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Cloning of *mnuA*, a Membrane Nuclease Gene of *Mycoplasma pulmonis*, and Analysis of Its Expression in *Escherichia coli*

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Membrane nucleases of mycoplasmas are believed to play important roles in growth and pathogenesis, although no clear evidence for their importance has yet been obtained. As a first step in defining the function of this unusual membrane activity, studies were undertaken to clone and analyze one of the membrane nuclease genes from *Mycoplasma pulmonis*. A novel screening strategy was used to identify a recombinant lambda phage expressing nuclease activity, and its cloned fragment was analyzed. Transposon mutagenesis was used to identify an open reading frame of 1,410 bp, which coded for nuclease activity in *Escherichia coli*. This gene coded for a 470-amino-acid polypeptide of 53,739 Da and was designated *mnuA* (for "membrane nuclease"). The MnuA protein contained a prolipoprotein signal peptidase II recognition sequence along with an extensive hydrophobic region near the amino terminus, suggesting that the protein may be lipid modified or that it is anchored in the membrane by this membrane-spanning region. Antisera raised against two MnuA peptide sequences identified an *M. pulmonis* membrane protein of approximately 42 kDa by immunoblotting, which corresponded to a trypsin-sensitive nucleolytic band of the same size. Maxicell experiments with *E. coli* confirmed that *mnuA* coded for a nuclease of unknown specificity. Hybridization studies showed that *mnuA* sequences are found in few *Mycoplasma* species, suggesting that mycoplasma membrane nucleases display significant sequence variation within the genus *Mycoplasma*.

Mycoplasmas are among the smallest free-living organisms known and are responsible for a number of respiratory and genital tract diseases in humans and animals (30, 31). The nutritional requirements of mycoplasmas (40), genomic sequence information (11) and biochemical studies (27) indicate that mycoplasmas lack most capacities for de novo synthesis of nucleotides. To compensate, they must encode enzyme activities and transport functions to facilitate the uptake of nucleic acid precursors either as free bases or as oligonucleotides (8). A membrane nuclease activity in mycoplasmas was initially reported in *Mycoplasma pulmonis*, a rodent respiratory and genital tract pathogen (19). Since this nuclease was located on the outer membrane surface of the organism, it could satisfy the need for purines and pyrimidines by the degradation of DNA or RNA in mucosal secretions. Nucleic acids could also be obtained from dead and dying cells of the respiratory tract to which the mycoplasma is attached. External membrane-associated nucleases have been identified in all *Mycoplasma* species tested, with most species appearing to produce multiple nucleolytic proteins (20). The divalent-cation requirements of these nucleases varied between species, suggesting that they may be produced by unrelated genes. Few studies of membrane-associated nucleases have been reported, although a *Mycoplasma penetrans* endonuclease has been recently purified and characterized (2). There are also two reports of mycoplasma nucleases that appear to be able to induce internucleosomal DNA degradation characteristic of apoptosis (24, 25).

To investigate this activity in *M. pulmonis* further, studies were initiated to clone and analyze one of its membrane nuclease genes. This process required the identification of nuclease activity in recombinant clones, Tn*1000* mutagenesis of the clones, and analysis of the results of that mutagenesis for loss of function. The expression of mycoplasma genes in *Escherichia coli* in a functional form poses particularly difficult problems. First, UGA codons are interpreted as tryptophan coding sequences in the genus *Mycoplasma* as opposed to stop codons in most other organisms (22). It is important to note that it is not possible to completely suppress all UGA codons in *E. coli* even in an efficient UGA suppressor background (36) because suppressor efficiency is context dependent (29). In addition, transcription and translation initiate aberrantly in *E. coli* within cloned mycoplasma sequences due to the high A+T content of the mycoplasma chromosome (12, 23). These two problems result in the loss of gene regulation and truncated gene products in *E. coli* either from premature termination at UGA codons or from internal transcription initiation. An additional problem is the stable maintenance of these sequences in *E. coli*. Expression of gene products, which may have a significant detrimental effect on *E. coli* may depend upon numerous variables such as product concentration, specific activity, and substrate specificity of the gene product. Differences between *E. coli* and mycoplasmas in codon usage may also play a critical role in the outcome of a project of this nature. To increase the chances of success, a novel screening strategy was used to identify cloned nuclease-coding sequences. In conclusion, we report here the successful cloning of a membrane nuclease gene from *M. pulmonis*, its DNA sequence, and an analysis of its expression in *E. coli*.

MATERIALS AND METHODS

Bacterial strains and plasmids. *M. pulmonis* CT and χ1048 were grown as previously described (20). These strains were originally obtained from M. K. Davidson. *M. gallisepticum* R (from Steve Geary), *M. hyopneumoniae* 232 (from R. F. Ross), *M. hyorhinis* GDL (from R. F. Ross), *M. capricolum* ATCC 27343, *M. fermentans* PG-18 (from S.-C. Lo), *M. fermentans* incognitus (from S.-C. Lo), *M. penetrans* (from S.-C. Lo), *M. hominis* 1620 (from L. D. Olson), *M. pneu-*

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TABLE 1. *E. coli* strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
E. coli		
LE392	F$^-$ *hsdR514* (r$_K^-$ m$_K^-$) *lacY1 supE44 supF58 galK2 galT22 trpR55 metB1*	
ISM612	[*leu*(UGA) *lacZ659*(UGA) *trpA9605*(UAG) *his29*(UAG) *ilv thyA metB argH rpoB rpsL prfB3*] pISM3001	21, 36
ISM614	LE392 [F$^-$ *hsdR514* (r$_K^-$ m$_K^-$) *lacY1 supE44 supF58 galK2 galT22 trpR55 metB1*] pISM3001	This study
ISM647	XL1-Blue [*recA1 lac endA1 gyrA46 thi hsdR17 supE44 relA1* (F′ *proAB$^+$ lacI^q lacZ*ΔM15 Tn*10*)] pISM3001	This study
DPWC	F$^+$ mating donor	Gold Biotechnologies
BW26	F$^-$ mating recipient, Kan^r	Gold Biotechnologies
CSR 603	(*recA1 uvrA6 phr-1*) maxicell strain	33
Plasmids		
pISM3001	*trpT* gene cloned behind a *lac* promoter in the vector pACYC184, Cm^r	36
pISM4170	6.6-kb *Sac*I fragment from phage 13-2B cloned onto pMOB, Ap^r	This study
pISM4172	7.0-kb *Sac*I fragment from phage 13-2B cloned onto pMOB, Ap^r	This study
pISM4175	2.2-kb *Cla*I fragment from pISM4172 cloned into pMOB, Ap^r	This study
pISM4176	2.6-kb *Cla*I fragment from pISM4172 cloned into pMOB, Ap^r	This study
pMOB	Cloning vector for Tn*1000* mutagenesis, Ap^r	Gold Biotechnologies

moniae ATCC 15531, and *A. oculi* ISM1499 (1) were also used in this study. Cultures were obtained from a stock culture maintained at −70°C, inoculated into fresh broth medium, and incubated statically at 37°C. *E. coli* strains (Table 1) were started from stock cultures and maintained in 1× or 2× Luria-Bertani broth, in superbroth (32 g of tryptone per liter, 20 g of yeast extract per liter, 5 g of NaCl per liter [pH 7.2]) or on Luria-Bertani agar media. Phage plates for screening genomic libraries on *E. coli* ISM612 consisted of superbroth base with superbroth soft-agar overlays. The plasmids constructed during the course of these studies are described in Table 1.

Isolation and manipulation of DNA. Mycoplasma chromosomal DNA was isolated from 1 liter of mid-log-phase culture as previously described (1). Plasmid DNA was isolated from *E. coli* by alkali lysis. DNA fragments used for cloning and as probes were isolated from agarose gels on GenElute agarose spin columns (Supelco). DNA probes were labeled with [α-^{32}P]CTP by using the Rediprime random-labeling system (Amersham, Arlington Heights, Ill.), and unincorporated nucleotides were removed by spinning the labeled probe through microspin S-300 HR columns (Pharmacia-Biotech).

Plasmids pISM4170 and pISM4172 were transformed into *E. coli* DPWC and mated with BW26 by the method described by Strathmann et al. (37). Recipient cells were selected on Luria-Bertani agar containing 50 μg of kanamycin per ml and 250 μg of ampicillin per ml. The Tn*1000* insertions in the plasmids resulting from these matings were mapped either by restriction digestion or by PCR. PCRs were performed in a 50-μl volume containing 1× buffer (GIBCO BRL), approximately 1 ng of plasmid DNA from alkali lysis, T7 primer and either primer 187 or primer 486 (5 pmol) (see below), 4 mM MgCl, 20 mM deoxynucleoside triphosphates, and 1 U of *Taq* polymerase. Cycling was performed as follows: one cycle of 5 min at 92°C and 30 cycles of 1 min at 92°C, 1 min at 55°C, and 2 min at 72°C. Tn*1000* inserts were also used as primer sites for DNA sequence analysis.

For hybridization, approximately 2 to 5 μg of mycoplasma DNA was digested with *Eco*RI and the fragments were separated on a 0.7% agarose gel and transferred to nylon membranes. The blots were hybridized with DNA probes by using Rapid-hyb buffer (Amersham). Hybridization was performed at 46°C (low stringency). The blots were washed in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS) three times for 15 min at room temperature and in 1× SSC-0.1% SDS twice for 30 min at 42°C.

Antisera. Antigen preparations containing semipurified nucleolytic proteins were prepared from SDS-polyacrylamide gel electrophoresis (PAGE) nuclease gels by using preparative spacers with the Bio-Rad Protean II electrophoresis unit. The running and renaturation conditions were adapted from standard conditions to obtain the maximal nuclease activity in preparative gels with the following modifications (20). The gels were washed for 1 h in incubation buffer (40 mM Tris, 0.01% casein, 0.04% β-mercaptoethanol [pH 7.5]), and nuclease digestion was allowed to proceed for 24 h. Areas displaying digestion of the DNA (nonfluorescing regions of the gel) were excised and minced with a 22-gauge needle. Two New Zealand White rabbits were immunized by injecting the acrylamide-protein slurry subcutaneously. The rabbits were boosted 2 weeks after immunization with the same antigen, and the antiserum collected at 4 weeks was tested for reactivity by enzyme-linked immunosorbent assay with *M. pulmonis* lysed whole-cell antigen (4) and by immunoblotting as described previously (39).

Hyperimmune antisera was also raised in BALB/c mice against two internal peptides of MnuA corresponding to amino acids 125 to 135 (QEKPKEKPGRK) and 320 to 331 (GSKGEGTTSVGV) of the predicted amino acid sequence. These peptides, designated the N-terminal and C-terminal peptides, respectively, were prepared with N-terminal cysteine residues to link them to maleimide-activated keyhole limpet hemocyanin (Pierce, Rockford, Ill.). The resulting peptide conjugates were then mixed with incomplete Freund's adjuvant and used to immunize mice. Antibody containing ascites fluid was then prepared from the

immunized mice by using SP2/0 myeloma cells as described by Luo and Lin (17). Antibody titers were determined by enzyme-linked immunosorbent assays with ovalbumin-peptide conjugates as the antigen. Immunoblot analyses were also performed with each of the ascites and with *M. pulmonis* whole cells as antigens.

Genomic library construction and screening. Chromosomal DNA from *M. pulmonis* was partially digested with *Sau*3A, and 9- to 15-kb fragments were isolated by using sucrose gradients (32). The fragments were partially filled in and ligated into *Xho*I-digested λGEM 12 arms as specified by the manufacturer (Promega Corp., Madison, Wis.). The recombinant phage were packaged and plated onto lawns of *E. coli* LE392. Single plaques were picked and amplified as previously described (21). The genomic library was screened by inoculating lawns of *E. coli* ISM612 with a 48-pin replicator as previously described (21). Rabbit anti-membrane nuclease antisera were used to develop the lifts and identify potential nuclease-expressing clones.

Nuclease detection assays. For nuclease detection, mycoplasma suspensions were produced as previously described (20). Nuclease activity in recombinant λ phage was measured in the following way. The opal suppressor strain *E. coli* ISM612 was infected with phage at a multiplicity of infection of 10:1, and the cells were shaken at 37°C for 2 h. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added (final concentration, 4 mM), and the shaking was continued for an additional 5 h. The cells were then harvested, resuspended in phosphate-buffered saline (PBS), and sonicated to disrupt them. Sonication was performed with a Branson Ultrasonicator at the maximum setting for the micro tip and 50% duration pulse for 20 pulses. Cellular debris was removed by centrifugation at 12,000 × *g* for 10 min, and the nuclease activity in the supernatant was monitored by the λ assay as described previously (20). Nuclease activities associated with recombinant plasmids were monitored in the *E. coli* ISM647 background. Transformed colonies were picked, grown overnight, harvested, resuspended in PBS, and sonicated prior to use. The SDS-polyacrylamide gel electrophoresis (PAGE) nuclease gel assay has also been described previously (20). In some SDS-PAGE nuclease gels, λ DNA (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) was used instead of salmon sperm DNA.

DNA sequence analysis. Plasmids for DNA sequencing were prepared by using Qiagen columns, and the sequencing was performed by the Nucleic Acid Instrumentation facility at Iowa State University by using cycle-sequencing protocols. The sequencing primers were oligonucleotides complementary to the transposon sequences adjacent to the inverted repeat ends. Primers 187 (5′-CAACGAAT TATCTCCTT-3′) (Gold Biotechnology) and 486 (5′-TCAATAAGTTATACC AT-3′) were used to sequence from Tn*1000* insertions (37). In some instances, the T7 primer (5′-AATACGACTCACTATAG-3′) and three other primers, 2590 (5′-GCGACACTGAGCCTACGAG-3′), 1145 (5′-GGTGTAGCTACTAAT AAAC-3′), and 890 (5′-GACCTTAGCCAAATGAAAC-3′) were used.

Sequence analysis was performed with MacVector software (Eastman Kodak). The hydrophilicity of the translated product was determined by the method of Kyte and Doolittle (16), and the secondary-structure predictions were made by the methods of Chou and Fasman (5) and Garnier et al. (10). The translated product was also analyzed for signal sequences and transmembrane domains with PSORT (available on the World Wide Web [27a]). PSORT uses the methods of McGeoch (18) and von Heijne (41) for signal sequence determination and the method of Klein et al. (14) for determining transmembrane domains.

Maxicell analysis. To confirm that the nuclease activity observed in recombinant *E. coli* was actually derived from *mnuA*, maxicell experiments were performed (33). Maxicell strain CSR 603 was transformed with pISM3001 and either pSK (negative control) or pISM4176 (*mnuA*) selecting for ampicillin and chloramphenicol resistance. Cells were grown overnight in Luria-Bertani broth and diluted to an optical density at 600 nm of 0.7. Samples (5 ml) were spread in a sterile petri dish and subjected to UV light at different time intervals (0 to 60 s) by being placed in a box containing three 15-W UV bulbs suspended 4 in. above
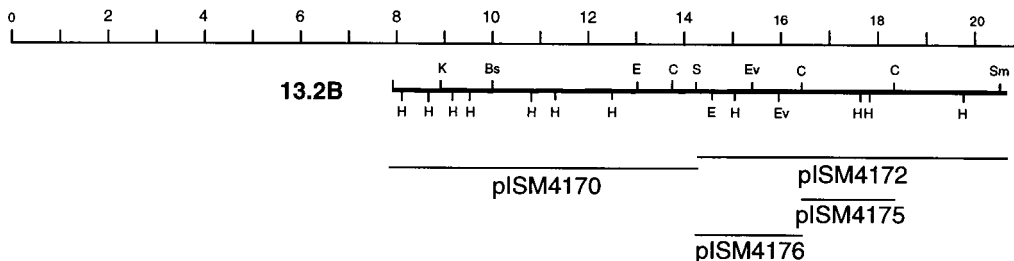
FIG. 1. Physical maps of the chromosomal inserts in recombinant phage. The subcloned regions and plasmid designations are also given. The scale at the top is in kilobases. Symbols: A, *Apa*I; B, *Bam*HI; Bs, *Bss*HII; C, *Cla*I; E, *Eco*RI; Ev, *Eco*RV; K, *Kpn*I; H, *Hin*dIII; P, *Pst*I; S, *Sac*I; Sm, *Sma*I.

the base. UV-inactivated cells (20 ml) were transferred to a 250-ml flask and incubated at 37°C with shaking for 5 h. IPTG (4 mM) was added to each culture, and the cells were shaken for 4 h at 37°C. The cells were pelleted by centrifugation, washed once with TS buffer (10 mM Tris, 140 mM NaCl [pH 7.5]), and sonicated to lyse them, and the cell debris was removed by centrifugation at 12,000 × *g* for 10 min. The lysate was then subjected to analysis by the λ and SDS-PAGE nuclease assays as described previously (20).

In some experiments, maxicell lysates were adsorbed with antisera to the MnuA C-terminal peptide in the following way. Antiserum was diluted 1:100 in PBS and adsorbed to nitrocellulose filters for 1 h. The filters were then blocked with 2% gelatin in PBS for 1 h, and then the maxicell lysates were adsorbed against the filters for 1 h at 37°C before being tested in the λ nuclease assay. An antiserum to an unrelated protein was adsorbed to negative control filters.

Immunoblot analysis. Immunoblots were prepared from *M. pulmonis* χ1048 whole cells or trypsin-treated cells by the method of Towbin et al. (39). To treat *M. pulmonis* with trypsin, cells were prepared from overnight cultures by being washed twice with PBS and then resuspended to a protein concentration of 3 mg per ml. The cell suspension was then treated with 100 μg of trypsin for 1 h, and samples were taken at 10-min intervals for analysis. Gels and nitrocellulose blots were prepared by using the Bio-Rad mini-Protean II system. A 10-μg portion of protein was loaded per well.

Nucleotide sequence accession number. The nucleotide sequence of the *mnuA* gene of *M. pulmonis* has been assigned GenBank accession no. U38841.

RESULTS

Identification of λ phage expressing mycoplasma nuclease activities. The genomic library of *M. pulmonis* representing 1,920 independent, recombinant λ with an average insert size of 10 to 12 kb was screened with rabbit hyperimmune anti-membrane nuclease antiserum as described previously (21). Although this antiserum was not raised against purified nucleases per se, it was useful to enrich for a population of recombinant phages of which a higher percentage expressed *M. pulmonis* nucleases than that of the original library. A total of 19 immunopositive phages were identified, 4 of which expressed nuclease activity by the λ assay. One of these phages, 13.2B, was chosen for further study based on the ability of a subcloned fragment to maintain nuclease activity in *E. coli*.

Cloning and functional analysis of the chromosomal fragments from nuclease-positive phage. The four chromosomal fragments from the nuclease-positive recombinant phage were subcloned, generating the plasmids described in Table 1 and Fig. 1. Each of the plasmids was transformed into the opal suppressor strain ISM612, which had no endogenous nuclease activity in our λ assay (data not shown). This step was necessary to read through internal UGA codons in the gene sequences in order to express functional gene products in *E. coli*. When ISM612 containing plasmid pISM4170 was analyzed for expression of nuclease activity, the activity was present in 10% or less of the independent colonies. This loss of phenotype was not the result of noticeable plasmid deletions or plasmid loss. Therefore, it was assumed that the nuclease gene in the cloned fragment was undergoing a high rate of mutation in the high-copy-number plasmid background, and this plasmid was not studied further. It is possible that the use of a low-copy-number

plasmid vector would enhance the stability of these fragments in *E. coli*, which will be examined in future studies.

Interestingly, all attempts to transform plasmid pISM4172 into strain ISM612 failed. Since this instability could arise from efficient opal suppression of internal UGA codons, resulting in high levels of mycoplasma nuclease expression in the *trpT-prfB3* background (36), less efficient suppressor strains were constructed. These consisted of pISM3001 derivatives of two *E. coli* strains lacking the *prfB3* mutant allele, LE392 and XL1-Blue. The resulting strains, ISM614 and ISM647, were screened for endogenous nuclease activity which might interfere with mapping of the nuclease structural gene by transposon mutagenesis. Both *E. coli* strains lacking the *prfB3* mutation supported the replication of plasmid pISM4172, but only ISM647 lacked nuclease activity as monitored by the λ assay (data not shown). More than 90 to 95% of the independent isolates of ISM647 pISM4172 contained nuclease activity (data not shown). Thus, the stable nuclease-positive background needed for transposon mutagenesis and mapping of the nuclease-encoding sequences was obtained.

To locate the nuclease structural gene within the 7.0-kb insert of pISM4172, the plasmid was subjected to Tn*1000* mutagenesis as described previously (37). The resulting insertions were restriction mapped, and selected plasmids representing insertions across the entire cloned fragment were screened for the loss of nuclease activity following transformation into ISM647. Insertions of Tn*1000* within a 1.0-kb region of pISM4172 knocked out nuclease activity (Fig. 2A). Plasmid pISM4176 containing the 2.6-kb *Sac*I-*Cla*I fragment of pISM4172 was nuclease positive in the ISM647 background, while the adjacent *Cla*I internal fragment in pISM4175 encoded no nuclease activity.

DNA sequencing and computer analysis. Analysis of the DNA sequence across the region that coded for nuclease activity identified an open reading frame (ORF), designated *mnuA*, of 1,410 bp coding for a 470-amino-acid protein of 53,739 Da (Fig. 3). Tn*1000* insertions upstream and downstream of this ORF failed to eliminate nuclease activity in the cloned sequence (Fig. 2). No Shine-Dalgarno-like sequence was found upstream of *mnuA*. Analysis of the predicted translated product of the *mnuA* DNA sequence shows an amino-terminal region rich in lysine residues followed by a 42-amino-acid hydrophobic region and another lysine residue which could serve as a membrane-spanning region to anchor the protein. The charged-hydrophobic-charged domains in the amino-terminal region resembled the principal features of bacterial signal peptides (28). The single cysteine residue was associated with a T-I-S-C motif near the amino terminus, previously reported to be a procaryotic prolipoprotein signal peptidase II recognition sequence (3, 42). The remaining portion of the molecule was extensively hydrophilic with substan-
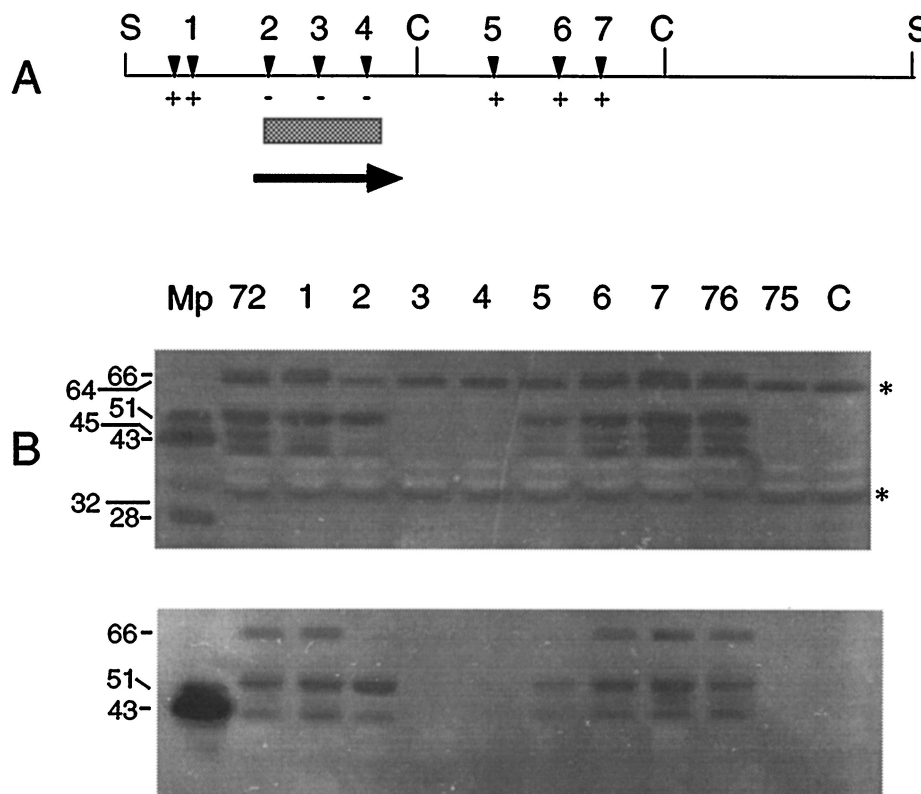
FIG. 2. Analysis of Tn*1000* inserts in pISM4172. Plasmid pISM4172 was subjected to Tn*1000* mutagenesis and analyzed as described in the text. (A) The locations of selected Tn*1000* inserts in pISM4172 are designated by inverted solid triangles. Below the map are the results of the λ DNA assay. Inserts that knocked out nuclease activity in the λ assay are designated −, and those that had no effect on nuclease activity are designated +. The bar indicates the minimal coding region encoding *mnuA* as defined by Tn*1000* mutagenesis. The arrow indicates an ORF determined by DNA sequence analysis and its direction of transcription. (B) Analysis of Tn*1000* inserts by an SDS-PAGE nuclease gel assay was performed as described in the text. The gels were digitized with a model 4900 high-performance charge-coupled device camera (Cohu, Inc., San Diego, Calif.) and a Macintosh IIci equipped with a Scion Corp. (Frederick, Md.) video board. The resulting TIFF file was cropped with and assembled in Adobe Photoshop and labeled in Aldus FreeHand. The apparent molecular weight of the nucleolytic bands is shown on the left in thousands. The asterisks indicate nucleolytic bands arising from *E. coli* with specificity for salmon sperm DNA. All lanes contained 5 μg of *E. coli* ISM647 protein except for the lane labeled Mp, which contained 116 ng (upper gel) or 175 ng (lower gel) of *M. pulmonis* protein. The upper gel contained salmon sperm DNA, and the lower gel contained λ DNA. Mp, *M. pulmonis* χ1048 antigen. The remaining lanes contain protein from *E. coli* ISM647 containing the corresponding plasmid (72, pISM4172; 76, pISM4176; 75, pISM4175; C, pMOB) or pISM4172 with Tn*1000* insertions (lanes 1 to 7) shown in panel A.

tial alpha-helical character. The estimated pI of the translated product was 9.04. There were five UGA codons coding for tryptophan (Fig. 3).

BLASTN and FASTA searches were performed on the *mnuA* sequence, and there were short stretches of amino acid similarity (10 to 15 amino acids) with the CpG methylase of *Spiroplasma citri*. A BLASTP search of the SwissProt database also indicated a significant homology at the amino acid level to an *M. pneumoniae* protein of unknown function, P01_orf474. There was also a low level of homology at the nucleotide level, which may explain the reaction of the *mnuA*-specific probe with *M. pneumoniae* DNA (see Fig. 6). These data suggest that ORF 474 in *M. pneumoniae* may code for a nuclease, possibly the nuclease identified in previous studies (20). There was no other significant homology to known DNA or protein sequences. There was also no apparent promoter region, but the upstream region was AT rich with several potential TATA boxes and possible ribosomal binding sites, which could account for the promoter activity observed in *E. coli*. Transcription termination most probably occurs at an inverted repeat sequence downstream.

Analysis of *mnuA* products produced in *E. coli*. Four products demonstrating apparent molecular masses of 66, 53, 45, and 39 kDa were expressed in *E. coli* ISM647 from the

chromosomal fragment containing *mnuA* (Fig. 2). Inclusion of different DNA substrates in the SDS-PAGE gels, i.e., heat-denatured salmon sperm DNA or λ DNA, allowed a clear differentiation between host-encoded nuclease activity and plasmid-encoded activity in this assay. The band at 45 kDa, however, was weak and was sometimes missing (Fig. 2B, lower panel, lanes 72 and 1). The 45- and 66-kDa bands appeared to be related, since the Tn*1000* insertion at position 175 of the *mnuA* gene sequence (insertion 2) knocked out the expression of both bands. The 53- and 39-kDa nucleolytic products produced with an insertion at position 2 (lane 2) may be derived from internal transcriptional initiation within the structural gene at a site downstream of this insertion. The 45- and 39-kDa products may result from premature truncation at the last UGA codon. Tn*1000* insertions located further downstream of insertion 2 but within *mnuA* (locations 3 and 4) knocked out all nucleolytic bands in the SDS-PAGE nuclease gels. Correlation of the λ assay results with the SDS-PAGE nuclease gel results shown in Fig. 2 shows that *mnuA* translation products which lack the amino terminus are inactive in whole-cell lysates. Some nuclease activity could be regained in these truncated products, however, in the SDS-PAGE nuclease assay (Fig. 2B, lower panel, lane 2).

To confirm that the nuclease activity was associated with the

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        +         +          +          +          +          +          +          +          +
                                                                      M   K   N   K   M   K   S   L   K
AATAATTGTTAAAACGCCTTTTTAACGCTTTGAAAAAGGCGTTTTTTAATTAAGCAATAATAAAATTATTGATATGAAAAATAAGATGAAATCGTTAAAA      99

 V   S   L   L   V   A   T   P   I   I   T   L   P   L   F   T   I   S   C   I   P   N   N   E   S   K   S   Y   Q   D   L   N   A
GTTAGTTTATTAGTAGCTACACCAATAATTACACTCCCATTATTTACAATTAGTTGCATTCCAAATAATGAATCTAAATCTTATCAAGATTTAAATGCC     198

 K   A   N   Q   A   A   I   L   L   K   Q   K   Q   I   N   S   N↓K   L   E   E   L   I   D   S   I   L   P   E   G   T   E   L
AAGGCTAATCAAGCGGCCATTTTGCTTAAACAAAAACAAATTAATAGCAATAAATTAGAAGAGCTAATTGACAGCATTCTACCCGAAGGAACTGAGTTA     297

 E   I   L   D   L   W   V   D   R   E   N   K   E   I   N   V   K   F   R   L   R   Y   K   D   R   R   Y   P   Y   S   S   D   V
GAAATTTTAGATCTTTGAGTGGATCGTGAAAATAAAGAAATTAATGTTAAATTCCGTCTTAGATATAAAGACAGAAGATATCCTTATAGTTCGGATGTC     396

 K   I   I   K   L   K   Y   E   S   I   L   E   N   L   K   P   Q   E   K   P   K   E   K   P   G   R   K   S   K   E   K   N   P
AAAATAATTAAGTTAAAGTATGAGTCTATCTTGGAAAACTTGAAACCGCAAGAAAAACCAAAGGAAAAACCCGGACGTAAGTCTAAAGAAAAGAACCCA     495

 K   T   Q   N   D   E   K   S   K   D   G   L   N   K   K   S   N   G   K   Q   K   M   K   W   G   H   W   N   V   L   K   L   S
AAAACCCAAAATGATGAAAGTCTAAAGATGGCCTTAATAAAAAGTCAAATGGAAAACAAAAAATGAAATGAGGACATTGGAATGTTCTTAAATTAAGC     594

 D   K   N   D   K   K   I   A   A   I   A   A   L   I   K   K   I   D   Y   D   V   V   G   L   T   E   V   F   S   E   E   A   V
GACAAAAATGATAAAAAAATTGCTGCCATTGCCGCTTTAATTAAAAAAAATTGACTATGATGTCGTTGGCCTAACGGAAGTATTTAGCGAAGAAGCCGTT     693

 K↓E   V   V   A   Q   L   N   K   L   A   G   S   G   T   Y   S   Y   V   A   S   K   K   L   K   G   S   Y   A   G   N   L   Q
AAAGAAGTCGTTGCTCAATTGAATAAACTAGCCGGCAGTGGCACATATTCTTATGTTGCATCAAAAAAAACTTAAAGGCTCATATGCTGGCAATTTGCAA     792

 A   E   H   V   G   V   I   Y   N   S   Q   K   I   K   T   I   P   F   E   K   T   K   N   N   K   K   E   I   G   Y   S   Y   I
GCTGAACATGTCGGAGTAATTTATAATAGCCAAAAAATTAAAACTATTCCATTTGAAAAGACTAAGAATAATAAAAAAGAAATAGGCTATAGTTATATA     891

 E   K   F   N   S   E   F   I   K   K   H   N   K   A   E   Y   V   R   P   P   Y   G   V   K   F   Q   W   L   K   G   K   K   E
GAAAAAATTCAATTCAGAATTTATTAAAAAAACATAACAAAGCCGAGTATGTACGACCACCATATGGTGTTAAATTCCAATGATTAAAAGGGAAAAAAGAA     990

 D   F   T   F   V   F   D   H   F   D   S   P   G   G   S   K   G   E   G   T   T   S   G   V   G   N   Q   E   L   A   E   A   R
GATTTTACATTTGTTTTCGACCATTTTGATAGCCCTGGCGGAAGCAAAGGTGAAGGAACAACTAGTGGAGTCGGCAATCAAGAGCTAGCAGAAGCTAGA    1089

 Q   L   A   N   V   L   D   H   F   D   E   I   D   G   E   N   E   D   I   F   F   S   G   D   T   N   I   K   A   G   K   E   K
CAACTTGCAAATGTACTTGATCATTTTGACGAAATTGATGGCGAAAACGAAGATATCTTCTTTAGCGGAGACACCAACATCAAAGCGGGCAAAGAAAAA    1188

 F   A   F   G   E↓E   L   F   K   K   Y   Q   S   A   F   K   D   N   L   E   F   A   T   S   L   G   I   K   A   G   N   Y   S
TTTGCTTTTGGTGAAGAATTATTCAAAAAATATCAATCCGCTTTTAAAGATAACCTTGAGTTTGCAACATCATTAGGAATAAAGGCCGGCAACTACTCA    1287

 Q   P   Y   D   K   M   F   T   K   T   K   F   K   T   S   N   H   G   I   Y   K   L   W   D   L   S   K   D   D   E   F   T   N
CAACCATATGATAAAATGTTCACAAAAACCAAATTTAAGACTTCAAATCATGGCATCTATAAACTATGAGACTTGAGTAAAGATGATGAATTTACAAAT    1386

 L   F   K   E   L   H   N   G   K   I   E   T   S   E   I   R   K   L   V   S   D   H   A   P   I   Y   S   E   V   E   F   E
CTTTTCAAAGAATTACATAATGGAAAAATTGAAACTAGTGAAATAAGAAAACTAGTATCTGATCACGCGCCTATATATAGTGAAGTTGAATTTGAATAA   1485
TTAAAAAAATATAAAAAAATCGATGCCTAACTTTGGTATCGATTTTAACTTT                                                1538
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FIG. 3. Nucleotide and deduced amino acid sequences of the *M. pulmonis mnuA* gene. An inverted repeat is doubly underlined at the 3′ end of the *mnuA* sequence. The single underline indicates peptides used to produce anti-MnuA antisera. (↓) indicates the sites of Tn*1000* insertions described in the manuscript. Boldface type indicates tryptophan-encoding UGA codons.

product of *mnuA* and that it was not acting as an activator of an *E. coli* gene, maxicell experiments were performed. Previous studies have shown that this approach is useful for the identification of plasmid-encoded proteins and that *E. coli* chromosomal genes are not expressed under these conditions (33). The maxicell strain CSR 603 was transformed with plasmids pISM3001 (opal suppressor) and either pSK (negative control) or pISM4176 (*mnuA*) and subjected to UV inactivation. Even short exposure times (15 s) under our experimental conditions reduced the viability of this strain to undetectable levels (data not shown). When lysates prepared from these cells were analyzed for nuclease activity by the λ assay, only strains containing pISM4176 (*mnuA*) showed measurable nuclease activity (Fig. 4). The variation in the size of the λ DNA substrate in the first two lanes of each dilution series in the lower panel of Fig. 4B is due to the high protein concentrations in those samples distorting the migration of the DNA in the gel. As the *E. coli* lysate is diluted twofold in the assay, this effect disappears. The nuclease activity could also be visualized on SDS-PAGE nuclease gels (Fig. 5). The amount of nuclease activity as measured by the λ DNA assay was independent of the UV dose, indicating that the nuclease was plasmid encoded
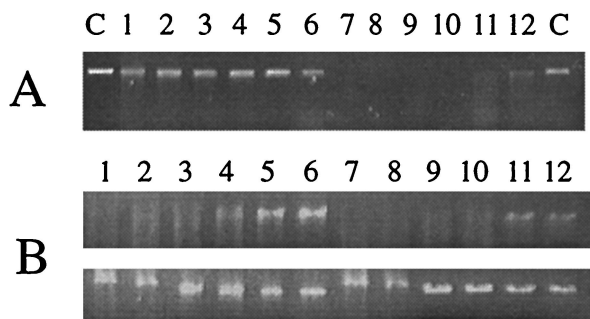

FIG. 4. Analysis of *E. coli* products by the λ assay. The preparation of *E. coli* extracts were described in Materials and Methods. Following treatment, the extracts were diluted twofold and incubated with λ DNA for 30 min. The reaction products were analyzed on a 0.7% agarose gel. (A) Analysis of CSR 603 extracts following UV irradiation to produce maxicells. Lanes: C, control λ DNA, 1 to 6, twofold dilutions of CSR 603 pISM3001 pSK maxicell extract; 7 to 12, twofold dilutions of CSR 603 (pISM3001/pISM4176) maxicell extract. (B) Analysis of ISM647 (pISM3001/pISM4176) and CSR 603 (pISM3001/pISM4176) extracts following adsorption with anti-MnuA C-terminal peptide antiserum. Upper panel, nonadsorbed extracts. Lower panel, extracts adsorbed with anti-MnuA C-terminal peptide antiserum bound to nitrocellulose. Lanes: 1 to 6, twofold dilutions of ISM647 (pISM3001/pISM4176) extract; 7 to 12, twofold dilutions of CSR 603 (pISM3001/pISM4176) extract.
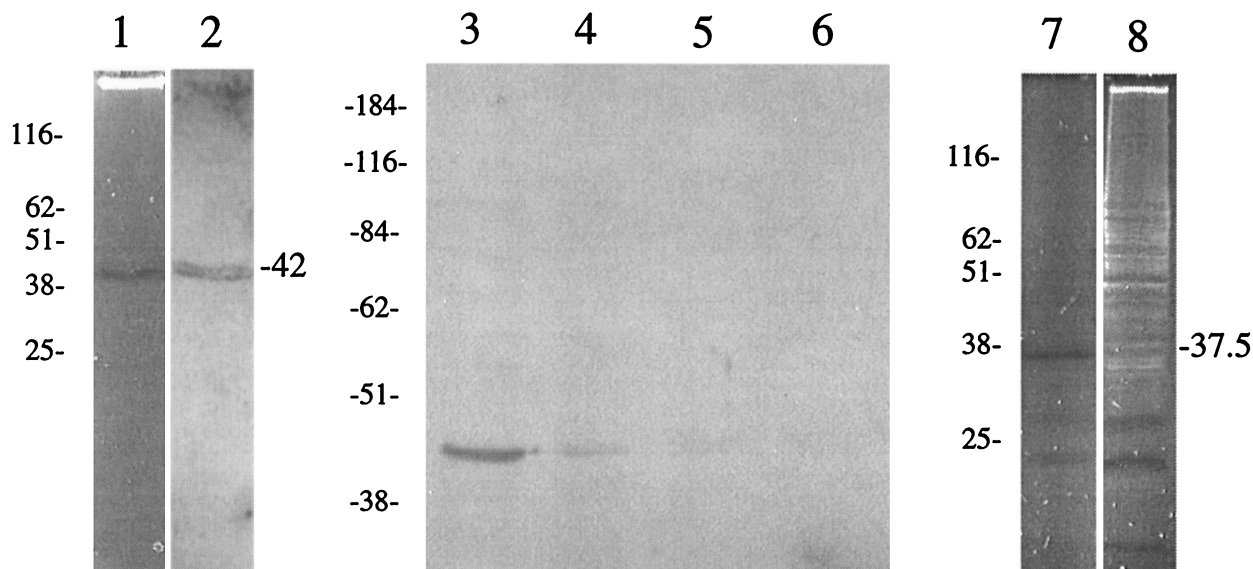
FIG. 5. Functional and immunoblot analysis of *mnuA* gene products. Lanes: 1, SDS-PAGE nuclease gel containing *M. pulmonis* antigen; 2, immunoblot analysis of an identical gel containing *M. pulmonis* antigen with anti-MnuA C-terminal peptide antiserum; 3 to 6, immunoblot analysis with anti-MnuA C-terminal peptide antiserum of *M. pulmonis* whole cells treated with trypsin for 0 (lane 3), 10 (lane 4), 20 (lane 5) and 40 (lane 6) min, respectively; 7, SDS-PAGE nuclease gel analysis of maxicell extracts from CSR 603 pISM3001 pISM4176; 8, SDS-PAGE nuclease gel analysis of maxicell extracts from CSR 603 pISM3001 pSK (negative control). Molecular weight markers are shown on the left of each panel (in thousands).

(33) (data not shown). Mouse antisera raised against the N-terminal peptide of MnuA reacted poorly to a single band of approximately 47 kDa on immunoblots with *M. pulmonis* antigen even though the enzyme-linked immunosorbent assay titers varied from 1:50,000 to 1:100,000 (data not shown). Antisera raised against the C-terminal peptide, however, reacted more strongly to the same band (Fig. 5). The nuclease band produced in CSR 603 (37.5 kDa) was smaller than the product produced in *M. pulmonis* (47 kDa). It is likely that this difference in size is due to the aberrant transcription-translation often observed in *E. coli* with mycoplasma gene sequences (12, 23) or in premature truncation due to inefficient suppression.

Presence of *mnuA*-like sequences in other *Mycoplasma* species. The internal *Eco*RV fragment of *mnuA* was used as a probe to determine if this gene was shared among 11 different *Mycoplasma* species. As shown in Fig. 6, there was cross-hybridization between the *mnuA* probe and chromosomal fragments from *M. hyopneumoniae* and *M. pneumoniae*. There is only one copy of *mnuA* in the *M. pulmonis* chromosome, and the gene is located in a similar location in both *M. pulmonis* CT and *M. pulmonis* χ1048, the latter being the strain from which *mnuA* was isolated.

DISCUSSION

This work represents the first cloning and analysis of a membrane nuclease gene, *mnuA*, from a mycoplasma. It seems reasonable that *mnuA* codes for a membrane-associated nuclease for the following reasons. First, all *M. pulmonis* nuclease activities observed in the SDS-PAGE nuclease gels have been shown previously to be associated with the membrane (19). This cloned sequence clearly produces a nuclease that survives the SDS-PAGE sample treatment in an identical fashion (19, 20). Not all nucleases survive this treatment and are visualized by the SDS-PAGE assay, since *E. coli* is known to contain numerous nucleases of various specificities. These nucleases are inactivated by our sample treatment and, fortuitously, do not interfere with the analysis of the *mnuA* gene products (Fig.

2). Second, the DNA sequence clearly shows a prominent membrane-spanning domain in the amino terminus and has a lipoprotein signal sequence that has been shown to function in *M. hyorhinis* (6). This has been considered by other investigators to be strong evidence that the cloned gene codes for a membrane-associated protein. Third, the MnuA-specific peptide antiserum that reacted with a single nucleolytic band on immunoblot analysis, and this band was sensitive to trypsin treatment of whole cells (Fig. 5). The inability to construct site-
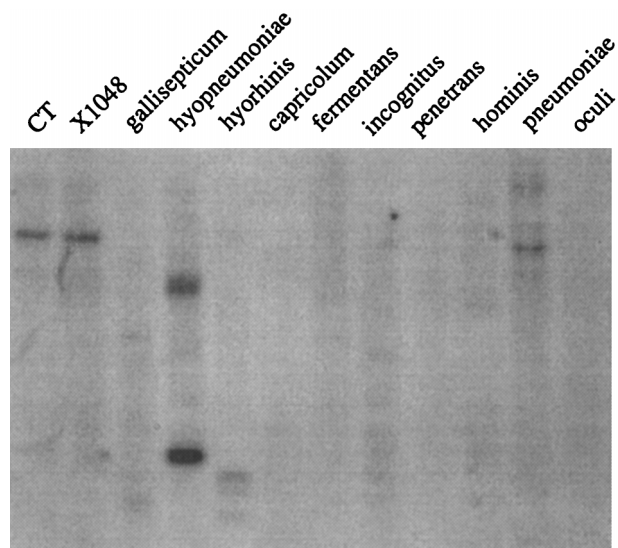

FIG. 6. Hybridization analysis of 11 *Mycoplasma* species with a *mnuA*-specific probe. The hybridization conditions are described in Materials and Methods. Lanes: CT, *M. pulmonis* CT; χ1048, *M. pulmonis* χ1048; gallisepticum, *M. gallisepticum* R; hyopneumoniae, *M. hyopneumoniae* 232; hyorhinis, *M. hyorhinis* GDL; capricolum, *M. capricolum* ATCC 27343; fermentans, *M. fermentans* PG-18; incognitus, *M. fermentans* incognitus; penetrans, *M. penetrans*; hominis, *M. hominis* 1620; pneumoniae, *M. pneumoniae* ATCC 15531, oculi, *A. oculi* ISM1499.

directed mutations in this *Mycoplasma* species limits the types of studies that can be performed to conclusively correlate the *mnuA* gene product in *E. coli* with one of the membrane nucleases in *M. pulmonis*. Our transposon mutagenesis studies, the immunoblot data with antipeptide antisera, and the sequence data, however, offer strong support for our hypothesis that *mnuA* codes for a mycoplasma membrane-associated nuclease.

E. coli has previously been a host for the expression of several nonspecific nucleases, such as those from *Staphylococcus aureus* (34), *Thermus filiformis* (9), and *Shigella flexneri* (7). The staphylococcal nuclease has been extensively studied (35) and is expressed at low levels from its native promoter in *E. coli* (34). The product is secreted either from its own signal peptide or with a lipoprotein signal sequence attached (26). Higher levels of activity can be obtained when the nuclease is expressed from a λ promoter (13), but in the absence of a functional signal sequence, expression of the nuclease was lethal (38). These studies indicate that expression of heterologous nucleases in *E. coli* is possible and is a convenient way to study these proteins but that their expression can be problematic and lead to lethal phenotypes.

The studies presented here clearly show that the level of active nuclease expression from the cloned fragments was low in *E. coli* (Fig. 2). At least 40 times as much protein was required from *E. coli* as from *M. pulmonis* to obtain comparable levels of activity on the SDS-PAGE nuclease gel (Fig. 2). The promoter activity that directed the expression of *mnuA* was associated with the mycoplasmal sequences, because neither λGEM 12 nor pMOB contains promoter-like sequences to regulate the expression of cloned genes. This is common in mycoplasmas (15) and is presumably due to the AT richness of the chromosomal DNA. Low-level expression of the nonspecific nuclease activity might be essential for *E. coli* and λ viability, since high-level expression could result in damaged chromosomal DNA or damaged λ concatameric DNA and an unsuitable packaging substrate. Thus, cloning strategies that would raise the levels of nuclease product within the cell, i.e., cloning into a high-copy-number cloning vector such as pKS or using a strong external promoter, would increase the likelihood of instability. Since *mnuA* contained five UGA codons (Fig. 3), expression was partially controlled by regulating suppressor activity in the ISM647 background in comparison to the ISM612 background, which has a chromosomal *prfB* mutation resulting in constitutive UGA suppression (21).

This low level of expression in *E. coli* created problems in immunoblot analysis with nuclease-specific antisera (data not shown). The reaction of anti-MnuA peptide antisera with *M. pulmonis* antigen was weak even with the C-terminal peptide antisera, suggesting that membrane nucleases in mycoplasmas are produced at low levels. In *E. coli*, the level of MnuA protein was below the level detectable by immunoblot analysis (data not shown), but this antiserum was capable of adsorbing out nucleolytic activity in ISM647 pISM3001/pISM4176 and CSR 603 pISM3001/pISM4176 lysates (data not shown).

Interestingly, both *E. coli* and *M. pulmonis* nuclease-banding patterns differed in their SDS-PAGE nuclease gel profiles as a function of the DNA substrate (Fig. 2). *E. coli* ISM647 contained nuclease bands of 64 and 32 kDa that digested salmon sperm DNA (Fig. 2B, upper panel, lane C) but not λ DNA (lower panel, lane C). This correlated with the results of the λ DNA assay; strain ISM647 had no measurable nuclease activity in that assay. An *M. pulmonis*-derived nucleolytic band of about 28 kDa was also absent in the gel containing λ DNA (Fig. 2B, lower panel, lane Mp). The lane was loaded with one-third more protein (175 ng) and showed much stronger bands in the 43-kDa region of the gel, but there was clearly no nucleolytic

activity in the 28-kDa region when λ DNA was used as a substrate. It is not known what distinguished the substrate specificity of these enzymes. This observation was reproducible and was observed with several different gels. Two possible explanations for the loss of activity with lambda DNA would be differences in the methylation pattern between eucaryotic and procaryotic DNA or single versus double strandedness of the template (the salmon sperm DNA was sheared and boiled prior to inclusion in the SDS-PAGE resolving gel). The *mnuA* gene products in *E. coli*, however, were unaffected by the differences in the DNA substrates. Thus, MnuA could be considered a nonspecific nuclease with a broad specificity.

It is possible that MnuA is the 51-kDa protein in the *M. pulmonis* banding pattern, considering the possibility that the single cysteine residue serves as a signal peptidase cleavage and acylation site (28, 42). The processed polypeptide would be 50,769 Da (not including the fatty acid side chain), but it is not known what effects, if any, DNA might have on the mobility of a DNA binding protein in DNA-containing resolving gels. All nuclease activities in *M. pulmonis* partition into the detergent phase during Triton X-114 fractionation (data not shown), suggesting that each nuclease has large regions of hydrophobicity or is a lipoprotein. It has been observed that mycoplasmal lipoproteins are improperly processed during expression in *E. coli*, and thus it is unlikely that the *mnuA* gene product has been processed correctly. This could affect its size or other biophysical characteristics.

These studies illustrate the difficulties in studying mycoplasma gene sequences in *E. coli*. Rarely can recombinant mycoplasma gene products be used to complement specific mutations in *E. coli* or be directly correlated with the gene product from the original host. Most often, the products are truncated prematurely or represent products from internal transcription initiation. For instance, Tn*1000* insertion 2 (Fig. 2) knocked out all nuclease activity in cell lysates but not in the SDS-PAGE nuclease assay, which lost only two of the four bands observed with the nonmutated plasmid. The presence of the two bands supports the hypothesis that internal transcriptional initiation within *mnuA* results in some of the products observed on SDS-PAGE nuclease gels because this insert is located behind the signal sequence of *mnuA*. These protein products were probably folded incorrectly in the cell lysate but regained a functional conformation during the denaturation and renaturation conditions of the SDS-PAGE nuclease gel assay. Tn*1000* inserts further downstream of this aberrant transcription initiation site knocked out all nuclease activity.

Antisera were used in this study to identify nuclease-containing recombinant phages and to correlate the *mnuA* gene product with nuclease activities. The rabbit antisera allowed us to enrich for a population of recombinant phages containing nuclease-positive recombinants. The 19 recombinant phages identified in the library represent a 100-fold enrichment, which significantly reduced the effort required to identify nuclease-positive recombinant clones. The immunoblotting results with the MnuA-specific peptide antisera directly correlated the *mnuA* gene product with a specific *M. pulmonis* nucleolytic band on SDS-PAGE gels (Fig. 5). These peptide antisera were also used to correlate the *E. coli* nucleolytic gene product with *mnuA*. This supports our hypothesis that *mnuA* codes for a membrane nuclease. The maxicell experiments further support this conclusion.

In summary, a membrane nuclease gene from *M. pulmonis* has been cloned and analyzed. It is not yet clear what role, if any, the MnuA nuclease plays in growth and virulence of *M. pulmonis*. These nucleases are fully capable of digesting mycoplasmal chromosomal DNA (data not shown), implying

that translocation of the *mnuA* gene product may be tightly coupled to translation (28). Further study of this activity could reveal basic mechanisms of gene expression and protein translocation in these cell wall-less bacteria.

ACKNOWLEDGMENT

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