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Membrane-Associated Nuclease Activities in Mycoplasmas

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Membrane-associated nucleases of various mycoplasmal species were investigated by using two nuclease assays. A lambda DNA assay was developed to measure nuclease activity associated with whole-cell suspensions, activity released from intact cells, and activity associated with detergent-disrupted cells. In most species, nuclease activities were entirely membrane associated, and disruption by a detergent had a stimulatory effect on these activities. All mycoplasmal species contained nuclease activity, but *Mycoplasma capricolum* was unusual because its activity was dependent upon magnesium and was inhibited by calcium. We developed a sodium dodecyl sulfate-polyacrylamide gel electrophoresis system that produced reproducible nuclease patterns, and this system was used to determine the apparent molecular weights of the nuclease proteins. An examination of 20 mycoplasmal species failed to identify common bands in their nuclease patterns. An examination of 11 *Mycoplasma pulmonis* strains, however, indicated that nuclease patterns on polyacrylamide gels may provide a means for categorizing strains within a species. Our results suggest that nucleases are important constituents of mycoplasmal membranes and may be involved in the acquisition of host nucleic acids required for growth.

Mycoplasmas, cell wall-less bacteria of the class *Mollicutes*, are among the smallest self-replicating organisms known and have been described as minimal living units (12, 15). These organisms are typically noninvasive animal pathogens that readily colonize mucosal surfaces and have immunomodulatory activities (3, 24–26), but are totally incapable of synthesizing typical eubacterial cell wall components, the major bacterial immunomodulatory components. It has been hypothesized that the selective association of host cell proteins with mycoplasmas could alter the immunoresponsiveness of a host (28). The presence of a small genome and the lack of numerous biosynthetic pathways correlate with growth requirements for macromolecular precursors such as phospholipids, cholesterol, nucleotides, and amino acids. Exchange of lipophilic probes from host cells to mycoplasmas has also been demonstrated (10, 29), but the mechanism(s) by which the mycoplasmas acquire these materials is unknown. Yet this mechanism must function efficiently, and it may contribute to immunomodulation and pathogenesis.

Unlike other eubacteria, mycoplasmas lack the biosynthetic capacity to synthesize de novo nucleic acid precursors (15). Nuclease activity in members of the *Mollicutes* has been proposed as the mechanism by which these organisms acquire the precursors required for their nucleic acids. Uptake of precursors could occur either as uptake of free bases or as uptake of small oligonucleotides. Therefore, a mechanism(s) must be present to induce release, degradation, and transport of host nucleic acids to intracellular sites. Transport processes have been identified in mycoplasmas (15, 18), but DNA and RNA degradation processes have not received adequate attention. Pollack and Hoffmann (14) and Roganti and Rosenthal (20) demonstrated that members of the genus *Acholeplasma* produce multiple proteins with nuclease activities, as indicated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). Minion and Goguen (11) found membrane-associated nucleases in *Mycoplasma pulmonis*. In addition, RNases are known to be present in mycoplasmas (8). Together, these activities are sufficient to produce all of the required nucleotides from undegraded DNA and RNA, as observed originally by Razin and Knight (19). This study was undertaken to expand our knowledge about the occurrence of membrane-associated nucleases and the proteins involved in various *Mollicutes* species. Our results suggest that nuclease activities are primarily associated with the membrane and may be essential for growth and survival (11, 20). This finding suggests that there is a unique mechanism of in vivo nucleotide acquisition.

MATERIALS AND METHODS

Mycoplasma growth and isolation. The mycoplasma strains used and the media used for cultivation are described in Table 1. Standard mycoplasma broth medium contained 25 g of PPLO broth (Difco Laboratories, Detroit, Mich.) per liter, 10% heat-inactivated (56°C, 30 min) GG-free horse serum (GIBCO/BRL, Grand Island, N.Y.), 5% fresh yeast extract (5), 0.5% dextrose, and 2.5 μg of Cefobid (Pfizer, Inc., New York, N.Y.) per ml; the pH was 7.8. Broth A was prepared as described previously (4). Friis medium contained 12 g of PPLO broth per liter, 12.6 g of brain heart infusion (Difco Laboratories) per liter, 20% heat-inactivated horse serum (GIBCO/BRL), 5% fresh yeast extract, 7.2% Hanks balanced salts solution (GIBCO/BRL), 0.5% dextrose, and 2.5 μg of Cefobid per ml; the pH was 7.6. SP4 medium was made as described previously (27) and was modified by adding 0.02% arginine. Frey's medium was prepared as described previously and was modified by adding 0.01% NADH (5). Cultures were obtained from a stock culture maintained at −70°C, inoculated into fresh broth, and incubated statically at 37°C. Cultures were maintained by serial passage. The total number of in vitro passages prior to testing could not be accurately determined.

Nuclease assay. Mycoplasma cultures were harvested at the mid- to late log phase of growth by centrifugation at 12,000 × *g* for 4 min, washed once with 0.01 M sodium phosphate–0.14 M sodium chloride (pH 7.3) (PBS), and resuspended at a

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TABLE 1. Mycoplasma strains and species, sources, losses in viability, and nuclease activities

Species or subspecies[a]	Strain	Host	Medium[b]	Source	Loss in viability (%)[c]	Nuclease activity[d]		
						Intact whole cells	Released[e]	Total[f]
Mycoplasma pulmonis	UAB6510	Rodent	P	M. K. Davidson	NL	1,250	±	20
	UAB5782	Rodent	A	G. H. Cassell	NL	1,250	±	20
	UABCT	Rodent	A	M. K. Davidson	ND	1,250	±	208
	UABT	Rodent	A	M. K. Davidson	NL	1,250	±	26
	JB	Rodent	A	G. H. Cassell	ND	1,250	−	1,250
	19612 (PG34)	Rodent	P	ATCC[g]	ND	833	±	416
	KD735	Rodent	A	K. Dybvig	NL	625	±	625
	X1048	Rodent	A	M. K. Davidson	NL	1,250	±	625
	ISM3050	Rodent	A	F. C. Minion	75	1,250	312	156
	M1	Rodent	P	M. K. Davidson	NL	156	625	78
	66	Rodent	P	M. K. Davidson	NL	78	416	20
Mycoplasma arthritidis	19611	Rodent	PA	ATCC	ND	104	±	96
Mycoplasma neurolyticum	19988	Rodent	F	ATCC	44	416	1,250	156
Mycoplasma hyopneumoniae	J	Swine	F	R. F. Ross	ND	833	−	625
	232A	Swine	F	R. F. Ross	ND	833	−	625
Mycoplasma hyorhinis	GDL	Swine	F	R. F. Ross	NL	208	833	156
Mycoplasma hyosynoviae	25591	Swine	FA	ATCC	ND	312	−	312
Mycoplasma dispar	SDO	Bovine	F	R. Rosenbusch	14	26	416	19
Mycoplasma bovis	79-27323	Bovine	F	R. Rosenbusch	ND	208	833	312
Mycoplasma bovoculi	C52	Bovine	F	R. Rosenbusch	NL	52	416	39
Mycoplasma capricolum	27343	Caprine	F	ATCC	ND	−	−	6,250[h]
Mycoplasma gallisepticum	19610	Avian	P	ATCC	14	416	833	20
	15302	Avian	P	ATCC	ND	833	833	52
	A5969	Avian	P	Hofstead	ND	208	±	20
Mycoplasma meleagridis	25294	Avian	FA	ATCC	ND	833	−	833
Mycoplasma gallinacium		Avian	P	Field isolate	ND	104	208	26
Mycoplasma fermentans	PG18		S	S.-C. Lo	ND	−	−	625
Mycoplasma fermentans subsp. *incognitus*		Human	P	S.-C. Lo	ND	416	1,250	39
Mycoplasma penetrans		Human	S	S.-C. Lo	ND	±	−	416
Mycoplasma hominis	1620	Human	PA	L. D. Olson	ND	32	208	39
Mycoplasma pneumoniae	15531	Human	P	ATCC	ND	±	−	416
Acholeplasma laidlawii	23206 (PG8)		F	ATCC	NL	104	208	20
	14192		F	ATCC	ND	208	±	20
Acholeplasma sp.	ISM1499		P	This laboratory	ND	104	±	10

[a] For every species and strain except *Acholeplasma* sp. strain ISM1499, which is a high-passage laboratory isolate that was passaged at least 150 times in vitro, the number of in vitro passages in our laboratory was less than 20. The actual numbers of passages before we received the strains are not known.

[b] P, standard PPLO broth medium; F, Friis medium; S, SP4 medium; FR, Frey's medium; PA, PPLO broth supplemented with arginine; FA, Friis medium supplemented with arginine.

[c] Percentage of viable cells lost during a 30-min incubation at 37°C in assay buffer supplemented with glucose. NL, no detectable loss in CFU; ND, not determined.

[d] Nuclease activity was determined subjectively as the number of nanograms of protein required to digest 250 ng of lambda DNA as described in Materials and Methods. The data are the means of at least three replicates. ±, a small amount of nuclease activity was present, but accurate measurement was beyond the sensitivity of the assay; −, complete lack of activity under all experimental conditions.

[e] Amount of nuclease activity released during a 30-min incubation at 37°C in assay buffer as described in Materials and Methods.

[f] Activity associated with detergent-solubilized whole cells.

[g] ATCC, American Type Culture Collection, Rockville, Md.

[h] Only 10 mM magnesium was included in the preparation. See Table 2.

concentration of 100 μg of protein per ml in PBS containing 1% dextrose, 2 mM $CaCl_2$, and 2 mM $MgCl_2$ (PBS-CM). In some studies, optimal buffer conditions were examined by altering the magnesium and calcium concentrations in the assay buffer. Protein concentrations were determined with the Bio-Rad dye reagent (Bio-Rad Laboratories, Richmond, Calif.) by using bovine serum albumin as a reference standard. To determine external activity, 25 μl of suspended cells, representing 2.5 μg of protein, was diluted in twofold increments in a 96-well plate with PBS-CM. An equal volume of lambda DNA (10 μg/ml of PBS; GIBCO/BRL) was added to each well, and the plate was then incubated for 30 min at 37°C. Lambda DNA digestion was stopped by adding 7 μl of borate sample buffer (7) to each well. Digestion products were analyzed by loading a Tris-borate agarose gel with 8 μl of sample per well, running the gel at 100 V for 45 min, and then staining it with ethidium bromide. Activity was expressed as the amount of mycoplasma protein that resulted in the complete loss of 250 ng of intact, full-size lambda DNA in 50 μl of PBS-CM at 37°C during a 30-min incubation period. The end point was determined by analyzing the reaction mixtures on agarose gels and identifying the dilution at which the undigested, full-length lambda DNA first disappeared (Fig. 1B, lane 2). Each species was tested at least three times by using different passages, and the mean value for nuclease activity was determined.

In order to determine whether the nuclease activity was released from intact cells or located on the external membrane surface, the following experiments were performed. Mycoplasma suspensions were incubated at 37°C for 30 min in assay buffer, the cells and membrane fragments were pelleted by centrifugation in a microcentrifuge at 12,000 rpm for 15 min, and 25 μl of each resulting supernatant fraction was diluted in

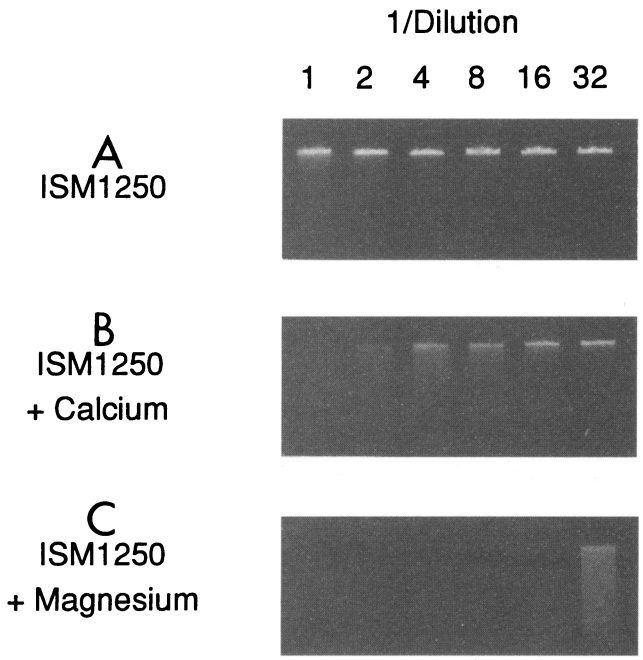

FIG. 1. Effects of calcium and magnesium on membrane nuclease activity in *Acholeplasma* sp. strain ISM1499. An overnight culture of strain ISM1499 was washed and prepared for the whole-cell nuclease assay as described in Materials and Methods. The beginning concentration of mycoplasma protein was approximately 5% of the normal concentration so that we could measure the effects of divalent cation addition. Mycoplasmas were added to the first well and serially diluted twofold with PBS. In parallel wells, calcium or magnesium was added to a concentration of 5 mM to the diluted cells. An equal volume of DNA substrate in PBS was then added to each well, and the plate was incubated for 30 min at 37°C. The digestion products were analyzed on a Tris-borate agarose gel.

twofold increments with PBS-CM and assayed as described above. CFU were measured before and after incubation to assess cell viability. Hexokinase activity (17) was measured in the supernatant fractions in parallel experiments from which dextrose was omitted to assess cell membrane integrity. Mycoplasma membranes were isolated by osmotic shock and were purified on sucrose gradients as described previously (16), and the purity of each membrane preparation was assessed by measuring its hexokinase activity (17). In other experiments, the total nuclease activity of each mycoplasmal species was determined by diluting 25 μl of suspended cells in PBS-CM containing 0.1% Tween 20 prior to addition of lambda DNA.

SDS-PAGE nuclease assay. To examine the variability in nuclease size, a modified SDS-PAGE system was used. The mycoplasmas were grown to mid-log phase, pelleted by centrifugation at 12,000 × *g* for 4 min, washed with PBS, resuspended in 40% glycerol, and stored at −20°C until they were used. Protein samples were prepared by mixing cells or supernatant 1:1 with SDS-PAGE sample buffer, and the preparations were incubated at 60°C for 15 min and centrifuged at 12,000 × *g* for 5 min) prior to loading. For some experiments, samples were boiled for 5 min prior to loading.

SDS-PAGE was performed by using a modification of the method of Laemmli (6) and a Bio-Rad mini-PROTEAN II unit. Sheared salmon sperm DNA (Sigma Chemical Co., St. Louis, Mo.) was incorporated into the 10% resolving layer at a final concentration of 10 μg/ml. The anode buffer contained (per liter) 3 g of Trizma base, 14.4 g of glycine, and 1 g of SDS (pH 8.3). The cathode buffer was 2× anode buffer supplemented with 0.15% SDS. The gels were electrophoresed at a current of no more than 10 mA per gel at 4°C until the dye front exited the gel. The optimal conditions for nuclease renaturation were as follows. The gels were washed by rocking them four times for 15 min in 100 volumes of incubation buffer (0.04 M Tris [pH 7.5], 0.01% casein, 0.04% β-mercaptoethanol), kept in incubation buffer overnight at room temperature, and then incubated statically for 8 h at 37°C in 100 volumes of incubation buffer supplemented with 2 mM $CaCl_2$ and 2 mM $MgCl_2$. The gels were stained with ethidium bromide (0.5 to 1 mg/ml) for 5 min, destained in water for 20 to 30 min, and subjected to UV illumination. Nuclease activity was detected as nonfluorescing regions on the gels. After photography, the gels were rinsed again with water and fixed in 50% ethanol–12% acetic acid for 1 h. The gels were then stained by using a silver staining kit (Accurate Scientific Chemical Co., Westbury, N.Y.) according to the manufacturer's directions. Silver-stained gels typically had a dark background because of the presence of DNA in the gels, but staining was sufficient to determine the positions of the molecular weight standards (GIBCO/BRL).

TABLE 2. Comparison of cation effects on membrane-associated nuclease activity

Organism	Nuclease activity in the presence of[a]:				
	2 mM Mg	5 mM Mg	10 mM Mg	2 mM Mg + 2 mM Ca	5 mM Mg + 5 mM Ca
M. pulmonis X1048	0	0	0	1,562	1,562
M. pulmonis UAB6510	0	0	±	1,562	1,562
M. pulmonis M1	390	390	390	390	781
M. capricolum	0	0	6,250	0	0

[a] The effects of different magnesium and calcium concentrations on the nuclease activities associated with whole cells were determined as described in Materials and Methods. Nuclease activity is expressed as the number of nanograms of protein required to digest 250 ng of lambda DNA. ±, a small amount of nuclease was present, but accurate measurement was beyond the sensitivity of the assay.

RESULTS

Nuclease activity comparison. To effectively compare levels of nuclease activity in different mycoplasma species, we developed an assay in which lambda DNA was used as a substrate and then the digestion products were analyzed on 0.7% agarose gels (Fig. 1). Divalent cations had a measurable effect on nuclease activity (Fig. 1 and Table 2). Addition of calcium resulted in a 32-fold increase (five-well difference) in *Acholeplasma* sp. strain ISM1499 activity, whereas addition of magnesium increased the activity 128-fold (Fig. 1). The combination of 2 mM magnesium and 2 mM calcium resulted in maximum activity in *M. pulmonis* and all of the other species (data not shown) except *Mycoplasma capricolum*. In *M. capricolum*, nuclease activity was detectable only when 10 mM magnesium was added, and the presence of calcium was inhibitory (Table 2). Interestingly, in two of the three *M. pulmonis* strains tested, X1048 and UAB6510, nuclease activity was observed only when calcium was present.

The results of the lambda nuclease assay are shown in Table 1. Nuclease activity was present in all of the strains and species tested, and the levels of activity varied in different strains and species. To control for nonspecific effects such as the association of medium components with the mycoplasma membrane

surface (2, 13, 30), broth was screened for nuclease activity before it was used. The medium controls were consistently negative. The assay sensitivity when DNase I (Sigma) was used was determined to be 0.0008 U (data not shown).

To determine whether significant proportions of the observed nuclease activities were cytoplasmic or membrane associated, assays were performed to measure (i) the amounts of nuclease activity secreted from the cells or released from the surfaces during incubation in assay buffer supplemented with glucose, (ii) the effects of incubation on cell viability, (iii) the amounts of nuclease and hexokinase activities associated with purified membranes obtained from selected species, and (iv) the total activities of detergent-solubilized cells. Several mycoplasmal species secreted or released nuclease activity into the assay buffer during incubation (Table 1), but incubation in assay buffer had no measurable effect on the cell viability of most species. The exceptions included *M. pulmonis* ISM3050, *Mycoplasma neurolyticum*, *Mycoplasma dispar*, and *Mycoplasma gallisepticum* 19610. Spontaneous lysis of cells with concomitant release of internal nucleases was ruled out as a factor in the appearance of soluble nuclease activity because less than 2% (0.18 to 1.68%) of the total cell hexokinase activity was released into the assay buffer by *M. pulmonis* X1048, *M. gallisepticum* 19610, *Mycoplasma bovoculi*, and *M. dispar* during incubation (data not shown). There was no difference in the percentage of hexokinase released between strains that also released nuclease activity (*M. bovoculi*, *M. dispar*, and *M. gallisepticum*) and *M. pulmonis* X1048, which released very low levels of nuclease activity.

Membranes were purified from *M. pulmonis* X1048, *M. gallisepticum* 19610, and *M. dispar* SDO, strains that represented species that released low, moderate, and high levels of nuclease activity into the assay buffer during incubation, respectively. No hexokinase activity was detected in the membrane preparations, but the nuclease SDS-PAGE patterns were very similar to the patterns obtained with whole cells (Fig. 2).

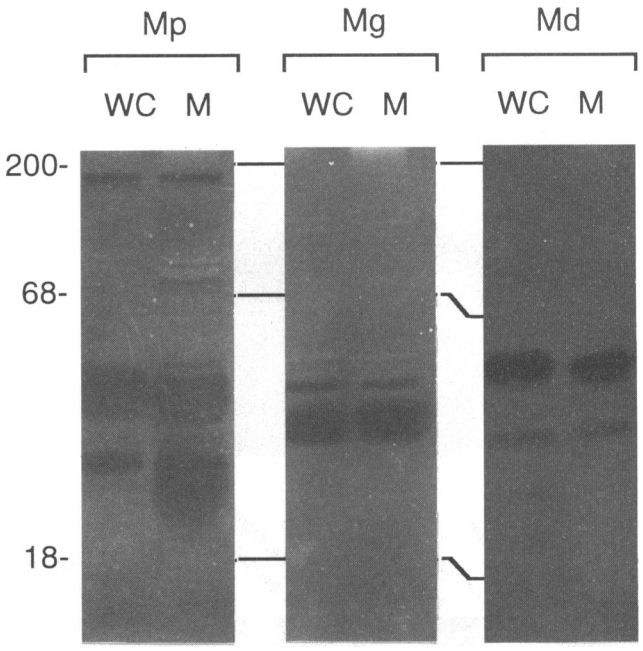

FIG. 2. Comparison of nuclease activities in whole cells and purified membranes as determined by the SDS-PAGE nuclease assay. Membranes were purified on sucrose gradients and the SDS-PAGE nuclease assay was performed as described in Materials and Methods. The amount of protein added to each lane was as follows: *M. pulmonis* X1048 (Mp), 5.4 μg of whole-cell protein (lane WC) and 1.7 μg of membrane protein (lane M); *M. gallisepticum* 19610 (Mg), 5.6 μg of whole-cell protein (lane WC) and 1.4 μg of membrane protein (lane M); *M. dispar* SDO (Md), 2.5 μg of whole-cell protein (lane WC) and 0.7 μg of membrane protein (lane M). The positions of size markers (in kilodaltons) are indicated on the left.

The activity of detergent-lysed cells was determined by incorporating Tween 20 into the buffers of the lambda assay, resulting in the lysis of the cell wall-less mycoplasmas. The results of this comparison are shown in Table 1. In many of the species, there was a dramatic increase in the levels of activity compared with the levels of activity in whole cells or cell lysates.

SDS-PAGE nuclease assay conditions. The SDS-PAGE nuclease assay was optimized for source of SDS, sample incubation temperature, running, renaturation, and storage conditions. Sequanol grade SDS obtained from Pierce Chemical Co., Rockford, Ill., and SDS obtained from BDH Chemicals, Ltd., Poole, England, were sufficiently pure to prevent permanent nuclease inactivation, supporting the findings obtained previously (5a, 9). No effect of sample incubation temperature (room temperature for 30 min, 37°C for 15 min, 60°C for 15 min, 100°C for 5 min) on banding patterns or nuclease activity was observed. The running gel temperatures, however, did have a dramatic effect on banding patterns and reproducibility (data not shown). When preparations were separated at a temperature greater than 10°C, nuclease activity was permanently inactivated. The most consistent results were obtained when the gels were run slowly in a Bio-Rad mini-PROTEAN II apparatus in a cold room with precooled buffers. The use of 0.25% SDS in the cathode buffer also increased the resolution of the bands, so 0.25% SDS was routinely used in this study. Variation in the renaturation temperature (4, 22, 30, or 37°C) or the addition of Nonidet P-40 (0.001 to 0.1%) had no effect on banding patterns. The presence of 0.2 M NaCl was inhibitory. Additions of proteins to the renaturation buffer were stimulatory in the following order: 1% Bacto Peptone > 0.01 to 1% casein > 0.01% bovine serum albumin > 0.01 to 1% gelatin > 1% egg albumin >> 1% skim milk. Concentrations of bovine serum albumin higher than 0.5% were inhibitory. The addition of β-mercaptoethanol (5×10^{-4} to 5×10^{-2} M) or dithiothreitol (5×10^{-6} M) significantly increased nuclease activity, as did the inclusion of magnesium and calcium. Nuclease activities were stable for several months when preparations were stored in 40% glycerol at −20°C.

Analysis of mycoplasmal nucleases by SDS-PAGE. The protein profiles of the nucleases from *M. pulmonis* strains are shown in Fig. 3A. The amounts of total protein in the lanes were adjusted in order to facilitate analysis. In general, 1 to 10 μg of protein was loaded into each lane, but the level of nuclease activity in strain 66 was significantly higher and less protein was required for analysis. The banding patterns of all of the strains tested except PG34 and JB were very similar. The most consistent feature of these patterns was the prominent triplet of nuclease activity in the 35- to 50-kDa range.

The nuclease patterns obtained for other mycoplasma species were variable. The three *M. gallisepticum* strains tested produced the same pattern of bands (Fig. 3B). The nuclease patterns of bovine and porcine species generally appeared to be similar, but there were no bands of similar sizes in the different species.

In general, the human mycoplasma species exhibited low levels of nuclease activity (Table 1). To detect nuclease activity

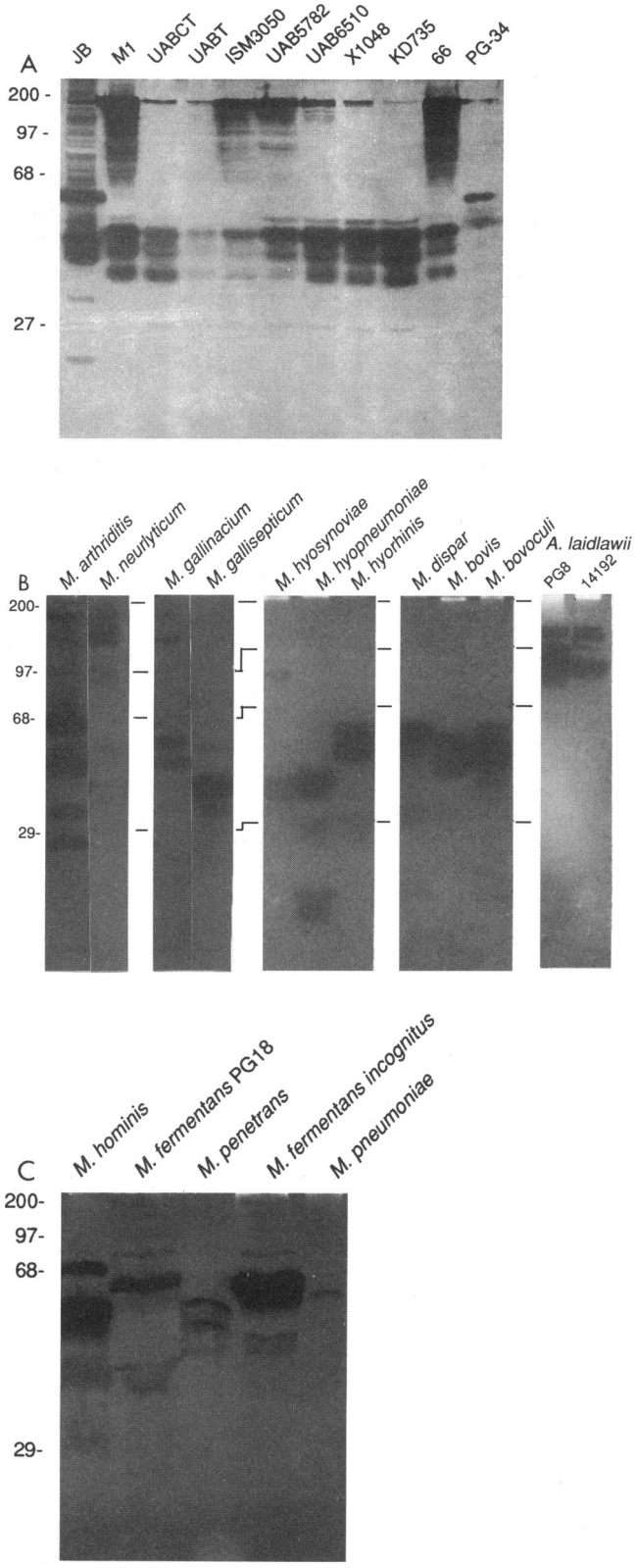

FIG. 3. Nuclease SDS-PAGE band patterns of various mycoplasma species. (A) *M. pulmonis* strains. (B) Various animal mycoplasmal pathogens and *Acholeplasma* species. (C) Human mycoplasma species. Washed mycoplasmas were suspended in 40% glycerol and stored at −20°C until they were used. For electrophoresis, cells were mixed 1:1 with SDS-PAGE sample buffer and incubated at 60°C for 15 min, and the proteins were separated on a 10% polyacrylamide gel containing 10 μg of DNA per ml. After electrophoresis, the gels were washed as described in Materials and Methods. To monitor digestion, gels were stained with ethidium bromide, subjected to UV illumination, and photographed with a Polaroid MP-4 camera and type 57 film. Species names and strain designations are indicated at the top of each gel. The positions of size markers (in kilodaltons) are indicated on the left.

in *Mycoplasma fermentans* PG-18 and *Mycoplasma penetrans* samples, more protein was loaded into each lane (24 and 57 μg, respectively). *M. fermentans* PG18 and *M. fermentans* subsp. *incognitus* produced similar patterns which differed primarily in the size of the lower-molecular-weight doublet (Fig. 3C). *Mycoplasma pneumoniae* exhibited very low activity as shown by the single band (Fig. 3C) and by the level of total activity observed after detergent solubilization (Table 1). Of the human species tested, *Mycoplasma hominis* exhibited the highest level of membrane-associated nuclease activity (Table 1).

DISCUSSION

Our data indicate that membrane-associated nuclease activities are a common feature of members of the class *Mollicutes*. The lambda assay detected nuclease activity in every species tested, confirming and expanding on previous reports of this activity in *M. pulmonis* (11) and in *Acholeplasma laidlawii* (20). The vast majority of activity in most mycoplasma species was cell and membrane associated, and little nuclease activity was released when organisms were incubated in assay buffer. This finding was confirmed by monitoring cell viability and hexokinase release. *M. capricolum* was unusual because its low level of nuclease activity was inhibited by calcium and required a high magnesium concentration (10 mM). Some species released nuclease activity into the extracellular environment during incubation although it was not clear whether this activity was the result of an active export process or a degradation product of membrane-associated activity. This soluble activity could be accounted for by cell lysis in only four of the strains tested; it could not be accounted for by cell lysis in the majority of species, in which no loss of viability occurred during incubation and only a small amount of released hexokinase was detected. If nuclease activity is secreted in some species, it may provide a convenient model system to study mycoplasmal protein translocation.

Despite careful adherence to the conditions described in previous studies in which SDS-PAGE analysis of nuclease-containing preparations was used (9, 20, 23), we were initially unable to obtain reproducible nuclease patterns in mycoplasma samples. Careful examination of the system variables revealed that control of the gel running temperature, the quality of the SDS, higher SDS concentrations in the cathode buffer, and addition of casein and β-mercaptoethanol to the wash buffer were major factors in increasing reproducibility. In addition, the stability of nuclease patterns during storage at −20°C enhanced the results significantly.

One unusual observation was the number and variability of the nuclease bands. Since mycoplasmas are often considered to be minimal living units, it was surprising that multiple nucleases were present. The presence in mycoplasma membranes of variable lipoproteins which exhibit size and charge variability has received considerable attention recently (1, 21, 22). Although the results of preliminary experiments indicate that the nucleases of *M. pulmonis* are not modified by palmitic acid (data not shown), the membrane-bound nucleases may exhibit

peptide length variability based on a mechanism similar to the mechanism observed with the variable lipoproteins.

It was hoped that the patterns could provide a way to differentiate between mycoplasma species, and our studies performed with multiple strains of *M. pulmonis* and *M. gallisepticum* indicated that most strains of a species could be categorized on the basis of the banding profile (Fig. 3A). The three *M. capricolum* strains tested had similarly low, but detectable, levels of activity (data not shown). The number of strains tested was limited, however, and additional studies will be required to expand these observations.

In conclusion, mycoplasmas produce multiple membrane-associated nucleases which may provide a mechanism for obtaining nucleotide precursors by degrading host chromosomal DNA. Whether this activity is actively involved in the pathogenic process is not known. Because of the requirement for nucleic acid precursors, this activity may be expected to contribute significantly to the survival of the organisms in hosts.

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