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Expression and Subcellular Location of a Leucine Aminopeptidase of Mycoplasma Gallinarum

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Abstract: Mycoplasma Gallinarum is a commensal with a host range that includes most poultry. This property of M. gallinarum may reflect unique mechanisms for colonization and persistence in various hosts. In previous studies a leucine aminopeptidase (LAP) gene candidate was cloned from a M. gallinarum recombinant genomic library and characterized. Here we evaluate the LAP gene from M. gallinarum at the protein level. A recombinant fusion protein was purified and employed as an antigen to immunize chickens to obtain polyclonal anti-LAP serum. The antiserum was utilized to identify the subcellular location by immuoblotting with Triton X-114 partitions of M. gallinarum proteins. Our results of these experiments show that the LAP gene product is located in the cytoplasmic fraction of the M. gallinarum cell.

Key words: Mycoplasma gallinarum, commensal, leucine aminopeptidase, subcellular location

Introduction

Aminopeptidases (APNs) are a group of enzymes with various functions. These enzymes are distributed widely among bacteria, and they can be expressed as membrane or cytosolic proteins, or they can be secreted from the cell (Gonzales and Robert-Baudouy, 1996). The basic function of bacterial APNs is to digest amino acids at the N-terminus of peptides derived from the extracellular environment or intracellular peptides (Gonzales and Robert-Baudouy, 1996). This degradation process may be accomplished by digestion with several different APNs (Miller and Green, 1983), which may be important during bacterial starvation (Reeve *et al*., 1984). Mycoplasmas are a class of prokaryotes that lack a cell wall and many of the enzymes for the major biosynthetic pathways for amino acids, fatty acids, and nucleic acid precursors (Himmelreich *et al*., 1997). However, mycoplasmas encode genes for proteases, nucleases and transport proteins (Razin *et al*., 1998). APN activity has been detected in the cytoplasmic extracts of a number of mycoplasma species (Vinther and Black, 1974; Neill and Ball, 1980; Ball *et al*., 1985). Shibata and Watanabe (1989) found that incubation of bradykinin with the cells of several mycoplasma species inactivated the vascular permeability activity of bradykinin, with the release of arginine from the mixtures. These results were confirmed and extended using the APN and carboxypeptidase enzymes isolated from one of the mycoplasma species, M. salivarium.

Mycoplasma gallinarum is one of the most frequently isolated mycoplasmas from poultry (Shimizu *et al*., 1979; Bencina *et al*., 1987; Poveda *et al*., 1990) and is generally considered a commensal (Yoder 1991; Taylor-Robinson and Cherry, 1972). Mycoplasma gallinarum is a non-fermentative and arginine-utilizing mycoplasma (Aluotto *et al*., 1970), with a requirement for at least 13 amino acids (Fischer *et al*., 1992). As with other non-fermentative mycoplasmas, arginine can be utilized as an energy source via the arginine dihydrolase pathway (Taylor *et al*., 1994). Mycoplasma gallinarum shows a strong arginine APN activity and a relatively low leucine APN activity (Ball *et al*., 1985). The activities of APNs have an important role in supplying required nutrients, and may play a role in the host adaptation of M. gallinarum to poultry. Our long-term goal is to identify the various proteases of M. gallinarum, and to understand the role of the protease system of this mycoplasma in host adaptation. In previous studies to identify and characterize M. gallinarum proteases, we found leucine APN (LAP) activity in cytoplasmic extracts of M. gallinarum and only weak activity associated with the membrane extracts. In addition, the gene that may be responsible for this enzyme activity was cloned and sequenced. The predicted amino acid sequence deduced from the open reading frame of the putative LAP gene showed 51% homology with the LAP protein of M. salivarium (Wan *et al*., in press). In this paper, we demonstrate M. gallinarum LAP gene expression at the protein level and the subcellular location of the gene product.

Materials and Methods

Mycoplasma: The M. gallinarum and M. gallisepticum F-strain used in these studies were kindly provided by Dr. S. H. Kleven (University of Georgia) and were propagated in Frey's medium supplemented with 12% swine serum (Frey *et al*., 1968) at 37 ^{0}C in anaerobic chambers.

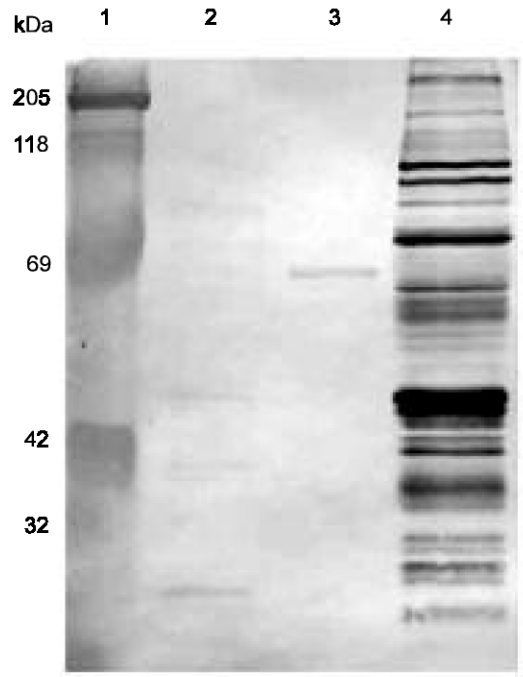

Fig. 1: Western blot with the rabbit anti-M. gallinarum antiserum.
1: Molecular mass standard.
2: trxA protein expressed from the pET32b(+) vector.
3: LAP-trxA fusion protein.
4: M. gallinarum whole cell lysate.

Mycoplasma whole cell lysates: Mycoplasma cells were collected from 24 h cultures by centrifugation, washed twice in PBS and incubated in lysis buffer at 37 °C for 30 minutes as described (Avakian and Kleven, 1990).

Phase partition of mycoplasma proteins with Triton X-114: The phase partition protocol was adapted from Wise *et al.* (1995). Briefly, cells from 5 ml cultures of M. gallinarum and M. gallisepticum F-strain (control mycoplasma) were harvested by centrifugation at 3,000 x g for 10 minutes, and then washed 3 times in phosphate buffered saline (PBS). The pellets then resuspended in 900 ul of PBS containing 100 mM phenylmethylsulfonyl fluoride (PBS-PMSF), to which 10% Triton X-114 was added to a final concentration of 1%. The mixtures were incubated on ice for 2.5 hours, and then centrifuged at 12,000 x g at 4 °C for 5 minutes to remove the unsolublized cells. The supernatants were collected and a sample was removed to represent the total protein fraction. The supernatant was then incubated at 37 °C for 5 minutes, and then centrifuged at 8,000 x g for 3 minutes to separate the detergent and aqueous phases. The detergent phase was partitioned 3 additional times as described above with 9 volumes of

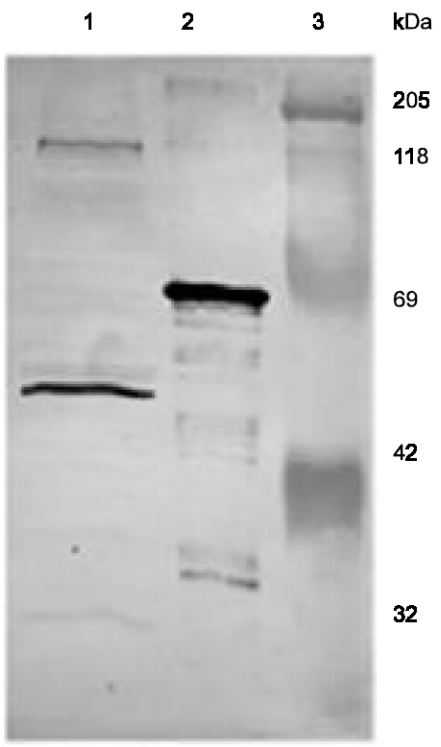

Fig. 2a: Western blot with the chicken anti - LAP-trxA antiserum.
1: M. gallinarum whole cell lysate.
2: LAP-trxA fusion protein.
3: Molecular mass standard.

PBS-PMSF. The aqueous phase was also partitioned 3 additional times with Triton X-114. The proteins were then collected by precipitation with trichloroacetic acid for analysis by Western blotting as described below.

Expression of recombinant M. gallinarum LAP protein in *E. coli*: The plasmid pET32b(+)-LAP contains the open reading frame of the M. gallinarum LAP gene (Wan *et al.*, in press). Plasmid pET32b(+)-LAP was introduced into *E. coli* strain AD494(DE3)pLysS cells by electroporation. Transformed cells were then incubated at 37 °C for 2 hrs in the presence of 3 mM IPTG to induce expression of recombinant protein. The LAP gene is expressed as a trxA fusion protein, termed LAP-trxA, to facilitate isolation of the recombinant protein from *E. coli* cell lysates. The 67 kDa LAP-trxA fusion protein expressed in inclusion bodies was purified with the His-Bind kit according to the pET system manual (Novagen, Madison, USA) and then employed as the antigen for immunization of chickens (see primary antibodies, below).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE): Protein samples were

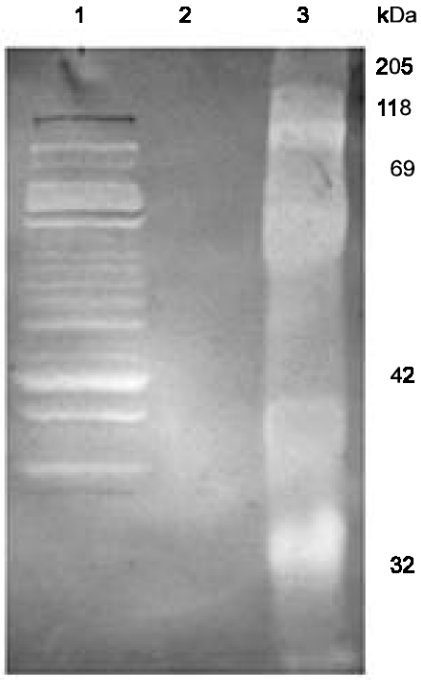

Fig. 2b: Western blot with the chicken pre-immune serum.
1: M. gallinarum whole cell lysate.
2: LAP-trxA fusion protein.
3: Molecular mass standard.

mixed with sample buffer (1:1) containing 5% 2-mercaptoethanol and heated at 95 °C for 5 minutes. Samples were then resolved on 0.7 cm thick 10% SDS-PAGE gels at 100 volts for 2 hr using the Laemmli buffer system (Laemmli, 1970).

Western blotting: Proteins were transferred to nitrocellulose membranes in transfer buffer (25 mM Tris, 192 mM glycine, 20% V/V methanol, pH 8.3) using a Mini Trans-Blot cell (Bio-Rad, Hercules, CA) at 100 volts for 1 hour (Towbin *et al.*, 1979). Proteins were then detected with various antibodies (see below) by a modification of the procedure described by Avakian and Kleven (1990). Membranes were blocked overnight at 4 °C in Tris buffered saline (TBS) containing 5% horse serum and 1% BSA. Membranes were then washed 5 times with TBS containing 0.05% Tween-20 and then incubated with primary antibodies diluted in TBS containing 5% horse serum and 1% swine serum for 1 hour at room temperature. Membranes were then washed 5 times and incubated with the appropriate secondary antibody diluted in TBS containing 5% horse serum, 1% swine serum, and 1% goat serum for 1 hour at room temperature. Membranes were then washed as above and developed with Sigma Fast NBT/BCIP (Sigma Chemical Co., St. Louis, MO) in water for 5 minutes. The molecular mass of proteins was estimated with the

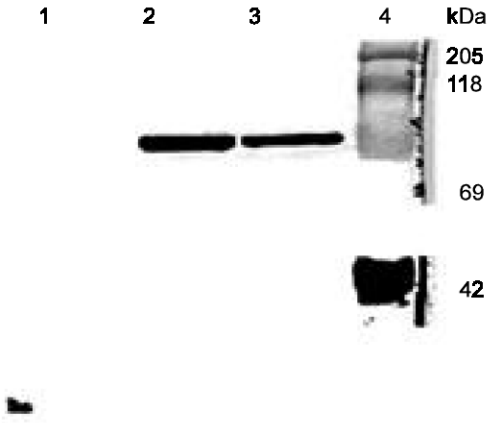

Fig. 3a: Western blot of M. gallinarum proteins fractionated with Triton X-114 and analyzed with the chicken anti - LAP-trxA antiserum.
1: Aqueous phase.
2: Detergent phase.
3: Total protein.
4: Molecular mass standard.

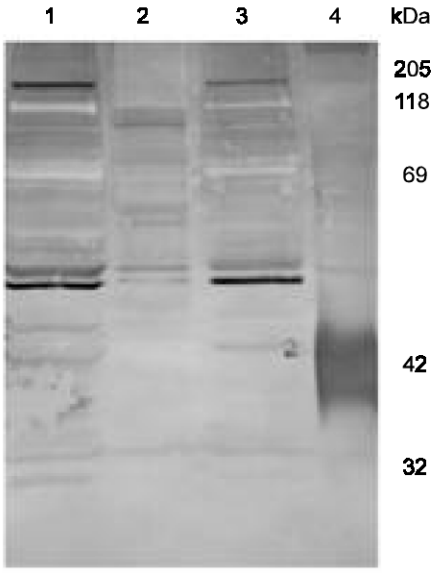

Fig. 3b: Western blot of M. gallisepticum F-strain proteins fractionated with Triton X-114 and analyzed with monoclinal antibody 6F10.
1: Aqueous phase.
2: Detergent phase.
3: Total protein.
4: Molecular mass standard.

Kaleidoscope prestained standards (Bio-Rad, Hercules, CA). Western blotting experiments were done at least twice.

Primary antibodies: The following antibodies were used in Western blotting experiments as detailed below.

Monoclonal antibody 6F10 is specific for a M. gallisepticum F-strain surface protein (Garcia and Kleven, 1994) (1/100 dilution). A rabbit antiserum raised against whole cell lysates of M. gallinarum (May *et al.*, 1988) (1/100 dilution). The primary antiserum specific for the LAP-trxA fusion protein was raised in mycoplasma-free hens. Briefly, two 4-week old white leghorn hens were bled from the wing vein and then immunized intramuscularly with 75ug recombinant LAP-trxA protein in Freund's complete adjuvant (1:1). The animals were then boosted by intramuscular injection of 280 ug recombinant LAP-trxA protein 2 weeks later. The chicken sera were collected one week after the second immunization. The anti - LAP serum was absorbed with an acetone powder of E. coli strain AD494(DE3)pLysS cell extracts to remove antibodies specific for the trxA vector-encoded protein (Harlow and Lane, 1988). The anti - LAP serum was used at a 1/100 dilution.

Secondary antibodies: The primary rabbit antibody was detected with a 1/100,000 dilution of goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma Chemical Co., St. Louis, MO); the mouse monoclinal 6F10 was detected with a 1/500 dilution of goat anti-mouse Ig alkaline phosphatase conjugate (Southern Biotechnology Associates, Birmingham, AL); and the chicken anti - LAP antibodies were detected with a 1/10,000 dilution of rabbit anti-chicken IgG alkaline phosphatase conjugate (Sigma Chemical Co., St. Louis, MO).

Results and Discussion

Expression of the M. gallinarum LAP gene: To determine whether the LAP gene is expressed as a protein, the LAP gene was transformed into *E. coli.* The LAP-trxA fusion protein produced was purified from *E. coli* cell lysates and identified by Western blotting using a rabbit antiserum raised against M. gallinarum whole-cell lysates. The rabbit antiserum not only recognized whole cell lysate proteins from M. gallinarum as expected (Fig. 1, lane 4), but also displayed weak reactivity to the 67 kDa LAP-trxA fusion protein (Fig. 1, lane 3), suggesting that the LAP gene is expressed in M. gallinarum.

To confirm LAP gene expression by M. gallinarum, the LAP-trxA fusion protein was then used to generate a polyclonal antiserum for Western blot analysis. The anti-LAP serum recognized the 67 kDa fusion protein as expected (Fig. 2a, Lane 2), and also recognized a protein in the 50 kDa range from the M. gallinarum whole cell lysate (Fig. 2a, Lane 1). The anti-LAP serum recognized an additional protein at the 118 kDa range (Fig. 2a, Lane 1), but this protein was also recognized by pre-immune sera (Fig. 2b, Lane 1).

The only other mycoplasma APN protein characterized to date is aminopeptidase My isolated from M. salivarium, which shows APN activity for both leucine and arginine substrates (Shibata and Watanabe, 1987). The native form of aminopeptidase My has a molecular mass of 397 kDa as estimated with gel filtration techniques, which then gives two subunits of 46 and 50 kDa with SDS-PAGE analysis (Shibata and Watanabe, 1987). The gene encoding the 46 kDa subunit of aminopeptidase My shows homology to other prokaryotic LAP, and therefore may encode the leucine APN activity of the enzyme (Shibata *et al.*, 1995). Mycoplasma gallinarum is similar to M. salivarium showing both leucine and arginine APN activity, and the 50 kDa product recognized by our antiserum may represent a subunit of a multi-specific APN. Additional experiments will be required to address that possibility.

Subcellular location of the M. gallinarum LAP protein: Aminopeptidase My is a membrane expressed enzyme (Shibata and Watanabe, 1987), and may function similarly to the surface expressed APNs of other prokaryotes (Blanc *et al.*, 1993). To determine the subcellular location of the LAP gene product, M. gallinarum proteins were fractionated with the detergent Triton X-114 (Wise *et al.*, 1995). The aqueous phase would contain hydrophilic cytosolic proteins, whereas the detergent phase would contain hydrophobic (membrane) proteins. The results of Western blot analysis with mycoplasma proteins fractionated using Triton X-144 is shown in Fig. 3. Mycoplasma gallisepticum F-strain expresses a 70 kDa membrane protein recognized by monoclinal antibody 6F10, and was used as a control for the procedure (Garcia and Kleven, 1994; Brown *et al.*, 1997). The 70 kDa protein recognized by monoclonal antibody 6F10 was present in the detergent phase and total protein fraction, but undetectable in the aqueous phase (Fig. 3a). In fractionation of M. gallinarum proteins, the LAP gene product was present in both the cytoplasmic phase and total protein fraction, with only minor amounts in the detergent phase (Fig. 3b).

We conclude that the M. gallinarum LAP gene product is a cytosolic enzyme, which is consistent with the predicted amino acid sequence analysis of LAP gene showing a lack of strong hydrophobic regions (Wan *et al.* in press). Given that mycoplasma APNs can be associated with the cell surface (Shibata and Watanabe, 1987) and expressed the cytosol (this report), it is likely that mycoplasmas possess a system of APNs similar to other prokaryotes, which would be responsible in providing amino acids for the cell (Mathew *et al.*, 2000). Therefore, identifying the specificity and mechanisms of peptide transport associated with the APN identified in M. gallinarum will be important for evaluating its role in host adaptation of this mycoplasma to poultry.

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