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**Characterization of membrane-associated nuclease
activity in *Mycoplasma pulmonis***

by

Karalee Jean Jarvill-Taylor

**A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY**

**Department: Microbiology, Immunology and Preventive Medicine
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Major Professor: F. Chris Minion**

**Iowa State University
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**Graduate College
Iowa State University**

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Karalee Jean Jarvill-Taylor

has met the dissertation requirements of Iowa State University

Signature was redacted for privacy.

Major Professor

Signature was redacted for privacy.

For the Interdepartmental Major

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For the Major Department

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For the Graduate College

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LIST OF ABBREVIATIONS

A.	<i>Agrobacterium</i>
aa	amino acid
AIDS	acquired immunodeficiency syndrome
AP	apurinic/apyrimidinic
ATP	adenosine triphosphate
B.	<i>Bacillus</i>
C.	<i>Corynebacterium</i>
dCTP	deoxycytosine triphosphate
DNA	deoxyribonucleic acid
E.	<i>Escherichia</i>
H.	<i>Haemophilus</i>
h	hour
Ig	immunoglobulin
IL	interleukin
IFN	interferon
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilobase
kDa	kilodaltons
L.	<i>Listeria</i>
LPS	lipopolysaccharide
M.	<i>Mycoplasma</i>
M	molar
μg	microgram
μM	micromolar
mA	milliamperes
mg	milligram
min	minute
ml	milliliter
mm	millimeter
mM	millimolar
N.	<i>Neisseria</i>
NK	natural killer

pmole	picomole
RNA	ribonucleic acid
rRNA	ribosomal RNA
<i>S.</i>	<i>Staphylococcus</i>
SDS-PAGE	sodium dodecyl sulfate- polyacrylamide gel Electrophoresis
subsp.	subspecies
TNF	tumor necrosis factor
tRNA	transfer RNA
UV light	ultraviolet light
Vlp	variable lipoprotein
Vsp	variable surface protein

CHAPTER 1. GENERAL INTRODUCTION

Introduction

Bacteria which belong to the family *Mollicutes* and genus *Mycoplasma*, referred to as mycoplasmas, are commonly found in nature. The term mollicutes will be used to refer to all members of the family, including the nonpathogenic *Acholeplasma* species. While many mycoplasma can be considered part of the normal commensal flora in vertebrates, others are well established pathogens of man and animals (219). The diseases resulting from mycoplasmal infections are usually chronic in nature with the host immune system playing an important role in development of pathological lesions. Areas of colonization are usually limited to the lungs, genital tract and joints. Diseases are often high in morbidity, but mortality is rare.

Although initially classified together because of their common lack of a cell wall, these prokaryotes have many other features in common. They are among the smallest and simplest bacteria known. Genetically, they contain small genomes, have one to two copies of rRNA genes, and have a limited number of tRNAs. Biochemically, they lack most metabolic pathways, exhibited by the inability to produce amino acids, fatty acids, cholesterol, and nucleotides. Hence, it is not surprising to find them in a parasitic relationship. Razin has written the following:

Living in the relatively constant environment of their host, mycoplasmas could do without the protection of a rigid cell wall. In this way the considerable number of genes involved in synthesis of the bacterial wall polymers were eliminated. On becoming parasites, mycoplasmas could do without many biosynthetic and degradative pathways and thus do without a considerable number of genes. In fact, mycoplasmas depend on their host for essentially the complete spectrum of amino acids, fatty acids, cholesterol and vitamins (169).

This gene absence includes the tricarboxylic acid pathway in all mycoplasmas and the glycolytic pathway for a select few (169). The recent completion of the sequencing of the entire genome of *Mycoplasma genitalium* verifies the lack of metabolic capabilities in the mycoplasmas (62).

In any parasitic relationship, the boundary between the parasite and the host is a battlefield. The parasite must acquire nutrients and the host must engage in defense. In mycoplasmal infections, the battlefield location is the membrane interface between the mycoplasma and the epithelial cell of the mucosal surface. Mycoplasmas maintain a tight adherence to this epithelial layer in spite of an active immune response by the host.

Consequently, the study of adherence has been a primary thrust of mycoplasma research. In spite of this activity, an understanding of actual adherence mechanisms from any mycoplasma species has remained elusive. Likewise, an understanding of the host defense strategy is lacking. It is well known that the host defense is ineffective at clearing the organism, and often the continued assault by the host defense system leads to a much more serious destruction of host tissues.

Adherence factors are the most well known and characterized proteins of the mycoplasma membrane. However, other activities must be present at the surface to acquire the necessary nutrients mycoplasmas need to survive and to evade itself from the immune system. Along with the adhesins, the membrane components shown in Table 1.1 have been identified. In no case have these functions been proven.

Table 1.1. Membrane activities of mycoplasmas

Activity	Putative Function
Mitogens	nonspecific stimulation of the immune system leading to a nonproductive host defense (146)
Variable lipoproteins	evasion of the immune system (231)
Capsules	antiphagocytic (217)
Fc, Fab binding proteins	alter antibody function (217)
Hemolysins	acquisition of cholesterol and phospholipids (83)
Phospholipases	acquisition of phospholipids (217)
Nucleases	acquisition of nucleotides (139)

This dissertation focuses on the membrane-associated nucleases of mycoplasmas. Mycoplasmas must acquire nucleotides from their surrounding environment, and it is hypothesized that the nucleases are involved in this acquisition process. Although mycoplasmas must have internal nucleases for DNA repair systems, and in a few species restriction-modification systems have been discovered (50, 195), membrane-associated nucleases had only been discovered in *Mycoplasma pulmonis* (139). This research has mainly study those nuclease activities associated with the membrane of the genus *Mycoplasma*. The following hypothesis has been examined:

Since purines and pyrimidines are essential mycoplasma nutrients and must be obtained from the host environment, nuclease activities are an important part of mycoplasma membranes. Consequently, it is proposed that mycoplasmas produce

multiple membrane-associated nucleolytic proteins as products of different structural genes. This redundancy would serve to reduce the likelihood of lethal mutations arising at high frequency in a single essential gene and thus prolong survival of the organism.

The specific aims of the project are as follows:

- To establish nuclease detection assays
- To examine the prevalence of membrane nucleases in various mycoplasma species
- To characterize the membrane nucleases of *M. pulmonis* biochemically
- To clone at least one membrane nuclease gene of *M. pulmonis* and characterize the sequence
- To search for similar sequences in other mycoplasmal species

Dissertation Organization

This dissertation has been prepared in an alternative thesis format, and contains five chapters. Chapter 1 is an general introduction and contains the literature review. Chapters 2 through 4 are manuscripts complete in themselves, with an abstract, introduction, materials and methods, results, discussion, and acknowledgments. The second chapter has been published and discusses the prevalence of nucleases among many different species, and characterizes the amount of activity present and the nucleolytic protein patterns by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The third chapter details the discovery of at least two nuclease genes from a genomic library and characterizes one gene with regard to its sequence and amino acid profile analysis. Finally, the fourth chapter describes the presence of the nucleotide excision repair system in mycoplasmas. A general discussion of this research is presented in Chapter 5. The references cited in the text are located after Appendix C. Appendix A and Appendix B report the biochemical analysis of the nucleases from *Acholeplasma oculi* and *M. pulmonis*. Appendix C is a listing of all plasmids constructed during this project.

Literature Review

An ideal parasite may be described as an organism which is able to survive and replicate itself in a dependent relationship with the host for an extended period of time with little or no apparent recognition by the host's immune defenses. These organisms must also be able to spread to other sites. In most aspects, mycoplasmas are ideal parasites. These organisms acquire most of their macromolecules from the host and live in a saprophytic relationship for

months or even years without elimination. Clinical symptoms resulting from most mycoplasmal infections often go unnoticed or result in only subtle physical changes.

The purpose of this section is to review the current knowledge of the mycoplasma infectious processes. Specifically, it is of interest to know why mycoplasmas exist in a parasitic relationship rather than being able to survive as true free living organisms. It is also of interest to study aspects of the host immune response against these organisms and the resultant tissue destruction associated with the disease. In many mycoplasmal species, described membrane activities are postulated to aid in maintaining and furthering continued survival in the host. The last section of the literature review will discuss the characteristics of several nonspecific bacterial nucleases which have been described and the nuclease activities found in mycoplasmas.

Mycoplasmas

General characteristics

Mycoplasmas contain essentially the minimal number of organizational units which are apparently necessary for cell growth and replication: a plasma membrane to separate itself from the exterior environment; ribosomes and a minimal number of tRNAs used to assemble proteins; and a circular double-stranded DNA molecule to provide the necessary genetic information (166, 169). Their genome sizes range from 580 kilobase pairs (kbp) for *M. genitalium* to 1350 kbp for *Mycoplasma mycoides* subspecies *mycoides* (20, 62). It has been held that only DNA polymerase III is used by mycoplasmas to replicate the chromosome (169) but in the DNA sequence of both *M. genitalium* and *Mycoplasma capricolum*, a DNA polymerase I homolog was found (19, 62). Most chromosomes contain one or two rRNA operons. In comparison, *Escherichia coli* contains seven rRNA operons (20). Only 29 of the potential 61 tRNA types have been found in *M. capricolum* (20), and 33 tRNAs, corresponding to 32 different anticodons, were recently identified in *Mycoplasma pneumoniae* (201). One of these tRNAs deserves attention. Mycoplasmas produce a tryptophan charged tRNA with the anticodon TCA (80, 233). In the universal genetic code, the UGA codon is a stop codon, has no corresponding tRNA, and is recognized by release factor 2 to bring about translation termination.

The gene organization of mycoplasmas and mechanisms of transcription and translation are similar to other eubacteria (20). Both monocistronic and polycistronic transcriptional units have been characterized, but repressor proteins have not been identified (20). Assuming that 20-30% of the genome consists of intergenic spacer regions, transcriptional signals, etc.,

the estimated coding capacity of mycoplasmas is between 400-500 proteins (145). The genome of *M. genitalium* encoded for 482 genes, comprising 88% of the genome, and it is thought to reveal the near-minimal set of genes required for independent life (73). This number of proteins is insufficient to supply all of the anabolic pathways.

Mycoplasmas contain a truncated respiratory system. They lack quinones and cytochromes, components of the electron transport system. In fermentative mycoplasmas, all ATP is generated through glycolysis. Nonfermentative mycoplasmas lack the glycolytic cycle and produce ATP by a 3-enzyme arginine pathway (91, 165).

There is also well documented evidence that mycoplasmas lack the ability to synthesize their nucleotides, phospholipids and cholesterol and many of the amino acids and vitamins (reviewed in (131)). All mycoplasmas lack the orotic acid pathway for pyrimidine synthesis and the enzymatic pathways needed for *de novo* synthesis of purine bases (165). Pyrimidine and purine phosphorylases are present to convert nucleotide phosphates to the corresponding free base (166). Mycoplasmas are also either partially or totally incapable of synthesizing long chain fatty acids (204). Some mycoplasmas, like *Mycoplasma gallisepticum*, even require preformed phospholipids for growth (176). In most prokaryotes, the plasma membrane fluidity is maintained by the preferential fatty acid synthesis and incorporation. However, membrane fluidity in mycoplasmas is influenced by the phospholipids they obtain from the environment and is regulated by the incorporation of cholesterol.

The deletion of the majority of metabolic and biosynthetic pathways is advantageous from an energy standpoint, but adapting to a parasitic mode of life has its disadvantages. An increased dependence on transport and acquisition processes is obviously required. In addition, mycoplasmas appear to establish a tight interaction with the host cell surface to possibly facilitate the acquisition of macromolecules.

Genome sequencing

Because of the relatively small genome size of mycoplasmas and their association with human disease, the genome of mycoplasmas are actively being sequenced in three different genome projects. The genome of *M. genitalium* has now been completely sequenced and the sequence deposited in the Genome Sequence Data Base (GSDB) (accession number L43967). Additional sequence similarity data and coordinates of the predicted coding regions are available at URL <http://www.tigr.org> (62). Information for the *M. capricolum* sequencing project, although not complete, has been deposited in the EMBL database with accession numbers from Z33005 to Z33376 (19). The genome of *M. pneumoniae* is also being sequenced and some sequence information has been deposited in GenBank. *M. genitalium*

and *M. pneumoniae* were selected for genome sequencing because of their association with human disease. *M. genitalium* appears to have the smallest genome among the mycoplasmas and would represent the minimal number of genes required by mycoplasmas to survive. By comparison *M. capricolum* has one of the larger genomes among the mycoplasmas and does not have many of the growth requirements of other mycoplasma species. Sequencing of this species would indicate the wide range of proteins that can be found in mycoplasmas, and the maximum information mycoplasmas may contain while still being classified as a mycoplasma.

The genomic sequencing of these species verifies many of the biochemical aspects of mycoplasmas known for years, such as truncated respiratory systems, and lack of amino acid, lipid and nucleotide metabolic pathways. The sequence data has also filled in informational gaps. For instance, DNA repair has not been studied in mycoplasmas, and the sequence information sheds light on the types of repair systems mycoplasmas use. It is tempting to assume that since the homologs for many *E. coli* or *Bacillus subtilis* proteins are present, these proteins must function in the same manner as they do in other bacterial species. The wisest course of action, however, is to use the information to guide research endeavors.

Goffeau in a commentary writes the following:

How rapidly will the new information be exploited at the biochemical level? Two facts might hinder such progress, which in principle could result in a definition of the minimal biochemical mechanisms and pathways required for life. One limiting factor might be the relatively small size of the scientific community actively working on molecular aspects of this bacteria (probably a few dozen compared to the thousands of molecular biologists studying yeast or *Escherichia coli*). Another difficulty is that the tools of classical or molecular genetics cannot be applied; no auxotrophic or other mutants are presently available for this parasite, which cannot grow on synthetic media. For further studies to take place, each of the *M. genitalium* genes should be over expressed in a heterologous host - a procedure that remains cumbersome (73).

Mycoplasma pathogenesis

Adherence factors

The prerequisite for mycoplasma parasitism and infection is attachment of the organism to the host cell surface (172). Invasion into the submucosal areas is a rare event. Adherence of mycoplasmas has been actively studied since their discovery, and the genetic and biological basis of adherence are the best studied areas in mycoplasmaology. Most adherence studies have been performed with the human pathogens *M. pneumoniae* and *M. genitalium*. These two species are closely related, and many of the adherence factors found in one have been later discovered in the other. Because *M. pneumoniae* and *M. genitalium* are both flask

shaped organisms and produce a specialized attachment tip, their adherence mechanisms are distinctly different than those of other mycoplasma species which have no similar structure.

The best characterized adhesin is the 170 kDa P1 protein of *M. pneumoniae* (reviewed in (10, 172)). This protein is densely clustered in the attachment tip with small amounts of the protein randomly distributed in the remainder of the cell membrane. The proline-rich C-terminus is believed to assist in the topological organization of the adhesin in the membrane (172). Mutants lacking P1 are unable to attach to epithelial surfaces and monoclonal antibodies directed towards P1 also block adherence (10). The sequence and genetic organization of P1 have also been well characterized. P1 is part of a three gene operon. Hybridization of P1 sequences with the genome indicated that as much as two-thirds of the structural gene for P1 could be found in multiples copies (211). Within these multiple copies are several different repetitive elements, and it has been proposed that rearrangements may occur by placing different copies of a repetitive element within the coding region of a structural gene (186). These repetitive elements appear to have a core element which is surrounded by other larger variable repetitive mosaics (58). Su *et al.* postulated that sequence diversity generated by genetic recombination within the structural gene for P1 and P1-related sequences may affect not only immunogenicity of P1 but, more importantly, the cytoadherence of *M. pneumoniae* and thus ultimately tissue tropism and pathobiology (211). Clearly, the importance of P1 to an infection by *M. pneumoniae* cannot be underestimated.

The finding of P1 positive nonadhering mutants of *M. pneumoniae* suggested that P1 alone was insufficient to mediate attachment. Using these mutants, various proteins have been shown to either be involved directly in adherence or involved in organizing P1 in the attachment tip. Cytoadherence mutants have been classified into the following groups. Class I mutants fail to produce five high molecular mass proteins designated HMW1 to HMW5 (10). They also fail to cluster the cytoadhesin P1 at the tip of the attachment organelle. These proteins have been shown to be part of a cytoskeleton-like scaffolding network in the mycoplasma cell (207, 208), and several of them are phosphorylated (44). Like P1, these proteins also are rich in proline (44, 152). Mutants of this class arise spontaneously and a potential regulatory locus important in their expression has recently been identified (75). Class II mutants lack the 30 kDa adhesin, which is also clustered in the attachment tip and has a proline rich C-terminus (10). Class III mutants lack proteins designated A, B and C (72, 85 and 37 kDa, respectively) (10). These proteins appear to be encoded in the same operon as P1 and are located near P1 in the tip structure (61, 106-108). Class IV mutants are represented by a single mutant which lacks P1 and proteins A, B, and C (10). This mutant has not been studied further. Research continues to define the gene products required for

attachment and construction of the specialized attachment tip. Recently, a proline rich 65 kDa protein was also identified as a component of the scaffolding in the attachment tip (161).

The primary receptors for *M. pneumoniae* are the sialoglycoconjugates on the host cell surfaces (reviewed in (95, 168)). However, neuraminidase treatment of these receptors does not completely abolish adherence, indicating that other types of receptors are also used by this pathogen. Other receptors implicated in *M. pneumoniae* adherence are sulfated glycolipids on metabolically active cells (97) and a sialic acid-free glycoprotein (68). Together, all of these receptors account for 90% of the attachment of *M. pneumoniae* to cell surfaces (61). Sialoglycoconjugates have also been demonstrated to be receptors for *M. genitalium*, *M. gallisepticum*, and *M. synoviae*.

Adhesins in other mycoplasma species have also been identified. In *M. genitalium*, a P1 like protein termed MgPa has been described and sequenced. It is similar both biochemically and genetically to the P1 protein. It has a proline rich C-terminus, is localized to the attachment tip and the chromosome has multiple copies of the gene sequence (39). The hydrophobicity plots of the C-termini of the two proteins are almost superimposable (40), and antibodies to one protein are capable of blocking adherence of the other (10). In addition, to the complete operon, nine repetitive elements that share sequence homology with the MgPa region are found in the genome, equaling 4.7% of the chromosome (62). Recent sequence data from different isolates suggests that recombination between these repetitive regions and the MgPa operon occurs. This recombination may result in antigenic variation within the MgPa protein and potential evasion of the host immune response (158). Homologues of the P30 and HMW3 have been discovered in *M. genitalium* (175), and P1-like adhesin genes have been described for *Mycoplasma pirum* (215), *M. gallisepticum* (45), and *Mycoplasma synoviae* (142).

Several potential adhesin factors in *M. gallisepticum* have been described; a 155 kDa protein was found which reacted to antiserum against P1 (172), an open reading frame encoding a 193 kDa protein was identified by PCR using P1-specific sequences (45), a 64 kDa lipoprotein appears to function in hemagglutination (60), and a 67 kDa surface protein termed pMGA (124) also seems to be involved in hemagglutination. There is the possibility that the 64 kDa protein, the 67 kDa protein and a 69 kDa protein isolated by lectin affinity chromatography (23) are identical and that the differences may be attributable to differences in experimental conditions (124). The pMGA appears to be a lipoprotein (126), and multiple copies of the pMGA gene are found in the *M. gallisepticum* chromosome (125). When four of these genes were analyzed, it was determined that mRNA was produced from all four genes with the mRNA coding for the predominant antigen on the cell being produced at

levels 20 to 40 times the amount of the others. Thus, it appears likely that there is some mechanism for transcriptional control in the selection of the pMGA gene expressed (72).

In contrast to the sialoglycoconjugates being the primary receptors for many of the above described adhesins, other mycoplasma species predominantly appear to use sulfated residues (113). *Mycoplasma hyopneumoniae* has been shown to bind to several diverse sulfated carbohydrate sequences in glycolipids, suggesting that there are several different receptors on the host cell surface (241). Further study of *M. hyopneumoniae* has implicated a 97 kDa protein (242) in adherence. *Mycoplasma hominis* has also been shown to bind primarily to sulfated glycolipids (153) and several potential cytoadhesins have been identified (76). *M. pulmonis* has been shown to bind to sulfated glycolipids (112) and adherence to red blood cells is blocked by sulfated residues (83).

Adherence of *Mycoplasma penetrans* appears to occur through fibronectin binding (71). This species has been associated with acquired immunodeficiency syndrome (AIDS) and is one of three mycoplasmas believed to reside intracellularly in the host (74). The binding to fibronectin appears to induce a cytoskeleton reorganization in the cell, potentially leading to host mediated internalization of the organism (71).

There are a few overriding themes presented by the study of the adherence factors of mycoplasmas. First, these organisms use more than one adhesin type to attach to the host cell surface thus extending their tissue and cell specificity. Second, a significant percentage of genomic information is used for the adhesins. The genes are repeated, at least partially, in all species examined at the genomic level. It is estimated that the P1 sequences comprise up to 6% of the chromosome in *M. pneumoniae* (172). It is assumed that further research will continue to find additional adhesins and cellular receptors.

Host immune response

Disease causing mycoplasmas have been found in virtually every mammal. Table 1.2 lists many of the major pathogens by host species and site of infection. Most mycoplasmas isolated from animals are not known to cause disease, and of those that do cause disease, the incident of disease may be sporadic (197). There is also a high degree of variability in pathogenesis among mycoplasma strains of the same species. Avirulent strains occur naturally, and there can be carriers with no apparent disease until the host is stressed. Simecka *et al.* has stated the following regarding pathogenesis:

The pathogenesis of mycoplasma disease is a complex process influenced by the genetic background of both the host and the organism, environmental factors, and the presence of other infectious agents. Although many virulence factors have been

Table 1.2. Diseases caused by mycoplasmas^a

Host	Organism	Type of Disease
Human	<i>M. pneumoniae</i>	Respiratory
	<i>M. hominis</i>	Urogenital
	<i>M. genitalium</i>	Urogenital, Respiratory
	<i>M. fermentans</i>	Urogenital
	<i>M. fermentans</i> subsp. <i>incognitus</i>	Intracellular, associated with AIDS
	<i>M. penetrans</i>	Intracellular, associated with AIDS
Cattle	<i>M. pirum</i>	associated with AIDS
	<i>M. mycoides</i> subsp. <i>mycoides</i> SC	Respiratory, arthritis
	<i>M. bovis</i>	Respiratory, mastitis arthritis
	<i>M. bovoculi</i>	Ocular
	<i>M. dispar</i>	Respiratory, mastitis
	<i>M. bovigenitalium</i>	Mastitis, Urogenital
	<i>M. bovirhinis</i>	Mastitis, Respiratory
	<i>M. californicum</i>	Mastitis
	<i>M. alkalescens</i>	Arthritis, Respiratory
	<i>M. canadense</i>	Mastitis
Sheep, goats	<i>M. agalactiae</i>	Arthritis, mastitis, systemic
	<i>M. capricolum</i>	Respiratory, arthritis, systemic
	F38 mycoplasma	Respiratory
	<i>M. capri</i>	Respiratory
	<i>M. mycoides</i> subsp. <i>mycoides</i> LC	Respiratory, arthritis, systemic
	<i>M. ovipneumoniae</i>	Respiratory
	<i>M. conjunctivae</i>	Conjunctiva
	<i>M. hyopneumoniae</i>	Respiratory
	<i>M. hyorhinis</i>	Arthritis
	<i>M. hyosynoviae</i>	Arthritis
Swine	<i>M. gallisepticum</i>	Respiratory, Sinuses, eggs, urogenital
	<i>M. meleagridis</i>	Airsacs
Poultry	<i>M. synoviae</i>	Airsacs, arthritis, eggs
	<i>M. iowae</i>	Airsacs, joints
	<i>M. equigenitalium</i>	Urogenital
	<i>M. equirhinis</i>	Throat, tonsils
Horses	<i>M. felis</i>	Respiratory
	<i>M. arthritis</i>	Arthritis
Rodents	<i>M. pulmonis</i>	Respiratory, Urogenital, Arthritis

^a taken from (95, 183, 197)

suggested for various mycoplasmas, there is no clear case of cause and effect between these factors and pathogenicity (197).

Also contributing to the pathology of mycoplasma infections is the host immune response. A lymphocytic infiltration is an integral part of lesion development (197). There is a balance reached between the host and the mycoplasmas which leads to a persistent long term infection in most situations.

Humoral immune response

The interaction of B cells with mycoplasmas can be divided into two categories, a specific response designed to bring about protection to the host, and a nonspecific response caused by the mitogenic properties of mycoplasma membranes. Humoral antibody responses cannot protect against mycoplasmal disease but they seem to prevent dissemination of infections to other tissues. Immune responses may also contribute to the development or exacerbation of disease (199). Although it was initially felt that antibodies were of primary importance in the clearance of the organisms, it has now been conclusively shown that development of an antibody response is usually insufficient to rid the host of mycoplasmas. In some situations, resistance to infection can be transferred passively with antibodies, as in protection of mice from *M. pulmonis*. However, even with the same species, passive transfer of antibodies does not work in other situations, as shown in the lack of protection to *M. pulmonis* in rats (78).

Antibodies of the IgM, IgG and IgA classes are produced during mycoplasma infections, but their production often occurs after the infection has established itself. In some cases, detectable levels of antibodies are not produced for weeks or even months after infection. IgM are detected initially, and the presence of IgM antibodies usually indicates an active disease rather than a prior infection or carrier state (199). The antibody class that appears second in the infection is dependent upon the host, the species, and route of infection. There may be some preferences for the production of particular subclasses of IgG, and the regulation and development of those subclasses may be important in defense mechanisms (197). The antibody at the local site may be much more important than a systemic immune response. The initial events in the development of immunity most likely occur either at the site of infection or within nearby lymphoid tissue (199). The most important functions for antibodies are in the activation of complement and in opsonization, both of which are actively used against the organism.

The extent of the humoral immune response may also play a role in the severity of the disease. With *M. pulmonis*, severity of disease appeared to correlate with antibody response.

Simecka *et al.* found that in LEW rats, which have more severe disease when infected with *M. pulmonis*, the antibody responses were greater than those found in the relatively resistant F344 rats (196, 198). Antibody responses may also contribute to disease through the formation of immune complexes, as has been suggested for mycoplasma-induced arthritis (225).

Cellular immune response

The lymphocytic infiltrate seen in the lesions of a mycoplasma infections can contain neutrophils, macrophages, natural killer (NK) cells, and T and B lymphocytes. Phagocytosis of mycoplasmas has been the subject of a recent review by Marshall *et al.* (127). Several factors appear to determine the capacity of the host to eradicate mycoplasmas. First, phagocyte activation, either induced directly or through cytokine production, is important in determining mycoplasmacidal activity. Second, the efficiency of phagocytosis varies with both the strain of mycoplasma and the individual host. Third, the nature and origin of a phagocyte influences its capacity to engulf and destroy mycoplasmas, probably as a result of differences in receptors or killing mechanisms. Finally, environmental factors may help modulate the host immune system (127). Mycoplasma antigen is probably sufficient to attract phagocytes to the site of infection, but even after recruitment of the cells, the antigen persists at the location. There is no evidence for phagocytosis of mycoplasmas by neutrophils or macrophages in the absence of antibody, although this point is subject to debate (127). Usually, after opsonization, phagocytosis occurs rapidly and the mycoplasmas are killed intracellularly (127).

The adherence of mycoplasmas to the membrane of the phagocytic cells appears to influence phagocytosis. This adherence may both stimulate the phagocyte and actually inhibit the uptake of other bacteria (reviewed by (197)). These observations may explain why mycoplasma infections activate macrophage function while also increasing susceptibility to other infectious agents. There is also evidence that macrophages are also able to kill surface-associated mycoplasmas through the release of hydrolytic enzymes and activated complement components (214).

Several components from mycoplasma membranes have been shown to induce the release of TNF- α , INF- γ , IL-1 and/or IL-6 from monocytes. Presumably, this stimulation also occurs *in vivo* with monocytes and macrophages. Faulkner *et al.* (53) undertook a study to examine the gene expression and production of various cytokines as they may be associated with pathogenesis in *M. pulmonis* infection in mice. IL-1 α and TNF- α were detected as early as 1 hour after infection. IL-1 β and IL-6 were seen at four to six hours after infection. INF- γ

was not evident until 24 hours post infection. It was suggested that these cytokines were produced by alveolar macrophages. Those studies implicated TNF- α production in the pathogenesis caused by *M. pulmonis*. TNF- α plays a role in the stimulation of inflammatory cytokine production, but it is not solely responsible for the acute inflammatory lesions. IL-1 may mediate, along with TNF- α , neutrophil recruitment. The role of IL-6 was unclear, but it was suggested that this cytokine may modulate the inflammatory response and may have both local and systemic effects during the disease progression. In a similar study, IFN- γ and TNF- α were detected 24 hours after infection and the results supported the idea that cytokines released from the lung tissue may be responsible for disease progression by influencing vascular permeability and accumulation of inflammatory cells (149).

The release of IFN- γ may account for the enhanced NK activity in mycoplasma infections (197). Alternatively, the mycoplasma mitogens may upregulate NK activity, as has been shown with the *M. arthritidis* mitogen (37). It has been suggested that NK cells may function in limiting the spread of certain mycoplasmas, rather than actual clearance from the site of infection (197). In studies using BALB/c mice or mice with severe combined immunodeficiency infected with *M. pulmonis*, clearance of mycoplasmas was inhibited significantly by antibodies directed to IFN- γ or NK cells (103, 104). This indicated that the NK cells may be the principle cell responsible for mycoplasmacidal activity in the respiratory tract of mice (41).

T cells appear to have conflicting roles in some mycoplasma diseases, and T cell subsets may have different effects on the pathogenesis of mycoplasma disease (197). Rats and mice deficient in T cells develop less severe respiratory disease than do normal animals after infection with *M. pulmonis* (43, 234). The role of T cells in a mycoplasma infection has not been extensively studied.

Cell components which affect the host immune response

The membranes of mycoplasmas contain many different components which not only give rise to antibodies in the host, but also influence immune cells. Many of the mycoplasma species have been shown to have mitogenic activity associated with their membranes (reviewed by (128, 146)). A partial list includes *M. pulmonis*, *Mycoplasma neurolyticum*, *M. hyorhinis*, *M. synoviae*, *M. gallisepticum*, *Mycoplasma arginini*, *Mycoplasma orale*, *M. hominis*, *M. pneumoniae*, *M. fermentans*, *M. fermentans* subsp. *incognitus*, and *M. penetrans* (54, 128). *Mycoplasma arthritidis* has been shown to secrete a mitogen belonging to the superantigen family (8), but it is the only mycoplasma identified with this characteristic. Mitogenic activity has been associated with lipoglycans, glycoproteins and an unidentified

lipid in *M. neurolyticum* (86) while proteins and carbohydrates are involved in *M. pulmonis* (147, 199). Using mitogenesis inhibiting monoclonal antibodies, Lapidot *et al.* determined that *M. pulmonis* mitogens are unique membrane complexes of variable molecular weights which are highly susceptible to heat and partially sensitive to reducing agents (105). Thus, it appears that different mycoplasmas exert their mitogenic activities via different cell components, and the organisms may express more than one type of mitogenic constituent. The membranes of *M. pulmonis* have been shown to be both mitogenic and to possess chemoattractant qualities for B cells but not T cells (185, 199). *M. hyorhinis* and *M. arginini* membranes are mitogenic for B-cells (162), whereas the superantigen from *M. arthritidis* is strictly a T-cell mitogen. The immune cell stimulated and the degree of stimulation may differ with each species of mycoplasma and the host involved.

Components of the mycoplasma membrane have also been shown to induce the release of cytokines (reviewed in (128)). These include interferon (INF)- γ , tumor necrosis factor (TNF)- α , interleukin (IL)-1 β and IL-6 (128). Species identified which stimulate release of TNF- α are *M. orale* (65), *M. capricolum* (190), *M. fermentans* (48, 72), *M. arginini* (77), *M. hyorhinis* (93), *M. mycoides* subsp. *mycoides* (178), and *M. gallisepticum* (128). Many of these species also induce either IL-1, IL-6 or nitric oxide. The membrane constituents have been characterized as membrane lipoproteins (77) or as low-molecular weight carbohydrate (235) in *M. arginini*. In *M. fermentans*, cytokine inducing activity is associated with a 48 kDa membrane protein (92) and a lipid structure (143, 144). Both a protein molecule and a glycan moiety from *M. hyorhinis* are responsible for inducing cytokine release (93). As was described for the mitogenic activities in mycoplasmas, different membrane components also appear to be responsible for stimulation of cytokine release from macrophages and monocytes, and each species of mycoplasmas may produce more than one type of activating substance. Although mycoplasmas do not contain lipopolysaccharide (LPS), the finding that many of the cytokine inducing factors contain lipid and lipoglycans may indicate that the membrane has LPS-like biological features. LPS is a potent virulence factor and a known inducer of cytokines (12, 28). Recent evidence indicates that stimulation by mycoplasma membranes uses some, but not all, of the pathways that LPS uses to stimulate cytokine release. Mycoplasmas and LPS appear to trigger the cells through different membrane receptors (164). It is probably a general phenomenon of mycoplasma membranes to be both mitogenic and induce cytokine release.

Another mechanism to circumvent the immune response is the production of Fc or Fab receptors. An Fc receptor has been discovered on *M. arginini*, a common tissue culture cell contaminant (109). This receptor did not appear to have antibody specificity and functions as

a lectin to bind the carbohydrate moiety of the antibody (109). A similar protein was found in *Mycoplasma salivarium*, with specificity towards IgG molecules (189). *M. arginini* and *M. hominis* have also been shown to express a protein which react with the Fab fragments of IgG molecules (2). Research in this area is lacking, and the role of these activities in virulence or immune system evasion has not been addressed (217).

Pathology associated with the infection

The pathogenic potential of mycoplasmas stems from the membrane-membrane interactions with host epithelial cells. Ciliostasis is seen in most infections. The mechanisms involved in cell injury are not well understood. It is clear that several mycoplasmas have the capability to directly cause cell injury. It is also possible that immune or inflammatory responses to the infection may directly contribute to cell injury through "innocent bystander" damage (197). Microscopic lesions generally contain phagocytic cells, show hyperplasia of the epithelium and a lymphoid response in the submucosa. There is a correlation between ciliostasis and tissue injury seen after infection with many mycoplasma species (197). Mycoplasma species can differ in the degree of ciliostasis induced, severity of tissue injury and type of tissue injury (6). Lymphoid hyperplasia and chronic inflammation in the region of the infection are characteristic of disease. In *M. pulmonis*, it is apparent that host responses are probably not the major cause of the initial epithelial damage (205). After initial damage, the evidence leans towards the host cellular immune responses affecting lesion development (90, 226). INF- α and IL-1 were found to contribute to the development of pneumonic lesions in *M. hyopneumoniae* infections (7). Rarely does any mycoplasma infection progress deeper than the epithelial layers. When infections extend beyond the initial area of infection, organisms can be detected in almost any body cavity depending upon the species. For example, the human pathogen *M. pneumoniae* has been implicated in the development of striatal lesions leading to acute neurologic dysfunction (187).

Mycoplasmas produce very few toxins, and of those known, none has been shown to have a direct effect on pathology. Also, it has been speculated that metabolic byproducts, such as hydrogen peroxide production or arginine metabolism, may contribute to pathology. Even though disease pathogenesis is similar between mycoplasma infections, the total package of virulence factors may result in each species having a distinctive progression of cell injury and disease (197).

In the last few years, three species of mycoplasmas, *M. fermentans* subsp. *incognitus*, *M. pirum* and *M. penetrans*, were discovered that appeared to violate many of the previously held dogma regarding mycoplasma pathology. These species were isolated from humans in

association with the AIDS. There continues to be ongoing debate about whether these species are contributors to AIDS, whether they alone are capable of causing AIDS, or if they only have a high prevalence in AIDS patients. The discovery of *M. fermentans* subsp. *incognitus* was unusual in that it was originally thought to be a virus capable of infecting NIH 3T3 cells (95). Evidence indicates that these three species can reside intracellularly in the cytoplasm of the host cell (206), and that they are invasive. *M. fermentans* subsp. *incognitus* has been linked with renal and central nervous system complications in patients with AIDS (11, 117) as well as with acute fatal respiratory disease in otherwise healthy individuals (118). In primates infected with *M. fermentans* subsp. *incognitus*, there was weight loss, leukocytosis, and periodic mycoplasma antigenemia. Mycoplasma infection was apparent in the spleens, livers, kidneys and brains of these infected animals. All animals infected died after a long term infection of 7-9 months (120). *M. penetrans* has been isolated from the urine of patients infected with HIV (119), and has also been shown to have the capacity to invade cells (116). AIDS-associated mycoplasmas have been the subject of several recent reviews which discuss their role in development of AIDS and associated pathology (16, 17, 114). Most mycoplasma researchers believe that mycoplasmas are at the least a cofactor in the development of AIDS.

Treatment and vaccination

For most mycoplasma infections, antibiotic treatment is recommended, but only partially effective. Antibiotics are helpful in reducing the severity of the disease, but usually are unable to clear the organism from the site of infection. Because they lack a cell wall, mycoplasmas are unaffected by those antibiotics which affect cell wall synthesis, such as ampicillin. Treatment of diseases caused by *M. pneumoniae*, the causative agent of atypical pneumonia, involve long term use of general antibiotics. However, Niitu reported that *M. pneumoniae* could be recovered for two to three months after clinically effective antibiotic treatment (151). In a patient systemically infected with *M. fermentans* subsp. *incognitus*, recovery was apparent after a six week treatment with deoxycycline (115). Unfortunately, antibiotic resistance in *M. pneumoniae* and other mycoplasmas has been reported (25), and, as with many other bacterial pathogens, is becoming an increasing problem in mycoplasma infections. Because of the general inadequacies of antibiotic treatment, development of vaccines are a more promising approach to the control of mycoplasma infections in humans and animals (52).

Many different types of vaccines have been tested over the years, including passive transfer of antibodies, formalin fixed mycoplasmas, temperature sensitive mycoplasmas, attenuated or killed mycoplasmas, etc. To date, the present conclusion is that none of the

commercial vaccines are able to prevent colonization of the organism. This situation is no better than treatment with antibiotics. Ellison *et al.* has stated the following in relationship to development of a vaccine for *M. pneumoniae*:

The current working concept for successful immunization can be stated simply as follows: preventing attachment prevents initiation of disease, and preventing tissue damage prevents or reduces the severity of disease. Several conditions must be met for immunization to be successful: (i) a vaccine must contain a protective immunogen(s), (ii) the antigens must be genetically and phenotypically stable, (iii) the route and presentation must elicit a protective respiratory mucosal response, and (iv) the vaccine must protect without inducing untoward toxic reactions or adverse immune abnormalities, including potentiation of disease (52).

Although there are approved vaccines for animal use, as for partial protection from *M. hyopneumoniae* or *M. gallisepticum*, there is not an approved vaccine for any mycoplasma causing human disease. There are several inherent problems in the development of vaccines. First, administration of some mycoplasma preparations may potentiate the disease. Potentiation has been observed with formalin-inactivated *M. pneumoniae* vaccines (203), inactivated *M. gallisepticum* (1) and with either *M. hyopneumoniae* antigens (184) or a whole-cell formalin-inactivated vaccine (96). Second, considering the prevalence of variable surface antigens found in mycoplasmas, it is unlikely that one strain or one type of protein preparation will be protective against other strains of the species. Finally, as with many other bacterial pathogens, mycoplasmas rapidly lose virulence upon *in vitro* passage. To be effective, vaccines must contain the virulence determinants produced during infection. Those determinants are difficult to characterize in mycoplasmas due to the complex nature of growth media, the poor *in vitro* growth of some species of mycoplasmas, and the length of time necessary to grow mycoplasmas in broth or on plates from clinical tissues, generally from 1 week to 1 month. Because of the inadequacies of vaccines to date, Brunner has stated that "the availability of a rapid diagnostic method and a mycoplasmacidal antibiotic are more pertinent goals than the development of a vaccine" (25).

Mycoplasma membrane proteins

While a considerable amount of research has focused on the adherence factors and surface antigens, little research has been done on other membrane proteins which may also be involved in parasitism. It is surprising that in *M. genitalium*, which like all mycoplasmas has only one membrane, has devoted 140 (30%) of its 482 genes to encode membrane proteins (73). Of the 96 genes coding for unknown proteins, most of them are membrane

proteins. These observations underscore the importance of membrane proteins to mycoplasmas and the general lack of information regarding these proteins.

Major surface antigens

Mycoplasmas produce a number of surface proteins which are immunogenic and are thought to play a role in evasion of the host immune response process. Many of the surface antigens of *Mycoplasma hyorhinis*, *Mycoplasma bovis*, *M. gallisepticum*, and *M. hominis* are grouped into protein families with similar transcriptional and translational controls, and similar protein motifs and characteristics.

The variable lipoprotein family of *M. hyorhinis* has been characterized in great detail (reviewed in (231)). There are six members known in this family, VlpA-VlpF (179, 238). It is hypothesized that this family, which undergoes both size variation and phase switching, generates extreme population diversity in *M. hyorhinis* (231). These proteins constitute the major membrane protein and surface coat of the organism, which is the primary interface with the external environment (33). Different strains of *M. hyorhinis* house different numbers of the Vlp genes in their chromosome, i.e. strain GDL-1 has all six of the genes, whereas SK76 contains only VlpA-VlpC (179, 238, 239). Phase switching of these genes is generated from a homopolymeric tract of adenine residues between the -35 and -10 regions upstream of the transcriptional start site (238). Addition or loss of adenine bases in this region control the on/off phenotypic switch of the protein. Each *vlp* gene can be transcribed alone or in any combination with other *vlp* genes in a non-coordinate manner (33).

The Vlp proteins are divided into three distinctive regions; region I is the membrane signal domain, region II is a constant domain and region III, the C-terminus, is the surface exposed domain. All of the Vlps have a homologous signal sequence of 30 amino acids and a prolipoprotein signal peptidase II recognition sequence, A/T I S C (239). Radiolabeling of VlpC demonstrated that during signal sequence cleavage, a lipid moiety is attached to the cysteine residue adjacent to the cleavage site serving to anchor the protein within the membrane (34). The remaining portions of the Vlp proteins are hydrophilic in nature. The size variation among the Vlp proteins is due to the direct identical repeated blocks of 36-39 bp within region III of each Vlp structural gene (33). Each Vlp has its own unique repeat unit with all of the repeat units being highly charged. The relative abundance of each Vlp is affected by the number of repeating units, i.e. the longer the protein, the less product is produced (33). Size variation occurs only in region III. Possible mechanisms of size variation leading to the precise deletion or insertion of the repeat sequences include homologous recombination and slipped-strand mispairing (231).

The variable surface antigens from *M. bovis* are designated Vsps - variable surface proteins. These proteins share the following characteristics with the Vlps: they are lipoproteins and are anchored by the N-terminal domain, they have regions of shared epitopes defined by monoclonal antibodies, the C-terminal region is surface exposed and has extensive repetitive structure, they undergo high-frequency size variation, they undergo high-frequency phase variation and combinatorial expression, and the proteins are the most abundant amphiphilic protein in these organisms (13). Unlike the Vlps, the Vsps can undergo size variation in region II of the protein (13), and the repeat units of region III are multiple sets of similar or nonsimilar repeat sequences, which generate nonequal ladder patterns upon enzyme digestion (13). To date, the Vsps have not been genetically characterized.

Using a monoclonal antibody that recognizes a common epitope among the Vsps, Yogev *et al.* discovered a shared epitope on a size-variant protein of *M. gallisepticum* (237). This finding led to the discovery of PvpA. Like the others described, PvpA varies in size and undergoes high-frequency phase variation. However, PvpA is not a lipoprotein, but rather has a typical prokaryotic signal peptide followed by a second hydrophobic domain (237). PvpA is also not a member of a gene family, but exists as a single chromosomal entity. There appears to be no genetic relationship between the Pvp proteins and Vsps. Yogev *et al.* (237) and other investigators (66) have also described other phase variant proteins from *M. gallisepticum*, but these have not been characterized or grouped into families.

Phase variant proteins have also been recently described for *M. hominis* (240). These proteins also undergo phase and size variation, with similar mechanisms to the Vlps. Furthermore, analysis of mycoplasmal DNA from synovial fluids indicates that variation occurs in vivo, suggesting that variation may be a mechanism for antigenic variation (240). Another research group also described antigenic variation involving lipid modified surface proteins in *M. hominis*. Size variation occurs not only within the same strain, but also between different strains isolated from human hosts. The authors raise the possibility that variation could be occurring in the human host (155, 156). The proteins investigated by the two research groups may or may not be identical. Other species isolated from humans have also been investigated for phase and size variation of surface markers, including *M. fermentans* (228).

The discovery of gene families encoding size- and phase-variant proteins in several different species of mycoplasmas indicates that surface antigen variation may be common. In *M. pulmonis*, a size variant surface exposed lipoprotein, termed the V-1 surface antigen, has been described. This antigen is thought to be involved in hemadsorption (200). At the amino acid level, the V-1 antigen is similar to the Vlps. Region I contains the membrane

prolipoprotein signal, region II appears to be a constant region, and region III consists of repeated units (14). The repeated units of most of the V-1 antigens are proline rich, much like the adhesin molecules described above (14). Genetically, the mechanism of regulation is unique among mycoplasmas. The V-1 antigens are encoded by the *vsa* gene locus. Phase variation is generated by DNA inversion of a 34 bp region of DNA termed the *vsa* box. For each of the various proteins, there is a *vsa* box at the beginning of region III (14). The area of inversion contains the N-terminal sequences. Thus, only one serotype of antigen is expressed at any given moment. All of the other *vsa* genes remain silent until an inversion event occurs to place the N-terminus in position to allow proper translation (14). DNA inversion in the *vsa* gene locus apparently is coordinated with the DNA inversion shown to be responsible for phase switching of the restriction-modification system, termed the *hsaI* locus (14). In *M. pulmonis* KD735, seven different potential structural genes have been identified in the *vsa* gene locus (14). In *M. pulmonis* CT, however, a much smaller gene locus was found containing only two different structural genes (200). One of the genes found in strain CT, the one encoding for the originally described V-1 antigen, was not described for strain KD735 (200). Future research must be done to correlate the results between the two research groups and to fully determine the genetic structure of the *vsa* locus.

One of the common features among the surface antigens is the presence of repeat sequences. In the Vlp and Vsp, these repeats are involved in size variation, are typically only 12-13 amino acids long and are located in the C terminal domain. Another family of surface exposed antigens is the Lmp proteins of *M. hominis*. These proteins have up to ten nonidentical repeated units of approximately 160 amino acids (101). The repeat units are not necessarily complete repeats as Lmp1 has 9 1/2 repeats. In comparison to the Vlp, region 1 of the Lmp proteins contains a nonlipoprotein hydrophobic signal sequence, region II is a central region without any repeated sequences, and region III contains the repeated domains (101). These proteins do not appear to undergo size or phase variation. Recent experiments implicate the Lmps in aggregation, a potentially helpful feature for *M. hominis* in a disease situation (84). The Vlp, Vsp, Vsa and Lmp proteins are all hydrophilic except for the membrane spanning domain.

The function of these varying surface antigens is unknown. For the V-1 antigen and the Lmp proteins, hemadsorption and aggregation are potential functions, respectively. It has been speculated that for the most part these antigens are involved in evasion of the host immune response. Structurally, they are all similar. Region I has either an attached lipid or a single membrane spanning domain, region II is generally constant while region III has repeat domains. Infected animals produce strong antibody responses toward the repeated units.

Because all known mycoplasmas are surface parasites, it is expected that many more families of surface antigens will be discovered.

Membrane-associated activities

Besides the surface antigens, other membrane activities are common to the mycoplasmas, one of which is hemolytic activity. Minion and Jarvill-Taylor established that the membranes of most mycoplasmas contain an activity which is capable of lysing trypsinized mouse red blood cells (138). It was also demonstrated that the hemolysin from *M. pulmonis* is inhibited by cholesterol and selected cholesterol derivatives (83). The activity was affected by heat treatment, thiol-reducing agents, thiol-activating agents and oxidizing compounds (83, 138), thus resembling the gram positive thiol-activated hemolysins (streptolysin, pneumolysin, and listeriolysin). The stereo-specificity in sterol recognition, however, was distinctly different than the pattern observed with the gram positive thiol-activated hemolysins (83). All but one of the gram positive thiol-activated hemolysins are secreted proteins, whereas the mycoplasma hemolysins are primarily associated with the membrane (136, 139). A hypothesis has been proposed that the hemolysin is involved in the acquisition of cholesterol based upon the observations that mycoplasmas have an absolute requirement for this molecule and that the probable receptor for the hemolysin is cholesterol (83, 135). Interestingly, *M. hominis*, and other arginine metabolizing mycoplasmas, *M. fermentans* subsp. *incognitus*, and *M. penetrans* were negative for the hemolytic activity as assayed in that study (138).

Both *M. fermentans* subsp. *incognitus* and *M. penetrans* are associated with AIDS and are believed to reside intracellularly. Shibata *et al.* undertook a study aimed at determining if these species were similar to the intracellular pathogen *Listeria monocytogenes* in the production of phospholipases (191). In *L. monocytogenes* it is thought that the phospholipase aids in releasing the organisms from the lysosome into the cytoplasm where the organism replicates (27). This study showed that both *M. fermentans* subsp. *incognitus* and *M. penetrans* contained a phospholipase C which was capable of hydrolyzing both phosphatidylinositol and phosphatidylcholine (191). Two other mycoplasma species, *M. hominis* and *M. arginini*, did not have phospholipase C activity (191).

Salman and Rottem have reported phospholipase A1 activity in *M. penetrans* (188). Other reports of phospholipases in mycoplasmas indicated that some species may have phospholipase A or lysophospholipase A activities (217). Considering that serum, a media component for all mycoplasmas, contains phospholipases (138), the presence of phospholipases in mycoplasmas needs additional investigation.

Another protein which may aid the entrance of *M. fermentans* subsp. *incognitus* and *M. penetrans* into the cytoplasm is a tyrosine phosphatase. Tyrosine phosphorylation is associated with alterations in receptor activity, cellular proliferation and modulation of the cell cycle (236), and is regulated by tyrosine kinases and tyrosine phosphatases (79). Protein tyrosine phosphatases have been found to have an important role in bacterial pathogenesis (18). Phosphatase activity directed toward tyrosine was found in *Mycoplasma faucium* and *M. fermentans* subsp. *incognitus* (192). Low levels of activity were determined for *M. pneumoniae*, *M. hominis*, and *M. buccale* while *M. orale* and *M. salivarium* were negative (192). The authors speculate that the tyrosine phosphorylation-dephosphorylation pathways in the host might be disturbed by these phosphatases which may play some important role in pathogenesis (192). In *Yersinia*, it was thought that the phosphatase might disrupt host signal transduction processes involved in bacterial killing (18).

One mycoplasma activity which has been hampering AIDS research is a membrane nuclease activity. It has been known for a many years that mycoplasmas contain both DNases and RNases, and these activities are believed to be involved in the acquisition of nucleotides (160, 174). Undegraded RNA and DNA can fulfill the requirement for nucleotide precursors for mycoplasmas. Nuclease activity has been found to be present in the particle-free supernatant of contaminated cell culture lines (51) and associated with the membrane (137, 139). It inhibits HIV reverse transcriptase activity (51), which is a measure of viral particle production and the most commonly used indicator for establishing the growth kinetics of HIV. Curing the cell lines of mycoplasmas removed the inhibitor, allowing for a more accurate assessment of HIV activity (51).

Nucleases

Nucleases in bacteria have been shown to function in protection of the organism, repair of damaged DNA and in recombination. Endonucleases are those enzymes which cleave bonds within a nucleic acid chain and may be specific for either RNA or DNA. Exonucleases cleave nucleotides one at a time from either the 5' or 3' termini, depending upon the specificity of the enzyme. With RNA substrates, the function of these enzymes is usually the degradation of mRNA. Other enzymes have been classified as either endonucleases or exonucleases, and some have now been determined to have other activities, such as glycosylases and AP (apurinic/aprimidinic) lyases. This section on nucleases will provide a brief review on DNA exo- and endonucleases from bacteria. Nonspecific bacterial nucleases or those thought to be involved in disease processes will be the focus of the final section.

Specific bacterial nucleases

Nucleases can be divided into the following types. Restriction endonucleases are those enzymes, which along with modification enzymes, are designed to protect resident DNA from attack by sequences of foreign DNA. There are both endo- and exonucleases which are involved in excising damaged DNA so that other enzymes can synthesize a correct replacement of the bases. Also involved in repair are the DNA polymerases, most of which contain both 3'-5' and 5'-3' exonuclease activities. Finally, nucleases are involved in DNA recombination. Bacteria can contain multiple types of each of these groups of nucleases, and many of them can be considered to be found in all bacteria. Restriction systems found in bacteria may be intrinsic or may have been introduced by a plasmid or phage. All species with a restriction enzyme also contain a DNA methylase activity with the same sequence specificity to provide host chromosomal DNA protection against the restriction enzyme. These enzymes are commonly used in biotechnology, many are readily available commercially and have been extensively studied. Table 1.3 describes the different types of restriction systems known.

There are several different mechanisms of DNA repair present in bacteria. These include photoreactivation (light repair), base excision repair, nucleotide excision repair and mismatch repair (to include both long patch and short patch repair). Following severe damage, the mistake prone SOS response is induced. Photoreactivation processes do not use nucleases.

Table 1.3. Restriction Systems^a

	Type II Enzyme	Type III Enzyme	Type I Enzyme
Protein Structure	separate endonuclease and methylase	bifunctional enzyme of 2 subunits	bifunctional enzyme of 3 subunits
Recognition site	short sequence (4-6 bp), often palindromic	asymmetrical sequence of 5-7 bp	bipartite and asymmetrical (e.g. TGA ∇ GTGCT)
Cleavage site	same as or close to recognition site	24-26 bp downstream of recognition site	nonspecific >1000 bp from recognition site
Restriction and methylation	separate reactions	simultaneous	mutually exclusive
ATP needed for restriction	no	yes	yes

^a taken from (110).

Most of the known nuclease can be assigned to the base excision repair systems, while a few nucleases play prominent roles in the other repair mechanisms. Of the original nine endonucleases described for *E. coli*, three are now known to be DNA glycosylases, two of the activities are contained in exonuclease III and one is an AP endonuclease (64, 110). The gene for endonuclease V has not been identified. Thus, most of the early discovered endonucleases function in base excision. Likewise, the function of several of the originally described exonucleases have been more accurately determined. For example, exonuclease II and exonuclease IV activities are found in DNA polymerase I (64). Endo- and exonucleases known to aid in base excision are: *recE* (originally termed exonuclease VIII), a 5'-3' double stranded DNA specific endonuclease; *recJ* (originally exonuclease VII), a single stranded specific 5'-3' exonuclease which also has deoxyribophosphodiesterase activity; and exonuclease I, a 3'-5' single stranded exonuclease (64, 110). Base excision is normally used to remove only damaged or incorrect bases, which explains the use of single strand specific nucleases.

The nucleotide excision repair system removes thymidine dimers or other damage resulting from ultraviolet light damage (64). UvrA is an ATPase, DNA binding protein which recognizes the damage and when combined with the two endonucleases, UvrB and UvrC removes a 10-13 base pair section of DNA. UvrD is the helicase which unwinds and releases the cut DNA. The gap is filled in by DNA polymerase I.

Mismatch repair is similar to the base excision repair but only the mismatched base is removed. This repair system, termed the *dam* repair system, uses the MutH, MutY, and MicA endonucleases to excise adenines from an adenine-guanine mismatch (110). The correct strand of DNA repaired during mismatch repair is determined by *dam* methylation; the newly synthesized, or nonmethylated, strand is the repaired strand.

As can be seen in the brief review of exo- and endonucleases of *E. coli*, each of the identified nucleases has a specific function, most of which relates to correction of duplication errors and repair of damaged DNA. Tight regulation of these processes prevent additional damage during the repair process. It can be assumed that many of these functions are common to both bacterial and eukaryotic systems.

Nonspecific bacterial nucleases

Several nonspecific nucleases from various species of bacteria, including mycoplasmas, have been discovered that are either membrane-associated or secreted. In most cases, these species are pathogenic, and the nuclease is a suspected virulence determinant. However, as in mycoplasmas, the actual relationship of the nuclease to the disease process is unknown. The

most well studied bacterial nuclease is the staphylococcal nuclease, also referred to as micrococcal nuclease, heat-stable nuclease or thermonuclease. This enzyme is a small (149 amino acid residues) extracellular enzyme produced by many pathogenic strains of *Staphylococcus aureus* (194). It is an extracellular phosphodiesterase which hydrolyzes RNA and DNA to 3'-phosphomononucleotides and is one of over 30 known extracellular proteins produced by this species (3). Two different forms of the enzyme are secreted. Nuclease A is the predominant form of 149 amino acids. Nuclease B has an additional extension of 19 amino acids at the amino terminus of nuclease A. Because of its small size and simple amino acid structure, staphylococcus nuclease has become one of the most extensively studied enzymes physiologically and biochemically. The advantages of working with this enzyme are that it is a small monomer with no cysteines, highly soluble in both its native and denatured forms, displays a simple reversible denaturation reaction plus a very high resistance to irreversible inactivation (194). Initially, the protein was used as a simple model system for analyzing protein structure and folding. As recombinant DNA techniques became popular, the nuclease was cloned and has been expressed in *E. coli* (193), *B. subtilis* (94, 132), *Corynebacterium glutamicum* (111), and *Saccharomyces cerevisiae* (159) to investigate protein secretion signals, identification of amino acids in the active site, over expression of the protein, protein structure and protein biochemistry. Initial studies of expression in *E. coli* indicated that the amount of nuclease activity synthesized in *E. coli* strains carrying the nuclease gene on a multicopy plasmid was much less than the amount produced from the genomic gene in *S. aureus* (193). The nuclease A form also appeared to be inefficiently processed from the nuclease B precursor form (193). The inefficiency of expression in *E. coli* led investigators to pursue secretion in *B. subtilis*. In this heterologous host, both the native promoter and the *Bacillus* promoter efficiently drove production of the nuclease, which is efficiently processed and secreted at high levels (94). It was in the *Bacillus* studies that the nuclease B form was determined to be the precursor of nuclease A with the additional amino acids functioning as the signal sequence (132). In another gram-positive organism, *C. glutamicum*, expression of the nuclease was not as high as in *Bacillus* using the native promoter. Placing the gene under the transcriptional control of the *tac* promoter increased expression by about 10-fold (111). The authors speculate that the expression is influenced by the G+C content of the organism, *S. aureus* has a low G+C content whereas *C. glutamicum* has a high G+C content (111). Since the initial studies in *E. coli* were performed with the nuclease gene using its native promoter, low expression may have been due to poor recognition by gram negative organisms of gram-positive promoters. Jing *et al.* expressed nuclease R, an analogue of nuclease A, in *E. coli* under the transcriptional control of the

lambda PR and PL promoters regulated by temperature sensitive repressors. High levels of expression were obtained, and there appeared to be little host dependence in different strains (85). The nuclease is translocated across the cytoplasmic membrane in all cases supporting the hypothesis concerning the conserved nature of protein transport among all organisms.

Because the nuclease is easily monitored and well studied, it was an ideal candidate to investigate the effects of the amino-terminal sequences in secretion. Using *S. cerevisiae*, three different derivatives of the nuclease were expressed, each with different amino terminal signals, the OmpA signal sequence from *E. coli*, a lipoprotein signal sequence, and one with no signal sequence (159). In previous work, a hybrid gene encoding the OmpA signal sequence fused to the mature portion of the nuclease was efficiently secreted and correctly processed in *E. coli*, whereas a nuclease lacking the amino-terminal signal sequence was not secreted from *E. coli* and was lethal in the presence of high concentrations of Ca^{2+} (212). In yeast, the nuclease lacking a signal sequence was synthesized at levels tenfold lower than the other derivatives. In contrast, the nuclease with the OmpA signal peptide and the nuclease with the lipoprotein signal sequence were synthesized at high levels (1-1.5% of the total protein) and a significant amount of the product was secreted into the yeast periplasm (159). High expression of nuclease in the yeast did not appear to be lethal or harmful as active nuclease was found in both soluble and membrane fractions of the cells (159). These results suggested that lower eucaryotes could decipher prokaryotic secretory information within the signal peptides.

The heat-stable nuclease is not the only nuclease produced by *S. aureus*. Vakil *et al.* found a heat-labile cellular nuclease which shares a number of properties with the heat-stable nuclease (221). In comparison, the heat-stable nuclease is stable to heating at 100°C for 15 minutes whereas the heat-labile nuclease is inactivated within 5 minutes of treatment. Both enzymes are monomers, prefer heat-denatured DNA as a substrate but can also use RNA as a substrate, have a pH optimum of approximately 10, have a temperature optimum in the range of 45°C to 50°C, and have an absolute requirement for Ca^{2+} ions which could not be substituted by other monovalent or divalent cations (221). Both the heat stable and heat labile nucleases are relatively inactive at pH 7.0 and only have moderate activity at 37°C. (221).

A thermonuclease also is produced by *Salmonella* and *Thermus*. In *Salmonella* this activity was discovered during PCR studies with crude cell extracts when reaction mixtures were held overnight at 4°C post reaction. Strains tested to be found with the nuclease were *Salmonella enteritidis*, *Salmonella typhimurium*, *Salmonella virchow*, *Salmonella dublin*, *Salmonella hadar* and *Salmonella agona* (70). Under similar conditions, amplified DNA

from *Listeria* and *Lactococcus* remained intact after storage at 4°C for up to several months. This indicated that not all bacteria produce comparable nucleases capable of withstanding the temperatures and conditions encountered during PCR cycling (70). The thermostable nuclease from *Thermus filiformis* was found by expressing DNA from *T. filiformis* in *E. coli*. The preferred substrate of this nuclease is single-stranded DNA, although double-stranded DNA can be digested. Nuclease activity increases with increasing temperatures up to 80°C, with low activity between 30°C and 40°C, and requires the divalent cations Ca^{2+} or Mg^{2+} for catalysis (57). This enzyme can convert supercoiled plasmid to nicked circular DNA and to linear DNA, indicating that it is primarily a nonspecific endonuclease. It is also able to digest lambda DNA to small fragments with 5' phosphates indicating the presence of exonuclease activity. These properties are similar in some respect to the endo-exonucleases found in *Neurospora*, *Aspergillus* and *Saccharomyces* (57). This particular activity was not described from *Thermus* prior to its expression in *E. coli*, and it is unknown if the protein is membrane-associated or secreted by *Thermus*.

Non-sequence specific endonucleases are produced by many different species of *Streptomyces*. These enzymes are located in the cell-wall cytoplasmic membrane space, have similar molecular masses, require Mg^{2+} for optical activity and are released after protoplast production (5). These enzymes differ in their patterns of DNA cleavage. They are believed to have a function in the recycling of the nucleic acids components before the formation of aerial mycelium (26).

In *Serratia marcescens*, a gram-negative enteric pathogen, the extracellular nuclease expression is regulated at the transcriptional level. The nuclease appears during the transition between exponential to stationary phase (31). This endonuclease demonstrated no strong base preference, is equally active against single and double-stranded DNA, has activity against both RNA and DNA, and produces short unequal 5' oligonucleotides (133). Two isoforms, SM1 and SM2, which are kinetically identical are produced by the organism (55). Unlike the staphylococcal nuclease, these proteins contain essential disulfide bonds. As these bonds do not form in the cytoplasm, the nuclease remains inactive in the cytoplasm (55). These nucleases have been expressed in *E. coli* (157).

Unlike all of the nucleases described, the nuclease gene from *Shigella flexneri* is located on a plasmid. This plasmid has been studied extensively because it is able to transfer to and replicate in a wide range of gram-negative bacteria (222). Derivatives of this plasmid have been used as cloning vectors. The most useful phenotype of this plasmid is its ability to completely suppress the ability of *Agrobacterium tumefaciens* to incite crown gall tumors on plants (121). During efforts to define the suppressing region of the plasmid, an open reading

frame was discovered with significant amino acid sequence homology to the staphylococcal nuclease. Subsequently, it was determined that an *E. coli* strain harboring the plasmid expressed nuclease activity. The protein has a potential signal peptide and insertions of *TnphoA* within the open reading frame resulted in alkaline phosphatase activity, confirming the presence of a functional signal sequence (35). Nuclease activity was also found when the plasmid was placed into *A. tumefaciens*. (35). The authors speculate that although the nuclease gene is not required for oncogenic suppression, it may play an accessory role involving the transfer of the T-DNA of the Ti plasmid from *A. tumefaciens* to plants (35).

In summary, there are different types of nucleases produced by pathogenic bacteria. Many of these are released into their surrounding environment. They are active on both RNA and DNA, and many of them are extremely stable biochemically. Their potential roles in pathogenesis however have not been studied or defined. The nucleases of mycoplasmas may potentially share similarities to many of these enzymes.

Nuclease activities found in mycoplasmas

Although not a nuclease, the DNA polymerase from several species of mycoplasmas has been characterized. Recently, DNA polymerase III was purified from *M. pulmonis* and the gene sequenced (9). It is believed that many mycoplasmas contain only DNA polymerase III (99). Early studies failed to identify 3'-5' exonuclease activity contained within the polymerase, an activity present in all other known DNA polymerase IIIs. Barnes *et al.* (9), however, showed that the polymerase from *M. pulmonis* did have nuclease activity. The authors proposed that mycoplasmas have at least two distinct DNA polymerases, depending upon which species is analyzed. One is an exonuclease containing polymerase such as that found in *M. pulmonis*. The other is a polymerase lacking exonuclease activity as those found in *M. orale*, *M. hyorhinis* or *M. mycoides*. Alternatively, those preparations which appear to lack exonuclease activity may be preparations of a polymerase which lacked a subunit responsible for exonuclease activity (9, 99). Exonuclease positive polymerases are approximately 165 kDa in size whereas those which are negative are around 100 kDa.

Restriction-modification systems have only been identified recently in mycoplasmas. A Type I restriction-modification system has been identified in *M. pulmonis*, termed the *hsdI* locus (50). An analogue of subunits for restriction, methylation, and sequence specificity was found in the predicted amino acid sequences. This locus is located on an invertible element much like the V-1 antigen, and the inversion of the element regulated expression. On one side of the *hsdI* locus is the *polC* gene.

A Type II restriction-modification system has been characterized in *M. fermentans*. (195). The endonuclease and the methyl transferase are specific for the sequence CAATTG and are encoded by divergent genes. The enzyme has the abbreviation *munI* and both the genes and amino acid sequence share similarity with *EcoRI* and *RsrI*. A second enzyme, termed *MfeI* has been discovered in *M. fermentans*. It is an isoschizomer of *MunI* (195). It is likely other restriction enzymes will be described in mycoplasmas. Many species have methylated DNA suggesting that they have restriction systems (46).

There are only a few studies which have addressed DNA repair systems in mycoplasmas. *M. buccale* possessed dark repair activity, but not light repair (4). However, *M. gallisepticum* apparently does not possess either light or dark repair (69). These studies were performed prior to 1980, and no more recent studies have been reported.

The *recA* genes from *M. pulmonis* and *M. mycoides* subsp. *mycoides* exhibit a high degree of homology with other *recA* genes of gram positive bacteria (87). Homologous recombination has been shown to occur in several mycoplasma species, including *M. pulmonis* and *M. gallisepticum* (29, 134). It is unknown if there is a LexA binding domain upstream of the *recA* gene as in *E. coli*, and *lexA* has not been discovered in mycoplasmas to date. The only known activity for RecA in mycoplasmas is in recombination.

Few nucleases have been discovered in mycoplasmas, and except for the preliminary report by Minion and Goguen of membrane associated nucleases in *M. pulmonis* (139), all of the nucleolytic activities were associated with "housekeeping" activities. None could be considered as potential virulence factors. This work extends the original observations by identifying and characterizing membrane associated nuclease activities in different mycoplasma species and by cloning and sequencing one nuclease gene from *M. pulmonis*. A *uvrB* homolog was also discovered as a consequence of these studies as part of a two gene operon with *mnuA*, the cloned membrane nuclease.

CHAPTER 2. MEMBRANE-ASSOCIATED NUCLEASE ACTIVITIES IN MYCOPLASMAS

A paper published in the Journal of Bacteriology¹

F. Chris Minion², Karalee J. Jarvill-Taylor^{2,3}, Diane E. Billings, and Eli Tigges

Abstract

Membrane-associated nucleases of various mycoplasmal species were investigated 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 different *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.

Introduction

Mycoplasmas, cell wall-less bacteria of the class *Mollicutes*, are among the smallest self-replicating organisms known and have been described as a minimal living unit (141, 166). They are typically noninvasive animal pathogens that readily colonize mucosal surfaces and have immunomodulatory activities (36, 210, 213, 218), but are totally incapable of synthesizing the typical eubacterial cell wall components, the major bacterial immunomodulatory components. It has been hypothesized that the selective association of

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²Associate Professor and Graduate Student, respectively.

³Primary Researcher.

host cell proteins with mycoplasmas could alter the immunoresponsiveness of a host (227). 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. The exchange of lipophilic probes from host cells to mycoplasmas has also been demonstrated (135, 229), 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 (166). 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 (166, 171), but DNA and RNA degradation processes have not received adequate attention. Pollack and Hoffman (160) and Roganti and Rosenthal (177) 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 (137) found membrane-associated nucleases in *M. pulmonis*. In addition, RNases are known to be present in mycoplasmas (123). Together, these activities are sufficient to produce all of the required nucleotides from undegraded DNA and RNA, as observed originally by Razin and Knight (173). 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 to growth and survival (137, 177). This finding suggests that there is a unique mechanism of in vivo nucleotide acquisition.

Materials and Methods

Mycoplasma growth and isolation

The mycoplasma strains and the media used for cultivation are described in Table 2.1. 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 (63), 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 (42). Friis medium consisted of 12 g of PPLO broth per liter, 12.6 g 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 Cefobid per ml, the pH was 7.4. SP4 medium was made as described previously (220) and was modified by adding 0.02% arginine. Frey's medium was prepared as described previously and was modified by adding 0.01% NADH (63). 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 x 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 concentration of 100 µg of protein per ml in PBS containing 1% dextrose, 2 mM CaCl₂ and 2 mM MgCl₂ (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 protein, were diluted in twofold increments in a 96-well plate with PBS-CM. An equal volume of lambda DNA (10 µg/ml in 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 the addition of 7 µl of borate sample buffer (122) 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 endpoint 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. 2.1B, 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.

Table 2.1. Mycoplasma strains, species, source, loss in viability and nuclease activity

Species ¹	Strain	Host	Media ²	Source or reference	Loss in Viability ³	Nuclease Activity		
						Intact Whole Cells ⁴	Released ⁵	Total ⁶
<i>M. pulmonis</i>	UAB6510	rodent	P	M. K. Davidson	NL	1250	±	20
	UAB5782	rodent	A	G. H. Cassell	NL	1250	±	20
	UABCT	rodent	A	M. K. Davidson	ND	1250	±	208
	UABT	rodent	A	M.K. Davidson	NL	1250	±	26
	JB	rodent	A	G. H. Cassell	ND	1250	-	1250
	19612	rodent	P	ATCC	ND	833	±	416
	(PG34)							
	KD735	rodent	A	K. Dybvig	NL	625	±	625
	χ1048	rodent	A	M. K. Davidson	NL	1250	±	625
	ISM3050	rodent	A	F. C. Minion	75	1250	312	156
<i>M. arthritidis</i>	M1	rodent	P	M. K. Davidson	NL	156	625	78
	66	rodent	P	M. K. Davidson	NL	78	416	20
	19611	rodent	PA	ATCC	ND	104	±	96
<i>M. neurolyticum</i>	19988	rodent	F	ATCC	44	416	1250	156
<i>M. hyopneumoniae</i>	J	swine	F	R. F. Ross	ND	833	-	625
	232A	swine	F	R.F. Ross	ND	833	-	625
<i>M. hyorhinis</i>	GDL	swine	F	R. F. Ross	NL	208	833	156
<i>M. hyosynoviae</i>	25591	swine	FA	ATCC	ND	312	-	312
<i>M. dispar</i>	SDO	bovine	F	R. Rosenbusch	14	26	416	19
<i>M. bovis</i>	79-27323	bovine	F	R. Rosenbusch	ND	208	833	312
<i>M. bovoculi</i>	C52	bovine	F	R. Rosenbusch	NL	52	416	39
<i>M. capricolum</i>	27343	caprine	F	ATCC	ND	-	6250 ^b	-
<i>M. gallisepticum</i>	19610	avian	P	ATCC	14	416	833	20
	15302	avian	P	ATCC	ND	833	833	52
	A5969	avian	P	Hofstead	ND	208	±	20
<i>M. meleagridis</i>	25294	avian	FA	ATCC	ND	833	-	833
<i>M. gallinacium</i>		avian	P	field isolate	ND	104	208	26
<i>M. fermentans</i>	PG18		S	S.-C. Lo	ND	-	-	625

<i>M. fermentans</i>								
<i>incognitus</i>		human	P	S.-C. Lo	ND	416	1250	39
<i>M. penetrans</i>		human	S	S.-C. Lo	ND	±	-	416
<i>M. hominis</i>	1620	human	PA	L. D. Olson	ND	32	208	39
<i>M. pneumoniae</i>	15531	human	P	ATCC	ND	±	-	416
<i>A. laidlawii</i>	23206 (PG8)		F	ATCC	NL	104	208	20
	14192		F	ATCC	ND	208	±	20
<i>Acholeplasma</i>	ISM1499		P	This laboratory	ND	104	±	10
<i>spp.</i>								

- ¹ For every species and strain, except for *Acholeplasma* spp. ISM1499 which is a high passage laboratory isolate representing at least 150 in vitro passages, the number of in vitro passages in our laboratory was less than twenty. Actual passages prior to our receiving the strains are unknown.
- ² P = Standard PPLO broth media; F = Friis media; S = SP4 media; FR = Frey's media; PA = PPLO broth with arginine; FA = Friis media supplemented with arginine
- ³ Loss in viability is the percent loss in viability during a 30 min incubation at 37°C in assay buffer supplemented with glucose. (NL) = no detectable loss in colony forming units. (ND) = not determined.
- ⁴ Whole cell activity was subjectively scored as the nanograms of protein in a whole cell suspension required to digest 250 ng lambda DNA as described in the Materials and Methods. Data represent the mean of at least three replicates. (±) indicates that a small amount of nuclease activity was present, but an accurate measure was beyond the assay sensitivity. (-) indicates a complete lack of activity under all experimental conditions.
- ⁵ Released activity is the amount of nuclease activity released during a 30 min incubation at 37°C in assay buffer as described in Materials and Methods. Data represent the mean of three observations.
- ⁶ 10 mM magnesium only. See Table 2.
- ⁷ Total activity represents the activity associated with detergent-solubilized whole cells.

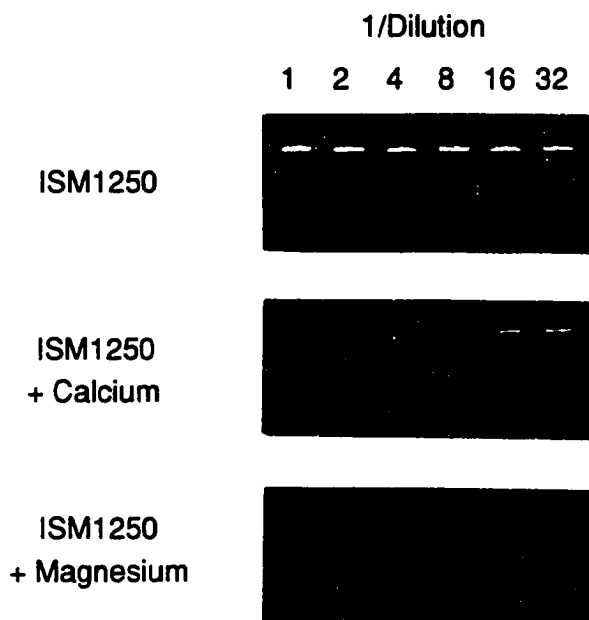


Figure 2.1. Effects of calcium and magnesium on membrane nuclease activity in *Acholeplasma* sp. strain ISM 1499. An overnight culture of strain ISM 1499 was washed and prepared for the whole-cell nuclease assay as described in Materials and Methods. The beginning concentration of mycoplasma protein 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 well, calcium or magnesium was added to the 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.

Mycoplasma suspensions were incubated at 37°C for 30 min in assay buffer, the cells and membrane fragments were pelleted by centrifugation in a microfuge at 12,000 rpm for 15 min, and 25 µl of the resulting supernatant fraction was diluted in twofold increments with PBS-CM and assayed as described above. CFU were measured before and after incubation to assess cell viability. Hexokinase activity (170) 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 (167), and the purity of each membrane preparation was assessed by measuring its hexokinase activity (170). In other experiments, the total nuclease activity of each mycoplasma species was determined by diluting 25 µl of suspended cells in PBS-CM containing 0.1% Tween 20 prior to the addition of the 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 x *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 supernate 1:1 with SDS-PAGE sample buffer, and the preparations were incubated at 60°C for 15 min and centrifuged at 12,000 x *g* for 5 min prior to loading. For some experiments, samples were boiled for 5 min prior to loading.

The SDS-PAGE was performed by using a modification of the method of Laemmli (102) 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 2x Anode buffer supplemented with 0.15% SDS. The gels were electrophoresed at a current of no more than 10 mAmp 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 (0.04M 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 hours at 37°C in 100 volumes of incubation buffer supplemented with 2 mM CaCl₂ and 2 mM MgCl₂. 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, gels were rinsed again with water and fixed in 50% ethanol, 12% acetic acid for 1 h. The gels were then stained 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).

Results

Nuclease activity comparison

To effectively compare levels of nuclease activities 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. 2.1). Divalent cations had a measurable

effect on nuclease activity (Fig. 2.1 and Table 2.2). Addition of calcium resulted in a 32-fold increase (5 well difference) in *Acholeplasma* sp. strain ISM1499 activity, whereas addition of magnesium increased the activity 128-fold (Fig. 2.1). 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 for *Mycoplasma capricolum*. In *M. capricolum*, nuclease activity was detectable only when 10 mM magnesium was added, and the presence of calcium was inhibitory (Table 2.2). Interestingly, in two of the three *M. pulmonis* strains tested, χ 1048 and UAB6510, nuclease activity was observed only when calcium was present.

The results of the lambda nuclease assay are shown in Table 2.1. Nuclease activity was present in all of the strains and species tested, and the level of activity varied in different strains and species. To control for nonspecific effects such as the association of media components with the mycoplasma membrane surface (22, 150, 232), broth was screened for nuclease activity before it was used. The medium controls were consistently negative. The assay sensitivity using DNase I (Sigma) was determined to be 0.0008 units (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 surface during incubation in assay buffer supplemented with glucose, (ii) the effects of incubation on cell viability, (iii) the amounts of nuclease and hexokinase activity 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 2.1), but incubation in assay buffer had no measurable effect on 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* χ 1048, *M. gallisepticum* 19610, *Mycoplasma bovoculi* and *M. dispar* during incubation (data not shown). There was no difference in the percentage of hexokinase release 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.

Table 2.2. Comparison of cation effects on membrane-associated nuclease activity

Organism	Nuclease activity in the presence of ^a :				
	2 mM	5 mM	10 mM	2mM Mg	5mM Mg
	Mg ^a	Mg	Mg	+ 2mM Ca	+ 5mM Ca
<i>M. pulmonis</i> χ 1048	0	0	0	1562	1562
<i>M. pulmonis</i> UAB6510	0	0	\pm	1562	1562
<i>M. pulmonis</i> M1	390	390	390	390	781
<i>M. capricolum</i>	0	0	6250	0	0

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

Membranes were purified from *M. pulmonis* χ 1048, *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. 2.2).

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 2.1. 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 (100, 130). 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 BioRad 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 protein 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} - 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.

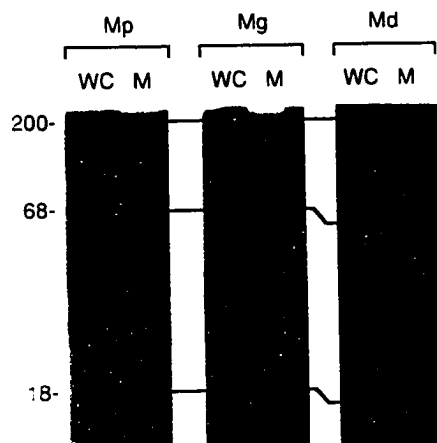


Figure 2.2. Comparison of nuclease activities in whole cells and purified membranes as determined by the SDS-PAGE nuclease assay. Membranes were purified on sucrose gradients and the SDS-PAGE nuclease assay was performed as described in Materials and Methods. The amount of protein added to each lane was as follows: *M. pulmonis* X1048 (Mp), 5.4 μ g of whole-cell protein (lane WC) and 1.7 μ g of membrane protein (lane M); *M. gallisepticum* 19610 (Mg), 5.6 μ g of whole-cell protein (lane WC) and 1.4 μ g of membrane protein (lane M); *M. dispar* SDO (Md), 2.5 μ g of whole-cell protein (lane WC) and 0.7 μ g of membrane protein (lane M). The position of size markers (in kilodaltons) are indicated on the left.

Analysis of mycoplasmal nucleases by SDS-PAGE

The protein profiles of the nucleases from the *M. pulmonis* strains are shown in Fig. 2.3A. The amounts of total protein in the lanes were adjusted in order to facilitate analysis. In general, 1-10 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 for PG34 and JB were very similar. The most consistent feature of these patterns was the prominent triplet of nuclease activity in the 35 - 50 kDa range.

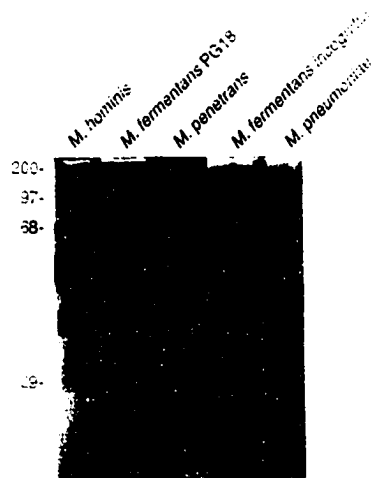
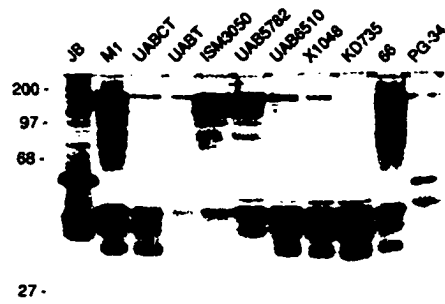
The nuclease patterns obtained from other mycoplasma species were variable. The three *M. gallisepticum* strains tested produced the same pattern of banding (Fig. 2.3B). The nuclease patterns of bovine and porcine species generally appeared to be similar, but there were no bands of similar size in the different species.

In general, the human mycoplasma species exhibited low levels of nuclease activities (Table 2.1). To detect nuclease activity 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. 2.3C). *Mycoplasma pneumoniae* exhibited very low activity as shown by the single band (Fig. 2.3C) and by the level of total activity observed after detergent solubilization (Table 2.1). Of the human species tested, *Mycoplasma hominis* exhibited the highest level of membrane-associated nuclease activity (Table 2.1).

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* (137) and in *A. laidlawii* (177). 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 concentrations (10 mM). Some species released nuclease activity to the extracellular environment during incubation although it was not clear whether this 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

Figure 2.3. Nuclease SDS-PAGE band patterns of various mycoplasma species. (A) *M. pulmonis* strains. (B) Various animal mycoplasma 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 designations are indicated at the top of each gel. The positions of size markers (in kilodaltons) are indicated on the left.



species, in which no loss of viability occurred during incubation and only a small amount of hexokinase release 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 (130, 177, 182), 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 (21, 180, 181). Although the results of preliminary experiments indicate that the nucleases from *M. pulmonis* are not modified by palmitic acid (data not shown), the membrane-bound nucleases may exhibit peptide length variability based upon a mechanism similar to the mechanisms observed with the variable lipoproteins.

It was hoped that the patterns could provide a means 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. 2.3A). 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 would 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 process of the organism in hosts.

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CHAPTER 3. CHARACTERIZATION OF A MEMBRANE NUCLEASE ENCODING GENE OF *MYCOPLASMA PULMONIS* AND ANALYSIS OF ITS EXPRESSION IN *ESCHERICHIA COLI*

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Karalee J. Jarvill-Taylor, Christina VanDyk and F. Chris Minion

Abstract

Membrane nucleases of mycoplasmas have been proposed to play an important role in growth and pathogenesis. As a first step in defining the function of this unusual activity at the cellular and molecular levels, a membrane nuclease gene of *Mycoplasma pulmonis*, designated *mnuA*, was cloned in *Escherichia coli*. To expedite antigen expression and functional analysis of cloned sequences, an opal suppressor host was used to overcome premature translational termination at UGA codons which code for tryptophan in mycoplasmas. Four nuclease positive clones from two different chromosomal DNA regions were obtained. This suggested that *M. pulmonis* contains at least two distinct nuclease-encoding genes. Insertional mutagenesis was used to locate a nuclease gene subsequently designated *mnuA* within a cloned 12 kilobase chromosomal fragment. DNA sequence analysis of the gene and its surrounding region identified an open reading frame of 1,410 base pairs with a deduced 470 amino acid polypeptide of 53,739 daltons. A prolipoprotein signal peptidase II recognition sequence was detected, and an extensive hydrophobic region in the amino terminal region suggests that the protein spans the membrane. Analysis of the gene products produced in *E. coli* showed two classes of products, full length and partial products that resulted from aberrant transcription and translation initiation. Some of these latter products were initially inactive, but became active (nuclease positive) after DNA-sodium dodecyl sulfate polyacrylamide gel electrophoresis. The *mnuA* gene products from *E. coli* showed no apparent DNA substrate specificity. However, one membrane nuclease in *M. pulmonis* failed to digest eucaryotic chromosomal DNA. This observation might provide additional approaches to the identification and analysis of other membrane nuclease genes from *M. pulmonis* and other mycoplasma species.

Introduction

The nutritional requirements of the mollicutes indicate that they lack most capacities for *de novo* synthesis of nucleotides. To compensate, mycoplasmas must encode enzyme

activities and transport functions to facilitate the uptake of nucleic acid precursors either as free bases or as oligonucleotides (56). Potent external membrane-associated nucleases have been identified in all mycoplasma species tested with most species appearing to produce multiple nucleolytic proteins (139). The membrane location of this activity 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 organism is attached. It has been shown that mycoplasmas can utilize external radiolabeled RNA and incorporate the labeled nucleotides into either DNA or RNA (154).

The divalent cation requirement of the nucleases varied. In *Acholeplasma oculi*, calcium was not as effective as magnesium in stimulating activity. The membrane nucleases of *Mycoplasma capricolum* were inhibited by calcium and were only active in the presence of a high concentration (10 mM) of magnesium. In *M. pulmonis*, strain differences in the cation requirements were observed; the membrane nucleases of strain UAB6510 and its rat passaged derivative χ 1048 were active only in the presence of calcium whereas magnesium was required for strain MI membrane nuclease activity (139). Thus, it is clear that although all mycoplasma species appear to contain these surface nuclease activities, there is variability in their cation requirements suggesting that they may not be encoded by related genes.

The membrane-nuclease activity in the mollicutes was initially reported in *M. pulmonis*, a rodent respiratory and genital tract pathogen (137, 197). Further analysis of this activity revealed that different strains of *M. pulmonis* produced remarkably similar nuclease banding patterns in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) nuclease gels (139). This species also expressed a higher amount of nuclease activity than the other mycoplasmal species tested (139).

The approach used to identify the structural gene for one of the membrane nucleases of *M. pulmonis* involved the production and use of anti-membrane nuclease antisera to screen a genomic library. The antisera was raised against semi-purified nuclease preparations obtained by preparative SDS-PAGE nuclease gels. Immunoreactive recombinant λ phage were then scored phenotypically for the expression of nuclease activity in an *Escherichia coli* opal suppressor host. The chromosomal DNA fragments were subcloned and restriction mapped. Tn1000 mutagenesis was then used to identify a region within one chromosomal fragment producing nucleolytic activity. The gene thus identified has been sequenced and has been designated *mnuA* for membrane nuclease, the first such gene identified in the mycoplasmas. Our results indicate that *M. pulmonis* contains at least two membrane nuclease genes. The DNA sequence, putative open reading frame and protein profile analysis are described for *mnuA*.

Materials and Methods

Organisms and media

M. pulmonis CT, *M. pulmonis* X1048, *Mycoplasma gallisepticum* R, *Mycoplasma hyorhinis* GDL, *Mycoplasma hyopneumoniae* 232, *Mycoplasma capricolum* 27343, *Mycoplasma fermentans* PG-18, *Mycoplasma fermentans* subsp. *incognitus*, *Mycoplasma penetrans*, *Mycoplasma hominis* 1620, *Mycoplasma pneumoniae* ATCC15531 and *Acholeplasma oculi* ISM1499 were grown as previously described (139). 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 3.1) were started from stock cultures and maintained in 1X or 2X Luria-Bertani broth, in superbroth (32 g tryptone, 20 g yeast extract, 5 g NaCl, per liter, pH 7.2) or on Luria broth agar media. Phage plates for screening genomic libraries on *E. coli* strain ISM612 consisted of superbroth base with superbroth soft agar overlays.

Isolation of *M. pulmonis* chromosomal DNA

Mycoplasma chromosomal DNA was isolated from 1 liter of mid log phase culture. The cells were centrifuged at 10,000 x *g* for 20 min and washed once in 0.01 M sodium phosphate - 0.15 M sodium chloride, (pH 7.4) (PBS). The pellet was resuspended in 7 ml of NET buffer (0.15 M NaCl - 0.08 M EDTA - 0.10 M Tris, pH 7.5) containing 1 mg of proteinase K (Sigma Chemical Co., St. Louis, Mo.) per ml. The cell suspension was incubated at 45°C for 30 min, one fifth volume lysis buffer (1% NP 40 - 1% Triton X-100 - 1% deoxycholate) was added, and the suspension was incubated overnight at 45°C. The mixture was then extracted 4 times with an equal volume of phenol/chloroform (1:1) (Amresco, Inc., Solon, Ohio) and once with chloroform/isoamyl alcohol (24:1) (Fisher Scientific Co., Pittsburgh, Pa.). DNA was dialyzed overnight at 4°C against TE buffer (10 mM Tris - 1 mM ethylene diamine tetraacetic acid, pH 8.0).

Antisera

Antigen preparations containing semi-purified nucleolytic proteins were prepared from a SDS-PAGE nuclease gel using preparative spacers with the Bio-Rad Protean II electrophoresis unit (Bio-Rad Laboratories, Richmond, Calif.). The running and renaturation conditions were adapted from standard conditions (139) in order to obtain the maximal nuclease activity in preparative gels. The nuclease gels were electrophoresed at constant

Table 3.1. Bacterial strains and plasmid descriptions

<i>E. coli</i> strains	Description	Ref.
LE392	F ⁻ <i>hsdR514</i> (r_k^- , m_k^-) <i>lacY1 supE44 supF58 galK2 galT22 trpR55 metB1</i>	
ISM612	(<i>leu</i> (UGA) <i>lacZ659</i> (UGA) <i>trpA9605</i> (UAG) <i>his29</i> (UAG) <i>ilv thyA metB argH rpoB rpsL prfB3</i>) pISM3001	(202)
ISM614	LE392 [F ⁻ <i>hsdR514</i> (r_k^- , m_k^-) <i>lacY1 supE44 supF58 galK2 galT22 trpR55 metB1</i>] pISM3001	This study
ISM647	XL1-Blue [<i>recA1 lac endA1 gyrA46 thi hsdR17 supE44 relA1</i> {F ^{proAB+} <i>lacIq lacZΔM15 Tn10</i> }] pISM3001	This study
DPWC	F ⁺ mating donor	Gold Biotechnologies
BW26	F ⁻ mating recipient, Kan ^R	Gold Biotechnologies
Plasmids		
pISM3001	<i>trpT</i> gene cloned behind a <i>lac</i> promoter in the vector pACYC184, Cm ^R	(202)
pISM4030	12.2 kb <i>SacI</i> fragment from phage 9.2C cloned into pKS	This study
pISM4060	4.7 kb <i>SacI</i> fragment from phage 13.5C cloned into pKS	This study
pISM4061	8.1 kb <i>SacI</i> fragment from phage 13.5C cloned into pKS	This study
pISM4070	3.7 kb and 7.5 kb <i>SacI</i> fragments from phage 14.11F cloned into pKS	This study
pISM4170	6.6 kb <i>SacI</i> fragment from phage 13-2B cloned onto pMOB, Ap ^R	This study
pISM4172	7.0 kb <i>SacI</i> fragment from phage 13-2B cloned onto pMOB, Ap ^R	This study
pISM4175	2.2 kb <i>ClaI</i> fragment from pISM4172 cloned into pMOB, Ap ^R	This study
pISM4176	2.6 kb <i>ClaI</i> fragment from pISM4172 cloned into pMOB, Ap ^R	This study
pMOB	Cloning vector for Tn1000 mutagenesis, Ap ^R	Gold Biotechnologies

current (no greater than 10 mA per gel) at 4°C. Following electrophoresis, the gels were washed four times in 100 volumes of incubation buffer (40 mM Tris - 0.01% Casein - 0.04% β -mercaptoethanol, pH 7.5) for 1 h each, and then incubated overnight in the same buffer at room temperature. Nucleolytic activities were identified by incubating the gel in renaturation buffer supplemented with 2 mM CaCl_2 and 2 mM MgCl_2 for 24 h, staining the gel with ethidium bromide, and then observing it under UV illumination. Areas displaying digestion of the DNA (nonfluorescing regions of the gel) were excised and minced using a 22 gauge needle. For each isolated band, two New Zealand White rabbits were immunized by injecting the acrylamide/protein slurry subcutaneously. Rabbits were boosted 2 weeks post immunization with the same antigen, and the antisera collected at 4 weeks was tested for reactivity by ELISA using *M. pulmonis* lysed whole cell antigen (30) and by immunoblot as described previously (216).

Genomic library

Chromosomal DNA from *M. pulmonis* was partially digested with *Sau3A*, and 9-15 kilobase fragments were isolated using sucrose gradients (122). The fragments were partially filled in and ligated into *XhoI*-digested λ GEM 12 arms according to the manufacturer's directions (Promega Corp., Madison, Wis.). The recombinant phage were packaged and plated onto lawns of *E. coli* strain LE392. Single plaques were picked using a sterile pasteur pipette and dispensed into 200 μl of buffer 100 mM sodium chloride - 10 mM magnesium sulfate - 50 mM Tris - 0.01% gelatin, (pH 7.5) (SM) in one well of a 96-well plate. To elute the phage, the plates were maintained at 4°C overnight on a shaking platform. The phage-containing supernatants were then plated in 10 μl volumes onto mini-lawns of *E. coli* LE392 in microtiter plates. After overnight incubation at 37°C, the mini-lawns were overlaid with 100 μl of SM buffer per well, the plates were incubated for 4 to 6 h at 4°C to allow for phage elution, the plates were centrifuged at 300 x *g* for 10 min, and the supernatant was removed and placed in sterile 96-well plates with an equal volume of SM buffer. Each microtiter plate was sealed with tape, and stored at 4°C.

Library screening

Screening of the genomic library was accomplished by inoculating lawns of *E. coli* ISM612 prepared in 85 mm petri dishes with the phage from the microtiter plates using a 48 pin replicator and growing the phage overnight at 37°C. Isopropyl- β -D-thiogalactopyranoside (Gold Biotechnologies, Inc., St. Louis, Mo.) - saturated nitrocellulose filters were placed on the plates, and the plates were incubated for an additional 5 to 6 h at 37°C. Nitrocellulose

lifts were screened for antigen positive plaques by incubating the filters in blocking solution (5% nonfat dry milk-0.10 M Tris-0.15 M NaCl-0.05% Tween 20, pH 7.5) for 2 h, and then with rabbit anti-nuclease serum diluted 1:100 in TS-Tween (0.10 M Tris-0.15 M NaCl-0.05% Tween 20, pH 7.5). The lifts were washed three times with TS-Tween, incubated with goat anti-rabbit immunoglobulin, alkaline phosphatase conjugate (Organon-Teknika, Durham, N.C.) diluted 1:1000 in TS-Tween for 2 h and then washed three times with TS-Tween prior to development with Naphthol AS-MX Phosphate and Fast Red (Sigma).

Recombinant DNA

Plasmid DNA was isolated by alkaline lysis (15). DNA electrophoresis was performed in Tris-acetate gels as described (122). Restriction mapping of the genomic fragments was performed initially using isolated λ DNA and then confirmed using the plasmid constructions. λ DNA was isolated from recombinant phage as described (122), digested with *SacI* (New England Biolabs, Inc., Beverly, Ma.) and ligated into *SacI*-digested pMOB DNA (Gold Biotechnologies). Plasmids pISM4175 and pISM4176 were constructed by digesting plasmid pISM4172 with *ClaI* and ligating to *ClaI*-digested, dephosphorylated pMOB. All plasmid constructions were confirmed by restriction analysis.

Nuclease detection assays

For nuclease detection, mycoplasma suspensions were produced as previously described (139). To determine the expression of nuclease activity by the recombinant λ phage, the opal suppressor *E. coli* ISM612 was infected with the phage at a multiplicity of infection of 10:1 and the cells shaken at 37°C. IPTG was added (40 mM final concentration) at 2 h, the cells were harvested by centrifugation after a 7 h incubation at 37°C, resuspended in PBS and sonicated prior to use. Nuclease activities associated with recombinant plasmids were monitored in the *E. coli* ISM647 background (Table 1). Transformed colonies were picked, grown overnight, harvested, resuspended in PBS and sonicated prior to use. Sonication was performed using a Branson Ultrasonicator at the maximum setting for the micro tip, 50% duration pulse for 20 pulses. The nuclease digestion of λ nuclease and the SDS-PAGE nuclease gel assays have been described (139). In some SDS-PAGE nuclease gels, λ DNA (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) was substituted for salmon sperm DNA.

Tn1000 mutagenesis

Plasmids pISM4172 and pISM4175 were transformed into *E. coli* DPWC and mated with BW26 according to method described by Strathmann (209). Recipient cells were selected on LB containing 50 µg per ml kanamycin and 100 µg per ml ampicillin. Colonies were picked, grown in 2X Luria broth, and the plasmids were isolated by alkaline lysis (15). Tn1000 insertions were mapped, oriented by restriction digests, and the plasmids analyzed for the loss of nuclease activity. This was accomplished by transforming the plasmids containing Tn1000 inserts into *E. coli* ISM647, preparing cell lysates and analyzing those lysates for nuclease activity as described above. Tn1000 inserts were also used as primer sites for DNA sequence analysis.

Hybridization studies

Chromosomal DNA was isolated as described above. Approximately 2 to 5 µg of DNA from each species was digested to completion with *EcoRI* and the fragments were separated on a 0.7% agarose gel. DNA fragments were transferred by the method of Southern to nylon membrane (Pall Biosupports) as previously described (122). Blots were treated with UV light for 3 min, baked at 80°C for 2 hours and hybridized with DNA probes using Rapid-hyb buffer (Amersham, Arlington Heights, Ill) as previously described (122). Hybridization was performed at 46°C. Blots were washed 3 times for 15 minutes in 2X SSC-0.1% SDS at room temperature and 2 times for 30 minutes in 1X SSC-0.1% SDS at 46°C. DNA probes were labeled using alpha labeled ³²P-CTP using the Rediprime random labeling system (Amersham) and unincorporated nucleotides were removed by spinning the labelled probe through microspin S-300 HR columns from Pharmacia-Biotech (Piscaway, NJ).

DNA sequence analysis

Plasmids for DNA sequencing were prepared using the midi plasmid isolation kit from Qiagen (Qiagen, Inc., Chatsworth, Calif.). All sequencing was performed by the Nucleic Acid Instrumentation facility at Iowa State University using cycle sequencing protocols. The sequencing primers were oligonucleotides complementary to the transposon sequences adjacent to the inverted repeat ends of Tn1000. Primer 187 (5'-CAACGAATTATCTCCTT-3') (Gold Biotechnology) binds to base pairs 39-63 of the transposon and provides sequence information outward from the end closest to the *SacI* site in the transposon (21). Primer 486 (5'-TCAATAAGTTATACCAT-3') binds to base pairs 5924-5947 of the transposon and provides sequence information outward from the opposite end. Three other primers were

constructed from sequencing data and used to sequence selected regions, primer 2590 (5'-GCGACACTGAGCCTAGAG-3'), primer 1145 (5'-GGTGTAGCTACTAATAAAC-3'), and primer 890 (5'-GACCTTAGCCAAATGA AAC-3'). Sequence analysis was performed using MacVector software (Eastman Kodak Company, New Haven, Conn.). The hydrophilicity of the translated product was determined by the method of Kyte and Doolittle (98) and the secondary structure predictions by the methods of Chou and Fasman (32) and Robson and Garnier (67). The translated product was also analyzed for signal sequences and transmembrane domains with PSORT (available on the World Wide Web at URL <http://psort.nibb.ac.jp>). PSORT uses the methods of McGeoch (129) and von Heijne (223) for signal sequence determination and the method of Klein et al. (88) for determining transmembrane domains.

Nucleotide sequence accession number

The nucleotide sequence of the *mnuA* gene of *M. pulmonis* has been assigned GenBank accession number U38841.

Results

Characterization of the anti-nuclease antisera

Antisera produced against SDS-PAGE purified membrane nucleases from *M. pulmonis* were used in immunoblot analysis of whole cells of *M. pulmonis* χ 1048 separated by a 10% SDS-PAGE nuclease gel. Two different immunoreactive profiles were seen using the antisera (data not shown). These crude antisera did not react specifically with a particular region of the blot, and none of the antisera were able to inhibit nuclease activities in *M. pulmonis* whole cells.

Identification and characterization of immunoreactive λ phage

The genomic library of *M. pulmonis* representing 1920 independent, recombinant λ with an average insert size of 10-12 kilobases (kb) was plated on *E. coli* ISM612 and screened as described above. In addition to the *lacI* controlled *trpT* opal suppressor on plasmid pISM3001 (202), a release factor 2 mutation, *prfB2*, is located on the chromosome in this strain. This combination is more efficient in expressing mycoplasma genes containing multiple internal UGA codons than strains containing only the *trpT* allele (202). Nineteen

Table 3.2. Antisera reactivity and nuclease activities of recombinant phage.

Phage Number	Rabbit Antisera No.		Nuclease Act. ^a
	89938	89786	
1.6G	+	-	-
3.6H	+	-	-
6.2B	+	-	-
6.3E	+	-	-
8.12F	+	+	-
9.2A	+	-	-
9.2B	+	+	-
9.2C	-	+	+++
9.3B	+	-	-
9.6G	+	+	-
9.10G	+	+	-
9.12E	+	+	-
11.2D	+	-	-
11.6E	+	-	-
13.2B	-	+	+++
13.5C	+	+	+
14.3D	+	-	-
14.7C	+	+	-
14.11F	-	+	+

^aThe nuclease activity was determined by screening λ lysates using the λ DNA nuclease assay as described in the Materials and Methods. Results were recorded as positive if any nuclease activity was observed in the cell lysates. (+) refers to a slight amount of detectable nuclease activity expressed by *E. coli* infected with the recombinant phage. (+++) refers to high nuclease activity expression.

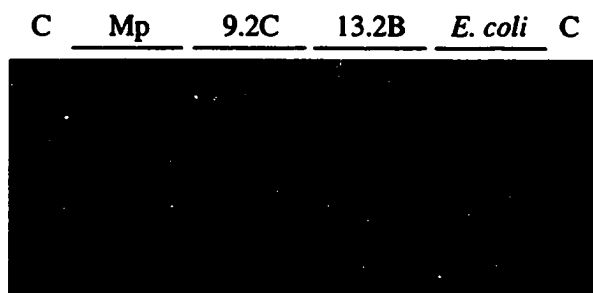


Figure 3.1. Analysis of recombinant phage 9.2C and 13.2B by λ assay. Phage lysates were prepared and examined for nuclease activity as described in the text. Undigested λ DNA is shown in the outer lanes (C). *M. pulmonis* antigen (Mp) was used as the positive control and *E. coli* ISM612 as the negative control. The three lane groupings represent increasing two-fold protein concentrations from left to right. The gel was digitized using a Cohu model 4900 high performance CCD camera (Cohu, Inc., San Diego, Calif.) and a Macintosh Iici equipped with a Scion Corporation (Frederick, Md.) video board. The resulting TIFF file was cropped with assembled in Adobe photoshop and labeled in Aldus Freehand.

immunopositive phage were identified in the library by the antiserum (Table 3.2). Four of the 19 immunoreactive phage expressed nuclease activity by the λ assay. The two demonstrating the highest level of activity are shown in Fig. 3.1. The inserts cloned within these positive phage were restriction mapped (Fig. 3.2). The chromosomal DNA from 9.2C, 13.5C and 14.11F were subcloned into pKS whereas the chromosomal DNA from 13.2B was subcloned into pMOB.

Cloning and functional analysis of the chromosomal fragment from phage 13.2B

In order to further analyze the plasmids, they were transformed into the opal suppressor strain ISM612 which had no endogenous nuclease activity in our assays (data not shown). When the plasmids containing chromosomal inserts from phage 9.2C and 13.5C (Fig. 3.2) were analyzed for expression of nuclease activity, the activity was present in less than 10% of the isolated colonies. This was not the result of loss of the plasmid. It was assumed that the nuclease genes were undergoing a high rate of mutation and these plasmids were not studied further. When pISM4170 was placed into strain ISM612, the same phenotype was obtained.

All attempts to transform plasmid pISM4172 into strain ISM612 failed. This was interpreted to be the result of plasmid instability in the *trpT-prfB2* background. To test this

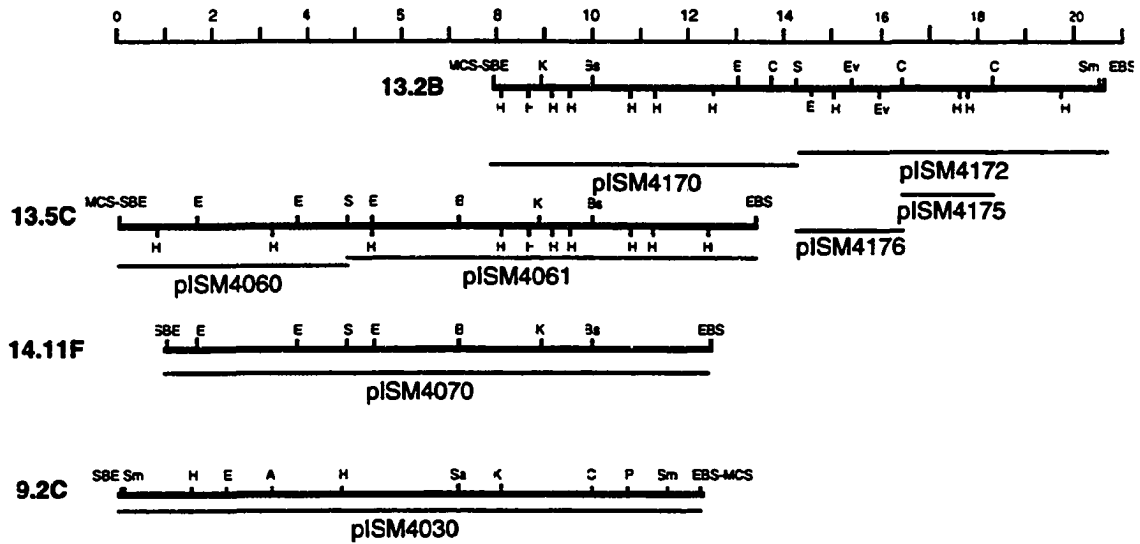


Figure 3.2. The physical maps of the chromosomal inserts in recombinant phage 13.2B, 13.5C, 14.11F, and 9.2C. The subcloned regions and the plasmid designations are also given. The sizes are given in kilobases. Symbols: A, *Apa*I; B, *Bam*HI; Bs, *Bss*HIII; C, *Cla*I; E, *Eco*RI; Ev, *Eco*RV; K, *Kpn*I; H, *Hind*III; P, *Pst*I, S, *Sac*I; Sm, *Sma*I.

possibility, two *E. coli* strains lacking the *prfB2* mutant allele, LE392 and XL1-Blue, were transformed with pISM3001. The resulting strains, ISM614 and ISM647, were screened for endogenous nuclease activity which might interfere with mapping the nuclease structural gene by transposon mutagenesis and examined for stable maintenance of pISM4172. Both *E. coli* strains lacking the *prfB3* mutation supported replication of plasmid pISM4172, but only ISM647 lacked the type of nuclease activity identified in our λ assay (data not shown).

To locate the membrane nuclease structural gene within the 7.0 kb insert of pISM4172, the plasmid was subjected to Tn1000 mutagenesis as described (209). 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 Tn1000 within a 1.0 kb region of pISM4172 knocked out nuclease activity (Fig. 3.3, Panel A, black bar). Plasmid pISM4176 containing the 2.6 kilobase *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 (Fig. 3.3)

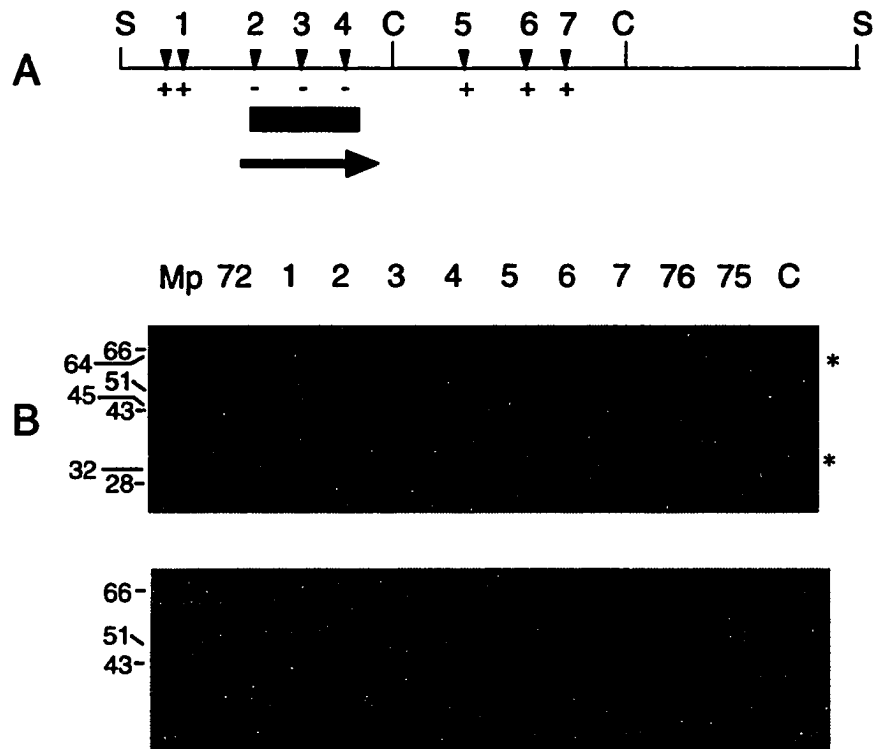


Figure 3.3. Tn1000 analysis of pISM4172. The plasmid pISM4172 was subjected to Tn1000 mutagenesis and analyzed as described in the text. (A) Shown are locations of selected Tn1000 inserts in pISM4172 are designated by inverted, filled triangles. Below the map are the results of the λ DNA assay. Inserts 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 Tn1000 mutagenesis. The arrow indicates ORF1 (*mnuA*) as determined by DNA sequence analysis and the direction of transcription. (B) Analysis of Tn1000 inserts by SDS-PAGE nuclease gel assay was performed as described in the text. The apparent molecular weight of the nucleolytic bands is shown on the left. (*) indicates nucleolytic bands arising from *E. coli* with specificity for salmon sperm DNA. All lanes contained 5 μ g *E. coli* protein from strain ISM647 harboring the indicated plasmid 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. Symbols: Mp, *M. pulmonis* χ 1048 antigen; 72, pISM4172; 76, pISM4176; 75, pISM4175; C, ISM647 (pMOB); the remaining lanes (1-7) contain protein from the strains harboring the correspondingly labeled Tn1000 insertions shown in panel A.

DNA sequencing and computer analysis

Approximately 5.0 kb of DNA sequence information was obtained from the region encoding the nuclease activity as shown in Fig. 3.4. An open reading frame (ORF) of 1410 bp was identified within this region encoding a 470 amino acid (aa) polypeptide of 53.7 kDa (Fig. 3.4). *Tn1000* insertions upstream and downstream of ORF1 failed to eliminate nuclease activity (Fig. 3.3). No Shine-Dalgarno-like sequence was found upstream of the *mnuA* ORF. There was also no apparent promoter region, but the region was AT rich with several potential TATA boxes. 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 rich region which could serve as a membrane spanning region to anchor the protein (Fig. 3.4). There was no typical cleavable signal sequence, but the charged - hydrophobic - charged domains in the amino terminal region resembled principle features of bacterial signal peptides (163). 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 (24, 239). The remaining portion of the molecule was extensively hydrophilic with substantial alpha helical character (Fig. 3.4). The estimated pI of the translated product was 9.04, and it had a calculated molecular mass of 53,738 daltons. Homology searches with the translated product revealed short stretches of homology (10-15 amino acids) with the CpG methylase of *Spiroplasma citri*, but no other homology with known DNA or protein sequences was observed. Several regions of the translated product of *mnuA* showed homologies to proteins with DNA binding characteristics. Among these was the DNA topoisomerase I of yeast, mouse and human, and the RNA polymerase beta of maize. No typical transcriptional terminator was identified.

Analysis of *mnuA* products produced in *E. coli*

E. coli containing pISM4172 expressed four distinct products of 66, 53, 45 and 39 kilodaltons (kDa) from the chromosomal fragment containing *mnuA* (Fig. 3.3). The band at 45 kDa, however, was weak and was sometimes missing (lower panel, lanes 72, 1 and 2). The 45 and 66 kDa bands appeared to be related since the *Tn1000* insertion at position 175 of the *mnuA* gene sequence (insertion 2) knocked out 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. *Tn1000* insertions located further downstream of insertion 2 (locations 3 and 4), knocked out all nucleolytic bands in the SDS-PAGE nuclease gels.

Figure 3.4. Nucleotide and deduced amino acid sequence of the *M. pulmonis mnuA* gene. Nucleotide coordinates are counted from the beginning of the sequence. The *mnuA* ORF begins at nucleotide 97 and extends through nucleotide 1486. An inverted repeat is underlined at the 3' end of the sequence. (↓) indicates a Tn1000 insertion site.

* * * * *
 ATAGAAATTGGCATAGAATTGGATAAAATAATTGTTAAACGCCTTTTAAACGCTTTGAAAAAGGCGTTTTTT 70
 M K N K M K S L K V S L L V A
 AATTAAGCAATAATAAAATTATTGATATGAAAAATAAGATGAAATCGTTAAAAGTTAGTTTATTAGTAGCT 140
 T P I I T L P L F T I S C I P N N E S K S Y Q D
 ACACCAATAATTACACTCCCATTATTTACAATTAGTTGCATTCCAAATAATGAATCTAAATCTTATCAAGA 210
 L N A K A N Q A A I L L K Q K Q I N S N↓K L E E
 TTTAAATGCCAAGGCTAATCAAGCGCCATTTTGCTTAAACAAAAACAAATTAATAGCAATAAATTAGAAG 280
 L I D S I L P E G T E L E I L D L W V D R E N
 AGCTAATTGACAGCATTCTACCCGAAGGAAGTGAAGTTAGAAATTTAGATCTTTGAGTGGATCGTGAAAAT 350
 K E I N V K F R L R Y K D R R Y P Y S S D V K I
 AAAGAAATTAATGTTAAATCCGCTCTTAGATATAAAGACAGAAGATATCCTTATAGTTCGGATGTCAAAAT 420
 I K L K Y E S I L E N L K P Q E K P K E K P G R
 AATTAAGTTAAAGTATGAGTCTATCTTGGAAGAACTTCAAACCGCAAGAAAAACCAAAGGAAAAACCCGGAC 490
 K S K E K N P K T Q N D E K S K D G L N K K S
 GTAAGTCTAAAGAAAAGAACCCAAAAACCCAAAATGATGAAAAGTCTAAAGATGGCCTTAATAAAAAGTCA 560
 N G K Q K M K W G H W N V L K L S D K N D K K I
 AATGAAAAACAAAAATGAAATGAGGACATTGGAATGTTCTTAAATTAAGCGACAAAAATGATAAAAAAAT 630
 A A I A A L I K K I D Y D V V G L T E V F S E E
 TGCTGCCATTGCCGCTTTAATTAAAAAAATTGACTATGATGTCGTTGGCCTAACGGAAGTATTAGCGAAG 700
 A V K↓E V V A Q L N K L A G S G T Y S Y V A S
 AAGCCGTAAAGAAGTCGTTGCTCAATTGAATAAACTAGCCGGCAGTGGCACATATTCTTATGTTGCATCA 770
 K K L K G S Y A G N L Q A E H V G V I Y N S Q K
 AAAAACTTAAAGGCTCATATGCTGGCAATTTGCAAGCTGAACATGTCCGAGTAATTTATAATAGCCAAAA 840
 I K T I P F E K T K N N K K E I G Y S Y I E K F
 AATTAAGAACTATTCCATTTGAAAAGACTAAGAATAATAAAAAAGAAATAGGCTATAGTTATATAGAAAAAT 910
 N S E F I K K H N K A E Y V R P P Y G V K F Q
 TCAATTCAGAATTTATTAAAAACATAACAAAGCCGAGTATGTACGACCACCATATGGTGTTAAATTCCAA 980
 W L K G K K E D F T F V F D H F D S P G G S K G
 TGATTAAAAGGGAAAAAGAAGATTTTACATTTGTTTTCGACCATTTTGATAGCCCTGGCGGAAGCAAAGG 1050
 E G T T S G V G N Q E L A E A R Q L A N V L D H
 TGAAGGAACAACCTAGTGGAGTCGGCAATCAAGAGCTAGCAGAAGCTAGACAACCTTGCAAATGTACTTGATC 1120
 F D E I D G E N E D I F F S G D T N I K A G K
 ATTTTGACGAAATTGATGGCGAAAAACGAAGATATCTTCTTTAGCGGAGACACCAACATCAAAGCGGGCAA 1190
 E K F A F G E↓E L F K K Y Q S A F K D N L E F A
 GAAAAATTTGCTTTTGGTGAAGAATTATTCAAAAAATATCAATCCGCTTTTAAAGATAACCTTGAGTTTGC 1260
 T S L G I K A G N Y S Q P Y D K M F T K T K F K
 AACATCATTAGGAATAAAGGCCGGCAACTACTCACAACCATATGATAAAATGTTCAAAAAACCAAATTTA 1330
 T S N H G I Y K L W D L S K D D E F T N L F K
 AGACTTCAAATCATGGCATCTATAAACTATGAGACTTGAGTAAAGATGATGAATTTACAAATCTTTTCAA 1400
 E L H N G K I E T S E I R K L V S D H A P I Y S
 GAATTACATAATGAAAAATTGAAACTAGTGAAATAAGAAAACTAGTATCTGATCACGCGCCTATATATAG 1470
 E V E F E
 TGAAGTTGAATTTGAATAATTAAAAAAATATAAAAAAATCGATGCCTAACTTTGGTATCGATTTTAACTTT 1532

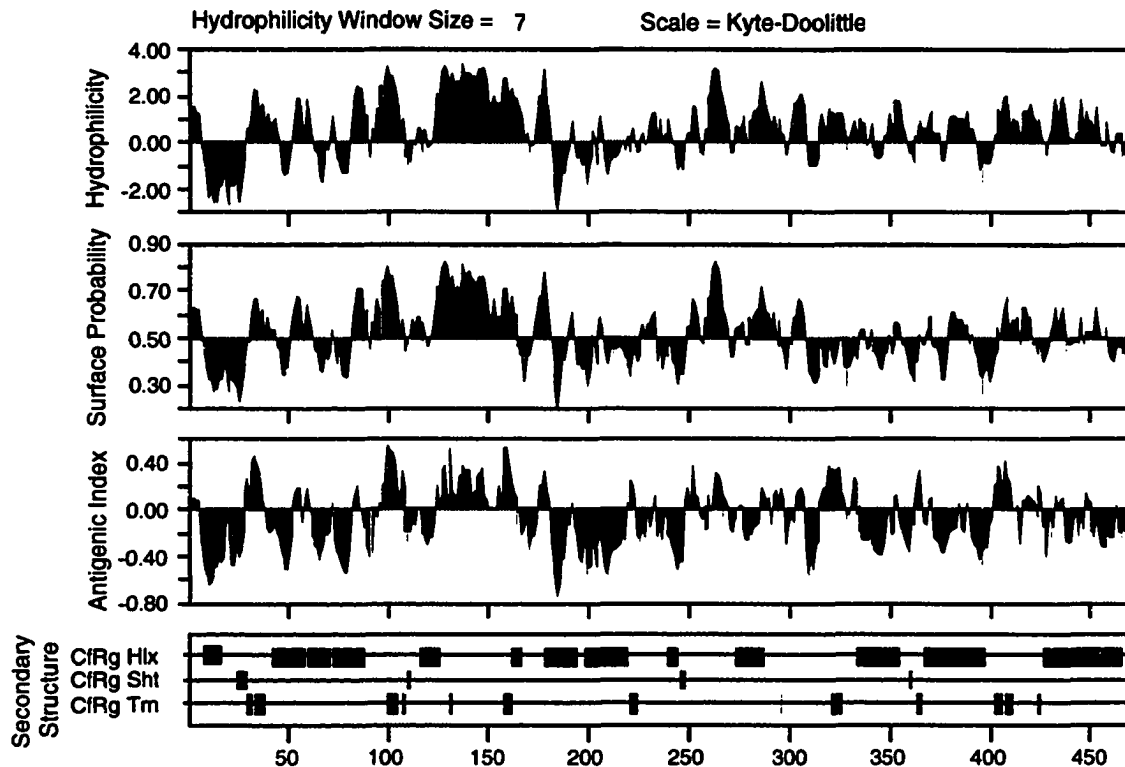


Figure 3.5. Predicted properties of the translated sequence of *mnuA*. All analyses were performed using MacVector software. The scale at the bottom of the figure indicates the amino acid residue number. The type of analyses are indicated on the left. Positive numbers indicate high probability for hydrophilic regions, surface exposure and antigenicity. Shown in the bottom panel are the regions of secondary structure that agree using the Chou-Fasman (Cf)(4) and Robson-Garnier (Rg)(9) methods. Hlx, α -helix; Sht, β -sheet; Trn, turn.

Correlation of the λ assay results with the SDS-PAGE nuclease gel results shown in Fig. 3.3 shows that *mnuA* translation products which probably 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. 3.3, lower panel, lane 2).

Presence of *mnuA* like sequences in other mycoplasma species

The internal *EcoRV* fragment of *mnuA* was used to determine if this gene was shared among mycoplasma species. As is shown in Figure 3.6, there was cross-hybridization between the *mnuA* probe and chromosomal fragments from *M. hyopneumoniae* and *M. pneumoniae*. There was only one copy of *mnuA* in the *M. pulmonis* chromosome and that the gene is located in a similar location in both *M. pulmonis* CT and *M. pulmonis* X1048, the latter being the strain from which *mnuA* was isolated.

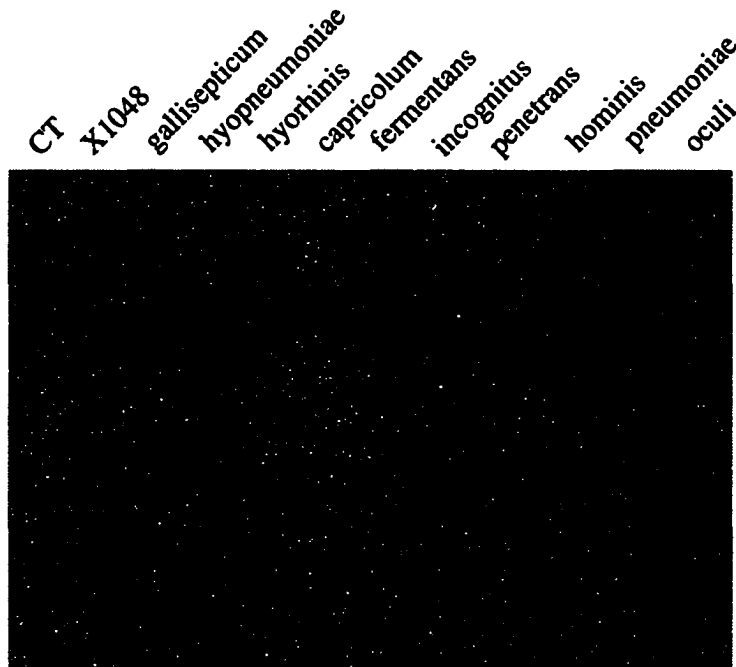


Figure 3.6. Hybridization of analysis of mycoplasma species with a *mnua* probe from *M. pulmonis*. The probe used for analysis was a *EcoRV* fragment from pISM4176. Hybridization conditions are described in the Materials and Methods. Lanes: CT, *M. pulmonis* strain CT; χ 1048, *M. pulmonis* χ 1048; 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* supsp. *incognitus*; penetrans, *M. penetrans*; hominis, *M. hominis* 1620; pneumoniae, *M. penumoniae* ATCC 15531, oculi, *A. oculi* ISM1499.

Discussion

This work represents the first cloning and analysis of a membrane-associated nuclease gene, *mnua*, from a mycoplasma. Identification and functional analysis of *mnua* was possible because of the suppression of UGA codons by *trpT176* resulting in expression of nucleolytic activity from cloned and mutated fragments. A highly efficient suppressor strain, ISM612, was unable to stably maintain any fragment containing membrane nuclease activity. This suggests that the higher levels of nuclease due to increased suppression efficiency of this strain as compared to strain ISM647 resulted directly in strain instability. The lower efficiency of UGA suppression in ISM647, however, would result in prematurely truncated protein products, some of which may contain nucleolytic activity (Fig. 3.3).

These studies clearly show that level of active nuclease expression from the cloned fragments was low in *E. coli* (Fig. 3.3). Also, expression was independent of vector-directed promoter activity. Neither λ GEM 12 nor pMOB contain promoters to regulate expression of cloned genes. Therefore, the promoter activity that directed the expression of *mnuA* was associated with the mycoplasmal sequences. This is common in mycoplasmas (89) and is presumably due to the AT richness of the chromosomal DNA. Low level expression of the nonspecific nuclease activity might be essential for λ viability since high level expression could result in damaged λ concatameric DNA and an unsuitable packaging substrate. Thus, cloning strategies that would raise the nuclease product levels 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 for instability.

E. coli has previously been a host for the expression of several nonspecific nucleases, such as those from *S. aureus* (26), *Thermus filiformis* (57) and *Shigella flexneri* (35). The staphylococcal nuclease has been extensively studied (194) and is expressed at low levels from its native promoter in *E. coli* (193). The product is secreted either from its own signal peptide or with a lipoprotein signal sequence attached (159). Higher levels of activity can be obtained when expressed from a λ promoter (85), but in the absence of a functional signal sequence, expression of the nuclease was lethal (212). All of these nucleases have been expressed in *E. coli*, indicating that expression of heterologous nucleases in *E. coli* is possible and a convenient way to study these proteins.

Mapping of the nuclease gene within the cloned fragment in pISM4172 involved random mutagenesis with Tn1000. Over 30 different insertions were analyzed, but only a portion are shown in Fig. 3.3 for clarity. Correlation of the insertion location with loss of nuclease activity identified a minimal coding region for the *mnuA* structural gene, minimized the amount of DNA sequencing needed for identification of the coding region, and confirmed the location of the ORF determined by computer analysis (Figs. 3.3 and 3.4).

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. 3.3). *E. coli* strain ISM647 contained nuclease bands of 64 and 32 kDa that digested salmon sperm DNA (Fig. 3.3, upper panel, lane C), but not λ DNA (lower panel, lane C). This correlated with our results with this *E. coli* strain in the λ DNA assay (see above). A *M. pulmonis* derived nucleolytic band of about 28 kDa was also absent in the gel containing λ DNA (Fig. 3.3, panel B, lower gel, lane Mp). The lane was loaded with a 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 present in the 28 kDa region when λ DNA was used as a substrate. It is

not known what distinguishes the substrate specificity of these enzymes. Two possible explanations would be methylation pattern differences between eucaryotic and procaryotic DNA or single vs. 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 DNA substrate differences.

These data suggest, but do not prove, that MnuA is the 51 kDa protein in the *M. pulmonis* banding pattern (Fig. 3.3). Considering the possibility that the single cysteine residue serves as a signal peptidase cleavage and acylation site (163, 239), the processed polypeptide would be 50,769 daltons in size (not including the fatty acid side chain). All nuclease activities in *M. pulmonis* partition into the detergent phase during Triton X-114 fractionation suggesting that each nuclease has large regions of hydrophobicity or are lipoproteins (data not shown). While this possibility seems plausible, there is no direct evidence supporting this hypothesis. For instance, it is not known what effects, if any, acylation might have on protein mobility in SDS-PAGE gels. Additionally, mobility of nucleases might be altered during SDS-PAGE in DNA-containing resolving gels as well.

It seems reasonable that *mnuA* does code for a membrane nuclease because all *M. pulmonis* nuclease activities observed in the SDS-PAGE nuclease gels are membrane associated (139). Also, the activity expressed in *E. coli* survived the sample preparation and SDS-PAGE analysis just like the membrane-associated nucleases reported earlier (137, 139) and had the same substrate specificity (Fig. 3.3). There may still be a possibility, however, that the cloned nuclease activity is unrelated to the membrane nuclease activity observed in *M. pulmonis* membranes. We have not been able to conclusively correlate the *mnuA* gene product in *E. coli* with one of the membrane nucleases in *M. pulmonis* due to our inability to construct site-directed mutations in this mycoplasma species.

Interestingly, Tn1000 insertion 2 (Fig. 3.3) knocked out nuclease activity in cell lysates, but not in the SDS-PAGE nuclease assay. The nuclease banding pattern lost two of the four bands seen with the nonmutated plasmid. The presence of the two bands confirms the hypothesis of internal transcriptional initiation because this insert is located behind the signal sequence of *mnuA*. The resulting protein products were probably folded incorrectly in the cell lysate, but regained a functional conformation during the denaturation and renaturation conditions of the assay.

To confirm the identity of the structural gene from *M. pulmonis* with a product produced by *E. coli*, further studies will be needed. Several different products are being produced, which may result from internal initiation and/or premature truncation. To confirm this suggestion, amino acid sequencing would need to be performed. However, purifying these

proteins may prove difficult as previous attempts to purify membrane nucleolytic activity from *M. pulmonis* have failed. This has been due to difficulties in purifying membrane proteins while maintaining functional activity. It is also likely that these nucleases are produced in low amounts, compounding the purification difficulties. A specific knockout mutation in *mnuA* in *M. pulmonis* would correlate the *mnuA* gene sequence with a specific gene product simply by comparing mutant and wildtype SDS-PAGE nuclease gel patterns. Two factors might hinder this approach, however; genetic tools in *M. pulmonis* are limited (47, 48), and *mnuA* may be essential to growth preventing the construction of a single knockout mutation.

It is evident that two different chromosomal regions of *M. pulmonis* express nuclease activities in *E. coli*. The restriction maps of the cloned fragments of are distinctly different (Fig. 3.2). These genes also show little homology at the nucleotide level since the *mnuA* gene sequence failed to hybridize to 9.2C phage DNA (data not shown) and to plasmids derived from 13.5C. The relative positions of the two regions on the chromosome could not be determined since a physical map of *M. pulmonis* was not available. It is clear, however, that the multiple bands observed in the nuclease banding pattern in *M. pulmonis* (139) is due at least in part to the expression of separate, unrelated genes in the chromosome.

Using an internal fragment from *mnuA*, there appears to be related genes to *mnuA* in other mycoplasma species. Analysis of 11 different species of mycoplasma indicated that homologous gene sequences are present in *M. hyopneumoniae* and *M. pneumoniae*. One copy of the gene was present in *M. pulmonis*. These three species of mycoplasmas are not closely related phylogenetically, but they are all respiratory pathogens.

It is not yet clear what role, if any, the MnuA nuclease plays in growth and virulence in *M. pulmonis*. These nucleases are fully capable of digesting mycoplasmal chromosomal DNA (data not shown) inferring that cotranslational translocation of the *mnuA* gene product may be occurring (163). Further study of this unique activity could reveal basic mechanisms of gene expression and protein translocation in mycoplasmas.

Acknowledgments

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CHAPTER 4. MOLECULAR ANALYSIS OF THE *uvrB* HOMOLOG IN *MYCOPLASMA PULMONIS*

A paper prepared to be submitted to Journal of Bacteriology
Karalee J. Jarvill-Taylor and F. Chris Minion

Abstract

Previous studies have described the cloning and analysis of a membrane nuclease gene in *Mycoplasma pulmonis*, *mnuA*. DNA sequence analysis upstream of the *mnuA* gene revealed a second open reading frame with a homology with *uvrB*, a component of the short patch repair excinuclease. The *uvrB* gene had 51% identity and 70% homology with the *uvrB* gene from *Haemophilus influenzae*, and contained a Walker type A nucleotide binding motif. An internal fragment from the *M. pulmonis uvrB* gene was used to probe chromosomal fragments of 11 mycoplasma species. The results indicated that many, but not all mycoplasma species have sequence homology to the *uvrB* gene of *M. pulmonis*. This also suggests that mycoplasmas have the capacity to repair DNA damage by the nucleotide excision repair system.

Introduction

The nucleotide excision repair system, sometimes referred to as dark repair or short patch repair, has been extensively characterized in several bacteria, most notably *Escherichia coli* (64). The critical enzyme for this process is the multi-component excinuclease composed of UvrA, UvrB, and UvrC subunits. UvrD helicase II and DNA polymerase I also participate in the process. The primary function of short patch repair is to recognize and remove thymidine dimers in UV damaged DNA, and damage resulting from exposure to various chemical agents or mutagens. The presence of the short patch repair is assumed in most bacterial species, and its absence results in an increased mutation rate and an inability to survive UV exposure.

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 (166, 169). As parasitic organisms, they are lacking a number of biosynthetic pathways (169). Early studies reported a dark repair system in *Mycoplasma buccale* (4), but not in *Mycoplasma gallisepticum* (69). This work was based upon survival after exposure to ultraviolet light. Further studies to identify and characterize a short patch repair system in mycoplasmas has

not been reported. Only recently have the genes of various proteins involved in DNA replication and repair, such as RecA and topoisomerase, been identified and characterized in mycoplasmas (49, 59, 87).

Mycoplasma pulmonis, a rodent pathogen, has been the subject of several genetic studies. The genes for RecA (87), DNA polymerase III (9), and a restriction-modification system (50) have been identified in this species. It was the first mycoplasma to be transformed using a clearly defined genetic element, Tn916 (48). Transformation studies using plasmids which integrate into the chromosome by homologous recombination indicated that *M. pulmonis* has an active recombination system that could be exploited for the introduction of specific mutations or genetic markers (134). Even with the genetic progress made over the last 10 years, there is still no chromosomal map available for this species and no transposon besides Tn916 has been found capable of transforming *M. pulmonis*.

Recently, a gene for a membrane nuclease gene from *M. pulmonis* was identified and characterized (82). During the course of those studies, it was determined that this gene, designated *mnua* for membrane nuclease, was located in a two gene operon. The upstream open reading frame was found to be 70% homologous to the UvrB proteins of *E. coli* and *Haemophilus influenzae*. The results of the analysis of *M. pulmonis uvrB* is described here.

Materials and Methods

Bacterial strains and plasmids

M. pulmonis CT, *M. pulmonis* χ 1048, *Mycoplasma gallisepticum* ATCC 19610, *Mycoplasma hyorhinis* GDL, *Mycoplasma hyopneumoniae* 232, *Mycoplasma capricolum* ATCC 27343, *Mycoplasma fermentans* PG-18, *Mycoplasma fermentans* subsp. incognitus, *Mycoplasma penetrans*, *Mycoplasma hominis* 1620, *Mycoplasma pneumoniae* ATCC 15531 and *Acholeplasma oculi* ISM1499 were grown as previously described (139). Cultures were obtained from a stock culture maintained at -70°C, inoculated into fresh broth medium and incubated statically at 37°C. *E. coli* strain LE392 [F^- *hsdR514* (r_k^- , m_k^-) *lacY1 supE44 supF58 galK2 galT22 trpR55 metB1*] was started from a stock culture and maintained in Luria-Bertani broth.

Plasmids pISM4170 and pISM4172 have been described elsewhere (see Chapter 3) (82). Plasmid pISM4170 contains a 6.6 kb *SacI* fragment from phage 13.2B cloned into pMOB (Fig. 3.2). Plasmid was constructed by cloning a 7.0 kb *SacI* fragment from phage 13.2B cloned into pMOB (Fig.3.2).

Isolation and manipulation of DNA

Plasmid DNA was isolated from *E. coli* by alkaline lysis (15). DNA fragments used for cloning and as probes were isolated from ethidium bromide stained agarose gels using GenElute Agarose Spin Columns (Supelco, Bellefonte, Pa.). The *uvrB* probe for the DNA hybridizations was isolated as a *SacI-HindIII* fragment from plasmid pISM4172. The fragment was labeled with the Rediprime random labeling kit (Amersham, Arlington Heights, Ill.) using alpha labeled ^{32}P -CTP (Amersham).

Plasmids pISM4170 and pISM4172 were transformed into *E. coli* DPWC and mated with BW26 according to the method described by Strathmann (209). Recipient cells were selected on LB containing 50 μg per ml kanamycin and 100 μg per ml ampicillin. Colonies were picked, grown in 2X Luria broth, and the plasmids were isolated by alkaline lysis (15).

Tn1000 insertions were mapped, either by restriction digest or by polymerase chain reaction. Polymerase chain reactions were done in a 50 μl volume containing 1x buffer (GIBCO, BRL), approximately 1 ng of plasmid DNA from alkaline lysis, T7 primer and either primer 187 or primer 486 (5 pmole) (see below), 4 mM MgCl₂, 20 mM dNTPs and 1 unit 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. Tn1000 inserts were also used as primer sites for DNA sequence analysis.

Hybridization studies

Chromosomal DNA was isolated from the mycoplasmal species described above. Approximately 2 to 5 μg of DNA of each species was digested with *EcoRI* to completion, and fragments were separated on a 0.7% agarose gel. DNA fragments were transferred by the method of Southern to nylon membranes as previously described (122). Blots were exposed to a transilluminator for 3 min, baked at 80°C for 2 h and hybridized with DNA probes using Rapid-hyb buffer (Amersham, Arlington Heights, Ill). Hybridization was performed at 42°C. Blots were washed in 2X SSC-0.1% SDS three times for 15 minutes at room temperature and in 1X Ssc-0.1% SDS twice for 30 minutes at 42°C.

DNA sequence analysis

Plasmids for DNA sequencing were prepared using Qiagen columns (Qiagen, Inc., Chatsworth, Calif.). All sequencing was performed by the Nucleic Acid Instrumentation facility at Iowa State University using cycle sequencing protocols. The sequencing primers were oligonucleotides complementary to the transposon sequences adjacent to the inverted

repeat ends. Primer 187 (5'-CAACGAATTATCTCCTT-3')(Gold Biotechnology) binds to base pairs 39-63 of the transposon and provides sequence information outward from the end closest to the *SacI* site in the transposon (209). Primer 486 (5'-TCAATAAGTTATACCAT-3') binds to base pairs 5924-5947 of the transposon and provides sequence information outward from the opposite end. The T7 primer (5'-AATACGACTCACTATAG-3') was produced by the Nucleic Acid Instrumentation facility at Iowa State University. Sequence analysis was performed using MacVector software (Eastman Kodak Company, New Haven, Conn.).

Nucleotide sequence accession number

The nucleotide sequence of the *uvrB* gene of *M. pulmonis* has been assigned GenBank accession number U59874.

Results

Sequencing of *uvrB*

During studies to identify genes coding for membrane nucleases in *M. pulmonis*, a lambda clone was identified with nuclease activity and its fragment subcloned into plasmid pISM4172 (See Chapter 3). Tn1000 mutagenesis was performed, and the mutants were screened for loss of nuclease activity in *E. coli* (82). DNA sequence analysis revealed an open reading frame coding for the membrane nuclease which was subsequently designated as *mnuA*. A potential upstream open reading frame was also identified with homology to *uvrB* genes in other bacteria. Since little is known concerning DNA repair systems in mycoplasmas, it was of interest to complete the sequence of this *uvrB* homolog and the rest of the *mnuA* operon. In order to complete the DNA sequence of the *uvrB* homolog, plasmid pISM4170, which contained the chromosomal region upstream of *mnuA*, was subjected to Tn1000 mutagenesis and inserts were localized by PCR. Those inserts located near the T7 promoter of pISM4170 were used for completion of the *uvrB* homolog sequence and the *mnuA* operon.

DNA sequence analysis

The complete sequence, with the corresponding amino acids, of the *uvrB* homolog is shown in Figure 4.1. The deduced amino acid sequence is also given. The gene codes for a protein of 75,878 daltons. There were no open reading frames of greater than 50 amino acids

Figure 4.1. Nucleotide and deduced amino acid sequence of *uvrB* from *M. pulmonis*. The coding region begins at nucleotide 201 and ends at nucleotide 2172. The underline indicates potential ribosomal binding sites.

+ + + + + +	
TCTTGAAATTTGCTTAATAACTTATAAAGATCATCGTCCGAAGACACACTTAAAAATGGCTCAAG	65
ATCAAAATTATTTATTTTATTTGTGCTCATTAAACGAACTCCATCAGACTTTTTTTTAGTAAAT	130
TTTAAAGAATATTAAATCGCTAATAACTTTTTGAATAACTAAGTTGATTAATAGTAAAATTAAGG	195
M E E K F V L H S P F A P S G D Q P E A	
AAGTTATGGAAGAAAAATTTGTATTACATAGTCCATTGCTCCTTCGGGCGATCAACCAGAAGCA	260
I K A L V D G I D E K K E H Q V L L G V T G	
ATCAAAGCACTAGTAGATGGCATTGATGAAAAAAGAACATCAAGTCCTTTTGGGAGTAACCGG	325
S G K T F T I A N V I A Q L N R P S L I I S	
TTCGGGTAAACATTTACTATTGCTAATGTTATTGCACAACTAAATCGTCCTTCTTTAATCATT	390
H N K T L A S Q L Y S E L K A L F P D N R	
GCCACAACAAAACTTTGGCAAGTCAACTATATAGTGAGCTAAAAGCGCTATTTCCAGATAACCGT	455
V E Y F V S Y F D F Y K P E A Y I P K S D L	
GTTGAATACTTTGTTTCGTATTTTGATTTTTATAAACCTGAAGCCTACATTCCTAAGAGCGATCT	520
Y I E K T S K N N K E L E A M R M S A I N A	
TTATATTGAAAAAAGTAGTAAAAACAATAAGGAATTAGAAGCGATGCGAATGTCAGCTATCAATG	585
L S I R K D T I V V A S V A A I Y G E S N	
CCTTAAGTATTAGAAAAGACACTATTGTTGTTGCCTCAGTTGCAGCTATTTATGGTGAAAGCAAT	650
P K H Y R Q N F F P I E V G M Q I D R K S L	
CCTAAGCACTATCGCCAAAACTTTTTTCCCATTGAAGTGGGTATGCAAATCGATCGAAAGAGTTT	715
L L K L S Q I G Y E R N R M E L N K G Q F D	
GCTACTAAAACTTTCGCAAATTGGATACGAAAGAAACCGCATGGAATAAATAAGGACAATTTG	780
V K G D S I E I C P G Y V S D T N I R I D	
ATGTTAAAGGTGACAGCATTGAAATTTGTCCCGGATATGTTAGTGATACTAATATTTCGAATTGAT	845
M F G N E I E A I T L I D P L S K N V E G S	
ATGTTTGGTAATGAAATTGAAGCTATTACTTTAATTGATCCTCTTTCGAAAAATGTTGAAGGGTC	910
R K N M T L F P A T T Y T V H E N T I Q N T	
AAGAAAAAATATGACGCTTTTCCCGGCCACTACTTATACCGTGCATGAGAATACCATTCAAATA	975
V D L I K Q E L S E R I E Y F K S H D K L	
CTGTAGATCTAATTAACAAGAGCTTTCTGAGCGGATTGAATACTTTAAATCGCACGATAAGCTT	1040
L E A Q R I K D R T L N D L D S L L E F G Y	
CTTGAAGCGCAAAGAATTAAGGATCGTACTTTGAACGATCTTGATTCACTACTAGAGTTTGGTTA	1105
T S G I E N Y S R Y L D G R A P G Q R P Y T	
CACTTCGGGAATTGAAAACACTCTAGATATCTAGATGGCAGAGCTCCAGGACAACGCCCTTATA	1170
L F D Y L P D D S V I F I D E S H L M I P	
CTTTGTTTGACTACTTACCAGATGATAGTGTTATTTTATTGATGAAAGTCACTTAATGATTCCC	1235

Q L H G M H N G D R A R K L S L V E Y G F R CAATTGCATGGTATGCATAATGGTGATCGTGCCAGAAAACCTAGTTTAGTTGAATATGGTTTTAG	1300
L P S A L D N R P L R F E E F A E F K F P K ACTACCATCTGCCCTTGATAATCGTCCATTACGTTTTGAAGAGTTTGCCGAATCAAATTTCTTA	1365
I Y I S A T P G D Y E L D L T H G E I V T AAATCTACATTTCCGCCACTCCTGGCGACTATGAACTTGATTTAACCCATGGCGAGATTGTTACT	1430
Q Y I R P T G L L D P I I E I H S R D N Q I CAGTATATTCGGCCAACTGGTTTGCTAGATCCTATTATTGAAATCCACAGTCGCGACAATCAAAT	1495
E D I Y D H L K E Q I A K K E R T L I L T T TGAAGATATTTATGACCATCTTAAAGAGCAAATAGCAAAAAAGAACGAACCTTGATTCTAACTA	1560
T K K N A E E L S L F L Q E K K I K S A Y CTACCAAGAAAAATGCTGAAGAGCTGTCTTTATTCTTGCAAGAAAAGAAAATCAAAGTGCTTAT	1625
I H D R F K I F E R N E I L K G L R M G K F ATTACGATCGTTTTAAGATTTTTGAACGTAACGAAATCTAAAAGGGCTTAGAATGGGCAAGTT	1690
D V V V G I N L L K E G I D L P E V S L I C CGATGTCTGTTGTTGAATTAATTTGCTTAAAGAAGGAATTGACTTACCAGAAGTTAGTTTGATTT	1755
V L N A D S T G L M R D T R S L I Q I V G GCGTTTTTAAACGCCGATAGCACTGGACTAATGCGTGACACTAGAAGCTTAATCCAAATTGTTGGT	1820
R A A R N D H G K V I F Y A N E I T S S M R AGAGCGGCGCGAAATGACCATGGTAAAGTTATTTTTATGCCAATGAAATAACAAGTAGCATGCG	1885
E C I E D N L F K R K I Q S E Y N E K H N I TGAATGCATTGAAGATAATTTATTTAAAAGGAAAATTCAAAGCGAATATAACGAAAAACATAATA	1950
I P K T I V K P I A P P I Q N G I L S E D TCATTCCAAAAACAATTGTAAAACCGATTGCTCCGCCAATTCAAATGGCATTCTTAGTGAAGAT	2015
H S K Y Y G E K D L S Q M K H N K K F I D Q CATTCAAATACTATGGTGAAAAAGACCTTAGCCAAATGAAACATAATAAAAAATTCATTGATCA	2080
M V R K M T Q L A K A N K F E E A I E I R D AATGGTTTCGAAGATGACTCAATTAGCTAAAGCCAACAAATTTGAAGAAGCAATTGAGATTCGTG	2145
Y L I E I G I E L D K ATTATCTAATAGAAATTGGCATAGAATTGGATAAATAATTGTTAAAACGC	2194

Figure 4.1 (Continued)

upstream of the *uvrB* homolog. Two potential ribosomal binding sites were identified upstream of the start codon (Figure 4.1). Termination of the operon most likely occurs after *mnuA* where an inverted repeat is located (data not shown). No UGA codons were found within the sequence of the homolog as there were no tryptophan coding codons present. This is also true of the *E. coli uvrB* sequence. BLASTN and FASTA homology searches were performed on the DNA sequence. Homology searches revealed that the reading frame was 51% identical and 70% homologous to the *uvrB* gene sequence of *H. influenzae* and 49% identical and 70% homologous with the *uvrB* gene sequence of *E. coli*. Numerous other *uvrB* genes were also found. Figure 4.2 shows the genetic and restriction map of the *mnuA* operon including the genes coding for *uvrB* and *mnuA* and their direction of transcription.

Comparison of the *M. pulmonis uvrB* protein to other species

The UvrB protein is a component of the ABC excinuclease, an endonuclease responsible for cleaving short patches of damaged DNA during short patch repair. One of the most notable features of this protein is the presence of a Walker type A nucleotide binding motif (64, 224). Figure 4.3 compares the amino acid sequence of the nucleotide binding sites of the *M. pulmonis* UvrB homolog with those amino acids determined from the *uvrB* genes in *E. coli*, *H. influenzae*, *Micrococcus. luteus*, *M. genitalium* and *Neisseria gonorrhoeae*. The nucleotide binding domain from *M. pulmonis* is identical to that of *H. influenzae* and *E. coli*. Surprisingly, the amino acid sequence of the *uvrB* gene from *M. genitalium* shows considerably more heterogeneity. Sequence analysis indicated that the *M. pulmonis* UvrB homolog is more closely related to *H. influenzae* and to *E. coli* than to *M. genitalium*. The *uvrB* homolog of *M. capricolum* has been partially sequenced, but the region needed for this comparison, the 5' two hundred amino acids, was not available.

Identification of *uvrB* sequences in other mycoplasma species

Previous reports have indicated that mycoplasmas may not contain short patch DNA repair systems. With the discovery of *uvrB* homolog gene sequences in *M. pulmonis*, it was of interest to screen other mycoplasma species for the presence of *uvrB* -like sequences particularly *M. gallisepticum* where previous studies have indicated that *M. gallisepticum* lacked dark repair (69). Using an internal fragment of *uvrB* as a probe (Fig. 4.2), chromosomal DNA from 11 different species of mycoplasmas were analyzed to determine the presence of *uvrB* like sequences. As is shown in Figure 4.4, *uvrB* like sequences were

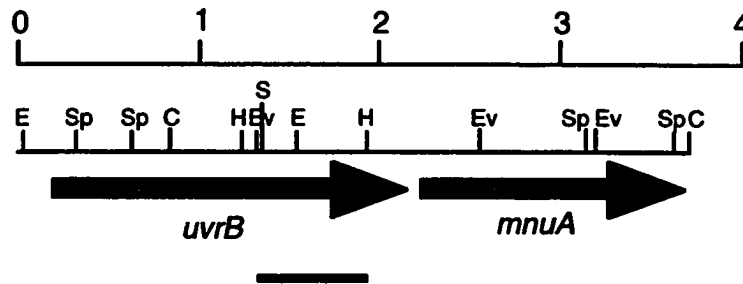


Figure 4.2. Genetic and physical map of the *mnuA* operon in *M. pulmonis*. The arrows indicate the coding regions for the *uvrB* and *mnuA* genes. Shown below the arrows is the region used for probing for *uvrB* homology in other mycoplasma species. The scale is in kilobases. Symbols: C, *ClaI*; E, *EcoRI*; Ev, *EcoRV*; H, *HindIII*; S, *SacI*; Sp, *SpeI*.

Species		Binding Domain												
Consensus Binding Motif		X	X	X	X				X	X	X	X	X	X
<i>M. pulmonis</i>	H Q V L L												A	Q L N R P
<i>H. influenzae</i>	H Q T L L												A	Q L N R P
<i>E. coli</i>	H Q T L L												A	D L Q R P
<i>N. gonorrhoeae</i>	Y Q T L L								Y		M		A	Q S G R P
<i>M. genitalium</i>	Q Q V L L	A				T			F				Q	N S Q L P
<i>M. luteus</i>	D V V L M	A				T			A	T	T	A	W	L E R L Q R P

Figure 4.3. Comparison of Walker type A nucleotide binding sites. The binding domains from the *uvrB* genes of various bacteria species were obtained from GenBank and are shown along with the consensus motif determined by Walker *et al.* (224). The light shaded region is the Walker type A consensus binding sequence. The darker shaded areas are regions of identity of other bacterial species with the *M. pulmonis* amino acid sequence within the binding motif.

observed in the two *M. pulmonis* strains tested, CT and χ 1048, and also in *M. gallisepticum*, *M. hyopneumoniae*, *M. hyorhinis*, *M. capricolum*, *M. fermentans*, and *M. hominis*. The probe failed to hybridize with *M. pneumoniae* and *A. oculi* 1499 DNA (Fig. 4.4).

Discussion

The nucleotide excision repair system repairs DNA that has been damaged by ultraviolet light or chemical exposure. Repair begins by recognition of the damaged DNA, mismatched bases, or modified bases by the multi-component excinuclease. The recognition of thymidine dimers or AP (apurinic or apyrimidic) DNA is initiated by ATP and two molecules of the UvrA subunit protein. The subunits UvrB and UvrC are then added to the complex which introduces two nicks in the DNA strand. These nicks are located 8 base pairs upstream of the DNA lesion and downstream four or five base pairs. The UvrD helicase II then

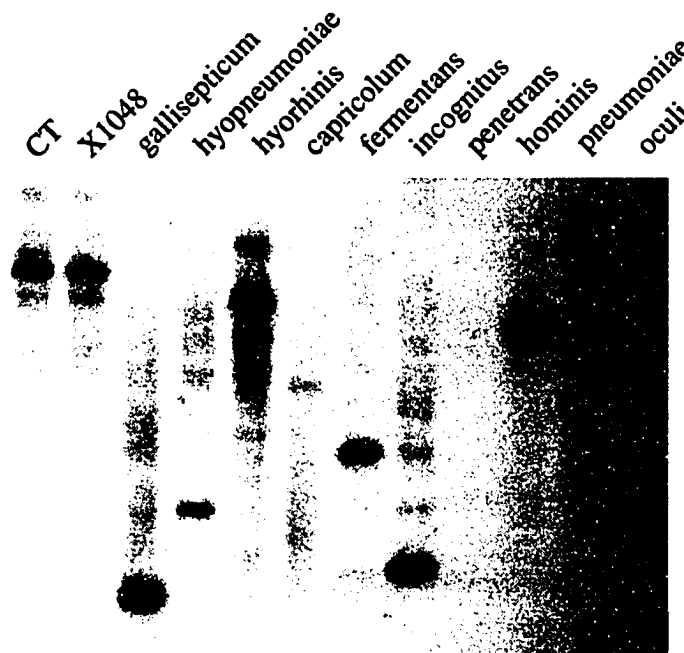


Figure 4.4. Hybridization of analysis of mycoplasma species with a *uvrB* probe from *M. pulmonis*. The probe used for analysis was a *SacI* - *HindIII* fragment from pISM4172. Hybridization conditions are described in the Materials and Methods. Lanes: CT, *M. pulmonis* strain CT; χ 1048, *M. pulmonis* χ 1048; 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* supsp. *incognitus*; penetrans, *M. penetrans*; hominis, *M. hominis* 1620; pneumoniae, *M. pneumoniae* ATCC 15531, oculi, *A. oculi* 1499.

unwinds the damaged DNA region and the gap is filled in by DNA polymerase I (64). The nucleotide excision repair system is assumed to be present in most organisms because of the need to repair damage done by exposure to UV light. With the small genome of mycoplasmas, the presence of any repair or biosynthetic pathway cannot be assumed, however. It was not determined if the *uvrB* product was functional in *M. pulmonis*. Previous studies by King *et al.* show that both *M. pulmonis* and *M. mycoides* possess *recA* gene sequences (87), but the products of those genes have also not been functionally characterized. It is interesting that the *uvrB* homologous fragment in *M. gallisepticum* is less than a kilobase in size (Fig. 4.4) suggesting that *uvrB* has been truncated. This correlates with the previous study by Ghosh *et al.* showing a lack of dark repair activity in this species (69). Although it is not known if any of the mycoplasma species showing *uvrB* homology have a functional nucleotide base excision system, *uvrA*, *uvrC* and *uvrD* homologs have been found in the mycoplasma sequencing projects in *M. genitalium* and *M. capricolum*.

Mycoplasmas are believed to have evolved from other procaryotes by degenerative evolution. Therefore, for mycoplasmas to have maintained a functional nucleotide excision repair system, selective pressure must have been exerted. The lack of cell walls and small genomes that are very AT rich should make mycoplasmas highly susceptible to DNA damage by UV radiation and thymidine dimer formation. In the absence of repair processes, thymidine dimers inhibit DNA replication resulting in cell death. Along with the photolyase, the Uvr excinuclease complex is a critical component in the thymidine repair process and long term survival in the environment.

The Uvr proteins are constitutively expressed at low levels in *E. coli* and are upregulated during the SOS response. This response is coordinated by the LexA protein. Upstream of the *uvrB* gene in *E. coli* is a LexA binding domain. There does not appear to be a LexA-like binding motif upstream of the *uvrB* homolog in *M. pulmonis*, however. Another protein which is upregulated by the LexA protein in *E. coli* is DNA polymerase I, which is required to fill in the gaps created by the removal of the thymidine dimers. Mycoplasmas are thought to use DNA polymerase III as their sole polymerase, and this protein and its promoter have been characterized for *M. pulmonis* (9). As is the case for *uvrB*, there does not appear to be a LexA binding motif upstream of the DNA polymerase III (9). It is also possible that a LexA homolog might be present in *M. pulmonis* with a different binding motif. A LexA homolog has not been found in *M. genitalium* for which the complete sequence is known or in *M. capricolum* for which about one half of the sequence has been reported. A SOS response in mycoplasmas has not been completely ruled out, but it appears unlikely. Further research will need to be done to directly answer this question.

In most cases, *uvrB* is monocistronic. In *M. pulmonis*, however, *uvrB* is the first gene of a two gene operon. This operon also codes for a membrane-associated nuclease gene, *mnua* (82). Although it may seem feasible for a nuclease to be used to recycle the nucleotides in the short oligonucleotide released upon thymidine dimer repair, this particular nuclease appears to be localized in the membrane. Another possible explanation for the inclusion of *mnua* in the two gene operon with *uvrB* is that the amount of UvrB is coordinately regulated with this membrane nuclease to ensure that an adequate supply of nucleotides is available for repair purposes. It is likely that the membrane nucleases function in acquiring nucleotides from the surrounding environment. In the absence of adequate levels of membrane nucleases and consequently purines and pyrimidines, gaps would remain in the DNA which would be lethal to the cell.

In summary, *M. pulmonis* contains a *uvrB* homolog which probably produces a functional protein, although this must be tested directly. These results suggest that many mycoplasma species contain *uvrB* homologs and possibly nucleotide excision repair systems. The deduced UvrB amino acid sequence is highly homologous to the *H. influenzae* and *E. coli* counterparts, indicating the conserved nature of these DNA repair proteins.

Acknowledgments

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CHAPTER 5. GENERAL CONCLUSIONS

General Discussion

Nonspecific nucleases are produced by many bacteria species, many of which are pathogenic. In these studies, nuclease activity was demonstrated to be present in all mycoplasma species tested (Table 2.1). Because of their inability to synthesize their own nucleotides, these nucleases are postulated to function in the acquisition of purines and pyrimidines rather than as a restriction barrier. The nucleolytic activity present in whole cell lysates is able to cause relaxation in supercoiled plasmid DNA (137), nick open circular forms to produce linear DNA, and digest linear plasmid and phage DNA into small fragments (137) possibly to single bases if the reaction is allowed to go to completion. The nucleases from mycoplasma appear to have endonuclease and/or exonuclease activities. The nuclease activity is also able to digest its own chromosomal DNA as well (134). Since many mycoplasmas contain methylases (46), it is probable that the nucleases are not inhibited by DNA methylation. Whether these activities are located within one protein or are the result of the interaction between multiple proteins is unknown. There are endo-exonucleases found in *Neisseria*, *Aspergillus* and *Saccharomyces* species (57). The staphylococcal nuclease and the nuclease identified from *Thermus* also show both endo and exonuclease properties (57, 194). It is reasonable to assume that the mycoplasmal nucleases may also contain both properties as both features would be beneficial to the acquisition of nucleotides.

Not only do all mycoplasmas produce nonspecific nucleases, the results indicate that these organisms localize the nuclease activity to their membrane. Most species appear to contain more than one nucleolytic protein (Figures 2.2-2.5). Although not specifically addressed by this study, it is likely that these nucleases are compartmentalized; they are located solely in the membrane and are either not present, not in the proper conformational state, or there is an inhibitor present to prevent their activity in the cytoplasm. In the case of the staphylococcal nuclease, the nuclease is released directly from the cell. In fact, most of the nonspecific nucleases discovered in bacteria have membrane signal sequences and are released directly from the organism. The presence of a nuclease on the membrane surface appears to be unique to the mycoplasmas, although the ones in *Streptococcus* are localized in the periplasmic space (5).

The presence of apparently more than one nucleolytic protein on the surface of mycoplasmas was unexpected, but there are several possible explanations. First, mycoplasmas may produce nucleases from multiple genes. This postulate is reasonable considering that mycoplasmas have assigned a considerable amount of their DNA to code for

adherence genes (62, 172). Recently, it has been suggested that recombination may occur between multiple copies of adhesin gene sequences (158). This recombination would likely result in adhesin antigenic variation and ensure that the adhesins are always produced. To address why there may be multiple genes and if recombination also occurs between the various genes it will be necessary to determine the function, relative position of each nucleolytic protein, and sequence each gene.

Second, in some mycoplasmas there may be only one gene responsible for the nuclease activity. (This was shown not to be true for *M. pulmonis* during this study). Mycoplasmas contain variable lipoproteins (Vlps) on the membrane surface that contribute significantly to the antigenic variability of the organism. Part of this variability is due to repeat units within the gene sequence. During DNA replication, slipped strand mispairing occurs within the coding regions for the repeated units which causes the insertion or deletion of the units and results in a situation where daughter cells produce a variety of different sized proteins (231). Alternatively, post-translational modification may be occurring prior to and concomitant with membrane translocation. One potential modification would be acylation. Posttranslational modification of proteins by proteolytic cleavage has not been reported in mycoplasmas.

The final alternative is a combination of the first two. There may be more than one nuclease gene with each gene producing more than one product. This occurs with the Vlps in a population of cells. The individual genes are altered between daughter cells, but there are also multiple genes (231). Considering the small genomes of mycoplasmas, altering both the protein products and having multiple copies of a gene type within the population is advantageous. The Vlps are thought to be involved in evasion of the immune response (231), but this has not been established experimentally.

Further studies in *M. pulmonis* were initiated to specifically address the mechanism for multiple membrane nucleases. Antisera was raised against nuclease containing bands from a preparative SDS-PAGE gel and used to screen a genomic library. 19 positive λ GEM 12 recombinant phage were obtained (Table 3.2). Because this antisera was produced against proteins in a gel slice as opposed to purified protein, it was necessary to more accurately identify the nuclease containing phage. This was accomplished by infecting *E. coli* strain ISM612 with the 19 recombinant phage and monitoring the lysates for nuclease activity. The ability of strain ISM612 to suppress opal stop codons in mycoplasma genes by inserting tryptophan residues has been well documented (140, 202). The strain was consistently negative for nuclease activity in our assay. Four of the nineteen phage generated nuclease activity. When single plaques from one of these purified phage were examined for nuclease activity, approximately 85% of the plaques produced nuclease activity indicating that the

nuclease activity was relatively stable when produced in a phage host. The chromosomal inserts from the four nuclease positive phage were subcloned and restriction mapped. Three of the chromosomal fragments were subcloned in two different plasmids due to an internal *SacI* sites. The insert in phage 13.2B was unstable in pKS but could be subcloned into pMOB. This may reflect a difference in plasmid number between pKS and pMOB. pKS is a high copy number plasmid with unregulated plasmid replication. pMOB is a derivative of pBR322 and probably produces 20-30 copies per cell.

Restriction mapping revealed that three of the four phage were from the same general chromosomal region. Phage 14.11F was a smaller version of 13.5C. The 3' end of 13.5C and the 5' end of 13.2B overlap (Figure 3.2). Phage 9.2C shared no homology with the others. Thus, there were at least two different regions of the *M. pulmonis* chromosome encoding for nuclease activity.

Expression of nuclease activity from cloned fragments from phage 13.5C and 9.2C was erratic. Occasionally, nuclease positive colonies were identified, but the expression was unstable and was lost upon subculture. Approximately 10% of isolated colonies at any given time expressed nuclease activity in these subclones, which was not true for the 13.2B subclones. Our explanation was that expression of nuclease activities in these subclones was high enough to select for spontaneous mutants at a high frequency. This may have resulted in a cell population of mostly mutants unable to express activity, without the loss of the plasmid in the strains. To try to rectify this problem, we reasoned that inadvertent nuclease expression might be the selective pressure that resulted in a high level of spontaneous mutants in our cell populations. This could have resulted from a leaky promotor in an efficient suppressor host. Thus, two approaches were apparent, reduce suppressor efficiency or tighten promoter regulation. The use of the alternative reduced suppressor efficiency hosts, ISM614 (LE392 with pISM3001) and ISM647 (XL-1 BLUE with pISM3001) were tested. Also tested was a protease negative strain, SOLR. Since both ISM614 and SOLR strains contained enough endogenous nuclease activity to interfere with our assay, they were not useful. ISM647 did not express nuclease activity, but the percentage of nuclease expressing colonies did not increase using this strain. The cloned nuclease genes, however, may not contain UGA codons and thus moving the plasmids into a less efficient suppressor background may not have been helpful. Next, reduction of promoter leakiness was attempted by the addition of glucose to the medium to induce catabolite repression. These fragments were cloned into pKS, which contains an IPTG inducible promoter for inducing gene expression. Addition of glucose also had no effect on the stability of nuclease expression.

It is likely that the nuclease genes are transcribed in *E. coli* from promoter-like sequences within the mycoplasma chromosomal DNA (89). It may not be possible to regulate expression of these genes until they have been completely sequenced. The observation that *mnuA* was stable when cloned into pMOB and that it does not contain its own promoter supports the view that *mnuA* may be expressed at lower levels than those associated with phage 9.2C or 13.5C and that lower levels of expression may result in more stable activity. The higher plasmid copy number of pKS may have led to strain instability with the other cloned fragments, presumably because of the higher gene copy number. Whether the level of nuclease expression from *mnuA* is low compared to the levels of nucleases in phage 13.5C and 9.2B cannot be determined because of the high rate of mutation.

The portion of the fragment from 13.2B cloned into pISM4172 contained the region responsible for active nuclease activity and was only expressed in a low suppressor efficiency host. At no time was pISM4172 capable of transforming ISM612, but the plasmid was stable in LE392 and the nuclease activity was expressed stably in ISM647. Thus, this plasmid and host were chosen for further study.

These results indicate that there are at least four membrane nuclease genes present in *M. pulmonis*. First, *mnuA* is present in pISM4172. A second gene must be present in the cloned fragment in phage 9.2C. The third gene must be located in the region where pISM4170 and phage 13.5C overlap as both of these demonstrate some nuclease activity in their plasmid form and 13.5C phage are positive. The final nuclease is substrate specific and is observed as a low molecular weight band in SDS-PAGE DNA gels using salmon sperm DNA as the substrate (Fig. 3.3). This latter nuclease would not have been discovered in our screening method because it does not recognize λ DNA. Additionally, this was the first time that a difference had been observed between the λ assay and the SDS-PAGE nuclease assay. It had been assumed previously that the two assays measured similar activities.

An important aspect of these studies is the use of *Tn1000* to identify the coding sequence of *mnuA* within pISM4172 as well as to functionally analyze the gene product. The *Tn1000* inserts were used to facilitate restriction mapping and DNA sequencing as well. Expression of nuclease activity by one of the plasmids mutated by *Tn1000* clearly shows that the nuclease is coded by the cloned fragment and is not an *E. coli* product that has been upregulated by the presence of the plasmid. Absolute proof will depend upon the availability of *mnuA* insertional mutants in *M. pulmonis*. Our current ability to mutate *M. pulmonis* is limited to the use of *Tn916* (48) and homologous recombination (134), although recombination has not been studied extensively. It may be technically difficult to transform

this species, and any transformation may be lethal if this particular nuclease gene is an essential gene.

There is other evidence, albeit circumstantial, that *mnuA* actually encodes a nuclease. First, there is the observation that one of the Tn1000 insertions in *mnuA* located near the N-terminus results in a product that can digest DNA. Colonies of this mutant were nuclease negative when analyzed using the lambda DNA digestion assay. Upon denaturation and renaturation during the SDS-PAGE nuclease assay, this mutant regained nuclease activity. Thus, the insertion is clearly in the structural gene for the nuclease. The products demonstrated in the SDS-PAGE nuclease assay presumably result from internal translational initiation. There would be no possibility of this protein upregulating a gene product after SDS- PAGE sample treatment. Second, the nucleases are membrane proteins and *mnuA* codes for a membrane protein according to the DNA sequence. As the Tn1000 insert near the N-terminus would not contain the membrane spanning domain and activity is observed only after denaturation and renaturation, proper conformation and membrane localization may also play an important part in nuclease activity.

A search of other species, using a fragment from *mnuA* as a probe revealed that there is only one copy of *mnuA* in *M. pulmonis* and there are similar sequences in other mycoplasma species. It should be pointed out that it is not uncommon for gene probes to fail to react with other mycoplasma species because of rapid changes in the DNA sequence due to a high mutation rate. Thus, the majority of the species did not react even under low stringency conditions. Because all mycoplasma species must acquire nucleotides and all species have nuclease activity (Table 2.1), it was not surprising to find a few with noticeable homology to *mnuA*. There is often similarity between proteins of similar function in mycoplasmas. For instance, proteins similar to the P1 adhesin of *M. pneumoniae* have been found in four other species of mycoplasmas (38, 45, 81, 215).

In all other bacterial species in which *uvrB* has been identified, it is monocistronic. The finding that *uvrB* was not monocistronic in *M. pulmonis* was surprising. There may be several advantages to having *mnuA* located downstream of this gene. The *uvrB* gene is constitutively expressed in other bacteria, and this would ensure that *mnuA* is also made constitutively. Also, *uvrB* is most likely an essential gene in *M. pulmonis* and there may be selective pressure to preserve this operon. Finally, the placement of these genes together may indicate they work cooperatively. As UvrB functions to repair the thymidine dimers, MnuA may ensure that there are enough nucleotides to repair the resulting gap in the DNA.

Hybridization studies using an internal fragment of *uvrB* also indicated that this gene is present in most other mycoplasma species. Notably, *uvrB* is present in *M. gallisepticum*,

which had previously been reported as lacking dark repair (69), but the fragment recognized is small and may represent a deletion in the chromosomal region where UvrB is located. The high homology of the *M. pulmonis*, *M. genitalium* and *M. capricolum uvrB* genes to *E. coli* indicated that they are most likely functional since all are approximately 70% homologous. The *uvrB* probe did not react with *M. pneumoniae* or *A. oculi* chromosomal DNA. Considering the similarity between the chromosomes of *M. genitalium* and *M. pneumoniae*, this was surprising. Both probes recognized the same restriction fragments for the chromosome of two *M. pulmonis* strains, χ 1048 and CT.

In summary, this work has shown that nucleases are common membrane components in mycoplasmas. In *M. pulmonis*, there may be multiple genes coding for membrane nucleases. One of these genes, *mnuA*, has been sequenced and analyzed. It encoded for a lipoprotein, which is capable of digesting different substrates, and is located in an operon downstream of *uvrB*.

Comparison of MnuA to Other Bacterial Proteins

MnuA is the first reported cloned nuclease from mycoplasmas. It has been known for over thirty years that these proteins exist, but none of the DNases or RNases have been studied at the molecular level. As one of the few membrane proteins studied, it is useful to compare this particular protein to the other membrane proteins characterized in mycoplasmas.

It is not unusual to find both adhesins and variable lipoproteins in mycoplasmas encoded by multiple gene copies. Membrane nucleases fall into this category. The variable lipoproteins and the MnuA protein share other features as well. Not only are there multiple genes located in the chromosome, they are both lipoproteins. In fact, the lipoprotein signal sequence present in the *vlp* genes of *M. hyorhina* is identical to that found in *mnuA* (34). Both types of proteins are extracted into the detergent phase upon Triton X-114 phase partitioning (21). They also have only one cysteine per protein, the cysteine located at the acylation site (34).

Even with their similarities, there are also notable differences between MnuA of *M. pulmonis* and Vlp of *M. hyorhina*. It does not appear that *mnuA* undergoes any form of slipped strand mispairing during DNA replication to disrupt transcription or gene expression as there are no repeated domains present in the DNA sequence. Another difference is the number of tryptophan residues present in these proteins. There are four UGA-encoded tryptophan residues in MnuA whereas there are no UGA codons located in the Vlp (238). Expression of Vlp in *E. coli* has been used as a model system for several years, but these

proteins do not need to overcome the codon usage difference between mycoplasmas and other bacteria. Using an opal suppressor host, translation of the *mnuA* mRNA proceeded through three of the UGA codons, with some product terminating at the fourth UGA codon. It is interesting that the truncated product was also expressed nuclease activity. This indicates that the active site is upstream of this UGA codon.

MnuA shares similarity with other gram-positive nucleases. Although this similarity does not translate into similarity at the nucleotide or amino acid level, MnuA is a nonspecific nuclease. All of these nucleases appear to have both endo- and exonuclease activity, and are capable of recognizing different types of DNA. The nucleases were discovered, for the most part, in pathogenic bacteria, and are considered to be virulence determinants. The actual role of MnuA in virulence is also unknown. Unlike the nucleases of gram positive bacteria, MnuA is located in the membrane and is thought to have a role in acquisition of nucleotides, which are required macromolecules for mycoplasmas. Because it is easily monitored, it may be a good model protein to study membrane localization and protein processing in mycoplasmas.

Recommendations for Future Research

There are many areas in this project which could be addressed with further research. Three different nuclease genes were cloned, but only one of them has been sequenced and characterized. Research on the other two may be difficult as the genes and/or the nuclease activity are unstable in *E. coli* when they are located on the plasmids constructed during this study. This may not be true if the genes are cloned into a low copy number vector. Alternatively, it may be possible to subclone smaller fragments containing nuclease activity through the elimination of unrelated regions producing instability. This might include promoter sequences if they are efficient in *E. coli* (89). One promoter containing sequence from *A. oculi* was a very strong promoter in *E. coli* (89). The pitfall of this approach is that it is completely unknown which internal fragments within the cloned sequences contain the activity and even slightly deleting a small region of the gene may prove to knock out the activity. Alternatively, mutagenesis could be performed on the phage clones. In their current form, the chromosomal fragments contained in the phage vector are very large and mutagenesis has proven difficult. However, if the fragments were used to screen a different phage library, say in λ ZAP, smaller chromosomal fragments may be identified which express nuclease activity and are more adaptable to mutagenesis strategies. This approach would probably be the wisest course of action as a *M. pulmonis* λ ZAP II library has already been constructed. Obtaining the correct fragments and screening the phage could occur relatively

quickly. There is always a chance, as with any genomic library, that the fragments are too small to contain the entire gene or that the gene was cleaved into two fragments. A potential third approach is to sequence the entire fragments. The obstacles with that approach is that it is not cost effective, the nuclease may not share homology with any other proteins and the proper reading frame may not be identified.

The expression of the staphylococcal nuclease in *E. coli* has defined many different types of experiments which could be performed with MnuA. Processing and signaling of mycoplasma proteins in exogenous hosts has been addressed with a few of the Vlp proteins of *M. hyorhinis*. However, alternative signal sequences and the results of having no signal sequence have not been studied. Based upon the observation that very low expression of the nuclease activity occurs in *E. coli*, experiments could be performed with other promoters to increase the level of expression as was also done with the staphylococcal nuclease. It may also be possible to change the signal sequence to produce a secreted protein. Additionally, specific mutations could be introduced to determine the functional domains of the nuclease. As *E. coli* may be an unsuitable host to produce large quantities of this protein, expression must be studied, stabilized and tightly controlled before any attempts at purification. Once the protein is purified, however, many other types of biochemical studies could be performed. These studies include determination of biochemical stability, nucleotide specificity, 3-dimensional conformation, etc.

Purification of nucleases from mycoplasmas has proven to be difficult. The growth of mycoplasmas is much more laborious than *E. coli* because they are grown in a very rich media, take days to grow and grow to a low density. The purification of membrane proteins has been achieved for very few mycoplasma proteins. There are the complications of media protein absorption to the mycoplasma membrane, extraction of the protein from the membrane and subsequent removal of detergents. Because MnuA is a lipoprotein, it is highly hydrophobic, complicating the purification process.

MnuA is thought to be involved in the acquisition process for nucleotides. It is unknown which nucleotides are recognized by MnuA. It is also unknown if this protein is essential to the survival of *M. pulmonis* both *in vitro* and *in vivo*. In order to determine these features, it will be necessary to mutate the gene in a virulent strain of *M. pulmonis* and then test for growth in the laboratory and for colonization and virulence in mice. The media used to grow mycoplasmas may need to be adapted as standard mycoplasma media contains sources of DNA and nucleotides. The amount of nucleotides provided in the media may need to be more accurately defined and appropriately adjusted to adequately address the role of this protein in the growth of mycoplasmas. This may prove impossible as a defined media for *M. pulmonis*

has not been described. If the nuclease activity is required for survival, *mnua* mutants will not be viable. Genetic approaches which allow for the controlled transcription of the gene may be the only way to determine if the gene is indeed essential.

The discovery of *uvrB* in *M. pulmonis* also leads to other potential areas of research. The other components of the nucleotide excision repair system have been sequenced in both *M. genitalium* and *M. capricolum*. However, they have not been identified in *M. pulmonis* nor have any functional studies been performed on any of these proteins from mycoplasmas. It would be interesting to determine if short patch repair actually functions in mycoplasmas as has been defined in other bacterial species.

There are also other potential areas of research which will become apparent when the above areas of research are addressed. Acquisition and membrane localization processes in mycoplasmas are completely undescribed. The potential role of this protein in nucleotide acquisition and possibly in virulence makes MnuA an ideal candidate for further study.

APPENDIX A. PREPARATIVE ISOELECTRIC FOCUSING OF THE MEMBRANE-BOUND NUCLEASES OF *ACHOLEPLASMA* USING THE ROTOFOR

Karalee J. Jarvill-Taylor and F. Chris Minion

Abstract

Acholeplasmas are atypical procaryotes which contain no cell wall having only a single limiting plasma membrane composed of up to 20% cholesterol as a limiting barrier. This increases the ratio of membrane lipid to total protein greater than that found in other procaryotes, and increases the difficulty of purifying membrane proteins. These studies establish the experimental protocols for partial purification of the membrane-bound nucleases of *Acholeplasma* ISM1499 using the Bio-Rad Rotofor, a preparative isoelectric focusing unit. The parameters examined include concentration and type of detergent used for solubilization, total protein concentration, and the concentration and pH range of ampholyte. Also, Triton X-114 partitioning and membrane preparations were investigated as sources for nuclease-enriched samples. Partial purification was obtained by maintaining the protein concentrations within a 10-20 mg range, incorporation of 5% ampholytes, and the use of the dialyzable detergent CHAPS.

Introduction

Preparative isoelectric focusing (PrIEF)¹ is rapidly gaining acceptance as a powerful separation system for protein isolation. In many cases, it is superior to column chromatography because of the establishment of an isoelectric point equilibrium producing a superior separation of protein mixtures. In addition, proteins can be isolated in their native, enzymatically-active state unlike some separation techniques such as sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) which employ denaturing conditions. The enzymatically-active fractions are easily identified using assays, or alternatively, the protein of interest can be identified through a variety of other techniques such as western blotting or enzyme-linked immunoassays using monoclonal antibodies. Preparative isoelectric focusing lends itself to a variety of post-focusing separation techniques for further purification if necessary.

¹ Abbreviations: SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; CHAPS, 3-[(cholamidopropyl)dimethylammonio]-1-propanesulfonate; PrIEF, preparative isoelectric focusing.

When applied to membrane proteins, the experimental parameters required for separation during PrIEF becomes more difficult to determine. In addition to the normal parameters such as protein concentration, pH range and concentration of carrier ampholyte, etc., the effects of the detergents on the separation and sample processing must also be considered. Protein solubilization is essential to purification of membrane proteins, and a wide variety of detergents can be used, each having its own specific advantages and disadvantages (148). Extraction of integral membrane proteins can often be an important first step in the overall purification scheme, but the experimental conditions needed to do this selectively while maintaining the native conformation of the protein and its enzymatic activity, can only be determined empirically, a difficult and time consuming exercise. Once separated by IEF, there is the additional complication that the detergent chosen may need to be removed prior to enzymatic assays and further purification. Detergent removal may also result in the loss of enzymatic activity or in protein precipitation.

Our studies focused on the purification of the membrane-bound nucleases from *Acholeplasma* using the commercially available Rotofor PrIEF unit from Bio-Rad Laboratories. The Rotofor is a refrigerated, cylindrical, constantly-rotating PrIEF unit. Proteins are separated into twenty discrete fractions along the pH gradient in free solution rather than in an insoluble matrix or density gradient. Microgram quantities of partially purified proteins can be isolated from crude extracts in four hours or less.

Acholeplasmas belong to a class of bacteria, the *Mollicutes*, that are grouped by their lack of a cell wall. Their single limiting membrane serves as the sole environmental barrier. This class of bacteria is pleomorphic in shape and is approximately 0.2 - 0.4 μm in diameter. The ratio of membrane lipid to total protein in mycoplasmas is greater than in other prokaryotes or in eucaryotes. During early attempts to purify the membrane nucleases of *Acholeplasma*, it was observed that the cell preparations contained a high amount of negatively-charged phospholipids that caused large insoluble aggregates to form during PrIEF. The nucleases were found in association with these aggregates because of their hydrophobic nature, preventing their purification.

In order to determine conditions necessary to purify these proteins, the amount of protein, the detergent type and concentration and the ampholyte range and concentration were varied. Triton X-114 phase partitioning and membrane isolation enrichment procedures were also performed as a preliminary step to purification. Efficient separation was obtained by preventing precipitate formation through careful control of the protein concentration and solubilization, increase in the amount of ampholyte and the use of a mixture of two ampholyte pH ranges.

Materials and Methods

Materials and chemicals

Ampholytes (Ampholine in the pH ranges 3.5 - 5.0, 4.0 - 6.5, 5.0 7.0, 3.5 - 9.5), CHAPS, Triton X-100, Triton X-114, salmon sperm DNA, phenol red, ethidium bromide, Trizma, glycine, and 2-mercaptoethanol were from Sigma (St. Louis, MO). Lambda DNA, GG-free Horse Serum, and glycerol were from GIBCO/BRL/Life Technologies (Grand Island, NY). PPLO broth base without crystal violet was from Difco Laboratories (Detroit, MI), and casein, methanol, ethanol, acetic acid, phosphoric acid, NaOH, NaHPO_4 , Na_2PO_4 , and NaCl were from Fisher Scientific Co. Fleishmanns Bakers yeast was the source of fresh yeast extract prepared as previously described (63). Cefobid was from Pfizer, Inc. (New York, NY). Ultra Pure Agarose was from Bio-Rad Laboratories (Richmond, CA). Chemicals for the preparation of the SDS-PAGE gels were purchased from Bio-Rad (Richmond, CA; acrylamide, N,N-methylene-bis-acrylamide) and Sigma (St. Louis, MO; N,N,N',N'-tetramethylethylene-diamine). Silver staining of SDS-PAGE gels was performed with a kit obtained from Accurate Chemical and Scientific Corp. (Westbury, NY).

Protein samples

Acholeplasma ISM1499 was grown to mid log phase in mycoplasma media (PPLO base without CV containing 10% GG-free horse serum, 5% fresh yeast extract, 1% dextrose, 25 $\mu\text{g/ml}$ Cefobid), centrifuged at $10,000 \times g$ for 15 min, resuspended in PBS (0.14 M NaCl - 0.01 M sodium phosphate, pH 7.3), and then centrifuged again. For whole cell preparations, the pellets were resuspended in water containing either 0.1 to 0.5% Triton X100 or 0.3 to 5% CHAPS. Cell debris was pelleted at $100,000 \times g$ for 30 min and the supernate was used for PrIEF. Nuclease activity was also extracted by Triton X-114 partitioning (230). In specific experiments, additional detergents were added to the Triton X-114 phase after partitioning (0.25 - 0.3% CHAPS or 0.1% Triton X-100).

For membrane preparations, cells were washed in 0.25 M NaCl, resuspended in 1/10 volume of 2 M glycerol and incubated for 15-30 min at room temperature. Cells were then rapidly injected into four volumes of warm (37°C) water using a syringe fitted with a 26 gauge needle, allowed to incubate at room temperature for 10 min, and then chilled rapidly on ice. Intact cells were pelleted at $7,500 \times g$ for 15 min, and membranes were removed by pelleting at $30,000 \times g$ for 30 min. Membrane proteins were solubilized by incubation in 4% CHAPS for 15 min and then diluted to 0.4% CHAPS with water prior to loading on the

preparative IEF cell. In some instances, the insoluble debris was pelleted after the initial incubation, although the presence of this material from membrane preparations did not seem to interfere with the separation.

Preparative IEF

The Bio-Rad Rotofor PrIEF cell was assembled and used according to manufacturer's directions. All separations were performed in a total volume of 50 mls with ampholyte concentrations ranging between 2 - 8% depending on the experiment. In most experiments, a mixture of ampholytes (pH 3.5 - 5.0 and pH 4.0 - 6.5) was used. Electrolyte solutions consisted of 0.1 M H_3PO_4 (anode) and 0.1 M NaOH (cathode). Separations were performed at 10-12 constant watts at 4°C for 2 - 3 hrs. Temperature was tightly regulated due to heat sensitivity of the nucleases. After separation, 20 fractions were collected by aspiration, and 200 μl of each fraction was dialyzed against 1 M NaCl for 1 to 2 hrs and then against water overnight prior to assaying for the nuclease activity.

Detection and analysis of nuclease activity

Nuclease activity was determined by testing each fraction in a lambda assay. Following dialysis of each fraction, 25 μl was diluted in 2-fold increments in a 96-well plate with PBS containing 2 mM CaCl_2 and 2 mM MgCl_2 . An equal volume of lambda DNA (10 $\mu\text{g}/\text{ml}$ in PBS) was added to each well and the plate was then incubated for 30 min at 37°C. Lambda DNA digestion was stopped by the addition of 7 μl of borate sample buffer (122) to each well. Nuclease activity was assessed by running the samples (8 μl of sample per well) on a 0.7% agarose gel in TBE buffer (122) at 100 V for 45 min and staining the gel with ethidium bromide.

Positive fractions were run on a SDS-PAGE nuclease gel to monitor active components in the fractions. A Bio-Rad mini-PROTEAN II apparatus was used with a 3% stacking gel and a 10% resolving gel (102). Salmon sperm DNA (10 $\mu\text{g}/\text{ml}$) was added to the resolving gel prior to polymerization. Anode buffer consisted of 3 g Trizma - 14.5 g glycine - 1 g SDS per liter. The cathode buffer was 2X anode buffer with the addition of 0.15% SDS. Gels were electrophoresed at a constant current of no greater than 7 mAmp per gel at 4°C until the dye front exited the gel. The gels were washed four times for 15 min each in 100 volumes of incubation buffer (0.04 M Tris pH 7.5, 0.01% Casein, 0.04% β -mercaptoethanol) with rocking. To allow for renaturation of the enzymatic activity, gels were maintained in incubation buffer overnight at room temperature. To assay activity, gels were placed in 100

volumes of incubation buffer with the addition of 2 mM CaCl_2 and 2 mM MgCl_2 and incubated statically for 18 hours at 37°C. Gels were stained with ethidium bromide (0.5 to 1 mg/ml) for 5 min, destained in water for 20-30 min, and subjected to UV illumination. Nuclease activity was detected as non-fluorescing regions in the gel. After photography, gels were rinsed again with water and fixed in 50% ethanol, 12% acetic acid for 1 hour. The gels were then silver stained to assess total proteins in each fraction and to determine apparent molecular weights.

Fractions were also analyzed for total proteins by standard SDS-PAGE. Dialyzed sample were precipitated by adding 10 volumes of acetone, placing at -20°C for several hours and pelleting by centrifugation at 100,000 x g for 10 min. Following centrifugation, protein pellets were resuspended in water and precipitated twice more by the addition of 10 volumes of absolute ethanol. For some applications, samples were precipitated three times with ethanol. The final pellets were placed in SDS-PAGE sample buffer and boiled for 5 minutes prior to analysis on a 10% SDS-PAGE resolving gel and silver staining. All gels were photographed using Kodak type 57 film. Photographs from these gels were scanned using a Hewlett-Packard Scan Jet IIc scanner and printed at 2400 dpi resolution.

Results

Determination of protein concentration resolvable in the Rotofor

The manufacturer recommends a protein concentration of 2 to 4 mg protein per component for separation in the Rotofor, and more than 2 g of total protein has been successfully focused. In initial experiments, focusing was performed with a total *Acholeplasma* whole cell protein concentration of approximately 25 to 50 mg, well within the manufacturer's guidelines. These preparations contained detergent solubilized proteins with the insoluble cell debris and membranes removed by ultracentrifugation. A large amount of gelatinous precipitate developed either upon the addition of the ampholites (2 to 4%) or after focusing was begun. During focusing, the precipitate focused near the anode electrode with some overflow in the adjacent fractions. Precipitates also tended to clog up the inner core of the unit which separated the various fractions, interfering with collection of the fractions. Most of the nuclease activity was associated with this precipitate. Upon analysis by SDS-PAGE, the precipitous material contained most of the protein from the preparation (data not shown).

Two different approaches were taken to avoid precipitation problems: lowering the protein concentration and increasing the ampholite concentration. When the protein concentration was reduced to 10 to 15 mg total protein and the ampholite concentration raised to 5%, most of the focusing experiments were completed with little or no development of a precipitate. In some cases, a granular precipitate developed, and nuclease activity was often found in these fractions, but it was not associated with the precipitated material. Experiments with protein concentrations below 5 mg were difficult to interpret because the nuclease activity was barely detectable. Ampholite concentrations greater than 6% increased the amount of heat generated which in turn inactivated the nuclease activity. All subsequent experiments were performed at a 5% ampholite concentration.

Solubilization of membrane proteins

Several parameters were considered when choosing the appropriate detergent to solubilize *Acholeplasma* whole cell preparations for preparative IEF. Included were the charge of the detergent, the ability of the detergent to extract the protein of interest, the retention of nuclease activity in the detergent-solubilized fraction, the ease with which the detergent could be removed, and the effect the removal of the detergent had on the enzymatic activity. Preliminary experiments were performed with Tween 20, Triton X-100, CHAPS, β -octyl glucoside, and Triton X-114 to examine the nuclease solubility and retention of activity in the detergent. The nucleases remained active in all of the listed detergents, but there were varying degrees of solubilization of each nuclease species as determined by the SDS-PAGE nuclease assay (data not shown). Both 0.1% Triton X-100 and 5% CHAPS were used to solubilize the nuclease activity in subsequent studies.

When cells from the same culture were compared in PrIEF, the detergent type used to solubilize whole cell preparations had little effect on the nuclease focusing (Figure A.1). The pH gradient was reproducible, and the nucleases focused in a single major peak with some residual activity present throughout other fractions in the gradient. Separations with Triton X-100 were not as reproducible as with CHAPS, however. Figure 2 compares two identical experiments with Triton X-100 extracted whole cells harvested on two separate days. Nuclease activity resolved into single peaks, but at two different isoelectric points (4.1 versus 5.5). Whole cells solubilized with CHAPS gave reproducible results with two peaks of activity (Figure A.2, lower panel). CHAPS-solubilized membrane preparations gave identical results to that in Figure A.2 (data not shown). Figure A.3 is a silver stained SDS-PAGE gel of the focused proteins from the CHAPS separation in Figure A.2.

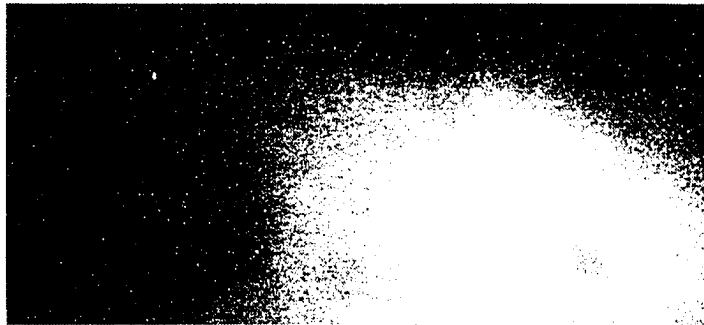
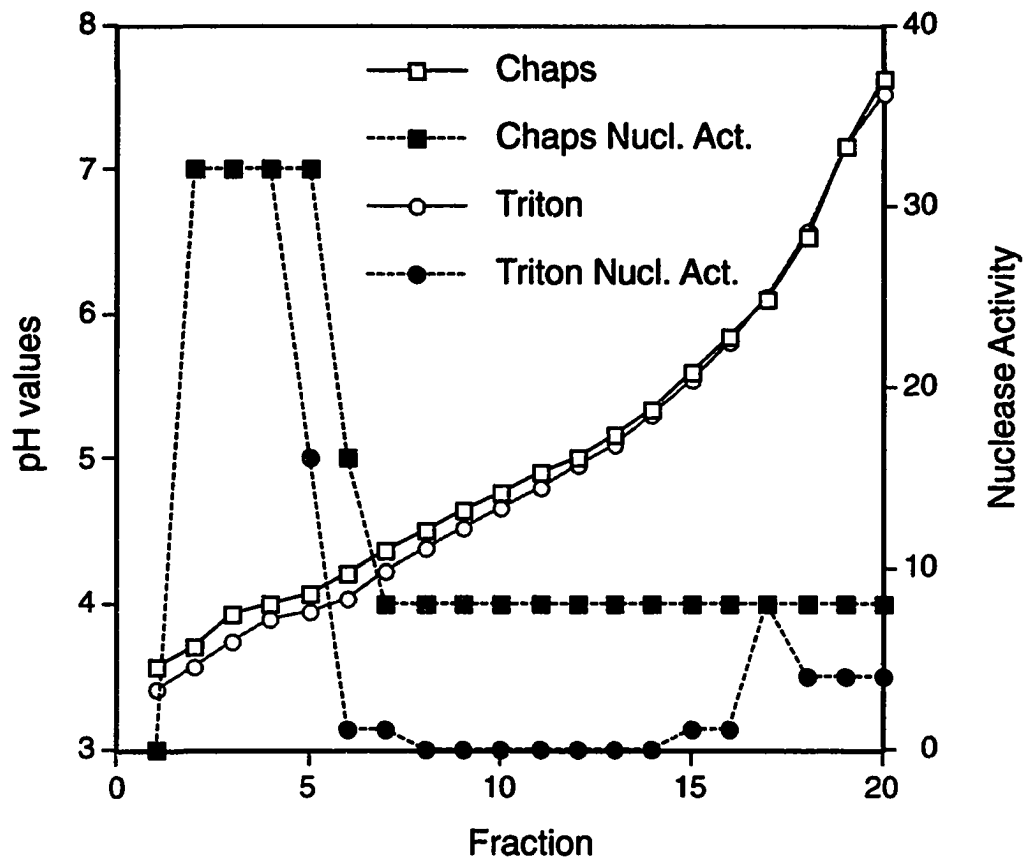


Figure A.1. Comparison of the effect of Triton X-100 or CHAPS solubilization on membrane nuclease Rotofor profiles. Whole cells isolated from the same culture were solubilized with either 1% Triton X-100 or 5% CHAPS. Prior to IEF, insoluble material was removed by ultracentrifugation. Nuclease activity was determined using the Lambda DNA assay and the IEF performed as described in the Materials and Methods. Ampholyte concentration was 3% 3.5 - 5.0 and 2% 4.0 - 6.5. Fractions 2-5 and 15-20 from the Triton X-100 separation were analyzed by the SDS-PAGE Nuclease assay and the results are shown below the graph.

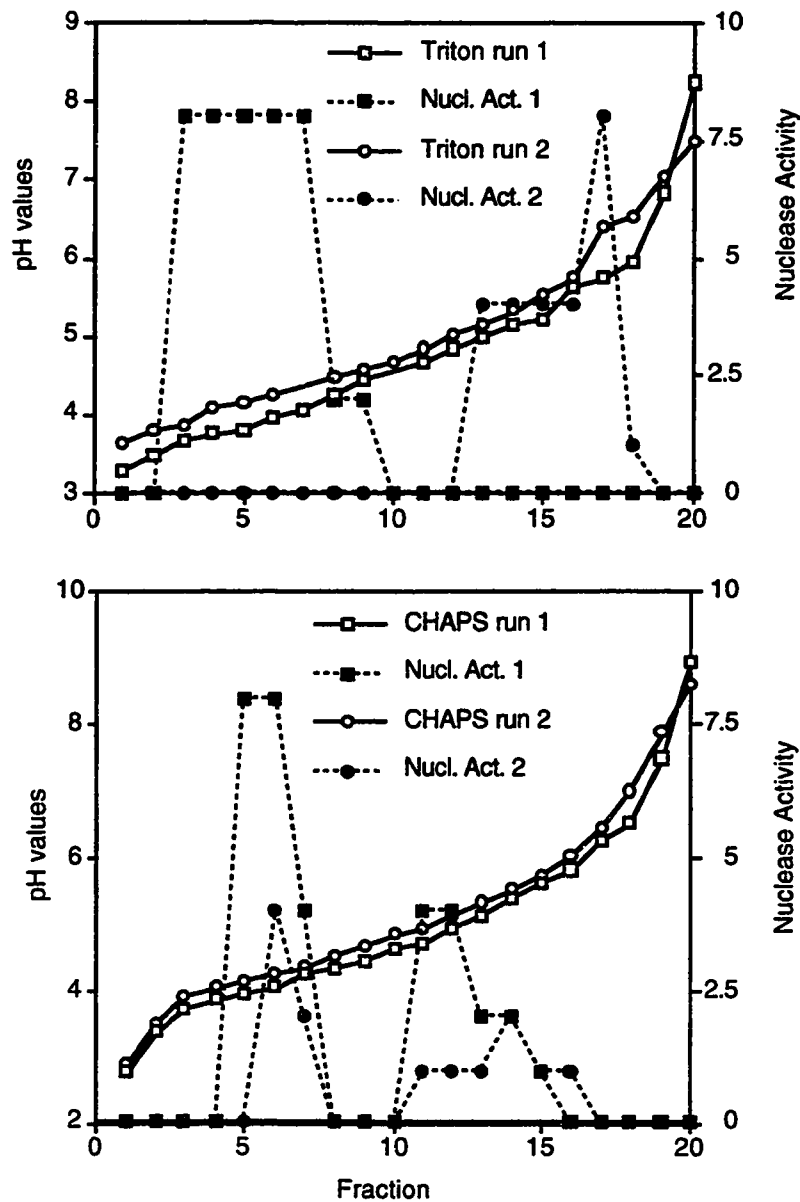


Figure A.2. Reproducibility of Rotofor separations of membrane nuclease activity. Whole cell preparations from two different cultures (separate days) were solubilized with 1% Triton X100 (Upper) or 5% CHAPS (Lower) and prepared for IEF identically. Nuclease activity was determined by the Lambda DNA assay. Shown is the nuclease and pH profiles of two runs of each detergent.

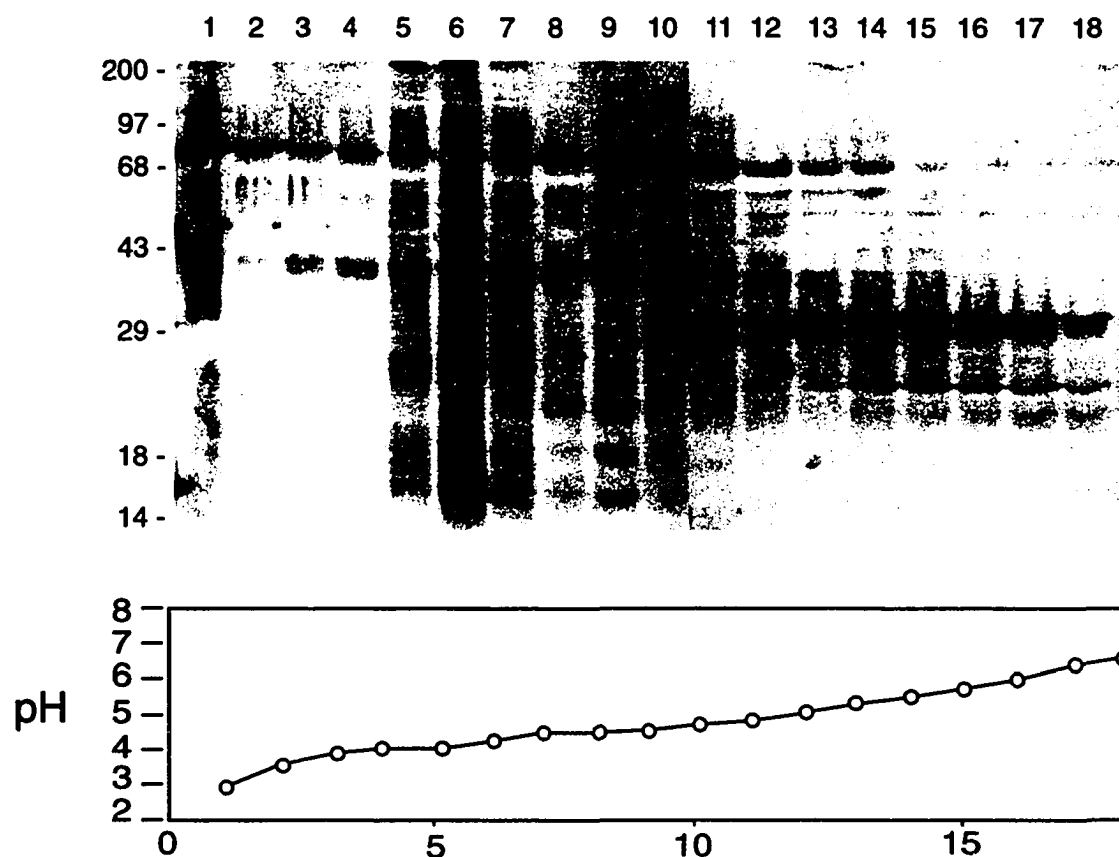


Figure A.3. SDS-PAGE analysis of fractions 1-18 of the CHAPS separation number 1 shown in Figure A.2. Fractions were precipitated with acetone and ethanol prior to separation on a 10% SDS-PAGE gel and silver staining. Beneath the gel is a graph of the pH value of each fraction.

Finally, the ease of removal is a major consideration when choosing detergents. The 0.2 mM critical micelle concentration for Triton X-100 prevented the use of dialysis for its removal. When SM-2 Biobeads were used in hydrophobic adsorption chromatography, all nuclease activity was bound to the beads (data not shown). When proteins in the Triton X-100 containing fractions were analyzed by SDS-PAGE, the detergent interfered with the proper separation and resolution normally expected with this technique. The bands were smeared and were difficult to visualize with protein stains (data not shown). To remove Triton X-100, the proteins were precipitated with acetone or ethanol which resulted in a loss of all nuclease activity. In contrast, CHAPS could be removed using an Amicon Centricon concentrator or by dialysis overnight in water. Proteins remained active in detergent-free solution and were easily visualized by SDS-PAGE.

Choice of ampholine pH range

The concentration and pH range of ampholytes affect the amount of protein that can be focused and the retention of the nuclease activity. In preliminary experiments using a pH range of 3.5 - 9.5, nuclease activity focused at approximately 4.2 (data not shown). When these fractions were refocused without the addition of additional ampholytes, the nuclease activity was found throughout the narrow pH gradient preventing further purification by IEF. Similar results were seen when a pH range of 4.0 - 6.5 or 5.0 - 7.0 was used (data not shown). The best focusing results were obtained with a mixture of ampholyte pH ranges: 3% 3.5 - 5 plus 2% 4 - 6.5 (data not shown).

Separation of Triton X-114 extracted proteins

Triton X-114 phase fractionation separates hydrophobic proteins from other less hydrophobic and hydrophilic proteins. The separation is based upon an unusually low cloud point temperature of Triton X-114. In preliminary experiments, the nuclease activity was found to partition exclusively into the detergent phase. Attempts to separate proteins from the undiluted detergent phase were unsuccessful. Triton X-114 concentrations greater than 2% in the Rotofor cell inhibited focusing and nuclease activity was found throughout the gradient (data not shown). By strictly regulating the temperature at 4°C, it was possible to focus samples with less than 2% Triton X-114 detergent, but individual fractions became biphasic upon collection and were difficult to manipulate experimentally. The addition of 0.3% CHAPS or 0.1% Triton X-100 to the Rotofor prior to focusing prevented phase fractionation of Triton X-114 and allowed normal focusing and handling.

Separation of detergent-solubilized membrane preparations

As a first step toward purification of membrane nucleases, the use of membrane preparations was also studied. Membranes were prepared by osmotic lysis as previously described, treated with 4% CHAPS, diluted to 0.4% CHAPS and focused in the Rotofor. *Acholeplasma ISM1499* membranes failed to completely solubilize in 4% CHAPS, but the insoluble material had no effect on IEF results and contained no nuclease activity (data not shown). The nuclease profile of the fractions was similar to that observed with whole cell preparations (Figure A.2).

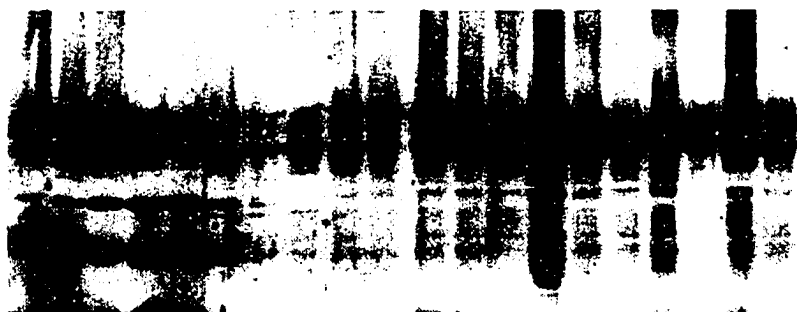
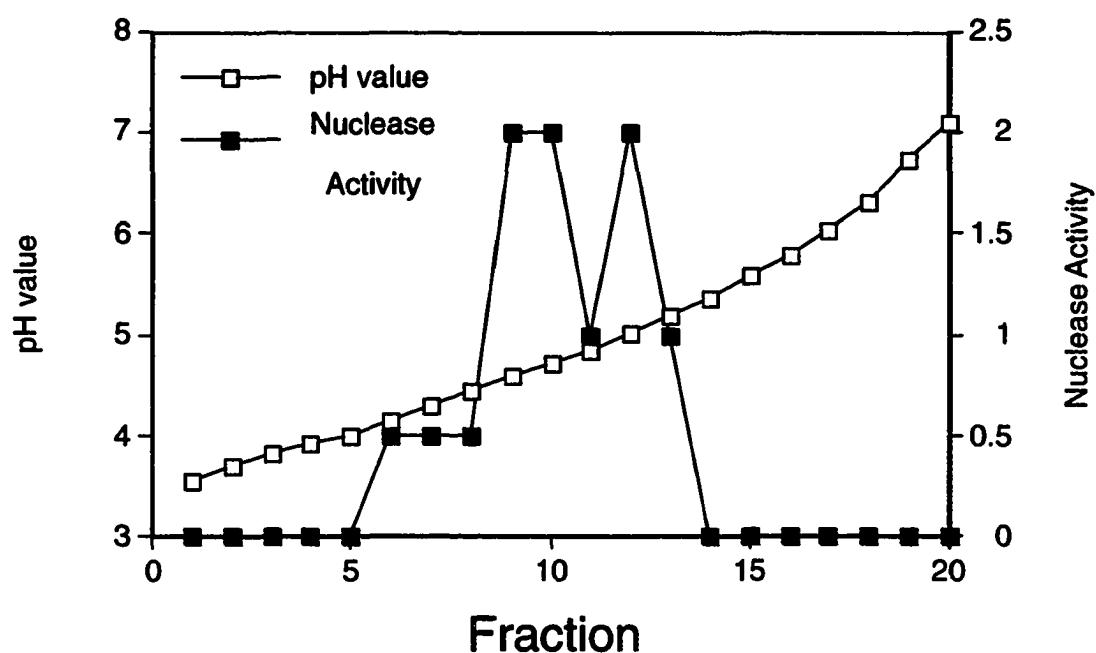


Figure A.4. Rotofor separation and the corresponding SDS-PAGE analysis using Triton X-114 extracted proteins. Whole cells were fractionated using Triton X-114 phase partitioning. Prior to separation, the detergent phase was diluted to 1% Triton X-114 with water and 0.3% CHAPS and 5% ampholytes were added. The upper panel shows the pH values and nuclease activity. Nuclease activity was determined by the Lambda DNA assay. A scanned image of silver-stained SDS-PAGE gels of fractions 1-20 is shown below. Fractions were precipitated with ethanol three times prior to separation.

Discussion

The results described in this study are the first reported attempts to partially purify any protein from a mycoplasma using the Bio-Rad Rotofor IEF cell. Problems were encountered during these studies because of the uniqueness of the organism involved. Achleoplasmas are much smaller than other replicating cells, and as such, have a higher membrane lipid to total cell protein ratio. Consequently, only lower protein concentrations could be focused without encountering large phospholipid aggregates which "trapped" nuclease activities and prevented their purification. The total protein concentration that could be loaded on the Rotofor from detergent-solubilized mycoplasmal preparations was 10 to 15 mg, much lower than that recommended for normal soluble proteins.

The purification of membrane proteins often is difficult and time consuming, but the Rotofor provides a simple, reproducible system for their isolation. The choice of detergent influenced many of the subsequent steps in the purification process. Since additional manipulation of the fractions was straightforward with the Rotofor, a detergent was chosen for solubilization of the membrane-bound nucleases that was uncharged, that could be removed easily without loss of activity, and did not interfere with subsequent analysis. The Triton series of detergents did not allow for ease of removal without the loss of activity, but CHAPS gave reproducible results and could be removed by dialysis. Other detergents such as β -octyl glucoside might also function well, but were not studied.

Ampholyte pH ranges and concentrations were chosen after empirical analysis. Excess heat generated at higher ampholyte concentrations inactivated nuclease activities, and therefore, concentrations above 5% could not be used to focus higher protein concentrations. Also, a mixture of ampholyte ranges gave the best results rather than a single range encompassing the pI of the protein of interest. This indicates that preparative IEF requires investigation of multiple parameters for each particular application.

Partial purification of the nuclease activities by Triton X-114 phase partitioning and membrane purification was also investigated. The nuclease activities associated exclusively with the Triton X-114 detergent phase and with purified membranes. The total number of proteins present in these preparations was lower than in whole cell preparations, which allowed a greater concentration of nuclease activity to be separated. The addition of a second detergent to Triton X-114 preparations greatly enhanced subsequent analysis of the Rotofor fractions. Since removal of the detergent was important in these experiments, CHAPS solubilized purified membranes is being used in purification of the membrane nucleases from *Mycoplasma pulmonis* (data not shown).

During these and related studies, additional information regarding the nucleases was obtained. The nucleases are hydrophobic, membrane-bound proteins which bind tightly to octyl sepharose, phenyl sepharose and many ion exchange resins (data not shown). Because of these properties and the observation that the nucleases focus together during preparative IEF, it is thought that they may form a membrane complex, possible with phospholipids. Unlike many other proteins, the nucleases also fail to stain after SDS-PAGE with either silver stains or Coomassie blue R. These proteins are present in all mycoplasmas tested, and protein profiles between different species demonstrate great diversity (data not shown).

In summary, the Rotofor is rapidly becoming an important tool in the generation of partially-purified, enzymatically-active protein preparations. In these studies, partially pure fractions of the *Acholeplasma* membrane bound nucleases were obtained. Optimal conditions consisted of protein concentrations of 10-15 mg per separation, use of CHAPS detergent to solubilize the proteins, a mixture of 3% 3.5-5.0 and 2% 4.0-6.5 ampholytes, and strict temperature regulation at 4°C.

Acknowledgments

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APPENDIX B. BIOCHEMICAL METHODS AND ANALYSIS OF MYCOPLASMAL MEMBRANE NUCLEASES

Introduction

This appendix will discuss the biochemical studies performed on the nucleases. Most of these studies were performed with *Acholeplasma oculi* 1499, with a relative few being applied to *M. pulmonis*. These experiments were not brought to completion and were not published. The purpose of their inclusion in this dissertation is to provide an overview of the biochemical nature of the nucleases. In general, mycoplasma membrane proteins are very difficult to purify and in these experiments, only partial purification of the nucleases was obtained. At no time were individual nucleolytic proteins separated into distinct fractions.

Materials and Methods

Heat and detergent treatment of mycoplasmas

Mycoplasmas were collected, pelleted at 10,000 x g for 15 min, washed in PBS, repelleted and finally resuspended in PBS. The ability of the nucleases to withstand heat treatment or detergent treatment was analyzed by the addition of differing amounts of SDS to the resuspended cells in combination with or without boiling of the sample for 5 minutes. Nuclease activity was monitored prior to treatment, immediately after boiling and 30 min after boiling. Activity was scored as follows: +++++ (greater than 1/32 dilution was seen to digest full length lambda DNA), +++ (1/16 dilution was capable of digesting lambda DNA), ++ (1/4th dilution was capable of digesting lambda DNA), + (negligible, but measurable activity present).

Several different detergents were also used to determine if they destroyed nuclease activity. These include SDS (varying concentration of 0.05 - 5%) octyl- β -glycoside (3-5%), Triton X-114 (1%), Triton X-100 (0.1%), CHAPS (4-5%). Detergents were added to the cell suspensions in PBS and nuclease activity was determined directly afterwards.

Membrane isolation

Mycoplasmas were grown overnight to an early mid log phase. Membranes are difficult to isolate from mycoplasmas obtained from mid to late log cultures. Organisms grown in 200 mls were harvested by centrifugation, washed twice with 0.25 M NaCl and resuspended in

1/10th volume of 2 M glycerol. Cells were incubated for 10 min at 37°C, minced several times through a 26 gauge needle and injected into 4 volumes of water at 37°C. The mixture was incubated at 37°C for 15 min and then cooled rapidly in an ice/water bath. Unlysed cells were removed by pelleting at 6,700 x g for 15 min. Membranes were harvested by pelleting at 31,000 x g for 30 min. Buffer was removed by aspiration, and the pellet was gently resuspended by swirling residual buffer in the centrifuge bottle. Membranes went into solution fairly easily, whereas any remaining pellet was most likely unlysed cells. The membrane suspension was loaded on top of a sucrose gradient (30/60%) and spun at 274,000 x g for two h under slow acceleration and slow deacceleration. The membrane band was removed, and washed once in PBS (10-15 mls) and twice in 1 ml of PBS. To maintain nuclease activity, membranes were resuspended in 40% glycerol and stored frozen. Large scale membrane preparations were also performed by increasing the above given volumes accordingly.

Trypsin treatment of whole cells prior to membrane isolation was achieved by resuspending the cells to approximately 1 mg protein per ml in PBS and treating with 500 µg/ml trypsin (T-1005, Sigma) for 1 h at 37°C. Cells were pelleted and washed in 0.25 M NaCl, and membranes were isolated as described above. Trypsin treatment was also done on isolated membranes by resuspending the membranes to approximately 1 mg protein per ml in PBS and treating with 500 µg/ml trypsin. Membranes were washed twice with PBS prior to use.

Triton X-114 phase fractionation

Mycoplasmas were pelleted from 250 ml of culture, washed once with PBS and resuspended in 1% Triton X-114 in TS buffer (0.01 M Tris - 0.15 M NaCl, pH 7.5) at room temperature. The suspension was incubated for 30 min at 4°C for equilibration of the detergent (mixed well to ensure complete distribution of the detergent), and then for 5 min at 37°C to induce partitioning. Phases were separated by centrifugation at 6,000 x g for 10 min at 20°C or higher temperature (rotor must be warm). If phases did not separate, equilibration and the 37°C incubation was repeated prior to centrifugation. The upper aqueous phase was removed and stored for later use. The lower detergent phase was diluted with an equal volume of distilled water, equilibrated for 30 min at 4°C and phase separation was induced by incubation at 37°C for 5 min. Phases were separated as before, the lower detergent phase was collected, and subjected to an additional phase partitioning. In specific instances, the upper phase was also partitioned by the addition of an equal volume of 1% Triton X-114, equilibration and induction of phase partitioning.

For specific applications, Triton was removed from the proteins by diluting the sample in 10 volumes of cold acetone, holding at 20°C for at least several hours to overnight, and pelleting the resulting precipitate at 31,000 x g for 20 min or in a microfuge for 15 min. An ethanol precipitation may also be performed instead, but ethanol is not as effective as acetone in removal of the detergent. To precipitate with ethanol, 10 volumes of ethanol were added to the protein mixture, held at 20°C for at least several hours and pelleting the resulting precipitate.

Column chromatography

Hydrophobic chromatography

Samples were prepared by disrupting washed whole cells in 5% CHAPS, diluting to 0.25% CHAPS and particulate matter was pelleted prior to use of the supernate. (0.25% is below the CHAPS critical micelle concentration of 0.39%). An octyl sepharose column was equilibrated with 0.1 M $(\text{NH}_4)\text{SO}_4$ - 0.05% CHAPS and the sample was loaded. The void volume of the column was approximately 1 ml. One ml fractions were collected with the following elution profile: 5 mls of 0.01M $(\text{NH}_4)\text{SO}_4$ - 0.05 CHAPS, 5 mls of 0.001 M $(\text{NH}_4)\text{SO}_4$ - 0.005% CHAPS, 5 mls of 0.01 M $(\text{NH}_4)\text{SO}_4$ with no detergent, 2 mls of 0.1% CHAPS, 2 mls of 0.25% CHAPS, and 1 ml of 5% CHAPS. Fractions were analyzed by the lambda assay to determine elution of the nucleases. In specific instances, beads of octyl sepharose were resuspended in 1% SDS and boiled to remove activity. The nucleolytic profile of the positive fractions were analyzed by the SDS-PAGE nuclease assay. Phenyl sepharose media was used as described for octyl sepharose.

Size fractionation

Mycoplasmas were harvested, washed, disrupted in 5% CHAPS and diluted to 0.25% CHAPS prior to use. The proteins were applied to a column with an approximately 16 ml void volume. PBS was used to elute the proteins and 4 ml fractions were collected. Fractions were analyzed for activity using the lambda assay.

Affinity chromatography

A group of dye based columns were obtained from Sigma. Samples for these columns were prepared by disrupting whole cells in 5% Triton X-100 and centrifuging at 100,000 x g for 30 min. The supernate was loaded onto the column and the columns were washed with 5

volumes of 0.10 M Tris - 0.15M NaCl - 5% Triton X-100. The column was eluted with the buffer plus 1 mg/ml DNA - 25 mM pyrimidines - 25 mM purines. Two ml fractions were collected and analyzed for activity by the lambda assay and the SDS-PAGE nuclease assay.

Antisera production

Rabbit hyperimmune antisera were produced against various *M. pulmonis* χ 1048 preparations. Rabbits were immunized i.v. with whole cells and membranes or subcutaneous with Triton X-114 detergent fraction or nuclease bands from SDS-PAGE gels. For i.v. immunizations the antigen was diluted to 1mg protein per ml in PBS. Rabbits were injected on day 1 with 1 ml, day 2 with 2 ml, day 3 with 3 ml, day 4 with 4 ml, and day 7 with 4 ml. The antisera was tested on day 11 and the animals were bled on day 18 and following days. For subcutaneous immunizations, rabbits were injected with pulverized gel slices (1.5 ml per rabbit total in 5 to 6 injection sites along the back). For Triton X-114 detergent fractions, 2.5 ml of 1:1 mixture with Freund's incomplete adjuvant also in 5 to 6 injection sites along back. These rabbits were boosted at 3 weeks with an identical amount of antigen. A test bleed was taken after 3 weeks and examined for anti-*M. pulmonis* activity by ELISA and immunoblot. The rabbits injected with the lower nuclease band were boosted a second time because of low activity. The antigen and corresponding rabbit number are given in Table B.1.

Results

Antisera analysis

Antisera produced against various mycoplasma preparations was analyzed by immunoblot against whole cell *M. pulmonis* lysate. The different antisera are listed in Table B.1 and the immunological response in each animal is demonstrated in Figure B.1.

Effects of heat and detergent treatments on nuclease activity

Treatment of whole cell mycoplasmas with increasing concentrations of SDS inhibited nuclease activity as the concentration increased (Table B.2). When the samples were boiled, there was no activity immediately after the heat treatment. However, after a 30 min incubation, a small amount of activity was seen in the lambda assay (Table B.3). These results indicate that the nucleases are relatively resistant to detergent treatment and can also renature after denaturation within a short period of time. Using the SDS-PAGE nuclease assay, there is no measurable difference between samples which are treated with SDS at room temperature for 30 min, 65°C for 15 min or those samples which are boiled for 5 min

Table B.1. Description of rabbit hyperimmune antisera

Rabbit Number	Antigen Description
90021	whole cells
90108	whole cells
90132	purified membranes
90117	purified membranes
90175	Triton X-114 extract, detergent phase
90174	Triton X-114 extract, detergent phase
89786	SDS-PAGE nuclease band, upper
90171	SDS-PAGE nuclease band, upper
90039	SDS-PAGE nuclease band, middle
89938	SDS-PAGE nuclease band, middle
89961	SDS-PAGE nuclease band, lower
89971	SDS-PAGE nuclease band, lower

(data not shown). The location of these proteins in the membrane may provide a means of protection from harsh detergent and heat treatments.

Treatment of mycoplasmas with different detergents at various concentrations also had no apparent effect on nuclease activity and stability. These detergents included Triton X-100, CHAPS, octyl β -glucoside and Triton X-114. Subsequent experiments were performed with CHAPS because of ease of removal. The effects of various storage conditions and precipitation methods is shown in Table B.3.

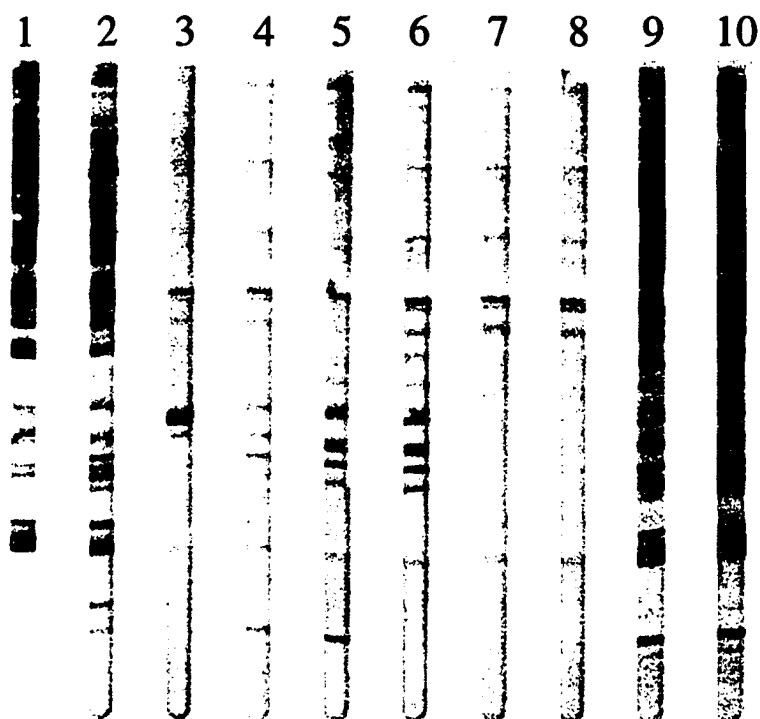


Figure B.1. Immunoblot analysis of *M. pulmonis* whole cell and subcellular fractions using different rabbit antisera. Antigen is *M. pulmonis* χ 1048 whole cells. Rabbit hyperimmune antisera was raised against various preparations. The antisera number is given for each lane. Lane 1, 90021; lane 2, 90117; lane 3, 89786; lane 4, 90191; lane 5, 90039; lane 6, 89938; lane 7, 89971; lane 8, 89961; lane 9, 90174; lane 10, 90175.

Table B.2. Sensitivity of nuclease activity to heat and SDS treatments

Buffer	Before boiling	After boiling	After 30 minute incubation
PBS	+++++	-	+
PBS - 0.05% SDS	+++++	-	+
PBS - 0.5% SDS	+++	-	+
PBS - 1% SDS	++	-	+
PBS - 5% SDS	+	-	+

Table B.3. Effects of purification of nuclease activity analyzed by Lambda and SDS-PAGE nuclease assay.

Treatment	Lambda Assay	SDS-PAGE
Triton X-114		
aqueous phase	-	not determined
detergent phase	+	+
Acetone precipitation	-	+
Ethanol precipitation	not determined	+
Boiling	-	+
1% SDS	+	+
0.25% CHAPS	+	+
4% CHAPS	+	+
Long-term storage 4°C	-	+
Long-term storage -20°C	+	+
Storage -70°C	-	not determined
Cibron Blue 3GA	+	-
Octyl Sepharose (1% SDS)	+	+
Phenyl Sepharose (1% SDS)	+	+
Sephadex S-200	+	+

Localization of nuclease activity

When mycoplasmas were subjected to Triton X-114 phase partitioning, nuclease activity was only detected in the detergent phase (Table B.3) indicating significant hydrophobicity and a probable membrane location. Analysis of the nucleolytic banding patterns after Triton X-114 partitioning was done using an SDS-PAGE nuclease gel after acetone precipitation. All of the nucleolytic proteins were present in the detergent phase (data not shown).

To further determine the location of the nuclease, trypsin treatment before and after membrane isolation was performed. Purification of membranes was verified by SDS-PAGE gels (Figure B.2). When membranes were isolated and analyzed by SDS-PAGE nuclease assay, all of the nucleolytic bands were present in the membranes (data not shown). Treatment with trypsin pre or post membrane isolation had no apparent effect on the nucleolytic banding patterns indicating that the nucleases are relatively resistant to trypsin treatment. This supports the suggestion that the nucleases are protected inside the membrane of mycoplasmas.

Column chromatography

Affinity chromatography was attempted using several different dye resins, octyl sepharose, and phenyl sepharose media. Nuclease activity bound to Cibron Blue 3GA, octyl sepharose and the phenyl sepharose media (Table B.3). The other dye resins did not absorb the nuclease activity. Activity, as measured by the lambda assay, was eluted from the Cibron Blue 3GA column using a buffer containing DNA and nucleotides. Unfortunately, these fractions displayed no activity when analyzed by SDS-PAGE nuclease assay. This may indicate that a cofactor was purified along with them in the fractions, which was lost during separation.

Attempts to purify the nucleases off of octyl sepharose or phenyl sepharose columns were unsuccessful. Nuclease activity bound tightly to both of these column materials and could only be eluted when the concentration of CHAPS was raised above the critical micelle concentration. Occasionally, a small amount of activity was observed in the void volume. Complete elution was achieved by treating the beads with 1% SDS for several hours or boiling. Fractions remained nuclease positive in the detergents. Analysis of the released material by SDS-PAGE nuclease assay and silver staining after SDS treatment indicated that the entire spectrum of nucleolytic proteins, as well as many other proteins, were tightly bound to the octyl sepharose resin (Figure B.2).

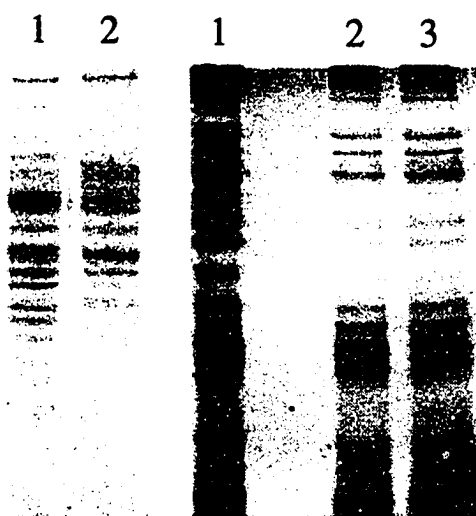


Figure B.2. SDS-PAGE analysis of membrane fractions and octyl sepharose adhering fractions. Left panel (Coomassie Blue stained): lane 1, *M. pulmonis* whole cells, lane 2, *M. pulmonis* purified membranes; Right panel (silver stained): lane 1, *M. pulmonis* whole cells, lanes 2 and 3, adherent fractions of *M. pulmonis* to Octyl Sepharose after standard elution. The fractions were eluted by SDS treatment of the beads.

Size fractionation of the nuclease activity was attempted using Sephadex S-200. All activity was eluted in the void volume. This was repeated several times with the various detergents at concentrations above and below the critical micelle concentration. These changes had no effect on the elution of the nuclease activity in the void volume.

Discussion

The experiments described here indicate that the nucleases are hydrophobic membrane proteins. The nucleolytic proteins partition exclusively into the detergent phase of Triton X-114 and are exclusively associated with membrane fractions. Proteins which are integral membrane proteins or lipoproteins are known to partition to the detergent phase, whereas many of the peripheral membrane proteins partition into the aqueous phase in the extraction. The discovery that MnuA has a membrane spanning domain and a prolipoprotein signal sequence correlates with these observations. The lipoproteins in mycoplasmas such as the Vlp are attached to the membrane by a fatty acid tail, with the remainder of the molecule usually being hydrophilic. This was also true of MnuA. Although these proteins may be hydrophilic in the C-terminus, they are relatively resistant to trypsin treatment. This indicates that they may either be in tight association with the membrane, buried in the membrane bilayer, or may not have an accessible trypsin cleavage site.

In the detergent treatments and in the column chromatography studies, it is possible that the nucleases were never completely separated from membrane components. This may explain why individual proteins were never resolved and why the proteins appeared to be in a complex when size fractionation studies were performed. Even when isoelectric focusing was performed, the proteins migrated together. Nonionic detergents may be unable to dissociate the strong hydrophobic forces attracting the nucleases and membrane components. In many respects, the results of these experiments would also be seen in attempts to purify a lipid molecule. Future attempts to purify the nucleases will need to consider the acylation of these proteins and possibly treat them as a lipid. It should be pointed out that none of the mycoplasma lipoproteins have been purified.

APPENDIX C. PLASMID CONSTRUCTIONS

Plasmid Designation	Parent Phage	Description of Construction
pISM4005	6.2B	4.8 kb <i>SacI</i> fragment cloned into pKS
pISM4010	6.3E	6.4 kb & 12.2 kb <i>SacI</i> fragments cloned into pKS
pISM4011	6.3E	12.2 kb <i>SacI</i> fragment cloned into pKS
pISM4012	6.3E	small <i>SacI</i> fragment cloned into pKS
pISM4013	6.3E	small <i>SacI</i> fragment cloned into pKS
pISM4020	9.3B	10.4 kb <i>SacI</i> fragment cloned into pKS
pISM4021	9.3B	10.4 kb <i>SacI</i> fragment cloned into pKS
pISM4022	9.3B	10.4 kb <i>SacI</i> fragment cloned into pKS
pISM4030	9.2C	12.2 kb <i>SacI</i> fragment cloned into pKS
pISM4040	9.10G	6.7 kb <i>SacI</i> fragment cloned into pKS
pISM4041	9.10G	8.2 kb <i>SacI</i> fragment cloned into pKS
pISM4042	9.10G	small <i>SacI</i> fragment cloned into pKS
pISM4050	11.6E	small <i>SacI</i> fragment cloned into pKS
pISM4051	11.6E	3.7 kb <i>SacI</i> fragment cloned into pKS
pISM4052	11.6E	5.5 kb <i>SacI</i> fragment cloned into pKS
pISM4060	13.5C	4.7 kb <i>SacI</i> fragment cloned into pKS
pISM4061	13.5C	8.1 kb <i>SacI</i> fragment cloned into pKS
pISM4062	13.5C	4.7 kb and 8.1 kb <i>SacI</i> fragments cloned into pKS
pISM4070	14.11F	3.7 kb and 7.5 kb <i>SacI</i> fragments cloned into pKS
pISM4071	14.11F	7.5 kb and 1.7 kb <i>SacI</i> fragments cloned into pKS
pISM4072	14.11F	3.7 kb and 7.5 kb <i>SacI</i> fragments cloned into pKS
pISM4073	14.11F	1.7 kb <i>SacI</i> fragment cloned into pKS
pISM4080	14.3D	3.7 kb <i>SacI</i> fragment cloned into pKS
pISM4081	14.3D	4.4 kb and 5.3 kb <i>SacI</i> fragments cloned into pKS
pISM4090	16.D2	2.2 kb and 3.1 kb <i>SacI</i> fragments cloned into pKS
pISM4091	16.D2	3.5 kb <i>SacI</i> fragment cloned into pKS
pISM4100	8.12F	3.5, 7 and 8 kb <i>SacI</i> fragments cloned into pKS
pISM4101	8.12F	8 kb and 12 kb <i>SacI</i> fragments cloned into pKS
pISM4102	8.12F	2.0 kb <i>SacI</i> fragments cloned into pKS
pISM4110	9.2A	2.2 kb and small <i>SacI</i> fragments cloned into pKS
pISM4111	9.2A	8, 10.4 and 12 kb <i>SacI</i> fragments cloned into pKS
pISM4112	9.2A	2.2 kb <i>SacI</i> fragment cloned into pKS

pISM4113	9.2A	10.4 kb <i>SacI</i> fragment cloned into pKS
pISM4114	9.2A	2.7, 3.2 and 8 kb <i>SacI</i> fragments cloned into pKS
pISM4120	9.2B	3.2 and 7.5 kb <i>SacI</i> fragments cloned into pKS
pISM4121	9.2B	2.5 kb <i>SacI</i> fragment cloned into pKS
pISM4130	9.6G	8 kb <i>SacI</i> fragment cloned into pKS
pISM4131	9.6G	4.5 kb <i>SacI</i> fragment cloned into pKS
pISM4132	9.6G	6, 8, and 10 kb <i>SacI</i> fragments cloned into pKS
pISM4140	9.12E	1.4 kb <i>SacI</i> fragment cloned into pKS
pISM4150	11.2D	1.8 kb <i>SacI</i> fragment cloned into pKS
pISM4160	14.7C	2.0 kb <i>SacI</i> fragment cloned into pKS
pISM4161	14.7C	1.7, 2.8 and 4 kb <i>SacI</i> fragments cloned into pKS
pISM4162	14.7C	1.3, 7 and 11 kb <i>SacI</i> fragments cloned into pKS
pISM4170	13.2B	6.8 kb <i>SacI</i> fragment cloned into pMOB
pISM4171	13.2B	6.8 kb <i>SacI</i> fragment cloned into pMOB
pISM4172	13.2B	6.6 kb <i>SacI</i> fragment cloned into pMOB
pISM4173	13.2B	2.2 <i>ClaI</i> fragments from pISM4172 cloned into pMOB
pISM4174	13.2B	2.2 <i>ClaI</i> fragments from pISM4172 cloned into pMOB
pISM4175	13.2B	2.2 <i>ClaI</i> fragments from pISM4172 cloned into pMOB
pISM4176	13.2B	2.6 <i>SacI</i> - <i>ClaI</i> fragment from pISM4172 cloned into pMOB
pISM4177	13.2B	2.6 <i>SacI</i> - <i>ClaI</i> fragment from pISM4172 cloned into pMOB
pISM4180	13.2B	pISM4176 with small <i>EcoRV</i> fragment deleted at end of the fragment (pISM4176 Δ 10)
pISM4181	13.2B	pISM4176 with large <i>EcoRV</i> fragment deleted at end of the fragment (pISM4176 Δ 8)
pISM4182	13.2B	pISM4181 with <i>tetM HincII</i> fragment from pISM1002 cloned into <i>HincII</i> site of MCS
pISM4201	13.2B	<i>SacI</i> - <i>ClaI</i> fragment from pISM4176 cloned into pG7ZCW (Wise)
pISM4202	13.2B	pISM4201 with <i>SacI</i> - <i>HindIII</i> fragment deleted using T4 DNA polymerase and blunt end ligation

ACKNOWLEDGMENTS

Throughout this study, I have been reminded that there is a Creator who grants to his children the knowledge of the creation in His timing. In His wonderful love and grace, God the Father sent His Son to this world and granted us a home to live in. I acknowledge that the study of science allows me to see small glimpse of the perfection of God, the intimate detail that He took with the creation and His infinite wisdom. I thank my Father and my Savior for Their unending faithfulness and for teaching me that I can never study so hard to extend beyond Their desire for me to learn.

Daily I am reminded that I have the faithful love and support of my husband, John Aaron Taylor. He has made sacrifices in his own career to allow me to pursue my dreams. Thank you, John, for loving me so much and standing beside me. I would have quit long ago without the reassurances and support in my home. We have been and will always remain a team dedicated to the study of the unknown.

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