGMKYDMSGAAI VCSTVLALAKNVKTNVVAAL TENLPGAKAQ 280
UUR_LAP      CFDGGYNVKTGSHRMWKF DMSGSAI VSMTRVALALNKEKVVAVCP LVLNLLAPEGQ 289
              .           .           .           .           .

MSA_LAP      LPDSVYTSMSGKTVEVNNTDAEGR LVLADRLY GATKLNATRLIDTATLTGTMLTALGQT 354
MGN_APN      LPENYVESMSGLSVEVTD TDAEGR LVLADGLY GATKLNATRLIDTATLTGTMLTALGHT 347
MPU_LAP2     LPDSVWKS MNGKSV EINN TDAEGR LVMVDAMTYAI RELKSTDLIDVATLTGAI VVALGNT 352
MPU_LAP1     IPDSVWKS MNGKTV EIVTD TDAEGR LVLADGITFAIRKLNATRVITATLTGTILYTLGTS 355
MPN_LAP      RPDDIQTA YNGKTV EIDNT DAEGR LVLADAI SYAAKDLKATQI IDVATLTGLMSYI LSTT 339
MGE_LAP      RPDDIKIAYNGKSV EIDNT DAEGR LVLADAI TYAAKDLAATHI IDVATLTGLMSYI LSTT 340
UUR_LAP      KPDDIVKSYNGKT IELDNT DAEGR LILADALTYAVRDLKASKLFDVATLTGAMIYALGDT 349
              .           .           .           .           .

MSA_LAP      YSGIYATSCKIWHQFEDA AKIAHEKVWRMPLHEDFNKTNKESLVADLNNYSNNEKSDCNT 414
MGN_APN      YSGIWS TNEGKWGLFEKAGQISHEKIWRMPLHDDFHQFNLASEVADLNNYNNSEKSDSNT 407
MPU_LAP2     YTGWSTND SKWELEKASKVAGEKIWRMPLDAEFAKGIKKS SVADLRNVDSGLGGSNS 412
MPU_LAP1     YTGWATNNTWKELLSAAKHD EPIWRLPLNEDYIESYKSKVADLVNWS SASKPDSNS 415
MPN_LAP      YTGIFSTCDM AWD AFKKAAC SAGEP VWR LPMHPDY LKPLQLTKLADLQNSTSARGAGSSR 398
MGE_LAP      YTGIFSTCDH QWESFKKAAC SAGEP VWR LPMHPDY LKPLQLTKLADLQNSTSARGAGSSR 400
UUR_LAP      YSGWATNNDI WNEVVVAADYAGELVWR LPPHNDFLKMLNS-NVADIANSVSDPRGGSSR 408
              .           .           .           .           .

MSA_LAP      AAMFLKEFTNNVPIHCDVAGTADKKGMGLGILVSTFVFEFGKSQQRNCESECDEQK CET 474
MGN_APN      AAMFLKQFTNNVDYI HCDVAGTADVGGKPGQVLIDTLVEFALLNEK----- 453
MPU_LAP2     AMFLNFPN EGV DHIHID IAGTANVGD S CTGMVKTLVEYLK----- 454
MPU_LAP1     AAMFLKEFT EKD FVHLDIAGTAEIEGEPQGV LKTLAQLALDI----- 459
MPN_LAP      AACFLAEFREGVPLIHCDI ASTASIQDLGQGV LVRTLYERAAQQAKE----- 445
MGE_LAP      AACFLAEFREGVSLIHCDI ASTASIENLGGQV LVRTLYERASQLANK----- 447
UUR_LAP      AACFLKEFT EGVYAHFDIAITADVGHKGTGVMLRTFYRIAQNQKFN----- 455
              .           .           .           .           .

```

Figure 3.10 Alignments of leucine aminopeptidases in mycoplasmas. The six active sites of leucine aminopeptidases identified in other leucine aminopeptidases were conserved in all leucine aminopeptidases from mycoplasmas (Burley et al. 1990; Kim and Lipscomb 1993; Strater and Lipscomb 1995a; Strater and Lipscomb 1995b).

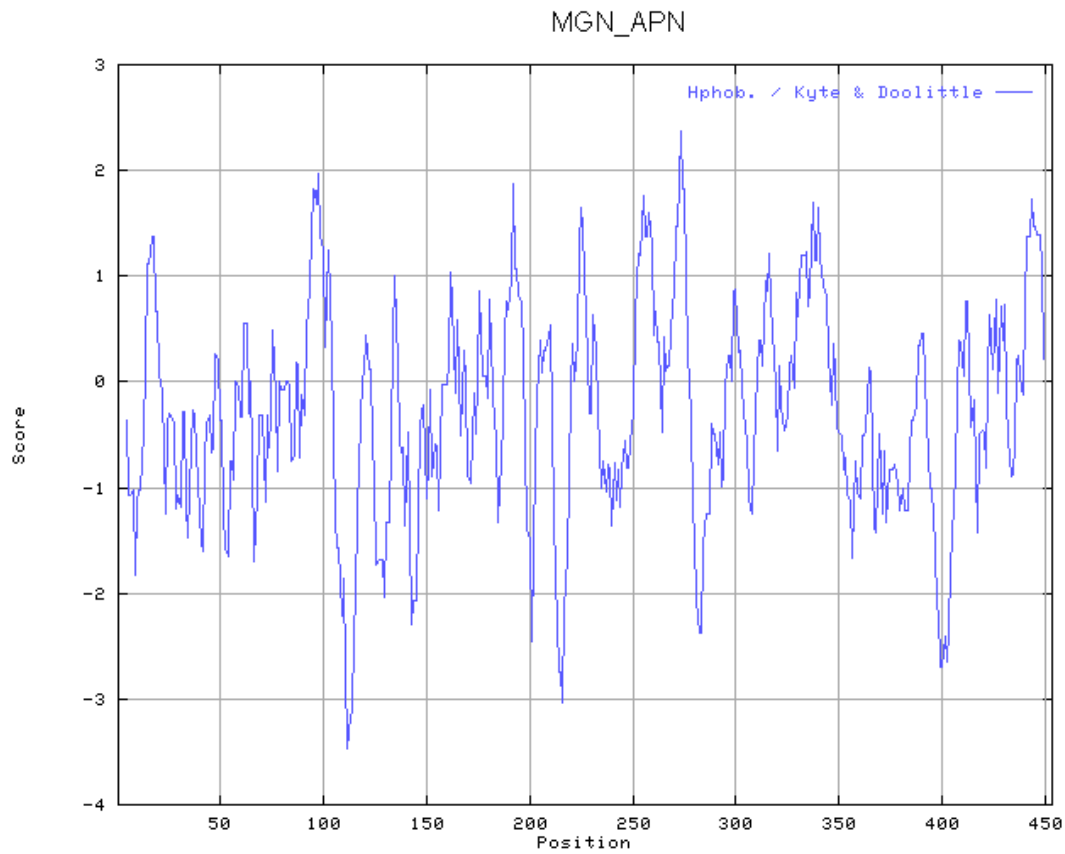


Figure 3.11 Hydrophobic plots for leucine aminopeptidase of *M. gallinarum*. It was drawn by ProtScale (<http://www.expasy.ch/cgi-bin/protscale.pl>) (Kyte and Doolittle 1982). It suggested that this aminopeptidase is a cytoplasmic protein because of lack of a hydrophobic domain for transmembrane region.

CHAPTER IV

EXPRESSION AND CHARACTERIZATION OF THE AMINOPEPTIDASE GENE OF *MYCOPLASMA GALLINARUM*

Abstract

Unlike most other host-specific mycoplasmas, *Mycoplasma gallinarum* exists as a commensal with a host range that includes most poultry and some mammals. This property of *M. gallinarum* may reflect unique mechanisms for its colonization and persistence in various hosts. Our previous studies cloned and characterized a leucine aminopeptidase (APN) from *M. gallinarum*, which was suggested to play a role in host colonization by supplying nutrition from peptide degradation and amino acid scavenging. This study evaluated protein expression of this leucine aminopeptidase gene. After mutation of the four TGAs encoded in the leucine aminopeptidase gene from *M. gallinarum*, the gene was ligated into the pET32b(+) expression vector and overexpressed in *E. coli* AD494(DE3)pLysS cells. The recombinant protein was purified and employed as an antigen to immunize chickens to obtain polyclonal anti-APN serum, which was utilized to identify the subcellular location by immunoblotting with Triton X-114 partitions. The results of these experiments confirmed our former conclusion that the leucine aminopeptidase gene product is located in the cytoplasmic portion of *M. gallinarum* cells with a weak interaction with the *M. gallinarum* cell membranes. Three samples were shown positive to *M. gallinarum* antigen using ELISA from 79 serum samples from chickens experimentally infected with *M. gallinarum*. Immunoblotting showed that there were a few proteins in

M. gallinarum recognized to the host immune responses and that leucine aminopeptidase was not able to provoke production of specific humoral antibody in natural infection. Our results suggest that the leucine aminopeptidase is not strongly immunogenic.

Introduction

Mycoplasmas are a class of unique gram-positive bacteria lacking a cell wall, and represent a distinct subgroup of pathogens for humans and animals (Razin et al. 1998). Many mycoplasmas have been found in the past two decades associated with human and animal diseases (Maniloff et al. 1992). For instance, *Mycoplasma genitalium* is an important pathogen for human nongonococcal urethritis (Deguchi and Maeda 2002). *Mycoplasma pneumoniae* causes community-acquired respiratory infections in children and adults (Hammerschlag 2001). *Mycoplasma gallisepticum* can cause chronic respiratory diseases in chickens. The co-infection of infectious bronchitis virus, Newcastle disease virus, or other disease in chickens has been reported to cause a major loss on many farms (Kleven 1998).

Colonization by mycoplasmas initially stimulates innate immune responses, followed by specific immune responses. Because the isolation of mycoplasmas is difficult and time-consuming, serodiagnosis has been used as an important means for diagnosis of mycoplasmal infection (Maniloff et al. 1992; Levisohn and Kleven 2000). In chickens infected intratracheally with *M. gallisepticum*, the humoral responses were detected as early as one week post infection by leukocyte migration inhibition (LMI) test or the hemagglutination-inhibition (HI) test (Chhabra and Goel 1980). The surface lipoproteins of mycoplasmas have been reported to be “preferential” targets of the host immune responses (Chambaud et al. 1999). Antigenic variation of these lipoproteins may play an important role in escaping clearance by the host immune systems. On the other hand, the cytoplasmic proteins of mycoplasmas may be presented to T cells

(Roitt 1997). After phagocytosis, mycoplasma cells will be degraded within the lysosome, and the degraded components may be presented to T cells MHC II. The T cells activate B cells for the production of specific antibody.

Mycoplasma gallinarum has been identified as a commensal in a wide range of vertebrate hosts, including poultry, cattle, pigs, and sheep (Shah-Majid 1996; Singh and Uppal 1987; Taylor-Robinson et al. 1968; Taylor-Robinson and Dinter 1968; Wang et al. 1990). *Mycoplasma gallinarum* was reported as one of the most frequently isolated mycoplasmas from poultry (Shah-Majid and Nihayah 1987; Kleven et al. 1978). Studies have shown that the colonization of the respiratory tract of poultry by *M. gallinarum* did not result in pathologic changes. In *in vitro* studies, Taylor-Robinson and Cherry (1972) found that *M. gallinarum* did not cause reduction of epithelial cell ciliary activity in tracheal organ culture.

However, *M. gallinarum* was reported to induce temporary airsacculitis when it infected chickens via aerosol or air-sac inoculation with the combination of a field strain of infectious bronchitis virus (IBV) or vaccination with IB and Newcastle disease (Kleven et al. 1978). *In vitro*, with infection with a Newcastle disease virus, *M. gallinarum* temporarily destroyed cilia on the epithelial surface of the tracheal organ culture, whereas microvilli were not affected by this infection (Shah-Majid 1996). Cilia resumed normal function after about one week.

Mycoplasma gallinarum does not induce a strong antibody response. In infected individuals with *M. gallinarum*, specific humoral IgG and IgM levels were very low and almost undetected (Varley and Jordan 1978a; Varley and Jordan 1978b; Bencina et al. 1991a; Bencina et al. 1991b; Bencina et al. 1992). The capability to persist in various hosts and to escape host immune responses suggested that some unique host adaptation mechanisms might be present in this mycoplasma.

In our previous studies, we cloned and characterized a leucine aminopeptidase from *M. gallinarum*. Our results showed that this enzyme is located in the cytoplasmic portion of *M. gallinarum* cells and may play a role in the nutrition supply of *M. gallinarum* for host adaptation (Chapter III). In this project, we generated recombinant leucine aminopeptidase by overexpression of this aminopeptidase gene in *E. coli*, and antisera were produced in chickens against this protein. Western blotting analysis with the mycoplasma proteins obtained by Triton X-114 partitioning demonstrated that this leucine aminopeptidase was not present in the internal membrane of *M. gallinarum*. We also identified three *M. gallinarum* positive serum samples after ELISA surveying of a group of serum samples from chickens experimentally infected with *M. gallinarum*. We found by Western blot analysis the chicken serum samples did not have specific antibodies for the leucine aminopeptidase of *M. gallinarum*.

Materials and Methods

PCR amplification of aminopeptidase gene

Primer APNFpET and APNRpET1 (Table 4.1) were used to PCR amplify the leucine aminopeptidase gene open reading frame from *M. gallinarum* genomic DNA. APNFpET and APNRpET1 have *Bam* HI and *Xho* I restriction sites, respectively, to facilitate cloning of the PCR product. In the PCR reaction, 20 pmol of both primers, and 50 ng *M. gallinarum* genomic DNA and 1 unit of DeepVent DNA polymerase (New England Biolabs, Inc., Beverly, MA) were used in a 50 µl volume. The PCR reaction was conducted at 94 °C for 10 mins, 30 cycles of 94 °C for 30 secs, 55°C for 30 secs, and 72 °C for 3 mins, then finally 72 °C for 5 mins.

Construction of the expression plasmid and site-directed mutagenesis

The *M. gallinarum* leucine aminopeptidase PCR products were blunt-end ligated into pT7Blue blunt ligation vector (Novagen, Madison, WI) to construct pT7Blue-APN plasmid (Figure 4.1). The site-directed mutagenesis was conducted according to the manufacturer's protocol using the multi-directed mutagenesis kit (Stratagene, Co., La Jolla, CA). The first round mutagenesis was conducted with primers 1b and 3 (Table 4.1) to mutate codons TGA to TGG at position 1009 and 2002 (Figure 3.3) to create plasmid pT7Blue-APN-FirstRoundMutation. These changes would create restriction endonuclease sites *Bsm* FI and *Bst* XI (Figure 4.1). These restriction endonucleases were used to screen plasmids containing the desired mutations. The mutations of plasmid pT7Blue-APN-FirstRoundMutation were then confirmed by nucleotide sequencing. The second round of site-directed mutagenesis of plasmid pT7Blue-APN-FirstRoundMutation was conducted with primers 2a and 4 (Table 4.1) to mutate codons TGA to TGG at position 1971 and 2047 (Figure 3.3) to create plasmid pT7Blue-APN-CompleteMutation. After screening by restriction enzyme digestion with *Hinc*II (New England Biolabs, Inc., Beverly, MA), the mutations pT7Blue-APN-CompleteMutation were confirmed by nucleotide sequencing. The APN gene with the expected mutations were released from plasmid pT7Blue-APN-CompleteMutation by digestion with *Bam* HI and *Xho* I. The APN gene was gel purified and ligated into the *Bam* HI and *Xho* I sites of pET32b(+) vector, creating plasmid pET32b(+)-APN (Figure 4.1). Nucleotide sequencing was used to confirm that the correct open reading frame retained after ligation into pET32b(+).

Induction of APN overexpression in *E.coli*

The plasmid pET32b(+)-APN were transformed into *E. coli* strains AD494(DE3)pLysS, BL21(DE3) or JM109, and plated on the appropriate selective media. Colonies were inoculated into 2 ml LB with appropriate antibiotics and incubated with agitation at 37 °C for 16 hrs. The 2ml culture was poured into 50 ml LB with appropriate antibiotics and incubated with shaking at 37 °C for about 3 hrs until the OD_{600nm} of the culture was 0.6. For induction, the concentration of IPTG was adjusted to a final concentration of 3 mM and incubation continued at 37 °C for 3 hrs, or 28 °C for 6 hrs, or 15 °C for 17 hrs.

Purification of recombinant proteins

The subcellular location of recombinant protein in *E. coli* was determined according to the pET system manual (Novagen, Madison, WI). *Escherichia coli* cells were collected by centrifugation of 50 ml culture at 10,000×g at 4 °C for 10 mins. The cell pellets were resuspended in 5 ml of 20 mM Tris-HCl pH7.4 with a final concentration of 100 mg/ml lysozyme. After incubation at room temperature for 10 mins, the suspension was sonicated 10 times on ice at the maximum setting for 15 secs with 30 secs intervals (Microson Ultrasonic Cell Distruptor, Model XL2000). The broken samples were then centrifuged at 14,000×g at 4°C for 10 mins, and the supernants collected as soluble fraction. The pellets were then washed twice by resuspending the cells in 5 ml 20 mM Tris-HCl pH7.4 followed by a centrifugation at 10,000×g at 4°C for 5 mins. Finally, the pellets were suspended in 5 ml 20mM Tris-HCl pH7.4 to represent the insoluble cytoplasmic fraction. Both the soluble and insoluble fractions were evaluated for the recombinant protein by SDS-PAGE gel electrophoresis.

After identifying the presence of expression products in the inclusion bodies, the inclusion bodies were purified using BugBuster protein purification kit (Novagen, Madison, WI).

A 200 ml cell culture was centrifuged at 10,000×g at 4°C for 10 mins, and 1 g cell pellets were resuspended in 5 ml BugBuster reagent with Benzonase (25 units/ml). The mixture was incubated at room temperature for 10 mins and then centrifuged at 16,000×g for 20 mins at 4°C. The cell pellets were resuspended further with 5 ml BugBuster reagent with lysozyme (200 µg/ml) and incubated at room temperature for 5 mins. After centrifuging at 16,000×g for 15 mins at 4 °C, the cell pellets were resuspended with 30 ml of 1:10 diluted BugBuster reagent. The cell pellets were collected again with centrifugation at 16,000×g for 15 mins at 4 °C. This washing step was repeated twice. Finally, the pellets were resuspended the pellet in 5 ml 1×binding buffer containing 6M urea. The collected inclusion body was further purified by His-Bind kit (Novagen, Madison, WI). The final products were eluted with 6 ml 1×elute buffer containing 6M Urea.

The 6 ml of inclusion bodies were injected into Certain Slide-A-Lyzer Dialysis Cassettes, whose membranes are 0.45 mil thick with a 10,000 MWCO (Pierce Chemical Co., Rockford, IL), and soaked into 400 ml of 0.1 M BBS with 0.15 NaCl and 4M Urea at 4 °C for 24 hrs. Then the dialysis cassette was transferred into 400 ml of 0.1 M BBS with 0.15 NaCl and 2M Urea and dialyzed at 4 °C for 12 hrs. Finally, the dialysis cassette was transferred into 400 ml of 0.1 M BBS with 0.15 NaCl at 4 °C for 12 hrs. The refolded proteins were then concentrated with Centricon-50 centrifugal filter (Millipore Co., Bedford, MA).

Digestion of recombinant proteins

To release the APN protein from the APN-trxA fusion protein, 10 µg recombinant APN-trxA protein was incubated with 0.1, 0.2, 0.5 or 1 unit enterokinase (Novagen, Madison, WI) at 22 °C for 16 hrs or 4 °C for 48 hrs, respectively. The digests were checked for APN protein released by analysis of SDS-PAGE gel electrophoresis.

Production of chicken anti-APN polyclonal antiserum

Two 4-week old mycoplasma free leghorn hens were immunized intramuscularly with 75 µg recombinant APN-trxA protein with Freund's complete adjuvant (1:1). These chickens were then administered a second intramuscular immunization with 280 µg recombinant APN-trxA protein after 2 weeks. Sera were collected one week after the second immunization. The serum samples were utilized to determine the subcellular location of the leucine aminopeptidase of *M. gallinarum* by Western-blot with Triton-X114 phase partitions of *M. gallinarum* proteins.

Phase partition of mycoplasma proteins in Triton X-114

The phase partition protocol was adapted from those presented by Bendjennat et al. (1997) and Riethman et al. (1987). *Mycoplasma gallinarum* cells were harvested by centrifugation of 1 liter of a 24 hrs culture at 40,000×g for 30 mins. The pellets were washed twice with 0.15 M NaCl and then resuspended with 4 ml 0.15 M BBS containing 0.15 M NaCl. Triton X-114 was added to the suspension to a final concentration of 1% and the solution was incubated at 4 °C for 3.5 hrs. The solution was centrifuged at 13,000×g at 4 °C for 1 min to remove the unsolubilized cells. The supernatants were collected and incubated at 37 °C for 10 mins to partition the hydrophobic and hydrophilic phases, and then centrifuged at 2,000×g for 5 mins. The aqueous phase was the hydrophilic fraction. The pellet was partitioned an additional time by adding the equal amount of water and then vortexed briefly. The final lower phase was the hydrophobic phase of *M. gallinarum* cells.

ELISA assay

An ELISA assay was developed to identify the presence of antibody in 79 serum samples collected from chickens experimentally infected with *M. gallinarum* by air exposure. Antigen

preparation was conducted according to methods described by Razin and Tully (1983). Briefly, mid-log phase mycoplasma cells were harvested by centrifugation at 34,000×g at 4°C for 30 mins and then washed twice with a 0.2 M Tris buffer. Following incubation in glycerol, cells were lysed and washed in 0.05 M NaCl/PBS. Each 96-well plate was coated with 2.5 µg antigen per well, fixed with 0.25% glutaraldehyde, and blocked with 3% BSA/PBS before ELISA testing. The chicken sera were applied to the appropriate wells and incubated at 37 °C for 3 hrs. Plates were then washed with TBS-0.05% Tween 20 and incubated 1.5 hrs with alkaline phosphatase conjugated rabbit anti-chicken IgG (Sigma Chemical Co., St. Louis, MO). Plates were then washed and developed with the chromogenic alkaline phosphatase substrate p-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, MO), and the color reactions quantitated at 405 nm.

SDS-PAGE

Protein samples were prepared by mixing the sample with sample buffer (1:1) containing 5% 2-mercaptoethanol (APPENDIX A). The preparation was incubated at 95 °C for 5 mins before loading on the gel. Two µg protein was loaded per well in the 10% SDS-PAGE gel, and electrophoresis conducted at 100 volts for 1.5 hrs. Following electrophoresis, the protein gel was stained in coomassie blue stain solution (Bio-Rad Laboratories, Hercules, CA) for 45 mins and then destained twice in distain solution for 45 mins (APPENDIX A).

Western-blot hybridization analysis

The Western-blot hybridization protocols were adapted from Harlow and Lane (1988). Proteins were separated under the same conditions as described above for the SDS-PAGE method. Immediately following by electrophoresis, the gel was soaked in 4 °C pre-cooled transfer buffer for 20 mins, and then proteins were transferred to nitrocellulose membrane (0.2 µm) (Bio-Rad

Laboratories, Hercules, CA) at 100 volts for 1 hr. The membrane was rinsed briefly in TBS and then blocked in TBS with 20% horse serum and 5% bovine serum albumin maintained at 4 °C for 16 hrs. The membrane was then washed five times, 5 mins each time, in TBS-0.05%Tween 20. After incubation with chicken serum samples or control serum at room temperature for 1 hr, the membrane was washed five times, 5 mins each time, in TBS-0.05%Tween 20. The NIH Donkey anti-*M. gallinarum* polyclonal antiserum was used as a positive control antisera and the SPF chicken serum (Sigma Chemical Co., St. Louis, MO) as a negative control antisera. Then the membrane was incubated with corresponding rabbit anti-chicken IgG alkaline phosphatase conjugate or rabbit anti-donkey IgG alkaline phosphatase conjugate (Sigma Chemical Co., St. Louis, MO) at room temperature for 30 mins. The membrane was rinsed with TBS-0.05%Tween 20 for 5 times, 5 mins each time. Finally, the membrane was developed with Sigma Fast 5'-bromo-4-chloro-3-indolyl phosphate/intro blue tetrazolium (Sigma Chemical Co., St. Louis, MO) solution for 15 mins and dried between layers of filter paper. The membrane was stored at room temperature shielded from light.

Quantification of protein and nucleic acid

Protein concentration was determined using the Pierce bicinchoninic acid protein assay kit (Pierce Chemical Co., Rockford, IL). Bovine serum albumin was used as a standard. The DNA concentration was determined by absorbance at 260 nm using GeneSpec I (Hitachi Genetic Systems).

Results

Mutagenesis of aminopeptidase gene of *M. gallinarum*

To express the leucine aminopeptidase gene in *E. coli*, the TGA codons encoding tryptophan had to be mutated to TGG for recognition as the codon tryptophan in *E. coli*. After ligation of APN gene into pT7Blue, the encoded four TGAs were mutated into TGGs. Table 4.2 shows the changes of restriction sites after mutation. Primer 1b generated a *Bst* XI site and Primer 3 generated a single *Bsm* FI. Primer 2a generated another *Hinc* II site. Primer 4 did not generate any new restriction sites and deleted an *Mbo*II restriction site. However, *Mbo* II could not be used to screen the mutants because several other *Mbo* II sites in the APN gene and the pT7Blue vector make differentiation difficult. Figure 4.2 shows the restriction site changes before and after site-directed mutagenesis. The nucleotide sequencing of the APN gene confirmed that the expected mutations had been generated.

Overexpression of aminopeptidase gene of *M. gallinarum* in *E. coli*

The expression of the APN-trxA fusion protein resulted in formation of inclusion bodies at 15°C, 28 °C, or 37°C for AD494(DE3)pLysS, BL21DE3, and JM109 (Kindly provided by Dr. Ma, Mississippi State University). Figure 4.3 shows APN-trxA fusion protein and trxA expression with induction with IPTG at 37 °C for 3 hrs. The APN-trxA is expressed as a 67 kDa fusion protein, and the trxA expression is expressed as a 23 kDa protein. Figure 4.4 shows the purified APN-trxA protein and its digestion products with enterokinase. The results showed that two fragments of 51 kDa and 16 kDa were generated from APN-trxA after digestion by enterokinase. The first fragment is associated with the leucine aminopeptidase of *M. gallinarum*, and the second fragment is the trxA protein encoded in the pET32b(+) plasmid. The reduced size

of trxA protein is due to the fragment within the multiple cloning sites which was digested and released before ligation.

Subcellular location of leucine aminopeptidase of *M. gallinarum*

To determine the subcellular location of leucine aminopeptidase of *M. gallinarum*, polyclonal chicken anti-APN-trxA sera were diluted to 1:1000 for Western-blot with phase partitions of *M. gallinarum* proteins. Figure 4.5 B is the result for Western-blot with pre-immune chicken sera 319, and Figure 4.5C shows the Western-blot for chicken sera 319 after immunization with APN-trxA recombinant protein. The results showed that the endogenous leucine aminopeptidase was present in the hydrophilic partition of Triton X-114. This supports the previous finding that the leucine aminopeptidase is a cytoplasmic protein probably having a weak interaction with the cell membrane (Chapter III).

ELISA screening of sera from chickens infected with *M. gallinarum*

Three samples were identified as positive for *M. gallinarum* infection from 79 sera from birds with *M. gallinarum* infection (Table 4.3). After comparison with the negative control of 0.22 and the positive control of 1.55, the three serum samples ranged from 0.67 to 1.05. Figure 4.6 shows the Western-blot results with *M. gallinarum* total protein. The results show that very few proteins were immunogenic. A 50 kDa protein showed the strongest reactivity with the samples from infected chickens. The results were confirmed by Western-blot with the NIH donkey anti-*M. gallinarum* positive control serum and SPF chicken serum as negative control serum. Chicken sera 59, 139 and 292 showed very similar results (Figure 4.6 D, E and F).

Detection of chicken humoral immune responses to leucine aminopeptidase of *M.*

gallinarum

To investigate the humoral immune responses to leucine aminopeptidase of *M. gallinarum*, Western blotting was used to analyze the chicken serum 59 for the presence of anti-APN antibody. Results demonstrated that the antibody in the chicken serum 59 against APN of *M. gallinarum* was not detectable. However, the NIH donkey anti-*M. gallinarum* polyclonal sera did recognize the expressed APN-trxA recombinant protein, and the result for normal chicken serum illustrated the background (Figure 4.7).

Discussion

We cloned and characterized a leucine aminopeptidase gene of *M. gallinarum*. Our results suggest that this gene might have a role in the nutrition of *M. gallinarum*. Leucine aminopeptidase activity was found both in the ultrasonic separated membrane and cytoplasmic fractions of *M. gallinarum* cells (Chapter III). In this chapter, we expressed the product of the gene and produced antibodies to characterize the gene product location.

The gene expression in the model bacterium, *E. coli*, has been utilized widely as a common and efficient means for “purification, location and functional analysis of proteins” (Sambrook et al. 1989). However, the expression of mycoplasma genes is more challenging than expression of genes from other prokaryotes (Minion 1998). First of all, UGA is used to encode tryptophan in the genus *Mycoplasma*, but serves as a stop codon in other prokaryotes (Inamine et al. 1990; Maniloff et al. 1992). Moreover, UGA is found to be a more frequent used tryptophan codon (Minion 1998). *Escherichia coli* cells were reported to have less than 3% UGA suppressor activity such that these UGAs will result in the expression of truncated products (Lewin 1997).

The second challenge is from the low Mol%G+C, 23-40%, in mycoplasma genomes (Razin et al. 1998). In *E. coli*, the AT rich regions are considered as the RNA polymerase binding regions, which may lead to unexpected transcription initiation within the target mycoplasma gene; therefore it is very difficult to control the expression of mycoplasma genes in *E. coli* (Knudtson and Minion 1994; Notarnicola et al. 1990; Jarvill-Taylor et al. 1999).

There are two ways of getting around the UGA tryptophan codon: the use of suppressor mutants of *E. coli* or mutagenesis of the mycoplasma gene. The *trpT176* allele has been used as UGA suppressor and can transport tryptophan into the UGA codon. But this function is UGA context-dependent and might not be efficient for some codons. Moreover, the efficiency will be much lower if there are multiple UGAs. In addition to *trpT176*, Smiley and Minion (1993) used another suppressor (*prfB3* or *rrsB* (SuUGA-delta C1054)), which influenced termination events to allow the read-through of multiple UGAs. ISM612, a constructed *E. coli* strain with *trpT176* and *prfB3*, was reported in the *M. pneumoniae* genomic library screening (Minion et al. 1995). In addition to *E. coli*, *Bacillus subtilis* with *prfB* was reported also to enhance the readthrough of UGA as tryptophan (Kannan and Baseman 2000).

We tried this procedure with no success. The leucine aminopeptidase gene of *M. gallinarum* was inserted into a fusion expression vector pTYB2 (APN-pTYB2) (New England Biolabs, Inc., Beverly, MA). APN-pTYB2 was then transformed into ISM943 (kindly provided by Dr. Minion, F. C., Iowa State University). ISM943 is a similar *E. coli* strain to ISM612 except it encodes T7 DNA polymerase, which allows using a T7 promoter plasmid for expression. However, the expression failed probably because the second UGA was too close to the third UGA (Minion, personal communication).

Therefore we mutated the four TGA codons to TGGs and cloned it into the pET32b(+) expression vector. To release the effects of His-Tag on the target protein, a stop codon was introduced at the end of the APN gene and before the His-Tag of C-terminus of trixA. We got high levels of expression in AD494(DE3)pLysS, BL21(DE3) or JM109 cells but the recombinant APN-trxA was found in inclusion bodies.

The purification of inclusion bodies resulted in a very high concentration of recombinant APN-trxA protein. After attempts to solubilize the inclusion bodies and refold the protein by dialysis, the recombinant protein did not show leucine aminopeptidase activity. This suggests that the APN-trxA fusion protein derived from inclusion bodies did not completely refold to its native functional structure. However, digestion of protein APN-trxA with enterokinase released a 51 kDa protein, which compares well with the products predicted from the APN open reading frame, and a 16 kDa protein, which is associated with trixA. Increasing the concentration of enterokinase resulted a more complete digestion of APN-trxA. We also found much of the proteins were degraded during enterokinase digestion, making it difficult to purify quantities of the recombinant protein.

Previous studies showed that the recombinant protein in inclusion bodies preserved their antigenicity and immunogenicity (D'Souza et al. 1994). To obtain a reagent for detection of the endogenous expressed APN, we generated the chicken anti-APN-trxA polyclonal antisera. The antisera obtained exhibited a very strong reaction with the APN-trxA recombinant protein and recognized with the aminopeptidase of *M. gallinarum*.

Jensch and Fricke (1997) compared seven isolation methods for their ability to release cytoplasmic proteins from the periplasm. Those methods included osmotic shock (Nossal and Heppel 1966), chloroform treatment (Ames et al. 1984), MgCl₂ treatment (Cheng et al. 1970), polymyxin treatment (Kimura et al. 1992), EDTA/lysozyme spheroblasting (Joannou and Brown

1992), $MgCl_2$ /lysozyme spheroblasting, and phase partition methods. Jensch and Fricke (1997) applied these methods on alanyl aminopeptidase, a periplasmic enzyme, and leucyl aminopeptidase, a cytosolic protein that is located close to the cytoplasmic membrane in cells of *Pseudomonas aeruginosa*. They concluded the Triton X-114 phase partition was the most efficient method to prevent the contamination of the periplasm with cytosolic protein.

The results of Western-blot experiments suggested that the leucine aminopeptidase was present in both cytoplasmic and membrane ultrasonic fractions but only in the hydrophilic partition by Triton X-114 partition treatment. These results are consistent with previous enzyme activity assays of the ultrasonic fractions of *M. gallinarum* cells, which demonstrated the presence of leucine aminopeptidase activity in both membrane and cytosolic fractions (Chapter III). Our results indicated that the leucine aminopeptidase of *M. gallinarum* is a cytoplasmic protein with a weak interaction with cell membrane. The peptide degradation and amino acid scavenging by aminopeptidases are reported to be important in the nutrition of prokaryotes (Tan et al. 1993; Morales et al. 2001). Because mycoplasmas lack the major genes to synthesize most macromolecular precursors, such as amino acids, lipids, and nucleic acid precursors, the aminopeptidase activity may play a more important role in the survival of mycoplasmas in their hosts (Maniloff et al. 1992; Razin et al. 1998). Thus the cloned leucine aminopeptidase might have a nutritional role in host adaptation of *M. gallinarum*. Our results suggest that ultrasonic treatment was less effective than Triton X-114 phase partition in releasing the cytosolic protein and removing the contamination of cytosolic protein from the cytoplasmic membrane.

Mycoplasma gallinarum generally has a low pathogenic potential (Shah-Majid and Nihayah 1987; Kleven et al. 1978). The infection of chickens with this species of mycoplasma does not stimulate a strong host immune response and the antibody level was reported to be undetectable (Varley and Jordan 1978a; Varley and Jordan 1978b; Benčina et al. 1991a; Benčina

et al. 1991b; Benčina et al. 1992). This suggests that *M. gallinarum* avoids recognition by the host immune system. Abu-Zhr and Butler (1976) showed that *M. gallinarum* did not develop a close association with the epithelium cells because mycoplasma cells appeared first in the lamina propria, which is different from most other mycoplasmas (Krause 1996; Razin et al. 1998). Our Western-blot results with chicken serum samples demonstrated the presence of limited specific humoral antibodies against *M. gallinarum* proteins but not against the leucine aminopeptidase of *M. gallinarum*. Similarly polyclonal antibody produced in donkey showed only weak specificity to the APN and this suggested that APN of *M. gallinarum* is not very immunogenic or expressed at very low levels. The use of our APN specific polyclonal antibody suggested the later might be the case because only weak bands were detected in *M. gallinarum* lysates.

The aminopeptidase and carboxypeptidase activity of several human mycoplasmas, including some pathogens, has been demonstrated to inactivate the inflammatory mediator bradykinin (Shibata and Watanabe 1989). In other prokaryotes, proteases have been shown to down regulate immune responses by the degradation of phagocyte LPS receptor CD14 and TNF•• (Calkins et al. 1998; Sugawara et al. 2000). Moreover, other studies have demonstrated that bacterial proteases possess the ability to suppress important neutrophil functions, such as the oxidative burst, in response to stimulation with phorbol esters (Kadowaki et al. 1994; Nakayama et al. 1995; Yoneda et al. 1990). The role of leucine aminopeptidase of *M. gallinarum* in its interaction with host immune systems is yet to be defined.

Table 4.1 Primers used for site-directed mutagenesis and PCR reaction

Name	Oligonucleotides
Primer 1b	5'-CGATTATAATAATTTACCATTTGAATGGATTCTAAAAAAATGGC-3'
Primer 2a	5'-CTTATTCTGGAATTTGTCAACAAATGAAGG-3'
Primer 3	5'-CAACAAATGAAGGAAAATGGGGACTTTTCGAAAAAGCAGG-3'
Primer 4	5'-GTCATGAGAAAATTTGGAGAATGCCATTACATG-3'
APNFpET	5'-GATATCGGATCCGATGAATAAATTGGAATTTG-3'
APNRpET1	5'-GCCGCACTCGAGCTATTTTTCATTAATAAAGC-3'

* Note: The bold letter is denoted for site-directed mutated site. They were designed to mutate A to G.

Table 4.2 Restriction sites changes from site-directed mutagenesis

Name	Restriction sites deleted	Restriction sites generated
Primer 1b	<i>AcsI</i> , <i>ApoI</i>	<i>BstXI</i>
Primer 2a	<i>BclI</i> , <i>BsiQI</i> , <i>FbaI</i> , <i>Ksp22I</i>	<i>HincII</i> , <i>HindII</i>
Primer 3	<i>MnII</i>	<i>BsmFI</i>
Primer 4	<i>MbolI</i>	None

*Note: The restriction enzymes labeled as bold were used to screen the mutations.

Table 4.3 ELISA results for chicken serum samples

Serum samples	Titer*
NIH Donkey anti- <i>M. gallinarum</i> sera	1.55
SPF chicken serum	0.22
59	0.67
139	1.05
252	0.77

*Note: the titer was the OD_{405nm} Unit.

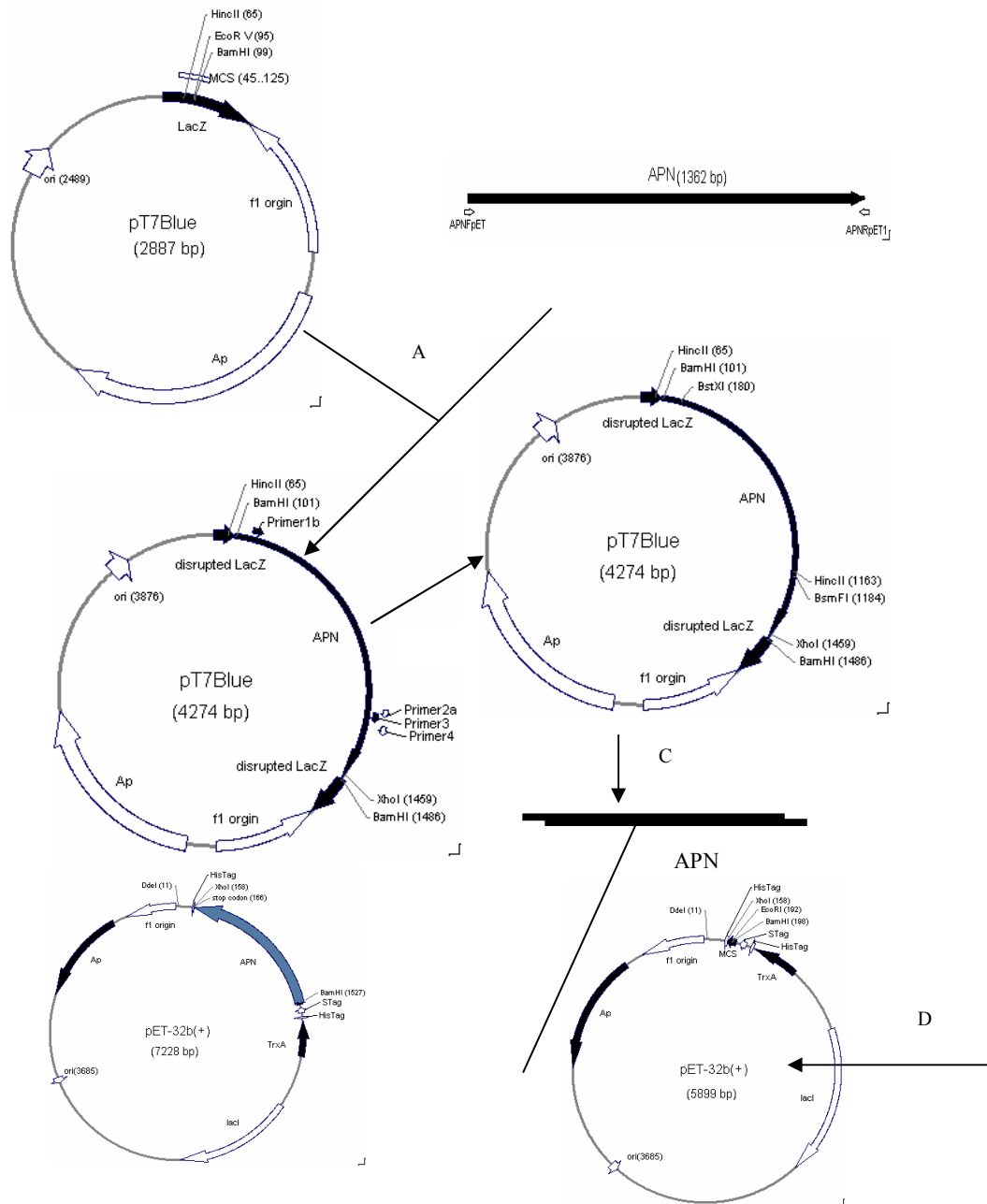


Figure 4.1 Site-directed mutagenesis and construction of expression plasmid. A. APN gene were replicated and inserted into pT7Blue plasmid to construct plasmid pT7Blue-APN; B. the first round site-directed mutagenesis with primer 1b and primer3 (plasmid pT7Blue-APN-FirstRoundMutation), and then the second round site-directed mutagenesis with primer 2 and primer 4 (plasmid pT7Blue-APN-CompleteMutation); C. The mutated APN gene was digested with *BamH* I and *Xho* I; D. The PCR products of mutated APN was inserted into pET32b(+)-APN expression vector (plasmid pET32b(+)-APN).

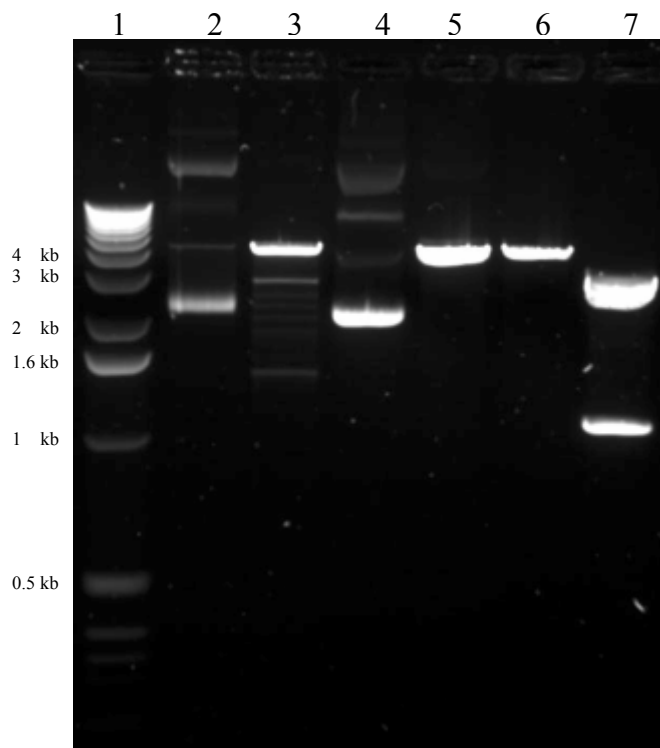


Figure 4.2 Mutants screening with restriction enzymes. Lane 1: 1 kb DNA marker; Lane2: pT7Blue-APN digested with *BsmFI*; Lane3: pT7Blue-APN-FirstRoundMutation digested with *BsmFI*; Lane4: pT7Blue-APN digested with *BstXI*; Lane5: pT7Blue-APN-FirstRoundMutation digested with *BstXI*; Lane 6: pT7Blue-APN-FirstRoundMutation digested with *HincII*; Lane 7: pT7Blue-APN-CompleteMutation digested with *HincII*.

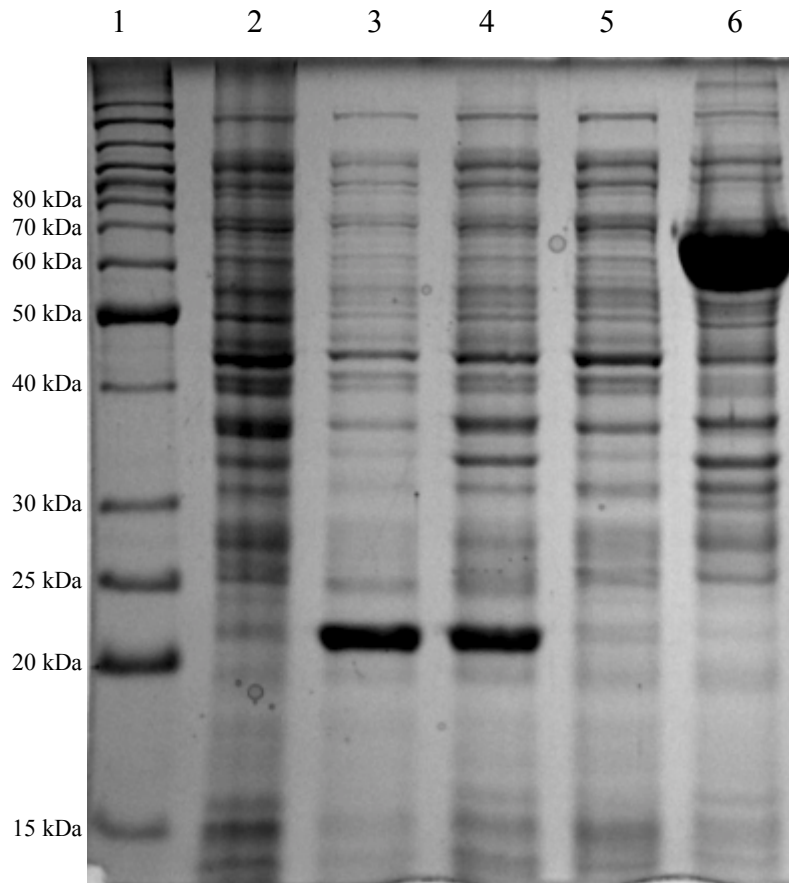


Figure 4.3 Overexpression of pET32b(+)-APN and control plasmid pET32b(+) in AD494(DE3)pLysS *E. coli* cells. The expression was induced with 3 mM IPTG and incubated at 37 °C for 3 hr. Lane 1: Protein molecular weight marker; Lane 2: AD494(DE3)pLysS cell control; Lane3: soluble fraction from pET32b(+) expression in AD494(DE3)pLysS; Lane 4: insoluble fraction from pET32b(+) expression in AD494(DE3)pLysS; Lane 5: soluble fraction from pET32b(+)-APN expression in AD494(DE3)pLysS; Lane 6: insoluble fraction from pET32b(+)-APN expression in AD494(DE3)pLysS.

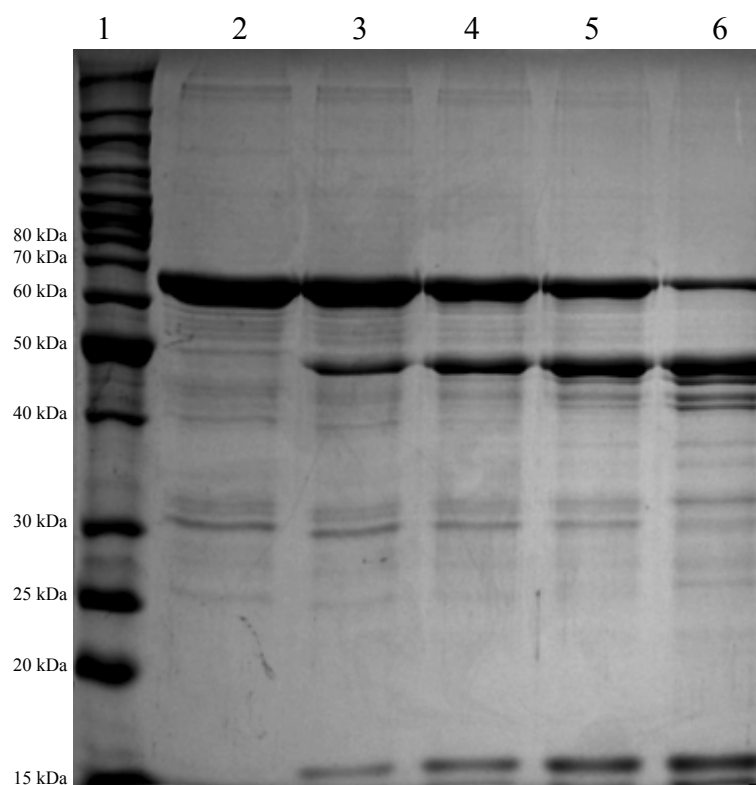


Figure 4.4 Digestion of APN-trxA fusion protein with enterokinase. The purified recombinant APN-trxA was digested with enterokinase at 22°C for 24 hrs. Lane 1: Bench marker; Lane 2: purified recombinant protein incubated with cleavage buffer; Lan3-6: 10 µg APN-trxA digested with 0.1, 0.2, 0.5 or 1 unit of enterokinase.

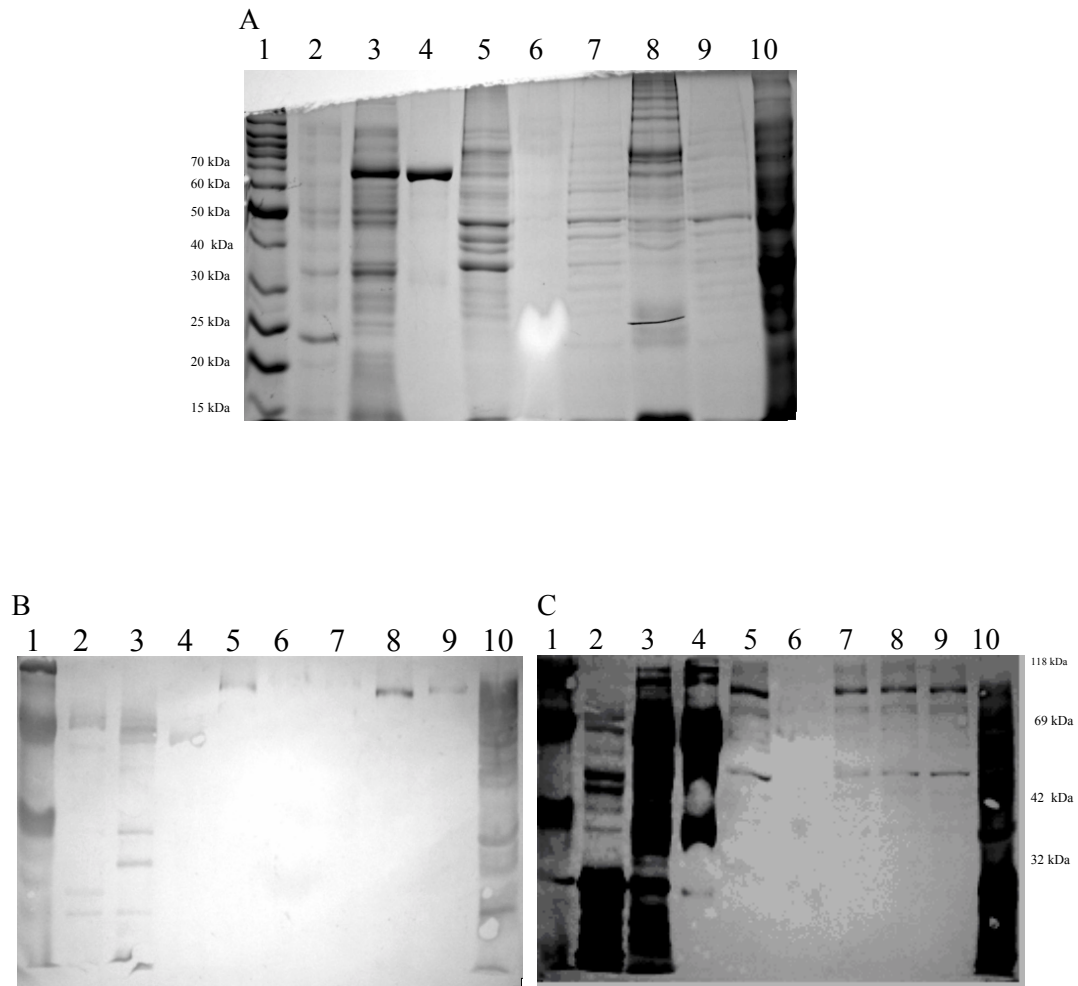


Figure 4.5 Subcellular location of leucine aminopeptidase of *M. gallinarum*. A. SDS-PAGE of the loaded protein samples; B. Western-blot results with chicken serum before challenging with APN-trxA recombinant protein; C. Western-blotting results with chicken anti-APN-trxA polyclonal serum. Lane 1: Protein molecular weight marker; Lane 2: AD494(DE3)pLysS cells with trxA recombinant proteins; Lane 3: AD494(DE3)pLysS cells with APN-trxA recombinant proteins; Lane 4: purified inclusion body (APN-trxA recombinant protein); Lane 5: *M. gallinarum* total protein; Lane 6: *M. gallinarum* Triton X-114 phase hydrophobic partition; Lane 7: *M. gallinarum* Triton X-114 phase hydrophilic partition; Lane 8: *M. gallinarum* ultrasonic membrane fraction; Lane 9: *M. gallinarum* ultrasonic cytosolic fraction; Lane 10: AD494(DE3)pLysS cell control.

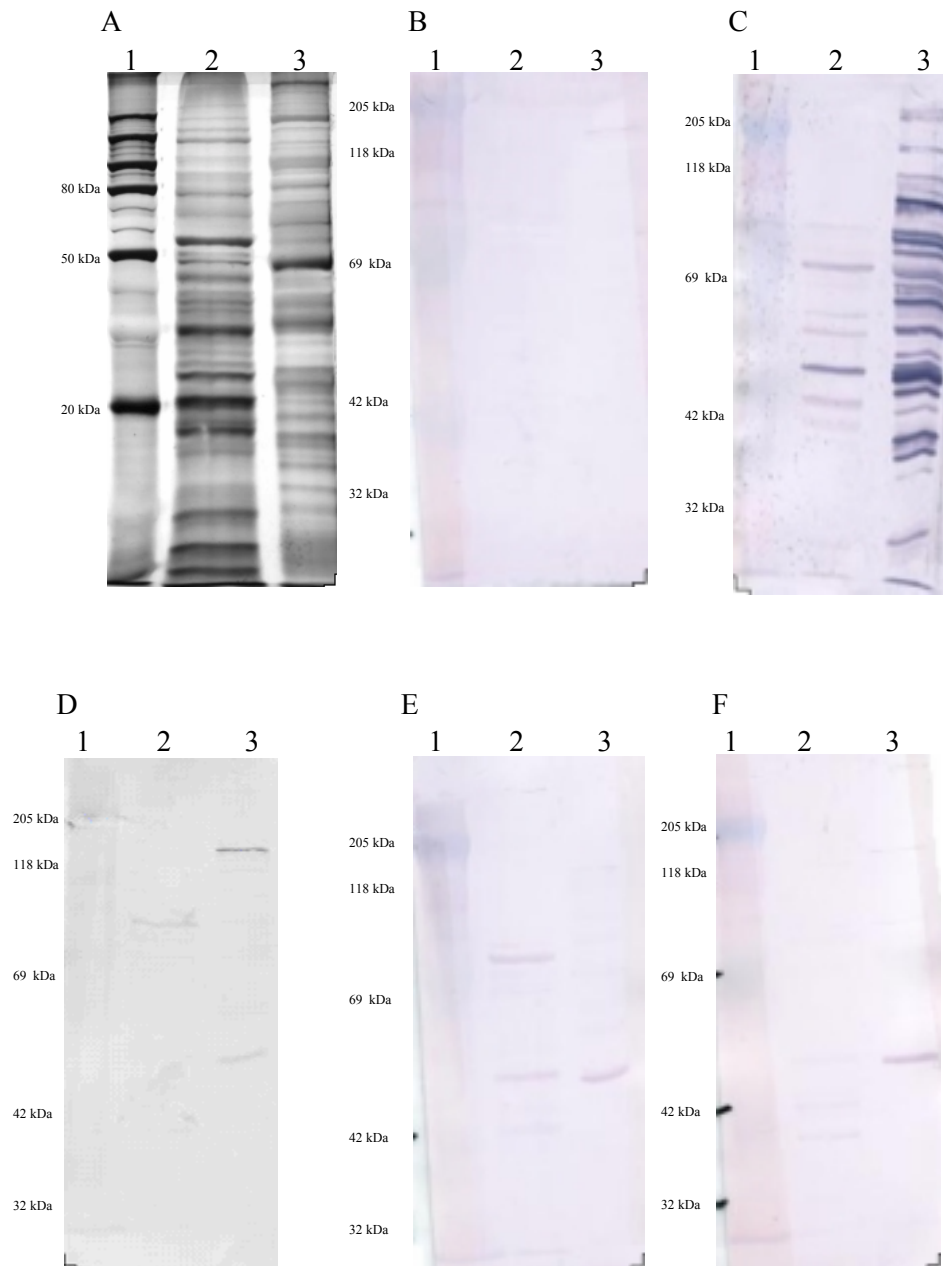


Figure 4.6 Western-blot hybridization results with chicken serum samples with *M. gallinarum* infection. A. SDS-PAGE for loaded protein samples; B. Results with SPF chicken negative control serum; C. Results with NIH Donkey anti-*M. gallinarum* polyclonal positive control serum; D. Results with chicken serum sample 59; E. Results with chicken serum sample 139; F. Results with chicken serum sample 292. Lane 1: protein molecular weight marker; Lane2. *M. gallisepticum* F strain total protein; Lane 3. *M. gallinarum* total protein.

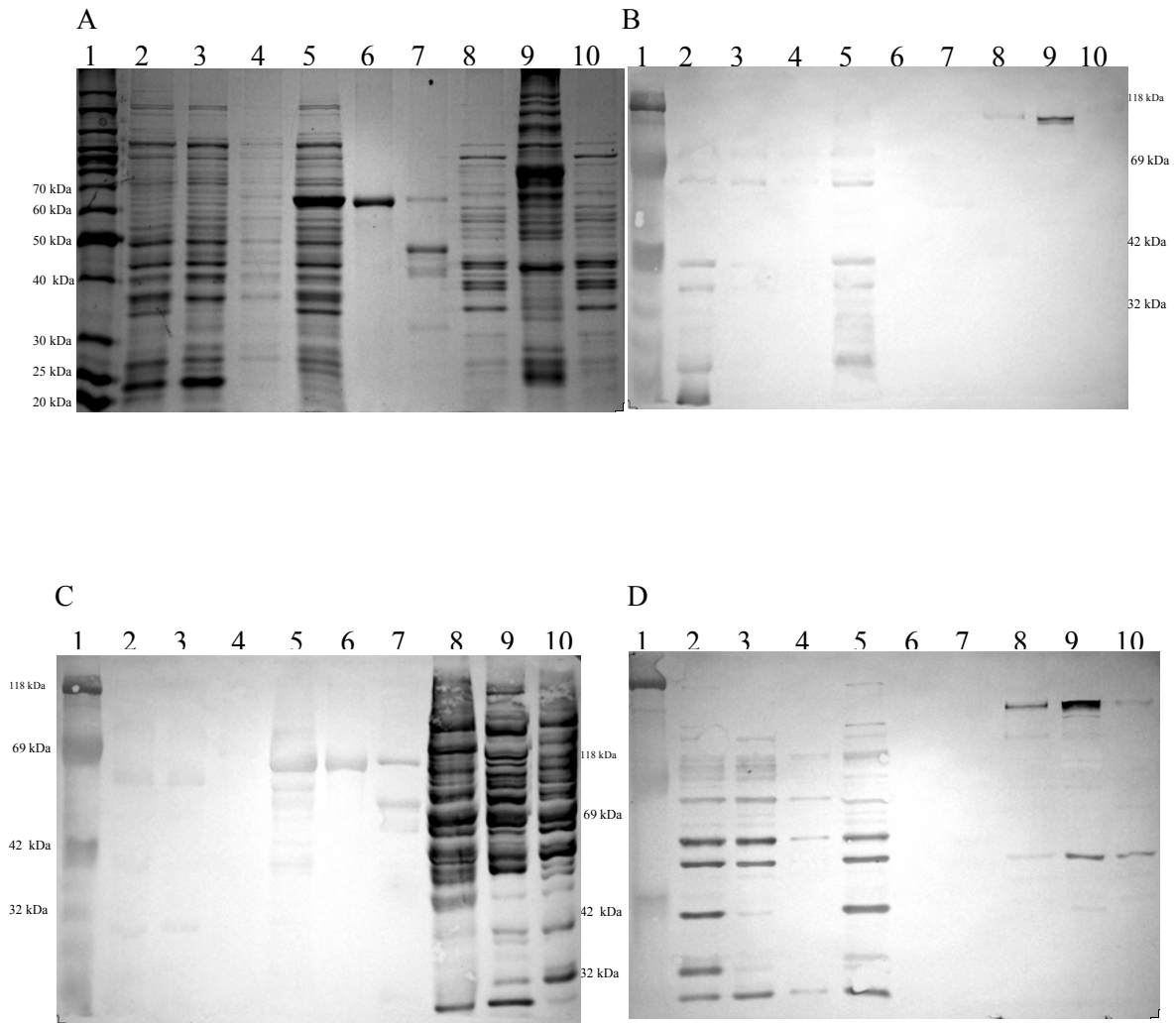


Figure 4.7 Western-blot hybridization results with chicken serum. A. SDS-PAGE for loaded protein samples; B. Results with SPF chicken negative control serum; C. Results with NIH Donkey anti-*M. gallinarum* positive control serum; D. Results with chicken serum sample 59. Lane 1: Protein molecular weight marker; Lane 2: AD494(DE3)pLysS insoluble proteins; Lane 3: AD494(DE3)pLysS soluble proteins; Lane 4: soluble protein of AD494(DE3)pLysS with APN-pET32b(+) after induction with 3 mM IPTG at 37 °C for 3 hr; Lane 5: insoluble protein of AD494(DE3)pLysS with APN-pET32b(+) after induction with 3 mM IPTG at 37 °C for 3 hr ; Lane 6: purified APN-trxA inclusion body; Lane 7: purified APN-trxA digested with enterokinase; Lane 8: *M. gallinarum* total protein; Lane 9: *M. gallinarum* membrane protein prepared by sonication; Lane 10: *M. gallinarum* cytoplasmic protein prepared by sonication.

CHAPTER V

CONCLUSION

Summary of results

A leucine aminopeptidase gene from *M. gallinarum* was cloned, sequenced, and characterized. The leucine aminopeptidase gene reported in this paper is the first protein coding gene cloned and characterized in *M. gallinarum*. This represents the second leucine aminopeptidase gene characterized in mycoplasmas after leucine aminopeptidase gene from *M. salivarium* (Shibata et al. 1995).

The cloned leucine aminopeptidase gene has a closer phylogenetic relationship to the leucine aminopeptidase of *M. salivarium* than do other mycoplasma aminopeptidase genes. The cloned leucine aminopeptidase gene conserved the enzyme active sites reported previously (Burley et al. 1990; Kim and Lipscomb 1993; Strater and Lipscomb 1995a; Strater and Lipscomb 1995b). An enzyme activity assay confirmed the presence of leucine aminopeptidase activity in *M. gallinarum*. The enzyme activity was enhanced effectively with Mn^{2+} and inhibited by EDTA. This suggests that the enzyme is a metallo-aminopeptidase. Subcellular location of leucine aminopeptidase with ultrasonic treatment indicated this enzyme is a cytosolic protein with a weak interaction with cell membrane of *M. gallinarum* cells. Thus this enzyme belongs to the family of leucine aminopeptidase (EC 3.4.11.1). The cloned leucine aminopeptidase gene has a 1,562 bp open reading frame, which is transcribed as a monocistronic message.

To further analyze the functions of the leucine aminopeptidase of *M. gallinarum*, the

encoded four TGA codons were successfully mutated into TGG to facilitate the overexpression of the cloned gene in *E. coli*. A very high level of expression was obtained after induction with IPTG, and the recombinant proteins were purified for production of antisera and investigation of host humoral immune responses against this protein in natural infection. Western-blot results showed that *M. gallinarum* stimulated the chicken to generate very limited specific antibodies against some *M. gallinarum* proteins but not against the leucine aminopeptidase of *M. gallinarum*.

The recombinant protein was used to immunize in chickens to generate polyclonal sera, which recognized a 51 kDa protein in *M. gallinarum*. Using the anti-sera, Triton X-114 phase partition confirmed the subcellular location of leucine aminopeptidase of *M. gallinarum*. *Mycoplasma gallinarum* is a non-host specific mycoplasma and its growth requires most of the standard amino acids. This suggests that the activity of leucine aminopeptidase may play a role in the nutritional supply during host adaptation of *M. gallinarum*.

Future work

Future work needs to evaluate the substrate specificity of the cloned aminopeptidase of *M. gallinarum*. An appropriate protein refolding method should be explored. More work is also needed to evaluate the role of leucine aminopeptidase *in vivo* and *in vitro* in host adaptation of *M. gallinarum*. If the aminopeptidase has a role in nutrition, it should be possible to produce an aminopeptidase activity negative mutant that can grow in amino acid supplying media. Based on our data, we can use a transposon to generate a leucine aminopeptidase activity negative *M. gallinarum* strain. We can compare the effects of this enzyme on the survival of *M. gallinarum* during amino acid starvation. In addition, understanding the regulation of gene expression of leucine aminopeptidase of *M. gallinarum* *in vitro* and in the host would also be another part of future work.

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APPENDIX

MEDIA AND BUFFER

Culturing media

<u>LB Medium (Luria-Bertani Medium) (pH7.0)</u>		<u>2XYT Medium (pH 7.0)</u>	
Bacto-tryptone	10 g	Bacto-tryptone	16 g
Bacto-yeast extract	5 g	Bacto-yeast extract	10 g
NaCl	10 g	NaCl	5 g
H ₂ O	1 litter	H ₂ O	1 litter
For plates add 12 g agar		For plates add 12 g agar	

Mycoplasma media (pH 7.7)

Part A

Frey Brot	22.5 g
Dextrose	3 g
H ₂ O	500 ml

After autoclaving Part A, mixed with Part B

Part B

Porcine serum	120 ml
Penicillin	0.64 g
Q.S. to 500 ml with sterile H ₂ O	

Southern-blot and Northern-blot hybridization buffer

Denature Buffer

NaOH	20 g
NaCl	87.5 g
Q.S. to 1 liter with H ₂ O	

Neutralization Buffer

Tris HCl	60.55 g
NaCl	175.5 g
Ajust pH to 7.4 with HCl and Q.S. to 1 liter with H ₂ O	

SDS-PAGE and Western-blot hybridization bufferRunning Buffer

Tris Base	3.03 g
Glycine	14.4 g
SDS	1.0 g
Q.S. to 1 liter with H ₂ O	

1.5M Tris (pH 8.8)

Tris Base	15.39 g
Tris HCl	3.69 g
Q.S. to 100 ml with H ₂ O	

Sample Prep Buffer

H ₂ O	4.8 ml
0.5 M Tris, pH 6.8	1.2 ml
Glycerol	1.0 ml
10% SDS	2.0 ml
0.1% Bromphenol Blue	0.5 ml

PBS

NaCl	8.0 g
KCl	0.2 g
Na ₂ HPO ₄	1.44 g
KH ₂ PO ₄	0.24 g
Q. S. to 1 liter with H ₂ O	

Transfer Buffer

Tris Base	3.03 g
Glycine	14.4 g
Methanol	200 ml
Q. S. to 1 liter with H ₂ O	

1.0 M Tris (pH 6.8)

Tris Base	0.8 g
Tris HCl	14.6 g
Q.S. to 100 ml with H ₂ O	

TBS (25 mM)

NaCl	8.0 g
KCl	0.2 g
Tris Base	3.3 g
Tris HCl	0.48 g
Q. S. to 1 liter with H ₂ O	

Distain solution

Ethenol	2.5%
Galactic acid	7.5%
dH ₂ O	90%