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Identification and Initial Characterization of a Putative *Mycoplasma gallinarum* Leucine Aminopeptidase Gene

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Abstract. Aminopeptidases (APN) may play a role in host colonization of *M. gallinarum.* Characterization of endogenous APN activity suggests that the leucine APN (LAP) of *M. gallinarum* is a metallo-aminopeptidase activated by Mn^{2+} and is present in the cytosol and possibly associated with the inner leaflet of the membrane. A 1.36-kb open reading frame (ORF) identified from overlapping genomic phage clones showed 68% nucleotide identity and 51% amino acid identity with the *M. salivarium* LAP gene. This ORF is expressed as a 1.5-kb monocistronic transcript and is present as a single copy in *M. gallinarum.* This gene sequence was modified to account for codon usage, and expression in *E. coli* produced a 51-kDa protein, which compares well with the product predicted from the ORF. This ORF is a strong candidate for contributing the LAP activity of *M. gallinarum* protein extracts.

Mycoplasma gallinarum has been identified as a commensal in a wide range of vertebrate hosts, including poultry, cattle, pigs, and sheep [27, 32, 33, 36]. This species was reported as one of the most frequently isolated mycoplasmas from poultry [3]. Generally, the colonization of the respiratory tract of poultry by *M. gallinarum* does not result in pathology or disease. However, *M. gallinarum* was reported to induce a temporary airsacculitis when it infected chickens by aerosol or air-sac inoculation with the combination of a field strain of infectious bronchitis virus (IBV) or vaccination for IBV and Newcastle disease [13]. *Mycoplasma gallinarum* does not induce a strong antibody response, and the humoral IgG and IgM levels are almost undetectable [2, 4].

Our long-term goal is to understand how mycoplasmas persist in various animal hosts. Mycoplasmas lack the major genes to synthesize most macromolecular precursors, such as amino acids, lipids, and nucleic acid precursors [14, 21]. Therefore, we hypothesize that various mycoplasmal proteases, which are important in nutrition of the mycoplasma cell, may play a role in adaptation of mycoplasmas to different host microenvironments. In particular, peptide degradation and amino acid scavenging by APN are reported to be important in the nutrition of prokaryotes [16, 31].

The *M. gallinarum* species provides an excellent model to study the proposed role of APN in host adaptation, as this mycoplasma is found different animal species. Previous studies have indicated that *M. gallinarum* possesses LAP activity [1]. In this project, our goals were to identify and characterize the LAP gene in *M. gallinarum* and to determine the subcellular location of the gene product. This information is required for future studies to address the function of mycoplasmal LAP in host adaptation.

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Materials and Methods

Mycoplasma strain and culture conditions. The *M. gallinarum* used (provided by S.H. Kleven, University of Georgia) was filter cloned three times and then cultured as described [18].

Mycoplasma protein preparation. The method for *M. gallinarum* protein purification was adapted from the methods described previously [17, 24]. Briefly, *M. gallinarum* cells were harvested from a 24-h culture and washed twice with 0.15 M saline. The cells were then sonicated on ice and centrifuged at 1000 *g* for 10 min. The supernatants were collected and used as the total protein extract. To separate cell membrane proteins from cytosolic proteins, the total protein extract was centrifuged at 114,000 *g* at 4°C for 2 h. The supernatants were filtered through 0.2-mm pore filters to yield the cytosolic proteins. The

pellets were suspended with 0.1 M BBS and then centrifuged at 2000 *g* at 4°C for 3 min to collect the supernatants as membrane proteins [19, 20]. Protein concentrations were determined by the bicinchoninic acid assay (BCA) kit (Pierce Chemical Co., Rockford, IL).

Aminopeptidase activity assay. LAP activity was assayed with the Sigma LAP diagnostic kit according to the manual's procedure (Procedure No. 251, Sigma Diagnostics, St. Louis, MO). The substrate-degrading products were quantified by absorbance at 600 nm, and activity per mg of protein was calculated. To evaluate cofactor requirements, the enzyme-substrate mixture was adjusted with a final concentration of 1 mM Zn^{2+}, Mn^{2+}, K^{+}, or Mg^{2+} before the LAP assay. To test the effect of EDTA on APN activity, the *M. gallinarum* protein preparations were incubated with a final concentration of 1 mM EDTA at 37°C for 15 min before the LAP assay.

Genomic library construction and library screening. Genomic DNA was isolated from 24-h broth cultures of *M. gallinarum* by using the QIAGEN genomic DNA isolation kit (QIAGEN, Inc., Valencia, CA) for construction of bacteriophage λZAPII library as described by Minion and coworkers [15]. The unamplified library had a titer of 6×10^6 pfu mL^{-1}, with less than 1% non-recombinants and an average insert size of about 5 kb. The genomic library was screened with oligonucleotide T7.1.854 (5′-ACAAATGAAGGAAAATGAGG-3′) which was end-labeled with fluorescein by using the Enzyme Chemiluminescent (ECL) 3′-oligolabeling kit according to the manufacturer's protocol (Amersham, Arlington Heights, IL). Duplicate lifts containing imprints of 30,000 pfu were hybridized for 16 h at 42°C with the oligonucleotide. Thereafter, the library lifts were washed at 42°C in 1 × SSC, 0.1% SDS, and then exposed to X-ray film for various time periods.

Nucleotide sequencing and sequence analysis. The DNA sequences of the inserts from phage library clones were determined by using the Big-Dye terminator reaction (Applied Biosystems, Foster City, CA), and reaction products were analyzed on an ABI Prism 310 Genetic Analyzer (Applied Biosystems). Nucleotide sequence data were analyzed with MacDNASIS Version 3.7 software (Hitachi Software Engineering America, Ltd., San Francisco, CA). Amino acid sequence prediction was based on mycoplasma codon usage with UGA encoding tryptophan [11]. Alignments of predicted amino acid sequence were conducted with the Clustal W version 1.81 program [34].

Northern and Southern blot analysis. A PCR product covering the entire ORF1 was amplified from genomic DNA and labeled with biotin by 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. Total RNA was prepared from 24-h cultures of *M. gallinarum* and *M. gallisepticum* with Trizol (Invitrogen Co., Carlsbad, CA), and 5 μg of glyoxal-treated total RNA was electrophoresed on 1% agarose gels [23]. *Mycoplasma gallisepticum* lacks LAP activity [1] and was used as a negative control. Northern blots were hybridized with the biotin-labeled probe according to the manufacturer's instructions (Ambion, Inc.). For Southern blots, 2 μg *M. gallinarum* genomic DNA was digested with *Cla*I, *Ear*I, *Nco*I, and *Pst*I for 24 h at 37°C and evaluated on 0.8% agarose gels as described [23]. *Hind*III-digested *M. gallisepticum* genomic DNA was used as a negative control.

Identification of 3′ terminus of mRNA. Total RNA was polyadenylated with poly (A) polymerase (Ambion, Inc.) prior to reverse transcription. Total cDNA was then synthesized from polyadenylated RNA primed with an $Oligo(dT)_{17}$ primer-adapter 5′-ATAGACTCGAGTCGACATCGA-dT17-3′ [8], and SuperScript II reverse transcriptase (Invitrogen Co.). The first-round PCR was conducted with a single primer, APN-F, for 30 cycles under conditions described before [18]. The products of the first-round PCR were then subjected to an additional 30 cycles of amplification with an internal gene-specific primer 1220S (5′-TTTAGTTTTAGCAGATGGAT-3′) and the adaptor-primer 5′-ATAGACTCGAGTCGAC ATCG-3′ [22]. The products of the second-round PCR were blunt-end ligated into pT7Blue vector for nucleotide sequencing.

Construction of the expression plasmid and multiple site-directed mutagenesis and expression of APN in *E. coli*. To clone a cDNA from cellular ORF1 transcripts, total cDNA was synthesized from DNase-treated RNA with random hexamers and SuperScript II reverse transcriptase (Invitrogen Co.). The cDNA was PCR amplified with DeepVent DNA polymerase (New England Biolabs, Inc., Beverly, MA) under conditions described previously [18]. The primers used were APN-F (5′-GATATCGGATCCGATGAATAAATTGGAATTT-TG-3′) and APN-R (5′-GCCGCACTCGAGCTATTTTTCATTT-AATAAAGC-3′). The underlined regions correspond to *Bam*HI and *Xho*I restriction sites, respectively, to facilitate cloning of the PCR product into pET32b(+) vector. For site-directed mutagenesis, the cDNA from ORF1 was blunt-end ligated into the pT7Blue vector (Novagen, Madison, WI) for site-directed mutagenesis according to the manufacturer's protocol with the multi-directed mutagenesis kit (Stratagene, Co., La Jolla, CA). The first-round mutagenesis was conducted with primer 1 (5′-CGATTATAATAATTTACCATTTGAATG**G**-ATTTCTAAAAAAAATGGC-3′) and primer 3 (5′-CAACAAAT-GAAGGAAAATG**G**GGACTTTTCGAAAAAGCAGG-3′) to mutate codons TGA to TGG at nucleotide positions 1009 and 2002. The second round of site-directed mutagenesis was conducted with primer 2 (5′-CTTATTCTGGAATTTG**G**TCAACAAATGAAGG-3′) and primer 4 (5′-GTCATGAGAAAATTTG**G**AGAATGCCATTACATG-3′) to mutate codons TGA to TGG at nucleotide positions 1971 and 2047. The underlined letters within primers 1 through 4 are designed to mutate A to G. After the mutations were confirmed by nucleotide sequencing, the cDNA was digested from pT7Blue and ligated into the *Bam*HI and *Xho*I sites of pET32b(+) vector, creating plasmid pET32b(+)-LAP for expression in *E. coli*.

Expression of recombinant protein. The plasmid pET32b(+)-LAP was transformed into *E. coli* strain AD494(DE3)pLysS cells and cultured in LB media with 1, 2, or 3 mM of IPTG at 37°C for 3 h. The purification of the fusion protein, termed LAP-trxA, was conducted with the His-Bind kit according to the pET system manual (Novagen). The purified protein was quantitated with the BCA kit and then analyzed in LAP assays as described above. To estimate the molecular mass of the recombinant protein expressed from the ORF1 cDNA, 10 μg of the LAP-trxA fusion protein was incubated with various levels of enterokinase (Novagen) at 22°C for 16 h, and then the reaction products were evaluated by SDS-PAGE.

Nucleotide sequence accession number. The GenBank accession number for the 3865-bp sequence containing ORF1 and ORF2 from *M. gallinarum* is AY080895.

Results and Discussion

Total protein extracts from *M. gallinarum* showed LAP activity, which is similar to a previous report [1]. The LAP activities for the membrane and cytosolic proteins were 3.65 and 0.96 OD_{600} units mg^{-1} protein, respectively. In evaluation of metal cofactors, the LAP activity of total proteins was enhanced by Mn^{2+}, followed by

Table 1. Effects of metallic cofactors on LAP activity of *M. gallinarum* cell extracts

Metal ion	LAP activity (OD_{600nm} unit mg^a protein)^{a,b} Leucine substrate
No metal ion	0.43 ± 0.03 a[c]
K^+	0.94 ± 0.06 b
Mg^{2+}	1.95 ± 0.06 c
Mn^{2+}	2.38 ± 0.06 d
Zn^{2+}	0.39 ± 0.05 a

[a] LAP activity is calculated by dividing the OD_{600nm} value by the test protein concentration.
[b] The values in the table are the activity mean values from three experiments ± standard error.
[c] Values with different letters are significantly different ($P \leq 0.05$).

Table 2. Effects of EDTA on LAP activity of *M. gallinarum* cell extracts

	LAP activity (OD_{600nm} unit mg^a protein)^{a,b} Leucine substrate
No metal ion	0.40 ± 0.01 a[c]
EDTA	0.00 ± 0.00 b
Mn^{2+}	1.26 ± 0.09 c
EDTA + Mn^{2+}	0.65 ± 0.07 d

[a] LAP activity is calculated by dividing the OD_{600nm} value by the test protein concentration.
[b] The values in the table are the activity mean values from three experiments ± standard error.
[c] Values with different letters are significantly different ($P \leq 0.05$).

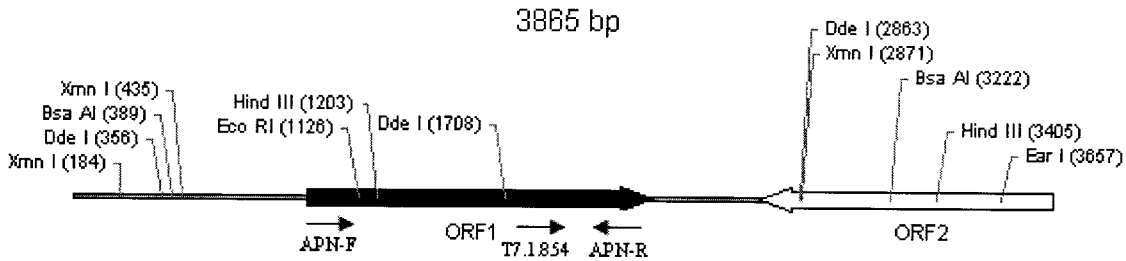

Fig. 1. Restriction map of 3865-bp fragment of genomic DNA deduced from overlapping phage clone inserts. Shaded box: ORF1 with the direction of transcription. Open box: ORF2 with the direction of transcription. Parentheses, the position of the restriction endonuclease sites in bp; small arrows indicate the position of oligonucleotide T7.1.854 used in genomic library screening, and oligonucleotides APN-F and APN-R used to PCR amplify ORF1.

Mg^{2+} and K^+ (Table 1). Zn^{2+} did not enhance LAP activity. EDTA inhibited enzyme activity, whereas Mn^{2+} not only enhanced LAP activity but also restored LAP activity inhibited by EDTA (Table 2). These results suggest that the LAP of *M. gallinarum* is a metalloaminopeptidase. The LAP activity of *M. gallinarum* protein extracts is similar to that of APN I of *E. coli*, in which activity is restored by Mn^{2+} and Mg^{2+} ions [35]. The *E. coli* APN is 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 [10, 35].

To identify the gene responsible for the LAP activity of *M. gallinarum* protein extracts, we initially obtained a short sequence during a library screen from an *M. gallinarum* genomic plasmid library under low stringency conditions with a degenerate oligonucleotide (5′-WTNGGNWTNGGNATHGGNWTNCCNATHG-3′; where W = A + T; H = A + T + C; and N = all four nucleotides used in oligonucleotide synthesis). The genomic plasmid sequence showed homology to the LAP of *M. salivarium* [26] and was subsequently used to design oligonucleotide T7.1.854 for screening of the phage genomic library. Library screening with oligonucleotide T7.1.854 identified six overlapping phage clones that were characterized by restriction endonuclease digestion. Comparison of restriction patterns of overlapping phage clones suggested that an 8-kb region of the *M. gallinarum* chromosome had been cloned. The inserts of the phage clones were completely sequenced with internal primers. A 3,865-bp sequence was obtained that contained two potential ORF (Fig. 1). Database searches indicated that the 1.36-kb 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 LAP proteins for other mycoplasmas showed amino acid identities of 51% with *M. salivarium;* 42% and 45% with *M. pulmonis* LAP1 and LAP2 respectively; 38% with *M. pneumoniae;* 36% with *M. genitalium;* and 36% with *U. urealyticum* (Fig. 2). In addition, the six amino acid residues conserved in the active site of other prokaryotic and eukaryotic LAP [5, 12, 28, 29] were conserved in the mycoplasma pro-

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Mgallinarum_LAP     --MNKL-EFLTKRDNNFLLVAVDYNNLPFEWISKKNGAISENLSKNEAYITIKEIK--NY 55
Msalivarium_LAP     --MELIKEIETSRNSSVLLKAIFEGDDAPTLLVKKQSQITEYLKENVAYVYLGKKSEFGY 58
Mpulmonis_LAP1      MKGKKVIKVSNEKEFPISLKLIFKQENMNDFLIQEDHQITEINSENTLYYFVKDKSKYDI 60
Mpulmonis_LAP2      ---MLLEKQTRERNNEMLLKAVFDSEAKIEGLIKTKNVITEFVEKNEAYVFLGKKEDFSR 57
Mpneumoniae_LAP     ------MKLN--KLF--DSKTVVFKKSDKYGKSDCCKTKCVEG-QIEFG--VKIPTDFP- 46
Mgenitalium_LAP     ------MRIN--KPFSDDSNTVVFVSSKTYG----VKEEAAHNPNVEFG--VVLPTDFP- 45
Uurealyticum_LAP    ------MTLNEKKEFVYELKAVEKNHN-LKGFEENTTEHVVFGGLYQKQQYLILTPDFDS 53
                           :       :          .                 :    .

Mgallinarum_LAP     DELVEFIKKNILNSQRAFQIDLNSFKNVLNINYEDILKAFISSIVFYQAKLFNKK--KEK 113
Msalivarium_LAP     KDAYEFARDLAENCARSYQLDLTTFVTEK-LCIKGVVDAFTKGINFSAFQYYNLKTFTKR 117
Mpulmonis_LAP1      KFFKKAASKIVENQNRNLNIDISSFSHNS-IALESIVKIFVEKYEFKHANLFKEKDKDDK 119
Mpulmonis_LAP2      EVWNSFLDSFIPSMKRSYQLDILSFETPS-YKLEDIIYDLYIKYDYIKADLFSHRKKESD 116
Mpneumoniae_LAP     -AFNRALVTFLKTQKNQLNVDLDSFVELYKAEN--LCYKTALKVVVASITFCETTPFTMK 103
Mgenitalium_LAP     -AFNRALVQFLKRKKTKLNINLDSLIELYKKNENSGCFHTAIKTVITSVTFCETTPFTMK 104
Uurealyticum_LAP    NELSRSLISFLEKSPKSVSVDLNSFLSLVPESR----HASLLNVVISALEYVEVTPFSLK 109
                                  .::: ::                          .

Mgallinarum_LAP     SKKTNFSLLFN--SDEDKEVFNKFNLIAENRNKIRNLQIMPENYCNSVELANYIVEDFSN 171
Msalivarium_LAP     VNENSLSFYLENISQDVLNVFKKALILVDAQNFARNLGVTPPNELNSEQLAEIIRKDFKK 177
Mpulmonis_LAP1      QYSINLYVSDKQKILVLETLLEKTLILTNALNTVRKLQIYPPNICNSEWLADFVYEDLKK 179
Mpulmonis_LAP2      ENKVSLTIWNN-ANRCFAEVYEKAKLISSLVNFARDLQTTPPNICNSEWMAKKVEEKAKS 175
Mpneumoniae_LAP     TEPQKNVEVAVKCDSEHTSLIKEYEVVGNYVNMARQLQDTPSDQLYPEEFVKRFEKAATG 163
Mgenitalium_LAP     TKPEKNVEVAVQCAVEYHNLVKEYETVGEYVNLARELQDTPSDLLYSEVFVKHFEKAASK 164
Uurealyticum_LAP    SKIETKNIHNLVVDQKYHDLINKLHVIAQSQTITRTLQDTPANLMTPGDFEERIKELFKD 169
                       .            : ::   : .  .  * *   * :   .  : . . :  .

Mgallinarum_LAP     LKNLKVTVLDKNMIEKLKMGLILSVNKGST---HEPRVVIVEYNGNKKSKDKIVFVG**K**GI 228
Msalivarium_LAP     YHNLKVKVLERKQIELLGMDLLLSVNKGSV---YEPRVVIIEYKGNPSSQEKTVLVG**K**GI 234
Mpulmonis_LAP1      IDSLKINIYNKSQIEDLGMNLFLSVNKGSA---YEPRLVSIEYNGDPSSEEKTVLVG**K**GI 236
Mpulmonis_LAP2      FENVEVKVLDKKAIEKHGMNLLLSVNKGSM---FEPRVVVLSYNGDSSSKERTSLIG**K**GI 232
Mpneumoniae_LAP     LG-VKITVLKQADLIKKKMGLLLGVNKGSE---REARLLVISYNNNKKSSETLALVG**K**GI 219
Mgenitalium_LAP     LP-VKIKVLKQSDLIKKKMGLLLGVNQGSE---REARLLVISYQANKNSKEKLAFVG**K**GI 220
Uurealyticum_LAP    LPEVKVSVLYRKDLEAKGMNAHVGVGKAAVSDKAQPRLVVVEYNNNPETNEKYAFVG**K**GV 229
                       :::.:  :  :    *.  :.*.:.:     :.*:: :.*: : .:.:   ::***:

Mgallinarum_LAP     TF**D**TGGINTKG-YHMSGMKY**D**MSGSAIVAYAVKTLAELKANVNVAAIMMITDNRQDGDAS 287
Msalivarium_LAP     TF**D**SGGYSLKPPKFMLGMKY**D**MSGSAIVAAVMKAIAQLKPNKNVSAIMCITDNRINGDAS 294
Mpulmonis_LAP1      TF**D**SGGYNIKT-SHMGGMKF**D**MSGAAIAAYALKAMAQLRAKSNFAALMLITDNRLNSVAN 295
Mpulmonis_LAP2      TF**D**SGGYSLKPAKSMVGMKY**D**MSGAAIVSAVVFALSALGVKRNVSAVLAITDNRVNGDAN 292
Mpneumoniae_LAP     TY**D**SGGMNIKTGDYMRGMKY**D**MSGAAIVCSTVLALAKNKVKTNVVAVAALTENLPGPHAQ 279
Mgenitalium_LAP     TY**D**SGGMNIKTGDYMRGMKY**D**MSGAAIVCSTVLALAKNKVKTNVVAVAALTENLPGAKAQ 280
Uurealyticum_LAP    CF**D**SGGYNVKTGSHMRWMKF**D**MSGSAIVSMTVRALALNKEKVNVVAVCPLVLNLLAPEGQ 289
                     :*:** . *    *  **:****:**.. .: :::    : *. *:  :. *     ..

Mgallinarum_LAP     LPENVYESMSGLSVEVTDT**D**A**E**G**R**LVLADGLYYGATKLKATTLFDVATLTGSVLTALGHT 347
Msalivarium_LAP     LPDSVYTSMSGKTVEVNNT**D**A**E**G**R**LVLADRLYYGATKLNATRLIDTATLTGTMLTALGQT 354
Mpulmonis_LAP1      IPDSVWKSMNGKTVEVTDT**D**A**E**G**R**LVLADGITFAIRKLNATRVITIATLTGTILYTLGTS 355
Mpulmonis_LAP2      LPDSVWKSMNGKSVEINNT**D**A**E**G**R**LVMVDAMTYAIRELKSTDLIDVATLTGAIVVALGNT 352
Mpneumoniae_LAP     RPDDIQTAYNGKTVEIDNT**D**A**E**G**R**LVLADAISYAAKDLKATQIIDVATLTGLMSYILSTT 339
Mgenitalium_LAP     RPDDIKIAYNGKSVEIDNT**D**A**E**G**R**LVLADAITYAAKDLAATHIIDVATLTGLMSYILSTT 340
Uurealyticum_LAP    KPDDIVKSYNGKTIELDNT**D**A**E**G**R**LILADALTYAVRDLKASKLFDVATLTGAMIYALGDT 349
                     *:.:  : .* ::*: :*******::.* : :.  .* :: ::  ***** :   *. :

Mgallinarum_LAP     YSGIWSTNEGKWGLFEKAGQISHEKIWRMPLHDDFHQFNLASEVADLNNYNNSEKSDSNT 407
Msalivarium_LAP     YSGIYATSCKIWHQFEDAAKIAHEKVWRMPLHEDFNKTNKESLVADLNNYSNNEKSDCNT 414
Mpulmonis_LAP1      YTGGWATNNKTWKELLSAAKKHDEPIWRLPLNEDYIESYKKSKVADLVNWSSASKPDSNS 415
Mpulmonis_LAP2      YTGVWSTNDSKWEKLEKASKVAGEKIWRMPIDAEFAKGIKKSSVADLRNVDYSGLGGSNS 412
Mpneumoniae_LAP     YTGIFSTCDMAWDAFKKAACCAGEPVWRLPMHPDYLKPLES-KLADLQNSTSVKGAGSSR 398
Mgenitalium_LAP     YTGIFSTCDHQWESFKKAACSAGEPVWRLPMHPDYLKPLQLTKLADLQNSTSARGAGSSR 400
Uurealyticum_LAP    YSGVWATNNDIWNEVVVAADYAGELVWRLPFHNDFLKMLNS-NVADIANSVSDPRGGSSR 408
                     *:* ::*    *  .  *.    * :**:*:. :: :      :**: *       ...

Mgallinarum_LAP     AAMFLKQFTNNVDYIHCDVAGTADVGGKPQGVLIDTLVEFALLNEK-------------- 453
Msalivarium_LAP     AAMFLKEFTNNVPYIHCDVAGTADKKGMGLGILVSTFVEFGKSQQRNCESCECDEQKCET 474
Mpulmonis_LAP1      AAMFLKEFTEEKDFVHLDIAGTAEIEGEPQGVLVKTLAQLALDI---------------- 459
Mpulmonis_LAP2      AMMFLNEFNEGVDHIHIDIAGTANVGDSCTGVMVKTLVEYLK------------------ 454
Mpneumoniae_LAP     AACFLAEFREGVPLIHCDIASTASIQDLGQGVLVRTLYERAAQQAKE------------- 445
Mgenitalium_LAP     AACFLAEFREGVSLIHCDIASTASIENLGQGVLVRTLYERASQLANK------------- 447
Uurealyticum_LAP    AACFLKEFTEGVPYAHFDIAITADVGHKGTGVMLRTFYRIAQNQKFN------------- 455
                    *  ** :* :     * *:* **.      *::: *: .       .
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Fig. 2. Alignment of the predicted amino acid sequence derived from *M. gallinarum* ORF1 with the proteins *M. salivarium* LAP (Accession No. D17450); *M. pulmonis* LAP1 (Accession No. NP_326206); *M. pulmonis* LAP2 (Accession No. NP_326557); *M. pneumoniae* LAP (Accession No. NP_110261); *M. genitalium* (Accession No. NP_073064); and *U. urealyticum* (Accession No. NP_078344). In the consensus line, "*" designates identical amino acids, and ":" designates conserved amino acids, and dashes indicate gaps introduced to maximize homology. Amino acid residues that are conserved in the active site of LAP from other prokaryotes and eukaryotes are highlighted in bold in the mycoplasma proteins.

teins and the protein predicted from ORF1: Lys 226, Asp 231, Asp 248, Asp 307, Glu 309, and Arg 311 (Fig. 2). Therefore, we conclude that ORF1 represents a strong candidate for the LAP gene of *M. gallinarum.*

A sequence representing a putative stem-loop structure is present in the 3′ end of the ORF1; this suggests a rho-independent termination of the transcript. The expected termination was confirmed at nucleotide positions 2311–2313 based on the sequencing results of cDNA clones obtained from the 3′ poly(A) tailing reaction (data not shown). This suggests that the LAP gene is expressed as a monocistronic message, which was observed as a 1.5-kb message on Northern blots (Fig. 3). To quantify the copy number of LAP genes encoded in *M. gallina-*

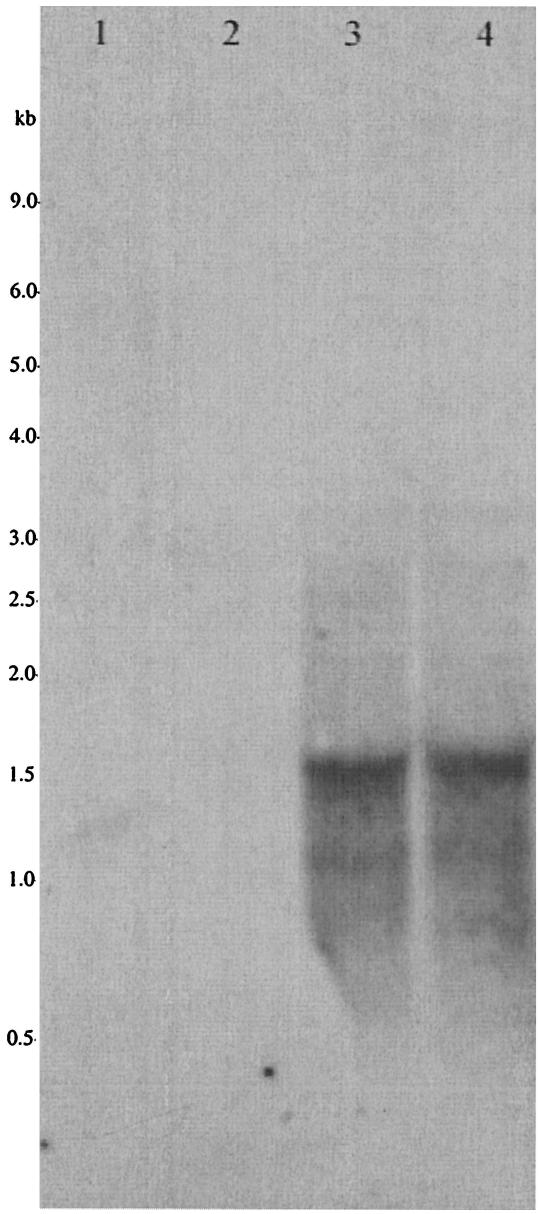

Fig. 3. Northern-blot hybridization analysis. Lanes: (1) molecular mass marker; (2) *M. gallisepticum* total RNA. Lanes (3) and (4): *M. gallinarum* total RNA. The blot was hybridized with the LAP biotin-labeled probe covering ORF1 that was amplified by PCR from *M. gallinarum* genomic DNA.

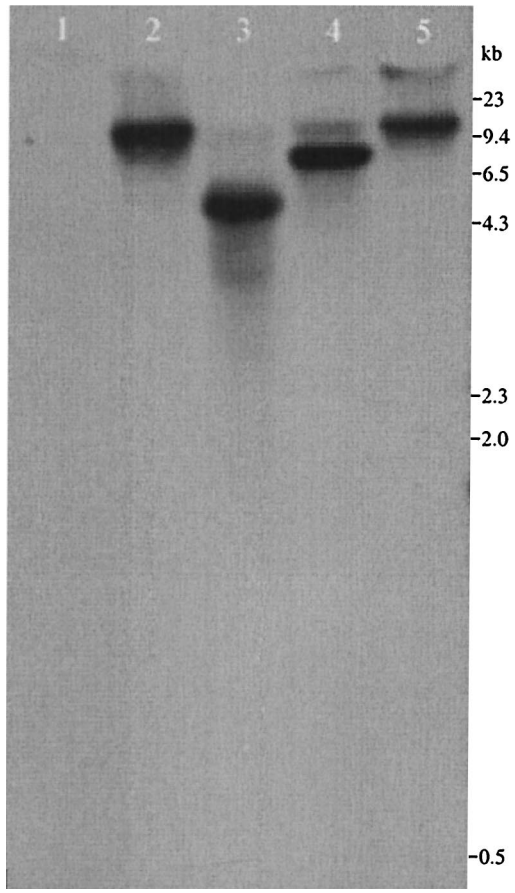

Fig. 4. Southern-blot hybridization analysis. The blot was hybridized with the LAP biotin-labeled probe covering ORF1 that was amplified by PCR from *M. gallinarum* genomic DNA. Lanes: (1) 2 μg *M. gallisepticum* F strain genomic DNA digested with *Hind*III; Lanes (2) through (5) contained 2 μg *M. gallinarum* genomic DNA digested with 10 units of *Cla*I, *Ear*I, *Nco*I, and *Pst*I, respectively.

rum, genomic DNA was digested with *Cla*I, *Ear*I, *Nco*I, and *Pst*I, and was hybridized with APN biotin-labeled probe. Hybridization to blots of *Cla*I, *Ear*I, *Nco*I, and *Pst*I digests resulted in only single bands being hybridized (Fig. 4). Therefore, the Southern blot experiments with restriction endonucleases flanking ORF1 suggest that the expressed LAP gene candidate is present as a single copy in *M. gallinarum* and may, therefore, be responsible for encoding both the cytosolic and membrane LAP enzyme activities of the cell.

To evaluate the function of the LAP gene product, we generated recombinant protein from ORF1 for evaluation in LAP assays. Within the ORF1, there are four TGA codons that are predicted to encode tryptophan in *M. gallinarum*, which is a common property of mycoplasma genes [11, 14]. To express the LAP gene candidate in *E. coli*, total cDNA prepared from *M. gallinarum* cells was amplified with ORF1-specific primers (APN-F and APN-R), and the product was ligated into a vector for site-directed mutagenesis. The TGA codons in the ORF1 cDNA were mutated to TGG for recognition as the codon for tryptophan in *E. coli*. The 67-kDa fusion protein, LAP-trxA, formed inclusion bodies at the various levels of IPTG used for induction. Digestion of LAP-trxA inclusion bodies with enterokinase released a 51-kDa protein, which compares well with the product

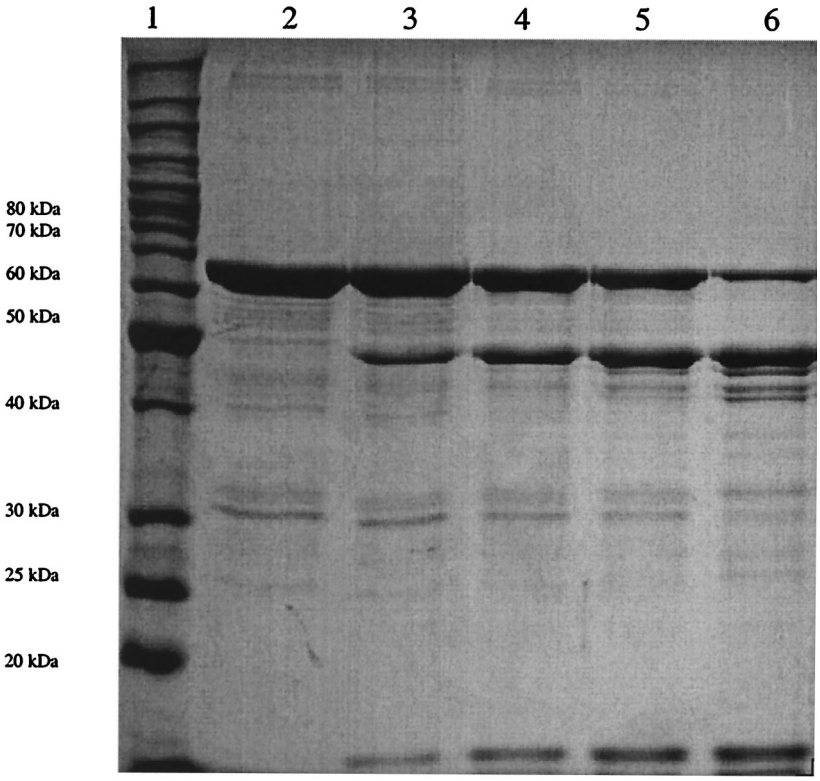

Fig. 5. Digestion of LAP-trxA fusion protein with enterokinase. The purified recombinant LAP-trxA was digested with enterokinase at 22°C for 24 h. Lanes: (1) molecular mass marker; (2) LAP-trxA incubated with cleavage buffer; Lanes (3) through (6) contained 10 μg of LAP-trxA incubated with 0.1, 0.2, 0.5, or 1 unit of enterokinase.

predicted from ORF1, and a 16 kDa protein, which is associated with trxA encoded in the pET32b(+) vector (Fig. 5). After attempts to solubilize the inclusion bodies and refold the protein by dialysis, the purified recombinant protein failed to show enzyme activity when evaluated in LAP assays (data not shown). This suggests that the recombinant protein did not adequately refold to its native functional structure. Further studies will be required to fully address this issue.

It is currently unknown how *M. gallinarum* survives in animals with little immune recognition. It is possible that a number of the proteins expressed on the cell membrane of *M. gallinarum* may be responsible for the ability of this species to escape clearance from the host immune responses of many different animals. For example, in other prokaryotes, proteases have been shown to downregulate immune responses by the degradation of TNF-α and the phagocyte LPS receptor CD14 [6, 30]. For mycoplasmas, APN activity has been detected in membrane preparations from *M. salivarium* and *M. laidlawii* with classical biochemical methods [7, 26]. The *M. salivarium* enzyme contains a strongly hydrophobic region indicative of a membrane-spanning region [26]. Membrane-associated LAP activity of *M. salivarium* has been shown to inactivate inflammatory mediators [25]. However, the predicted amino acid sequence of the *M. gallinarum* LAP ORF1 lacks an obvious transmembrane region based on hydropathy plot analysis (data not shown). Therefore, it is possible that the endogenous LAP of *M. gallinarum* may be associated with the inner leaflet of the cell membrane. The possible association of *M. gallinarum* LAP with proteins on the inner leaflet of the cell membrane is interesting and warrants further study. In this case, the LAP could be in close proximity to a membrane transport system and could function in removal of the N-terminal amino acid (leucine) from transported peptides. This would render the peptides susceptible to further degradation within the cell [10]. In this manner, the LAP candidate described in this study may have an important role in degradation of intracellular proteins for nutrition of the cell [9].

From the information obtained in this study, we plan to evaluate the role of the LAP product in survival of *M. gallinarum* in different microenvironments. These studies will require evaluating the substrate specificity of the LAP gene product and to generate antiserum specific to the product to formally validate the subcellular location of the enzyme in the cell.

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