Functional characterization of a surface protein of *Mycoplasma bovoculi*

by

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TABLE OF CONTENTS

SUMMARY

Mycoplasma bovoculi is known to cause conjuntivitis in cattle. A 94kDa surface protein, designated as p94, is an interesting protein with pleotropic effects for the mycoplasma. Studies performed herein identify the protein as a surface-located NADH oxidase of *M. bovoculi* . Monoclonal antibody (M25.5), specific for p94, inhibits the production of hydrogen peroxide and NADH oxidase activity of *M. bovoculi* . Affinity chromatography followed by gel filtration chromatography demonstrated that NADH oxidase activity is present in the affinity purified p94 and that the protein is a multimer of 94kDa. The enzyme activity is found in the membrane as multimers of p94 and in the cytoplasm as low molecular weight moieties. The NADH oxidase(s) of *M. bovoculi* accept electrons from ferricyanide, menadione and DCPIP but not cytochrome C.

CHAPTER 1. GENERAL INTRODUCTION

Several different microorganisms have been isolated from the eyes of cattle with Infectious Bovine Keratoconjunctivitis (IBK). These included rickettsia-like organisms, bacteria and chlamydia [123]. However, initial studies showed no evidence for mycoplasmas in IBK. It was only later that several species of mycoplasmas were identified from conjunctival smears of cattle with bovine keratoconjunctivitis [35]. Of the mycoplasmas that were isolated from cattle with IBK, *Mycoplasma bovoculi* was the most frequently isolated together with *Moraxella bovis* [32, 53]. It was first characterized by Langford and Leach [59] on the basis of its cultural, morphological, biological and serological properties. Since then, several authors have found *M. bovoculi* associated with bovine conjunctivitis [75, 32, 95, 53, 8].

In separate attempts, Friis and Pederson [32] and Rosenbusch and Knudtson [95] have shown that instillation of *M. bovoculi* cultures on the conjunctivae of cattle resulted in conjunctivitis. *M. bovoculi* has been shown to play a significant role in predisposing cattle to ocular colonization with *M. bovis* and *M. ovis* [94, 96]. Studies on the immune responses of cattle infected with *M. bovoculi* revealed a primary IgM and IgG response in serum and a predominant IgA response in lacrimal fluid [104]. Cattle given *M. bovoculi* antigen parenterally prior to infection developed more severe conjunctivitis than did naive animals [93].

Many different membrane proteins of *M. bovoculi* have been identified with the aid of monoclonal antibodies. Of these, a 94kDa protein (p94) was found to be the most interesting one with pleotropic effects for the mycoplasma. The 94kDa protein is a surface associated protein that is randomly distributed on the periphery of the cell [103] and is recognized by early systemic immune responses of cattle infected with the organism [104, 7]. It is a trypsin sensitive protein involved in the attachment of *M. bovoculi* to conjunctival cells and to cornea [103]. In addition, it is the only target for the action of complement-independent metabolism inhibiting antibodies [7].

In view of the importance of this protein to *M. bovoculi*, it was of interest to further study p94. The objectives of this research are to define a function for the protein and to characterize the protein.

CHAPTER 2. LITERATURE REVIEW

In reviewing the literature pertinent to the research performed, a general overview of *Mollicutes*, with special emphasis on their membrane proteins and metabolic inhibition phenomena is presented. This is followed by a review of reactive oxygen species produced during aerobic respiration by bacteria.

General Characteristics of Mollicutes

The name mycoplasmas is used rather loosely to denote any species included in the class *Mollicutes*. Mycoplasmas are the smallest self replicating procaryotes devoid of cell walls [85]. They have a characteristic fried egg appearance on solid medium and are filterable through membrane filters of 220-450nm pore diameter. The genome size differs from other procaryotes in its small size and low guanine plus cytosine content. The mycoplasma cell contains only the minimum number of organelles essential for cell growth and replication, such as the plasma membrane, ribosomes and a double stranded deoxyribonucleic acid.

The classification of *Mollicutes* was formally established by Edward and Freundt [27]. *Mollicutes* is the only class in the division *Tenericutes* (wall-less bacteria) of the kingdom *Procaryotae*. The class *Mollicutes* consists of a single order *Mycoplasmatales*. Based on nutritional and morphologic criteria, these organisms were subdivided into

three families *Mycoplasmataceae*, *Acholeplasmataceae* and *Spiroplasmataceae*.

Mycoplasmataceae and *Acholeplamataceae* are pleiomorphic with cells that are small coccoid to long branched filaments [30]. Some of the mycoplasma species like *M. pneumoniae* and *M. pulmonis* possess tip-like structures which aid in locomotion and also in attachment. The genome of mycoplasmas is typically procaryotic in consisting of a circular double-stranded DNA molecule. The genome size is about 5×10^8 daltons. The family *Mycoplasmataceae* is the only one that is subdivided into two genera, *Mycoplasma* which does not hydrolyse urea and *Ureaplasma* that possesses the enzyme, urease.

Acholeplasmataceae consists of a single genus *Acholeplasma* whose members are unique from other families in not requiring sterols for growth [30]. *Spiroplasmataceae* differs from other two families in possessing a helical morphology and undulating motility of the filaments. It includes a single genus, *Spiroplasma*. The genome size is the same as *Acholeplasma*, about 1×10^9 daltons.

Two genera recognized as members of the class *Mollicutes*, but whose taxonomic position is uncertain, are *Anaeroplasma* and *Thermoplasma* [85]. The genus *Anaeroplasma* includes organisms that resemble the typical mycoplasmas but have an absolute requirement for anaerobic conditions. The wall-less, non-parasitic procaryotes isolated from heated coal refuse piles were included in the genus *Thermoplasma*. The members of this genus are unique in having a monotrichous flagellum and swimming motility, peculiar DNA, RNA and minimal nutritional requirements.

Many criteria are used for distinguishing species and strains of mycoplasmas. For example: genomic DNA G+C content, DNA methylation patterns, DNA-DNA or DNA-RNA hybridization pattern, genomic maps, cell protein electrophoretic profiles

and serologic properties including growth inhibition by specific antiserum [30].

Various species of mycoplasmas have been isolated from humans, animals, plants and insects [85]. Several species have been found to occur in more than one host, although the majority of mycoplasmas show a rather specific host and tissue specificity. Most members of the class *Mollicutes* other than *Thermoplasma* are parasites and many are pathogens. Mycoplasmas rarely invade the bloodstream and tissues. They adhere to and colonize epithelial lining of the respiratory and urinogenital tracts of infected animals and can therefore be regarded as surface parasites. There are many factors involved in the pathogenicity associated with the mycoplasmas, for example, hydrogen peroxide, the end product of respiration in mycoplasmas, or the capsular galactan of *M. mycoides* subspecies *mycoides* have been incriminated in the pathogenicity of mycoplasmas.

Membrane Proteins of Mycoplasma

Mycoplasmas are convenient models for studying some basic problems of cell biology because of their simple structure and limited biochemical activity. Mollicutes lack a cell wall and outer membrane. Plasma membrane is the only membrane that envelops the cells and is exposed to the surrounding environment, except for some species that have capsule. Proteins comprise roughly two thirds of the mass of the membrane, the rest is lipid [85]. As in other bacteria, mollicutes have integral and peripheral membrane proteins. The integral membrane proteins are embedded deeply into the lipid bilayer and are mostly bonded by hydrophobic interactions between the lipid hydrocarbon chains and the amino acid side chains. Their release from the membrane requires detergent solubilization, protein denaturation or the use of

organic solvents [37]. Peripheral proteins are bound by electrostatic interactions and are released more easily by mild treatments such as EDTA or by changes in pH [122]. TX-114 phase fractionation has been used in many instances to separate and identify integral membrane proteins from peripheral or cytoplasmic proteins [89].

About 50-80% of the total dry weight of a mycoplasma membrane is protein [86]. By two dimensional gel electrophoresis, approximately 320 individual proteins were detected in *Acholeplasma laidlawii*, of which 140 were found to be associated with the membrane [6]. Two -dimensional, denaturing gel electrophoretic analysis also revealed $300 - 355$ cellular proteins in *M. mycoides* and *M. capricolum* [52, 92]. In contrast, a recent analysis has revealed aprroximately 1800 polypeptides in *E. coli*, at least 200 of which are of membrane origin [5]. Each electrophoretic band may not be a representative of a single protein since complex membrane proteins can disassociate into subunits during processing. The electrophoretic patterns of mycoplasma membranes represents a much more stable characteristic of a given species than the composition of its membrane lipids. The molecular weights of the membrane proteins may range from 15,000 to over 200,000, values that are comparable to those of other biological membrane proteins.

The membrane proteins of mycoplasmas perform a variety of functions. Apart from their structural and catalytic properties, they also function in electron transport, export signaling, attachment and immunologic activities of mycoplasma cells. The localization of membrane proteins in mycoplasmas has been made easy by the labeling of external proteins using the lactoperoxidase mediated iodination technique [4]. Based on this study, it was concluded that more proteins were exposed on the inner face rather than on the outer face of the membrane. Trypsin treatment of the

cells followed by iodination studies confirmed that most of the proteins exposed on the outer surface are of high molecular weight. Other techniques used to study the disposition of membrane proteins included crossed immunoelectrophoresis [49], freeze fracture [100] and immunocytochemistry [99].

Mycoplasma membranes have been popular tools in biomembrane research because of their ease in isolation and also because they can be obtained free of other organelle contaminants [85]. Osmotic lysis is the simplest and the gentlest way to isolate cell membranes. Mycoplasma cells are sensitive to osmotic shock because they lack cell walls. However, there are some species that are resistant to hypo-osmotic shock like *M. gallisepticum* and *M. pneumoniae* [83]. Unlike bacterial protoplasts, mollicutes are quite capable of resisting osmotic lysis [87]. The plasma membrane possesess high tensile strength depending on the amount of cholesterol present, which in turn depends on the aging of the culture [51]. To overcome these difficulties, alternative methods such as preloading the cells with glycerol, using digitonin or alkali treatment have been used to increase the internal osmotic pressure and for the subsequent lysis of cells. Mechanical disruption of cells including ultrasonic oscillation, French press or an X press have also been used. Sonic oscillation is very efficient but has the disadvantage of breaking the membranes into small pieces which are difficult to recover by centrifugation. Alternate freezing and thawing has also been used to disrupt mollicute cells [83]. Mycoplasma cells are able to resist lysis by utilizing membrane ATPases. *M. gallisepticum* cells suspended in NaCl solution are easily lysed by adding dicyclohexylcarbodiimide, an ATPase inhibitor which enhances swelling of the cells [101]. The purity of the membrane preparation is usually tested by isopycnic centrifugation, electron microscopy and the presence of enzyme markers [83].

Mycoplasma membrane proteins are usually designated as 'p' followed by the molecular size of the protein. For example, a 101kDa protein from *M. hyorhinis* is known as p101 [127]. Horowitz et.al. identified seven different surface antigens from *M. pulmonis* that were recognized by the host during natural infection [40]. A 64kDa membrane lipoprotein of *M. gallisepticum* was identified as a haemagglutinin and antisera raised against it inhibited haemagglutination of chicken erythrocytes. The protein was found to be immunodominant and involved in the process of attachment to tracheal epithelium [29]. Of all the membrane proteins that have been characterized so far, P1 protein from *M. pneumoniae* was the most thoroughly studied. The protein localizes in high concentrations at the terminal attachment organelle of wild-type *M. pneumoniae* and is also detected less densely elsewhere on the mycoplasma surface. Monoclonal antibodies directed against surface proteins of *M. pneumoniae*, especially P1, blocked attachment to chicken erythrocytes, but they did not have any effect on thymidine incorporation [74]. Certain *M. pulmonis* surface membrane proteins possessed chemoattractant activity for rat B lymphocytes and macrophages [98]. Most of the mycoplasma membrane proteins are associated with lipids. Twenty different integral membrane proteins of *M. hyorhinis* were found to be associated with lipids by monoclonal antibody binding. One monoclonal antibody against p23 from *M. hyorhinis* was also found to mediate complement-dependent mycoplasmicidal activity [14]. Surface protein antigens of *M. hyopneumoniae* were found to be covalently attached to lipid by HPLC, and were selectively modified by lipids [124]. Multiple structural variants of the V-1 antigen of *M. pulmonis* could be found within a single strain [121]. The variation was due to the presence of multiple subunits within the V-1 structure which aggregate due to hydrophobic interactions. The Vlp

system of *M. hyorhinis* defines antigenic and structural variation of surface proteins [97]. A variation also occurs for the P1 protein. The presence of size variant antigens may contribute to differences in the pathogenicity of mycoplasmas.

Only 20 proteins have been purified so far from mycoplasma membranes, most of whose functions are still unknown or hypothetical. Using Tween-20, six integral membrane proteins of *A. laidlawii* were selectively purified [39, 49, 48]. These proteins included the $t_{1a}, t_{1b}, T_2, T_3, T_{4a}$ and T_{4b}. Polypeptide t_{1a} was later shown to express antigenic determinants both on the internal as well as on the external surfaces of the membrane. A major membrane protein, spiralin, was purified from *Spiroplasma citri*, using surfactants [125]. *M. pneumoniae* cytadhesin P1 is another integral membrane protein of known function which has been purified to homogeneity [44]. A *M. genitalium* protein (MgPa) that shares antigenic determinants and sequence similarities with P1 was purified using the same procedure used for P1 [66]. Four membrane proteins of known function have been purified from mollicutes. These are an NADH oxidase and an ATPase from *A. laidlawii*, and aminopeptidase and arginine specific carboxypeptidase from *M. salivarium* [88, 61, 107, 108]. *M. pneumoniae* has a membrane glycoprotein of 60kDa which contains 7% carbohydrate. [50]. A protein which is also believed to be glycosylated has been purified from the membrane of *Spiroplasma citri* [109]. However, it is not completely clear as to whether these are true membrane proteins or serum glycoproteins that are present in the culture medium and can tightly bind to the cell surface. Contamination of mycoplasma membranes with serum proteins has always been a problem in antigen preparation [54]. Also, the composition of the growth medium and the aging of the cells play an important role in expression of the proteins, and therefore in membrane composition.

Some of the genes of the purified proteins have been cloned and sequenced. The peripheral proteins seem to lack hydrophobic segments whereas the integral proteins contained one or many transmembrane segments [122]. Several of the proteins have typical bacterial signal peptides. The signal peptide of P1 is processed whereas other proteins like p101 from *M. hyorhinis* do not cleave their signal peptide and use it as a hydrophobic anchor [43, 127]. The study of protein translocation across single plasma membrane is hampered by the lack of mutational systems in mycoplasmas and also by the presence of UGA (Trp, in mycoplasmas) codons in the recombinant genes, which makes it difficult to express these genes in *E. coli*. Evidence was presented that *M. hyorhinis* possess an equivalent to the high affinity periplasmic binding protein dependent transport system similar to gram negative bacteria. A protein (p101), also from *M. hyorhinis*, was identified recently as a protein involved in export function by sequence analysis [34, 127]. Later a p37 protein from the same organism was identified from the sequence of the p37 gene, as being part of a homologous, high affinity transport system [24].

Metabolic Inhibition in Mycoplasmas

The metabolic inhibition (MI) test is one of the most useful serologic techniques for determining antibody titers in serum and for the identification of mycoplasma species. Several authors had described growth inhibition of mycoplasma by specific antiserum [26, 18]. Jensen initially observed that specific antiserum inhibited the reduction of tetrazolium by *M. pneumoniae* in broth medium [46]. Purcell et al. and Taylor-Robinson et al. modified the test to include substrates such as glucose, arginine or urea in the medium and used them as indicators of growth [82, 113]. The test

is based upon inhibition of growth by specific antisera and is detected as an inhibition of pH change due to the production of ammonia (non-fermenting mycoplasma) or acid (fermenting mycoplasma). The highest dilution of antiserum which prevented pH change (color change) was considered as the MI antibody end point. To identify mycoplasmas that do not produce acid or hydrolyse arginine, metabolize urea or reduce tetrazolium, a modified test was developed based on phosphatase production [38].

The test has been used to detect antibody in the sera of many host animal species. It is considered to be more specific and more sensitive than agglutination, complement-fixation or precipitation [41]. A major disadvantage of this test is that some mycoplasmas fail to produce antibody in their natural host that is detectable by MI test [41]. Washburn developed a new technique for determining anti-mycoplasma metabolic inhibition which determines the end point titer by comparing the absorbances of mycoplasma cultures at 560nm of the test material as well as the control [119].

It was demonstrated that the MI titer was enhanced when unheated guinea pig serum was added. The serum might provide a source of complement. The MI test conducted with *M. pneumoniae* suggested that complement components C3, C8 and other components in the complement cascade are required to demonstrate MI activity [20]. Complement independent metabolic inhibition using monoclonal antibody against specific membrane proteins was shown in *M. bovoculi* and *M. hominis* [7, 28]. In *M. hominis*, it was also demonstrated that the $F(ab)_2$ structure of the monoclonal antibody had metabolic inhibition potential and decreased viability and MI observed was accompanied by mycoplasma agglutination by the monoclonal antibody. Stud-

ies with *M. arthritidis* indicated that more than one protein may be involved in metabolic inhibition, one an integral membrane protein, the other probably a protein of cytoplasmic origin [120].

There is a good correlation between the growth-inhibition and metabolic inhibition reactions suggesting that growth inhibiting antibody is closely related to or identical to metabolism inhibiting antibody [113]. The metabolic inhibition test has been modified many times to identify specific species of mycoplasma or to overcome infrequent or inconsistent inhibitions [90].

Bacterial Respiration and Reactive Oxygen Species

The bacterial aerobic respiratory chain functions in the translocation of protons out of the cytoplasmic membrane which generates a proton motive force to drive the synthesis of ATP [126]. The cytoplasmic membrane of bacteria contains electron transfer systems, the ATP synthetase and carrier proteins for solute transport. Most of the energy required for metabolism is provided by the oxidation-reduction reactions of respiratory substrates through the electron transport chain. A terminal oxidase finally transfers the electron to oxygen. Bacterial respiratory systems have different terminal oxidases to transfer electrons to oxygen. One group of terminal oxidases are the cytochrome oxidases, the other are quinol oxidases.

During the process of carbohydrate metabolism, the coenzymes NAD^+ and $NADP^+$ are reduced to NADH and NADPH [79]. The reducing power of NADH is transferred to oxygen via different intermediate carriers such as the Fe-S cluster-containing flavoproteins, quinones or cytochromes. During this passage of electrons to oxygen, part of the energy is used to synthesize ATP, which involves the ATP

synthetase. Mollicutes differ from other procaryotes in not having any quinones or cytochromes. The major components of their respiratory chain are the NADH dehydrogenase and NADH oxidase. These activities may both be mediated by the same protein.

The transfer of electrons to oxygen and the reduction of oxygen to water can produce oxygen species with reactive properties [9]. Oxygen is not toxic because of its own reactivity, but its reduction to water tends to proceed by a series of single electron transfers which generate reactive intermediates such as the superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH^-). Oxygen radicals may also be produced during ionizing radiations, redox reactions or when macrophages and neutrophils produce bursts of these radicals during immune response.

Bacteria possess defense mechanisms for each of the reactive oxygen species. Many of the proteins required for bacterial defense against oxidative damage have been identified [112]. They include superoxide dismutases, catalase, NADH dehydrogenase, glucose-6-phosphate dehydrogenase, etc. Although bacteria do not possess glutathione peroxidase, they do have hydroperoxide reductase and glutathione reductase which play a role in protecting against oxidation. Hydroperoxide reductase converts the hydroperoxides to their corresponding alcohols and glutathione reductase maintains a pool of reduced glutathione, which in turn can serve to maintain the reduced state of cellular proteins. Glucose-6-phosphate dehydrogenase activity was found to increase under superoxide generating conditions. It was suggested that oxidatively stressed cells have an increased requirement for NADPH, which is generated by the action of this enzyme.

The univalent reduction of oxygen results in the formation of O_2^-. It can also

be produced by the univalent oxidation of H_2O_2 [9]. Several oxidative enzymes have been shown to produce O_2^-. These include xanthine oxidase, aldehyde oxidase and dihydroorotic dehydrogenase. The superoxide radical either attacks DNA directly or causes the secondary generation of other radicals which attack DNA. The key defense against superoxide radical is superoxide dismutase. This enzyme catalyses the reduction of superoxide to H_2O_2 and oxygen. Hydrogen peroxide itself is toxic to cells. It may be generated directly by divalent reduction of oxygen or by dismutation of superoxide radical. It is also the primary product of the reduction of oxygen by numerous oxidases. Hydrogen peroxide is known to be cytotoxic. *Streptococcus sanguis* excretes H_2O_2 in sufficient amounts to prevent the growth of other organisms [36]. *Pneumococci* respiring in the presence of glucose and lactate accumulate H_2O_2 in the medium which sometimes results in autotoxicity [106]. Unlike O_2^-, H_2O_2 can freely diffuse through biological membranes causing DNA damage, membrane disruption and release of calcium ions leading to activation of calcium dependent proteases and nucleases. The enzymes involved in scavenging H_2O_2 are catalase and peroxidase. They convert H_2O_2 to water and oxygen.

Some of the damage caused by H_2O_2 is due to a reaction of H_2O_2 and O_2^- with iron salts resulting in a highly reactive intermediate, the hydroxyl radical [9]. The mixture of iron salts and H_2O_2 is called Fenton's reagent. The reactions taking place due to this, result in the decomposition of H_2O_2 by way of radical intermediates. The hydroxyl radical is the most potent of all the reactive intermediates. Experiments conducted with cell free extracts of *E. coli* have demonstrated that proteins exposed to hydroxyl radical are more susceptible to proteolytic degradation [22]. The primary defense against this radical are the superoxide dismutases and catalases which

scavenge the superoxide radical and hydrogen peroxide.

Hydrogen Peroxide Production in Mycoplasma

Since the discovery that the hemolysin of *M. pneumoniae* and other mycoplasmas is hydrogen peroxide [110], the virulence of mycoplasma species has been attributed to reactive oxygen species, especially in those cells that maintain close host cell association. Local tissue damage and subsequent disorganization of the epithelial membrane has been observed due to close association of the pathogenic mycoplasmas within the host. Somerson et al. first reported on the identification of *M. pneumoniae* and *A. laidlawii* hemolysin as hydrogen peroxide [110]. They also suggested that the toxicity of *M. pneumoniae* for erythrocytes and the respiratory tissues was due to hydrogen peroxide. Later, Cole et.al. used the benzidine blood reaction to test hemolysis and peroxide production in several species of mycoplasma [19]. Based on their test they concluded that the major hemolysin of mycoplasma species was peroxide.

Production of H_2O_2 during the metabolism of glucose and glycerol has been demonstrated in vitro by a number of mycoplasma species and has led to speculation concerning the role of oxidative damage in pathogenesis [71]. The production of H_2O_2 appears to be a characteristic of a flavin terminated electron transport chain in mycoplasmas. In *M. pneumoniae*, H_2O_2 is produced during the oxidation of glucose by one of the two NADH oxidases present in the cell free extracts [64]. In *M. mycoides*, large amounts of H_2O_2 was produced during glycerol oxidation in the presence of glycerophosphate oxidase [91]. The first test of the role of peroxide and host catalase in virulence was reported by Brennan and Feinstein [13]. *M. pulmonis* grew better

in culture medium containing catalase than without it, which suggested that large amounts of H_2O_2 produced was probably toxic to the cells. Even though *M. pulmonis* induced severe pneumonia in acatalic mice, fewer pneumonias were observed which suggested that H_2O_2 secretion by *M. pulmonis* in catalase deficient mice was suicidal to mycoplasma. Cherry and Taylor-Robinson showed that the cytopathic effects of *M. mycoides* subspecies *capri* on chicken tracheal organ cultures was due to hydrogen peroxide production and the cytopathic effect could be delayed by the addition of catalase [16]. *M. pneumoniae* inhibits host cell catalase in several different types of human cells. Also, the production of H_2O_2 has been associated with increased lipid peroxidation in infected fibroblasts [3]. Amar et al. found that exogenous H_2O_2 was not required when lactoperoxidase was used to iodinate the membranes of these organisms. *M. hyorhinis* which was closely associated with murine L cells in culture supplied enough H_2O_2 to iodinate these cells, obviating the necessity for any exogenous H_2O_2 [60].

Even though many mycoplasma species produce H_2O_2, catalase has been detected in mycoplasmas only in a few cases. Jacobs and coworkers demonstrated the susceptibility of mycoplasma to the bacteriocidal peroxidase-H_2O_2 -halide system of human polymorphonuclear neutrophils [45]. Low et al. reported the lack of catalase in *M. pneumoniae* [63]. Other authors also have failed to identify catalase activity in several mycoplasma species [110, 62]. However, low level catalase activity was detected in *M. arthritidis*, *M. bovigenitalium* and *M. pulmonis* by Meier and Habermehl [70]. Even the presence or absence of superoxide dismutase has not been confirmed in mycoplasma. However, the pathogenic mycoplasmas seem to be resistant to the effects of oxidative molecules. These pathogens may have evolved additional mech-

anisms to protect themselves from their self generated oxidative species, like altered permeability to oxygen species or modification of enzymes to reduce sensitivity to H_2O_2 and other oxygen radicals [115].

Superoxide Dismutases

Superoxide dismutases (SOD) are enzymes that catalyze the dismutation of superoxide radicals to hydrogen peroxide and molecular oxygen. The enzyme was first discovered by McCord and Fridovich. It was purified from bovine erythrocytes as an enzymic function for erythrocuprein [69]. There are three distinct classes of SOD's found in nature based on their metal content. Most prokaryotes, including bacteria and blue green algae contain only manganese or iron containing SOD. Some species contain only a single type of metalloenzyme while other species contain two metalloenzyme forms. Hybrid enzyme containing both functional iron and manganese as cofactors have been found [17]. Although exceptions exist, most gram-positive bacteria usually contain only manganese containing SOD (Mn-SOD) while gram-negative bacteria contain only the iron (Fe-SOD) form or both forms. The eukaryotes contain all classes of SOD. The Mn-SOD and Fe-SOD are found in all species of eukaryotes that do or do not contain the Cu-Zn SOD. In instances where both Cu-Zn SOD and Mn/Fe SOD's are present, the Cu-Zn dismutases have been shown to be cytosolic and Mn forms to be associated with the mitochondria. Dismutases from closely related species do show immunological cross reactivity, but more distant relatives do not cross-react [111].

The discovery of SOD led to the proposal that superoxide radical was an important agent of toxicity of oxygen and SOD was an essential defense. In support

of this assertion, all facultative aerobes were found to have SOD, while aerotolerant aerobes had less SOD and the obligate anaerobes had no SOD. But, some anaerobes have been found to have SOD in small amounts. For example, *Clostridium acetobutilycum* has only $1 - 6\%$ as much SOD as do aerobically grown *E. coli.* In the anaerobe *Selenomonas ruminantium*, levels of oxygen which inhibited growth also caused increases in SOD [31]. It was demonstrated for certain bacteria that exogenously added SOD provided protection for the organism. *Nocardia* produces and secretes a superoxide dismutase, a case which explains the organism's unusual resistance to the oxidative burst of polymorphonuclear phagocytes [10].

The Cu-Zn dismutases are a remarkably conserved family with respect to their structural properties. The purified enzyme was shown to be a dimer with a native molecular weight of around 32,000 to 33,000 daltons containing nearly 2.0g atoms of copper and 2.0g atoms of zinc per mole. The two identical subunits are joined by non-covalent interactions and each subunit has an intrachain disulfide bond. The active site contains an atom of copper and an atom of zinc. The Cu-Zn SOD is the most stable of the metalloenzyme SOD's. The activity is not inactivated by sodium dodecyl sulfate or by 8M urea. Only 50% of the activity is lost at 67°C. The Mn/Fe dismutases are not as uniform in basic properties such as molecular weights, subunit structure and metal content. Some are dimers while others are tetramers. The metal range may be from 0.5 to 1g atom per mole of subunit polypeptide. Only one type of metal is present, manganese or iron. The subunit molecular weight may range from 19,000 to 22,000g per mole. The Mn/Fe SOD's are less stable than Cu-Zn SOD's, but they are not grouped as labile enzymes. Dismutases from thermophilic bacteria are more stable than Mn-SOD from mesophiles [111].

Superoxide dismutases were first reported in mollicutes by Petkau and Chelack [78]. Early studies showed some mycoplasma and ureaplasma species lacking SOD [55, 56]. Superoxide dismutases from five acholeplasma species were found to be immunologically heterogenous which suggested evolutionary diversity of SOD's. Recently, Meier and Habermehl found SOD in cell extracts from 21 strains of mollicutes including mycoplasma, acholeplasma and ureaplasma strains. The cell extracts were dialysed for 24hrs and then heated at 60°C. Since SOD's are resistant to heating, other enzymatic or chemical reactions that interfered with the SOD assay were inactivated or removed [70].

Reduced Nicotinamide Adenine Dinucleotide Oxidase

Reduced nicotinamide adenine dinucleotide oxidase (NADH oxidase) have been found both in eukaryotes and prokaryotes [72]. In bacteria, they have been identified in aerobes as well as in anaerobes. The enzyme mediates direct transfer of electrons from NADH to molecular oxygen without intermediate electron carriers. Utilization of oxygen as an electron acceptor results either in the production of H_2O_2 or H_2O. NADH oxidase activity may be assayed either as the increase in oxygen consumption induced by the addition of NADH as measured with an oxygen electrode, or from the oxidation of NADH as measured spectrophotometrically by the decrease in absorbance of NADH at 340nm.

The NADH oxidases of plants and animals are intrinsic to the plasma membrane [72]. The enzyme appears to be resistant to the action of cyanide, catalase and superoxide dismutase. The NADH oxidase of eukaryotes is not a peroxidase and may function as a terminal oxidase to link transfer of electrons from NADH to oxygen at

the plasma membrane. Mammalian NADH oxidase isolated from the liver plasma membrane contains two subunits of molecular weight 32 and 68kDa. The enzyme is an intrinsic membrane protein. However, the plant plasma membrane NADH oxidase exhibits a site for NADH oxidation at the external membrane surface. The NADH oxidase activity was found to decrease when lipid cofactors were extracted from the membrane. Other than NADH and quinones, no other cofactors were identified for this enzyme. The activity was inhibited by quinone analogs such as piericidin, the flavin antagonist atebrin and gangliosides such as G_{M3}. Highly purified preparations of plasma membranes of plants contain two types of NADH oxidases designated as NADH oxidase I and NADH oxidase II. The type I enzyme is cyanide, catalase and SOD insensitive and the final product appears to be H_2O. The type II enzyme is inhibited by cyanide, catalase and SOD. Using different orientations of plasma membrane vesicles, studies were conducted to determine the orientation of NADH oxidase within cells. Based on this, it was concluded that the oxidase is capable of accepting electrons from NADH from either surface of the plasma membrane despite the fact that physiological access to NADH must be via the cytoplasm [72].

According to Morre et al, the functional significance of NADH oxidase is unknown but some relationship to growth and growth control is indicated [72]. Studies with NADH oxidation in the presence or absence of ascorbate radical revealed an accelerated NADH oxidation in response to a synthetic growth hormone. With the plant membranes, the auxin growth factors stimulated the oxidase directly. Antibodies raised against auxin receptor from maize inhibited the NADH oxidase of plasma membranes from seedling soybean shoots to the same extent as growth. With liver plasma membranes, the NADH oxidase activity is stimulated by EGF, insulin and

vasopressin [72]. Recent evidence implicating the oxidase in the control of animal cell growth has come from studies of antiproliferative effects of retinoic acid and calcitrol [73]. Oxygen is a limiting factor for growth in mammalian cells but relationships between oxygen for growth and plasma membrane NADH oxidase have not been investigated.

Characterized NADH oxidases of bacteria are flavoproteins and are either soluble or membrane associated. A H_2O_2 producing NADH oxidase was purified from *Bacillus megaterium* [102]. The enzyme is dimeric with a molecular weight of 52,000 daltons for each subunit. FAD was bound non-covalently and functioned as a prosthetic group for the enzyme. Even though obligate anaerobes have no obvious use for oxygen consuming enzymes for metabolism or energy generation, NADH oxidases have been identified in anaerobes such as *Streptococcus* spp. [105]. The NADH oxidase from *Clostridium thermohydrosulfuricum* and *Thermoanaerobium brockii* has been found to be a thermostable enzyme that contains FAD and iron-sulfur [67]. The protein is hexameric and produces superoxide radicals and H_2O_2 from NADH. A H_2O forming NADH oxidase was purified from *Streptococcus faecalis* [105]. The enzyme does not contain any metal and is a single subunit enzyme of molecular weight 51kDa, with FAD as its prosthetic group. The enzyme is inhibited by sulfhydryl reagents. Under anaerobic conditions, a H_2O_2 forming NADH oxidase has been detected in the same organism. The H_2O forming enzyme was demonstrated as sharing structural connections between NADH oxidase and NADH peroxidase. Limited amino acid sequence of the two proteins were found to be 40% identical in the active site cysteinyl peptide sequence [2]. Schmitdt et al. suggest that in the presence of the enzyme the organism would be able to utilize a variety of substrates and more energy could be

produced via substrate linked phosphorylation [105]. The NADH oxidase of *Thermus thermophilus* HB8 is a monomeric protein of 25kDa with FAD present in 1:1 ratio to the polypetide chain. Superoxide radical was not found to be an intermediate in the NADH oxidation reaction. The enzyme is quite stable at elevated temperatures of 80°C. The N-terminal amino acid sequence of the *Thermus thermophilus* HB8 enzyme was used to synthesize a oligonucleotide probe and the the gene encoding the H_2O_2 forming enzyme was cloned and sequenced [77, 76].

Sometimes, NADH oxidases are a means for bacteria to modify their way of growth and energy metabolism or to take advantage of oxygen in the environment. *Leuconostoc* grows faster in the presence of glucose and oxygen. The faster growth rate was correlated with the induction of NADH oxidase in the organism [65]. Likewise, in *Desulfovibrio desulfuricans* and *Streptococcus mutans*, the growth was positively correlated with the activities of NADH oxidase and superoxide dismutase [1].

The localization of enzymes in mollicutes was systematically studied by Pollack et al [81]. They separated the membrane and supernatant fractions by osmotic lysis of cells followed by ultracentrifugation. Based on their studies, they concluded that the NADH oxidase activity in *Acholeplasma* was localized on membranes and in *Mycoplasma,* it was detected in the soluble fraction. However, studies with *M. gallisepticum* strain 293 and *M. hominis* strain 07 indicated that the activity was primarily associated with the membrane or the particulate fraction of the cell [117, 116]. Hence, it may be said that in mycoplasma species these activities could be near or loosely associated with the membrane or generally distributed in the cytoplasm [80]. NADH oxidase activity has not been generally detected in *Ureaplasma* spp. The presence of NADH oxidase in *Anaeroplasma* has not been confirmed [68].

Although NADH oxidase activities have been detected in many mollicute species, very little progress has been made in the isolation and purification of the enzyme. In *M. pneumoniae*, cell free extracts were examined for NADH oxidase activities [64]. The activity was found to be in the soluble fraction. Two types of NADH oxidase activities were identified. One of the activities was found to be FMN dependent and H_2O_2 producing. The other activity did not require any flavin cofactors. Jinks and Matz first purified an NADH dehydrogenase from *Acholeplasma laidlawii* membranes [47]. They reported that this dehydrogenase contains only trace amounts of iron. FMN was identified as its prosthetic group. The enzyme consists of one major and two minor bands by SDS-PAGE analysis. The enzyme could utilize various electron acceptors such as ferricyanide, menadione and dichlorophenol indophenol (DCPIP), but not oxygen or cytochrome C. The NADH dehydrogenase was found to be insensitive to respiratory chain inhibitors. It appears to be an integral membrane protein.

Reinards also purified a NADH oxidase from *Acholeplasma laidlawii* membranes using 3% TX-100 and subsequent chromatographic steps [88]. The enzyme was found to be a copper containing iron-sulfur protein with FMN at its prosthetic site. The enzyme was composed of three subunits of molecular weights 65kDa, 40kDa and 19kDa. It utilized oxygen as an electron acceptor producing H_2O_2 in nearly stoichiometric amounts. The enzyme also used other electron acceptors including ferricyanide, DCPIP, and cytochrome C. Metal chelating and mercurial agents were shown to inhibit the activity of the enzyme. Superoxide dismutase inhibited the enzyme activity indicating the presence of superoxide radicals as intermediates in the electron transfer from NADH to all electron acceptors. NADH oxidase was purified

from the cytoplasmic fraction of *M. capricolum* [56]. The molecular weight of the single subunit enzyme was determined to be 72.5kDa by gel filtration chromatography. The enzyme contained FAD, but no copper, zinc, molybdenum, manganese or iron. The enzyme could accept electrons from ferricyanide besides oxygen, but not DCPIP, Cytochrome C or methylene blue. The transfer of oxygen was not inhibited by superoxide dismutase and iron-sulfur clusters were found to be absent.

Fermentative mycoplasmas are described as having a flavin terminated respiratory chain [79]. This characterization is based, in part, on the absence of cytochromes and quinones and on the presence of flavins. Mollicutes apparently pass electrons directly from NADH to molecular oxygen by way of a flavoprotein without lipoquinones or cytochromes, mediated by an oxygen dependent NADH oxidase. This reaction requires oxygen as an electron acceptor for all except the strict anaerobic anaeroplasmas. It is not clearly understood whether this oxidation of NADH is coupled to ATP synthesis. NADH oxidation may serve simply to supply NAD^+ or in association with the membrane bound ATPase maintain pH and/or proton gradients. In *Acholeplasma laidlawii*, it has been suggested that a transmembrane electrochemical potential capable of driving transport can be produced by respiration and ATP hydrolysis. The NADH oxidase of *Acholeplasma laidlawii* is a multisubunit complex enzyme that is capable of carrying out the separation of protons and electrons, thus establishing a transmembrane potential. However, this theory has not yet been confirmed. Substrate phosphorylation is a major mechanism for the production of ATP in mollicutes [79]. Studies with whole *M. gallisepticum* cells showed the blocking of energy yielding metabolism due to the inhibition of NADH oxidase and lactate dehydrogenase by copper complexes. The authors suggested that the inhibition could be due to the

entrance of copper in the mycoplasma cell via a carrier mechanism followed by the inhibition of both NADH oxidase and LDH by free copper, ultimately resulting in the inhibition of glycolysis [33].

In summary, H_2O_2 has been implicated as one of the virulence factors in the pathogenicity of mycoplasmas. In mycoplasmas, H_2O_2 is produced during the metabolism of glucose or glycerol. Some of the enzymes involved in H_2O_2 production are superoxide dismutases and NADH oxidases. Superoxide dismutases are key defense against superoxide radicals whereas NADH oxidases have been correlated with growth and energy metabolism of the organism. The 94kDa surface protein is an interesting protein with many properties for *M. bovoculi*. The N-terminal sequence of the protein was found to have homologies with various superoxide dismutases and also to various regions of the mitochondrial genome especially to those containing the NADH dehydrogenases, cytochrome oxidases and ATPases. Hence, it was our interest to find out whether p94 is an enzyme involved in H_2O_2 production and to identify it as a component of the metabolic pathway of *M. bovoculi*.

CHAPTER 3. MATERIALS AND METHODS

Mycoplasma

Mycoplasma bovoculi strain FS8-7, initially isolated from the eyes of Iowa cattle with infectious bovine keratoconjunctivitis was cloned twice by filtering through a sterile 0.45 micron Millipore membrane (Millipore Corp., Bedford, MA) and plating onto modified Friis agar [57]. Single colonies were isolated, grown in modified Friis broth [57] with 20% fetal calf serum (GIBCO laboratories, Grand Island, NY) and used at passage 9 and 10.

Mycoplasma bovigenitalium, PG 11 (type strain) and *Acholeplasma laidlawii* cs392, also grown in modified Friis broth, were used as experimental controls.

Monoclonal Antibodies

Three different monoclonal antibodies were used. Two of these (M25.5 and M7.3) were developed against surface proteins of *M. bovoculi.* The third (M29.6) was against a surface protein of *Moraxella bovis.* Mouse-mouse hybridomas and monoclonal antibodies were developed in our laboratory as described previously [118]. They were used as serum-free hybridoma supernates and were concentrated (35X) using a vaccum concentrator.

Polyacrylamide Gel Electrophoresis and Immunoblotting

Discontinuous PAGE was performed with or without SDS as described previously by Laemmli [58]. The gels were made with 10% separating gel and 4% stacking gel. A Hoeffer SE 600 series vertical slab gel unit was used to electrophorese protein samples. The procedures used are as outlined in the Hoeffer catalogue (pp 89-93, 1983 Hoeffer Scientific Instruments, San Francisco, CA). *M. bovoculi* samples containing $50-100\mu$g protein were loaded into 0.75cm wide lanes. Native gels containing 1% TX-100 (Pierce, Rockford, IL) were run at 80V (constant voltage) with cooling (running water) and SDS-PAGE gels at 80mA (constant current). A set of known molecular weight standards (Pharmacia Chemicals, Piscataway, NJ) was used to estimate the molecular weights of the protein bands on SDS-PAGE gels. A molecular weight standard was made using seven different known molecular weight proteins at 1μg per lane (α- lactalbumin, Chicken egg albumin, BSA monomer, BSA dimer, Urease trimer, Urease haxamer and carbonic anhydrase) (Sigma Chemical Co., St. Louis, MO) for native gels. The procedure used for determining molecular weights in a non-denaturing system was a modification of Bryan [15] and Davis [21].

The western blot technique described previously [114] was used to detect transferred proteins. The proteins electrophoresed on native as well as on SDS-PAGE gels were transferred onto nitrocellulose using a transfer apparatus (Bio-Rad Laboratories, Richmond, CA). The gels were rinsed three times in transfer buffer containing 25mM sodium phosphate buffer, and 20% methanol, pH6.8. Transfer was performed at 19mA for 18-24hrs. The nitrocellulose membrane was washed three times with wash buffer (0.15M NaCl, 0.01M Tris buffer pH8.6, and .05% Tween 20) for a total of 30min with continuous shaking. A blocking reaction was performed with 1%

skim milk in wash buffer. Transfers were incubated with monoclonal antibody 25.5 (0.2mg/ml) at room temperature (RT) for 3-4hrs with continuous shaking and then washed three times. Radiolabeled (^{125}I) goat anti mouse IgG at $250 - 300\mu$Ci/ml (ICN Biomedicals, Costa Mesa, CA) was incubated for 1hr at RT and the membrane was washed as above. Autoradiography was done using Kodak XAR-5 film (Eastman Kodak Co., Rochester, NY) at -70^{o}C.

Assay for Hydrogen Peroxide Production

Hydrogen peroxide was assayed by its horseradish peroxidase- mediated oxidation of phenol red [25]. *M. bovoculi* cells grown for 24hrs at 37°C were centrifuged at $27,000\times$g. The pellet was washed once with phosphate buffered saline (PBS) and resuspended in PBS at 3mg/ml of protein. An isotonic phenol red solution (PRS) containing 140mM NaCl, 10mM potassium phosphate buffer (pH 7.2), 55mM glucose, 0.01% phenol red and 0.05% horseradish peroxidase (Sigma Chemical Co., St. Louis, MO) was subsequently added to the suspension of cells in a ratio of 1:1 by volume. When reacting the cells with monoclonal antibodies, equal protein concentration (3mg/ml) of each monoclonal antibody was added and the antigen-antibody complex was incubated at 37°C for 30min prior to the addition of PRS. The mixture containing cells and PRS was kept in a 37°C water bath. Appropriate aliquots were taken out at 10min intervals. These were centrifuged as before. The pH of the supernatant was adjusted to 12.5 with 1N NaOH and then assayed for the presence of H_2O_2 as increased absorbance at 610nm using a Spectronic 601 spectrophotometer (Milton Roy Inc., Rochestor, NY).

For H_2O_2 assay with trypsinized cells, the cells were grown and washed as above.

Trypsin (Difco Laboratories, Detroit, MI) at a final concentration of 100μg per ml in PBS was added and the mycoplasma cells were incubated at 37°C for 1hr. The cells were then centrifuged in a microcentrifuge for 5min, washed once with PBS and used immediately for the assay.

Preparation of Samples for Enzyme Analysis

TX-114 Phase Fractionation of Proteins

M. bovoculi components were separated into hydrophobic and hydrophilic fractions using the method of Bordier [12]. Log phase cultures of *M. bovoculi* were centrifuged for 10min at 600×g to remove debris, and the resulting supernatant was centrifuged for 45min at 16,000×g. The pellet was washed once with PBS and resuspended in the same buffer at a final concentration of 2mg/ml. Pre-washed, condensed TX-114 detergent (Sigma Chemical Co., St. Louis, MO) was added to give a final concentration of 1% wt/vol and the mycoplasmas were solubilized for 30min at 4°C. Insoluble components were removed by centrifuging at 4°C for 5min at 12,000×g. The TX-114 solubilized material was placed in a microtube and incubated for 5min at 37°C to induce rapid condensation of the detergent. The suspension was then centrifuged at 22°C for 5min at 16,000×g. The upper aqueous layer was separated from the detergent layer. Both fractions were brought to 1% wt/vol TX-114 by adding either the detergent or the buffer. Phase partitioning was repeated 8 more times to get the desired purity.

Osmotic Lysis

The procedure for osmotic lysis was as described in [84]. Briefly, *M. bovoculi* cells grown to early log phase were pelleted at 16,000×g for 45min. The pellet was washed once with Tris-NaCl buffer (0.25M Tris, 0.15M NaCl) and resuspended in 5ml of 2M glycerol. The cells were then incubated for 15min at 37°C. The cells were injected into 200ml of prewarmed (37°C) deionized water using a 25 gauge needle and incubated at 37°C for 30min. Unlysed cells were removed by centrifuging at 600×g for 10min. The supernatant was centrifuged at 100,000×g for 1hr. The pellet was separated from the supernatant fraction and washed once with buffer.

Sonication

M. bovoculi cells were grown in modified Friis broth for 48hrs at 37°C. The cells were harvested by centrifuging at 16,000×g for 45min. The pellet was washed once with PBS or Tris-NaCl (0.25M Tris, 0.15M NaCl) buffer and resuspended in the same buffer. The cells were then subjected to sonication using a Braunsonic 1510 sonicator for 3min at 100W on ice. The cell lysate was used as such or ultracentrifuged at 100,000×g for three hours. The pellet obtained after centrifugation was washed once with buffer. The supernatant was used without further processing. sectionEnzyme Assays

Superoxide Dismutase (SOD)

The superoxide dismutase assay was performed according to McCord and Fridovich. [69]. The reaction mixture contained 0.1mM cytochrome C (Sigma Chemical Co., St. Louis, MO), 0.5mM xanthine (Sigma Chemical Co., St. Louis, MO), 50mM

potassium phosphate buffer (pH7.8), in a total volume of 3ml, and sufficient amount of xanthine oxidase (Sigma Chemical Co., St. Louis, MO) to produce an increase of absorbance at 550nm of 0.025 per min. The assay mixture was usually standardized with 100μl of xanthine oxidase (6×10^{-7}M). An increase of 0.01 per minute was taken to be 1unit of SOD activity. For assaying samples containing SOD, the buffer was replaced with the sample. The samples were either whole cells of *M. bovoculi* resuspended in PBS or cell lysate obtained after sonication. Cell lysates of *M.bovigenitalium* and *A.laidlawii* obtained and assayed in the same manner as *M. bovoculi* were included as controls. Data is expressed as mean±standard deviation of three different batches of cell samples.

NADH Oxidase and NADPH Oxidase Assay

Both the assays were performed according to Pollack et al. [81], with slight modifications. The NADH and NADPH oxidase activities were measured by a decrease in absorbance at 340nm and 25°C. The reaction mixture contained 10mM Tris buffer, 0.169mM NADH or NADPH (Sigma Chemical Co., St. Louis, MO) and $100 - 200\mu$g of cell protein. The protein was obtained from intact *M. bovoculi* cells or sonicated cell lysate. The reaction was started by the addition of either NADH or NADPH and the changes in absorbance were recorded over a 4-5min time period. *A. laidlawii* cells prepared in the same manner as *M. bovoculi* cells were used as a positive control. The negative control included the non-enzymic oxidation of NADH. For inhibition studies with M25.5, 1ml (2mg/ml) of the whole cell suspension was incubated with 0.5ml (3mg/ml) of monoclonal antibody at 37°C for 30min prior to measuring the NADH oxidase activity. Data is represented as mean decrease in absorbance at 340nm of

four different batches of cell samples, plus or minus the standard deviation. Superoxide dismutase (420U) (Sigma Chemical Co., St. Louis, MO), sodium cyanide (1mM) (Sigma Chemical Co., St. Louis, MO), and catalase (340U) (Sigma Chemical Co., St. Louis, MO) were added to the reaction mixture to study the resistance of NADH oxidase activity to these substances. A reaction mixture containing *M. bovoculi* cells and NADH was assayed for the production of H_2O_2 as increased absorbance at 610nm. Data is represented as mean$\pm$ standard deviation of two different batches of sample.

Adenosine Triphosphatase Activity (ATPase)

The ATPase assay was done as described previously [23]. The reaction mixture contained 0.25ml of ATPase assay buffer (1.25μM of $MgCl_2$, 1.1μM of NaCl, 50μM of Tris-HCl (pH8.0)), 0.1ml of 12.5μM ATP (Sigma Chemical Co., St. Louis, MO) and 0.65ml of sample with or without M25.5 (3mg/ml). The samples were intact cells of *M. bovoculi* or cell lysate of the same organism obtained by sonication. The mixture was incubated at 37°C for 15-30min, then 1ml of 10% TCA was added. The precipitated proteins were removed by centrifugation at 16,000$\times$g for 5min. The supernatant was assayed for the amount of phosphorus released. Specific activity was expressed as micrograms of phosphorus released per min per milligram protein. Data is represented as mean of three different cell samples plus or minus the standard deviation. Control assay included all of the reaction mixture but no test sample.

Alternate Electron Acceptors for NADH Oxidase

NADH oxidase activity was tested for its ability to accept electrons from substances other than oxygen. The reagents for the assay used were the same as for

the NADH oxidase assay. In addition, the reaction mixture contained 0.2mM of each of the following: Ferricyanide (405nm) (Sigma Chemical Co., St. Louis, MO), menadione (340nm) (Sigma Chemical Co., St. Louis, MO), dichlorophenolindophenol (600nm) (Sigma Chemical Co., St. Louis, MO) and cytochrome C (550nm) (Sigma Chemical Co., St. Louis, MO).

Native Gels for Enzymes

Native Gel for Superoxide Dismutase (SOD)

Native polyacrylamide gels were run with 50μg to 60μg of protein per lane, obtained from Tx-114 phase separation, osmotic lysis or sonication. *E. coli* Mn-SOD (Sigma Chemical Co., St. Louis, MO) at 2-3 units of enzyme activity was used as a positive control. The gel was stained [11] in a solution containing nitroblue tetrazolium (NBT) and riboflavin (Sigma Chemical Co., St. Louis, MO) in the dark and later illuminated with a flourescent light to visualize the bands containing SOD activity.

Native Gel for NADH Oxidase

M. bovoculi cells grown in modified Friis broth were harvested by centrifugation (16,000×g for 45min), washed once with Tris-NaCl buffer and resuspended in the same buffer (2mg/ml of protein). The cells were solubilized with a variety of non-ionic detergents. The nonionic detergents used were 0.5% of Triton X-100, CHAPS and NP-40 (Pierce, Rockford, IL). Triton X-100 at concentrations of 1% and 3% was later used to solubilize the mycoplasma. Subsequent to solubilization with detergents (1hr at room temperature), the cells were centrifuged at 100,000×g for 1hr to re-

move insoluble components. Samples containing 100μg protein per lane were then electrophoresed on native gels. The gel was stained in a solution of NADH-NBT [64] for 90min in the dark. A Western blot analysis of similar gels was performed using M25.5.

Native Gel for NADH Oxidase with Affinity Purified p94

The protein p94, of *M. bovoculi* was affinity purified as described previously [7]. Affigel10 (Bio-Rad Laboratories, Richmond, CA) beads were reacted overnight with serum-free M25.5 at 4°C. *M. bovoculi* protein was prepared by solubilizing cells with 0.5% TX-100 for 1hr at room temperature. The unlysed cells were removed by ultracentrifugation (100,000×g for 1hr). The supernatant was then reacted with the M25.5-bound beads for 3hrs at room temperature. The beads were recovered by centrifugation and were loaded onto a column. The column was washed overnight with Western blot wash buffer. The protein was eluted with 5M $MgCl_2$. Dialysis was performed against the wash buffer to remove 5M $MgCl_2$. The affinity purified sample obtained was then concentrated 20X and loaded onto a 10% non-denaturing gel along with whole *M. bovoculi* cell lysate and stained for NADH oxidase activity as before.

An immunoblot analysis was also performed to confirm the identity of the p94 protein. The protein sample was electrophoresed on 7.5% SDS-PAGE and transferred onto nitrocellulose as described before. The transferred sample was reacted with M25.5 or polyclonal antibody against whole *M. bovoculi* proteins.

Native Gel for NADH Oxidase in Membrane and Supernatant Fractions

M. bovoculi cells were grown and harvested as before. The cells were washed once with Tris-NaCl buffer and resuspended in the same. The cells were sonicated for 5min on ice at 100W. Unlysed cells were removed by centrifuging at 500×g for 10min. The supernatant was then centrifuged at 100,000×g for 3hrs. The pellet was washed once with buffer and then solubilized with 1% TX-100 for 1hr at room temperature. The supernatant was subjected to ultracentrifugation as before, to remove any residual pellet fraction. The pellet and supernatant fractions were electrophoresed on a 10% non-denaturing gel and stained for NADH oxidase activity as before. Whole *M. bovoculi* cell lysate obtained by 1% TX-100 solubilization was used as a positive control.

Gel Filtration Chromatography

To determine the native molecular weight of proteins with NADH oxidase activity, gel filtration chromatography was performed using Sepharose CL-6B beads (Pharmacia Chemicals, Piscataway, NJ). The known molecular weight markers used to calibrate the column included cytochrome C, albumin, β-amylase, apoferritin and thyroglobulin. Whole *M. bovoculi* cells lysed with 1%TX-100 as well as affinity purified p94 were applied to the column separately. Approximately 1ml of sample was applied to the column containing 10ml of gel bed for affinity purified sample and 20ml of gel bed for the whole cell lysate. The column was washed with either Western blot wash buffer or buffer with TX-100 instead of TW-20, depending on the sample used and eluted fractions of approximately 0.33ml were collected using an automatic fraction collector (LKB, Bromma, Sweden). The NADH oxidase activity in each fraction

was determined as decrease in absorbance at 340nm.

Determination of the pI of p94

M. bovoculi cells were grown in modified Friis Broth (1L) and harvested by centrifugation (16,000×g for 45min). The cells were washed once with PBS and resuspended in 5ml of distilled water. The cell suspension was solubilized with 0.5% Triton X-100 for 1hr at 4C. Insoluble components from the cell suspension were removed by centrifugation at 100,000×g for 1hr. Five millilitres of the ampholyte solution (pH3.5-9.5) (Sigma Chemical Co., St. Louis, MO) and 40ml of distilled water were added to the supernatant to bring the total volume to 50ml. Isoelectric focusing of the sample was performed using a Rotaphor™ preparative isoelectrofocusing cell (Bio-Rad Laboratories, Richmond, CA) with 0.1M H_3PO_4 and 0.1M NaOH as buffers at the cathode and the anode ends. The gel was run for 4-5hrs at 11-12W. After electrofocusing, 20 samples with different pH values were collected and dialyzed for 18hrs against water. The samples were then run on a 7.5% SDS-PAGE and immunoblotted using M25.5.

CHAPTER 4. RESULTS

Production of Hydrogen Peroxide

Intact *M. bovoculi* cells resuspended in PBS released H_2O_2 into the medium. The H_2O_2 production increased over time for at least an hour. As shown in Fig 4.1, monoclonal antibody 25.5, specific for p94 of *M. bovoculi* inhibited the production of H_2O_2. Trypsin treated cells of *M. bovoculi* showed decreased production of H_2O_2 when compared to untreated cells (Fig 4.2.)

Enzyme Assays

Superoxide Dismutase (SOD)

Superoxide dismutase activity was found in *Acholeplasma laidlawii* and *Mycoplasma bovigenitalium*. *Acholeplasma laidlawii* cell lysate had 18-20U of activity per mg protein and *Mycoplasma bovigenitalium* had 2-3U per mg protein (Table 4.1.). *M. bovoculi* cell lysate as well as intact cells did not show the presence of SOD activity.

NADH Oxidase and NADPH Oxidase

Intact cells of *A. laidlawii* and *M. bovoculi* oxidized NADH. The NADH oxidase activity of *M. bovoculi* was specifically inhibited by M25.5. (Table 4.2.). This blocking

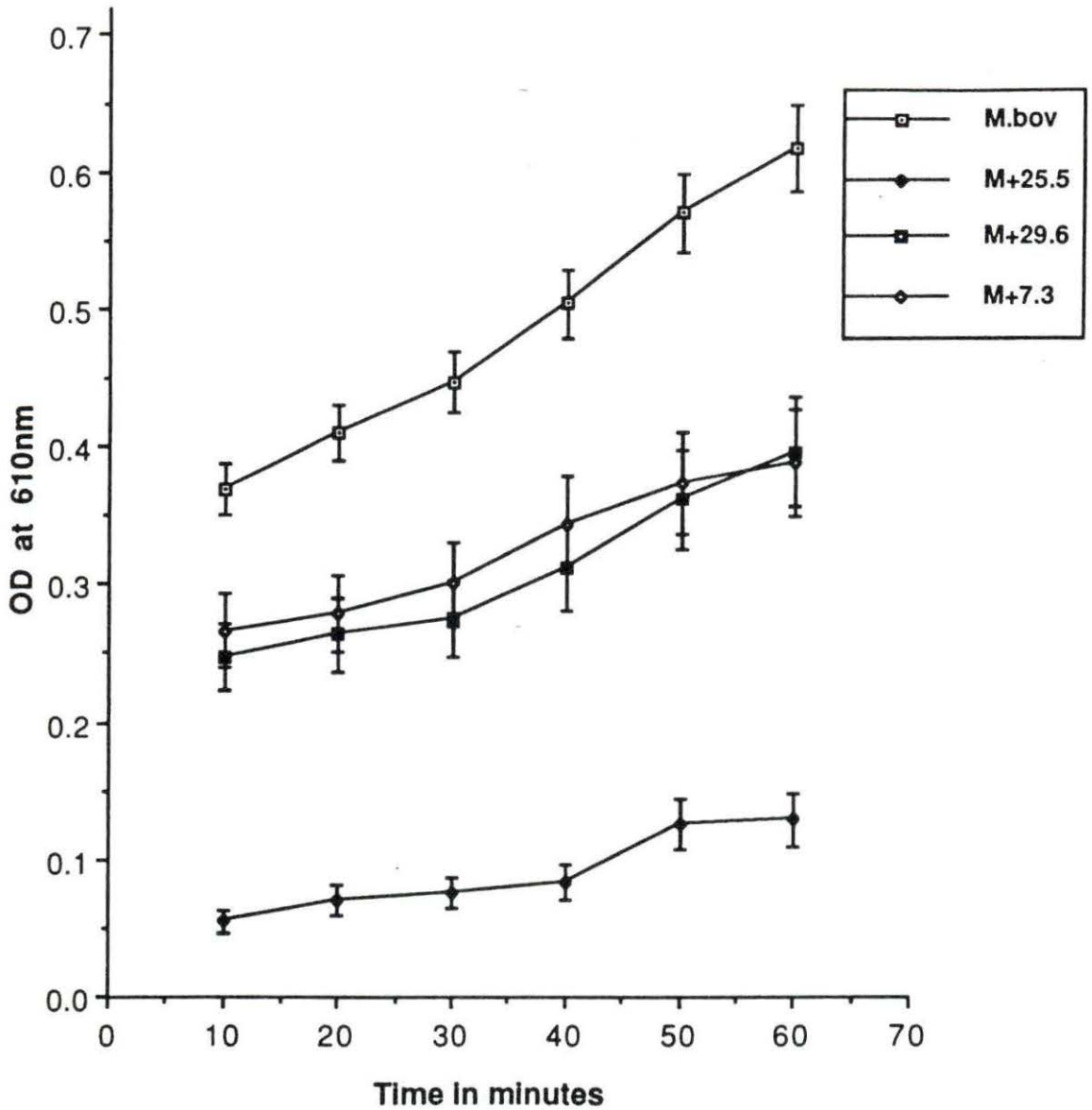


Figure 4.1: Blocking effect of monoclonal antibody 25.5, specific for p94 and non-specific monoclonal antibodies 29.6 and 7.3, on the production of H_2O_2 by *M. bovoculi* cells as measured at 610nm. M.bov=*M. bovoculi* cells, M+25.5= *M. bovoculi* cells+M25.5, M+29.6=*M. bovoculi* cells+M29.6, M+7.3=*M. bovoculi* cells+M7.3.

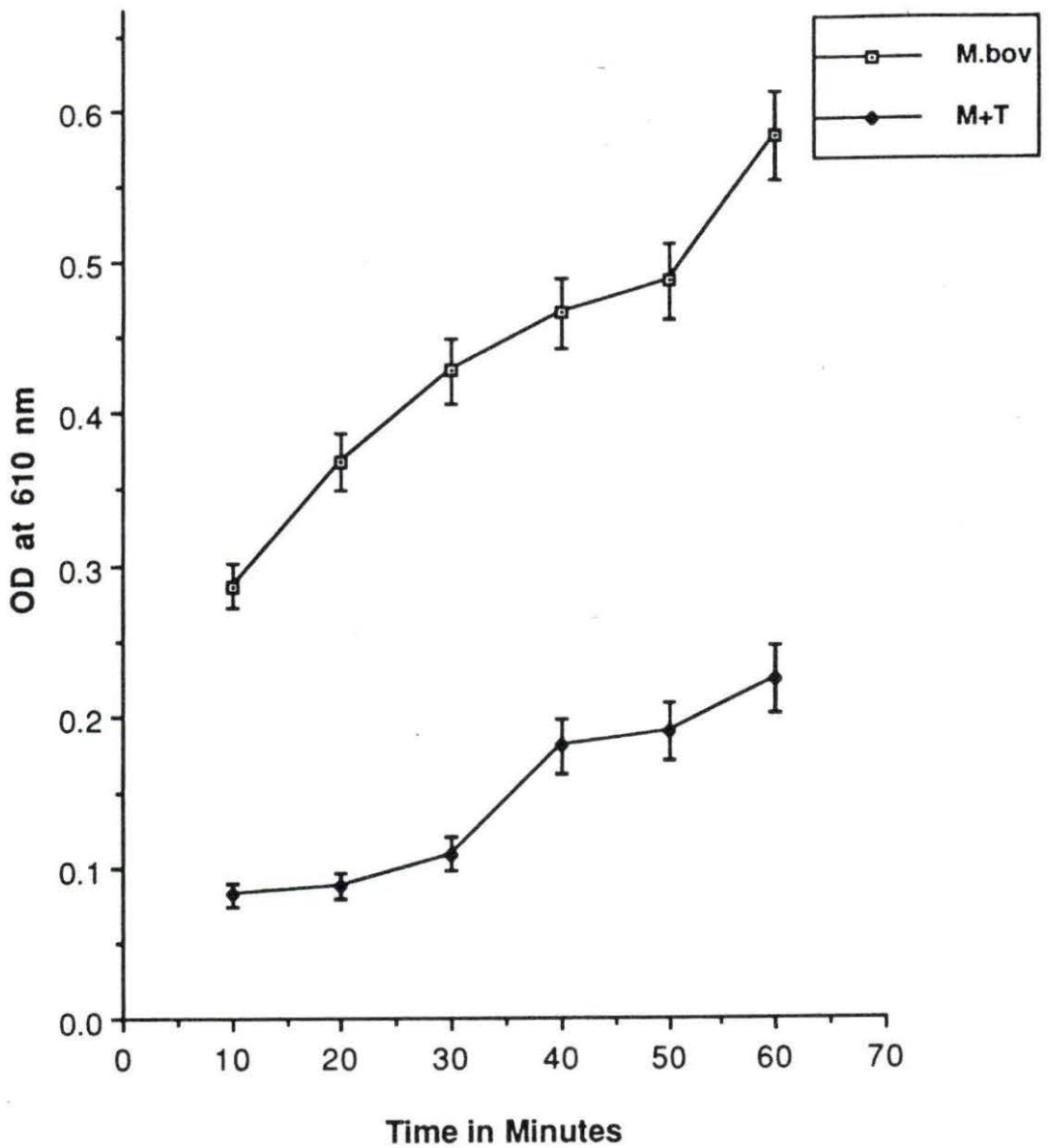


Figure 4.2: Decrease in H_2O_2 production as measured at 610nm by *M. bovoculi* cells subsequent to treatment with trypsin as compared to untreated cells. M.bov=*M. bovoculi* cells, M+T=*M. bovoculi* cells treated with trypsin.

Table 4.1: Specific SOD activities in three mycoplasmas expressed as units per mg protein.

Mycoplasma	Cell lysate	Intact cells
A.laidlawii	18±2	ND
M.bovigenitalium	2±0.5	ND
M. bovoculi	0	0

ND = not determined

effect was not seen with *A. laidlawii* NADH oxidase. NADPH oxidase activity was found only in the cell lysate of *M. bovoculi* and not in the cell lysate of *A. laidlawii.* Superoxide dismutase and catalase did not affect the NADH oxidase of *M. bovoculi.* Cyanide inhibited the NADH oxidase to 30% of the total activity (data not shown). The production of H_2O_2 increased by 50% when measured at 610nm with the addition of NADH to the assay mixture as compared to a mixture containing *M. bovoculi* cells alone (Table 4.3.).

Table 4.2: Specificity of monoclonal antibody 25.5 for NADH oxidase of *M. bovoculi.*

cell sample	*A. laidlawii*	*M. bovoculi*
whole cells	0.18±0.02	0.34±0.05
whole cells+M25.5	0.12±0.01	0.02±0.003

Adenosine Triphosphatase Activity (ATPase)

ATPase activity was found on intact cells as well as in cell lysate of both *M. bovoculi* and *A. laidlawii* (Table 4.4.). Monoclonal antibody 25.5 added to a mixture

Table 4.3: Hydrogen peroxide produced by *M. bovoculi* during the oxidation of NADH.

Cells+NADH	Cells	NADH
0.21±0.02	0.1±0.01	0.02±0.004

containing only intact cells did not inhibit ATPase activity.

Table 4.4: Adenosine triphosphatase activity of *M. bovoculi* and *A. laidlawii.*

Mycoplasma	Whole cells	Whole cells + M25.5	Cell lysate
A.laidlawii	0.68±0.15	0.62±0.1	0.5±0.1
M. bovoculi	0.6±0.2	0.6±0.16	0.4±0.08

Alternate Electron Acceptors for NADH Oxidase

The NADH oxidase(s) of *M. bovoculi* can accept electrons from ferricyanide, menadione and DCPIP but not cytochrome C (Table 4.5.).

Table 4.5: Alternate electron acceptors for NADH oxidase of *M. bovoculi*

Substance	% NADH oxidase activity
Control	100%
Menadione	91%
Ferricyanide	57%
DCPIP	98%
Cyt C	1%

Native Gels for Enzymes

Native gel for Superoxide Dismutase

Samples obtained from TX-114 phase separation, sonication or osmotic lysis were run on native gels and stained for SOD activity. None of the samples tested showed any band for SOD activity. Only the positive control (*E. coli* Mn-SOD) was seen as a clear distinct band on the gel (data not shown).

Native Gel for NADH Oxidase

Native gels stained for NADH oxidase showed several activity bands. Activity was seen as an intensely staining slow migrating band and two distinct fast migrating bands. Although 0.5% of the non-ionic detergent was sufficient to solubilize *M. bovoculi* cells and show activity on the gel, the bands were more clearly visualized when 1-3% detergent was used to solubilize cells (Fig 4.3, Fig 4.4). The tentative molecular weights for bands with activity on native gel are as follows: 190kDa, 150kDa, 33kDa, and 18kDa.

To determine whether the NADH oxidase activity was due to p94, M25.5 was reacted with proteins transferred onto nitrocellulose from similar gels as above. The reactivity of M25.5 on the Western blot corresponding to the gel with 0.5% detergent is seen as a streak rather than as sharp bands with more intense reaction observed at a position on the gel that has no activity (Fig 4.5). However, sharper bands are observed on the immunoblot of the gel corresponding to Fig 4.4. The monoclonal antibody detected the slow migrating band and one of the fast migrating bands on the activity gel (Fig 4.6).

Figure 4.3: Native Gel for NADH oxidase activity with detergent solubilized *M. bovoculi* samples. Lane A: cells solubilized with 0.5% CHAPS, lane B: cells solubilized with 0.5% NP-40, lane C: cells solubilized with 0.5% TX-100.


A
B
C

Figure 4.4: Native gel for NADH oxidase activity containing cell samples solubilized with 1% TX-100 (lanes A and B) and 3% TX-100 (lanes C and D).


A
B
C
D

Figure 4.5: Immunoblot of cells solubilized with different non-ionic detergents, electrophoresced on a 10% native gel, reacted with M25.5 and I^{125}-labeled goat anti-mouse IgG. Lane A: cells solubilized with 0.5% CHAPS, lane B: cells solubilized with 0.5% NP-40, lane C: cells solubilized with 0.5% TX-100.

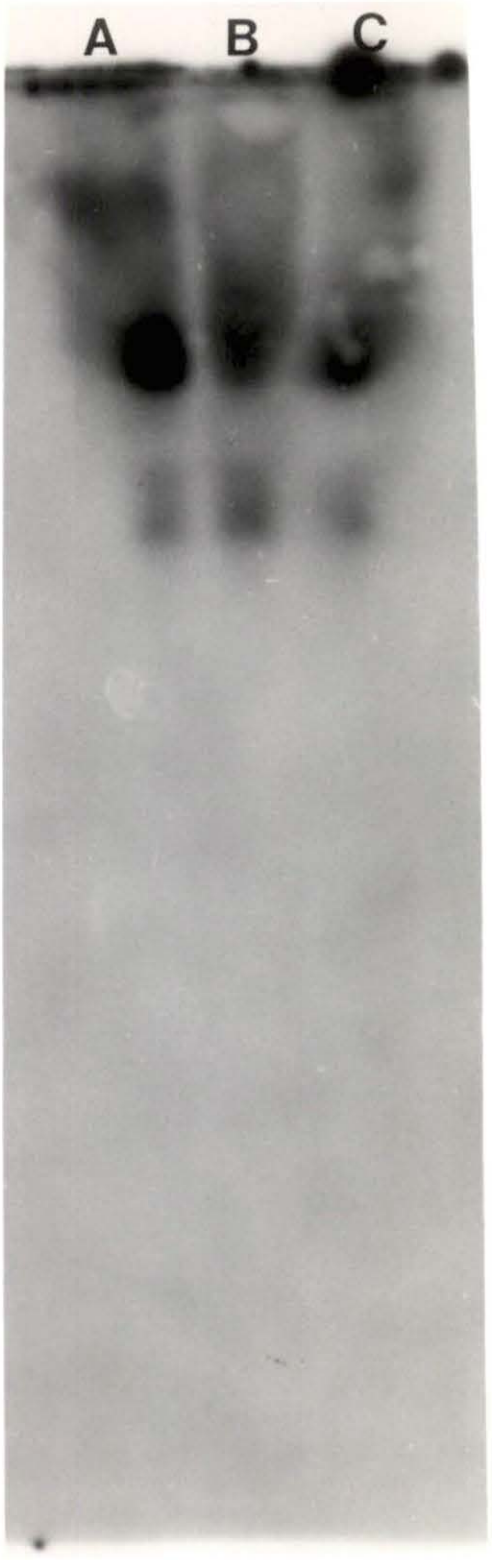
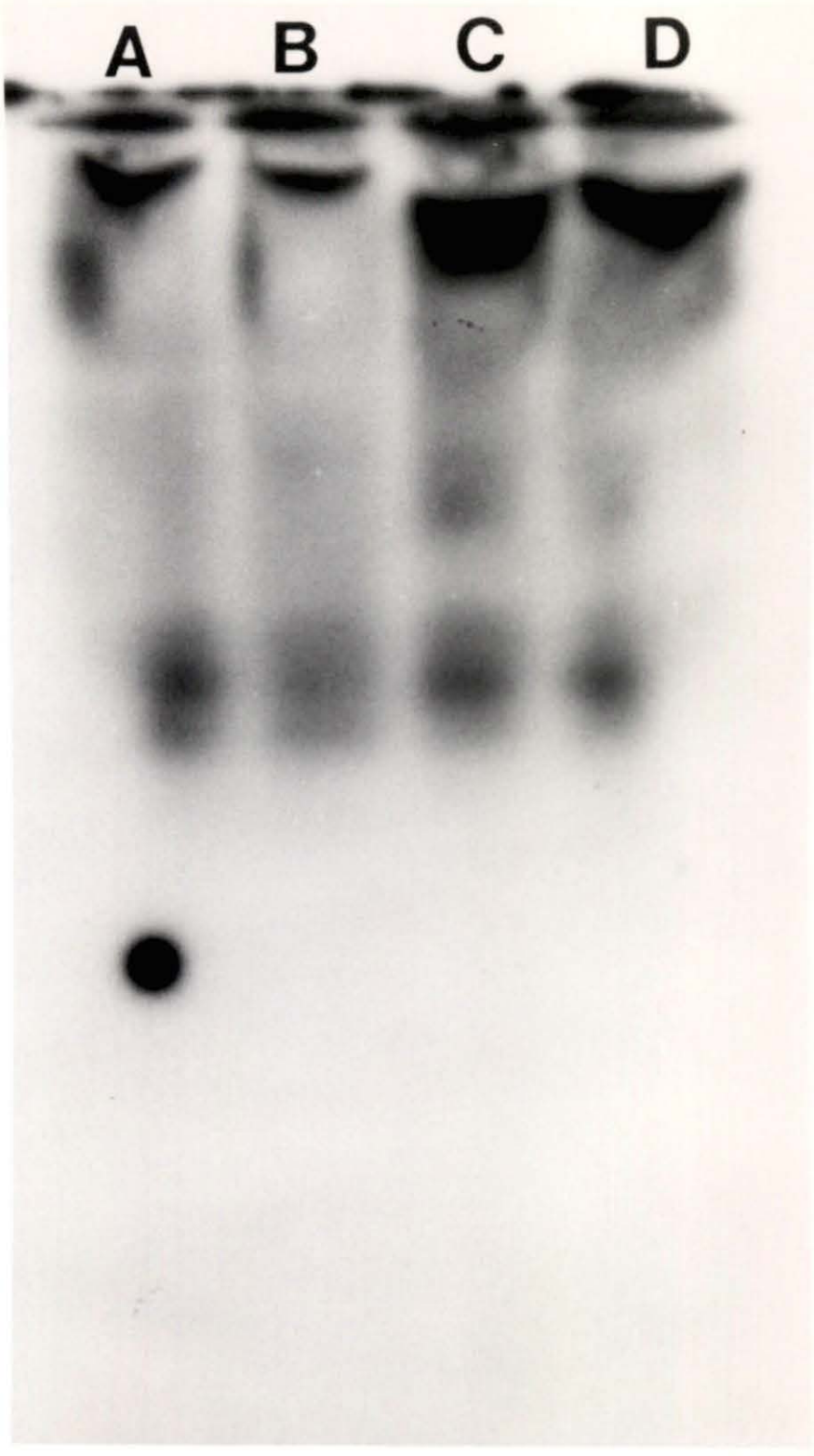

A
B
C

Figure 4.6: Immuunoblot analysis of TX-100 solubilized cells electrophoresced on a 10% native gel, reacted with M25.5 and I^{125}-labeled goat anti-mouse IgG. Lanes A and B: cells solubilized with 1% TX-100, lanes C and D: cells solubilized with 3% TX-100.


A
B
C
D

Native Gel for NADH Oxidase with Affinity Purified p94

NADH oxidase activity was detected in the affinity purified p94 sample as seen in Fig 4.7. The activity was seen as a diffuse and slow moving band. The control activity with whole *M. bovoculi* cell lysate shows slow and fast migrating activity bands. The immunoblot analysis of affinity purified p94 sample on SDS-PAGE indicates the presence of a single band reactive to M25.5. The polyclonal antibody detects an additional band of 39kDa besides p94 (Fig 4.8).

Native Gel for NADH Oxidase in Membrane and Supernatant Fractions

The whole cell lysate of *M. bovoculi* showed similar bands as before. The membrane fraction contained only the slow migrating band, whereas the supernatant fraction contained both the slow and fast migrating bands (Fig 4.9).

Gel Filtration Chromatography

Gel filtration chromatography resolved several activity peaks. The activity peaks and the corresponding molecular weights of the fractions with activity are as shown in Fig 4.10, Fig 4.11 and Table 4.6. NADH oxidase activity was found to reside in 630kDa and 187kDa fractions, as separated by gel filtration. This value compares with 190kDa calculated for activity in native gels. There was only one difference observed in A_{280} peaks and NADH oxidase activity peaks with affinity purified p94. The A_{280} gave three peaks at corresponding molecular weights of 630kDa, 380kDa and 187kDa (data not shown).

Figure 4.7: Native gel for NADH oxidase activity in affinity purified sample. Lane A: affinity purified p94, lane B: whole cell lysate.

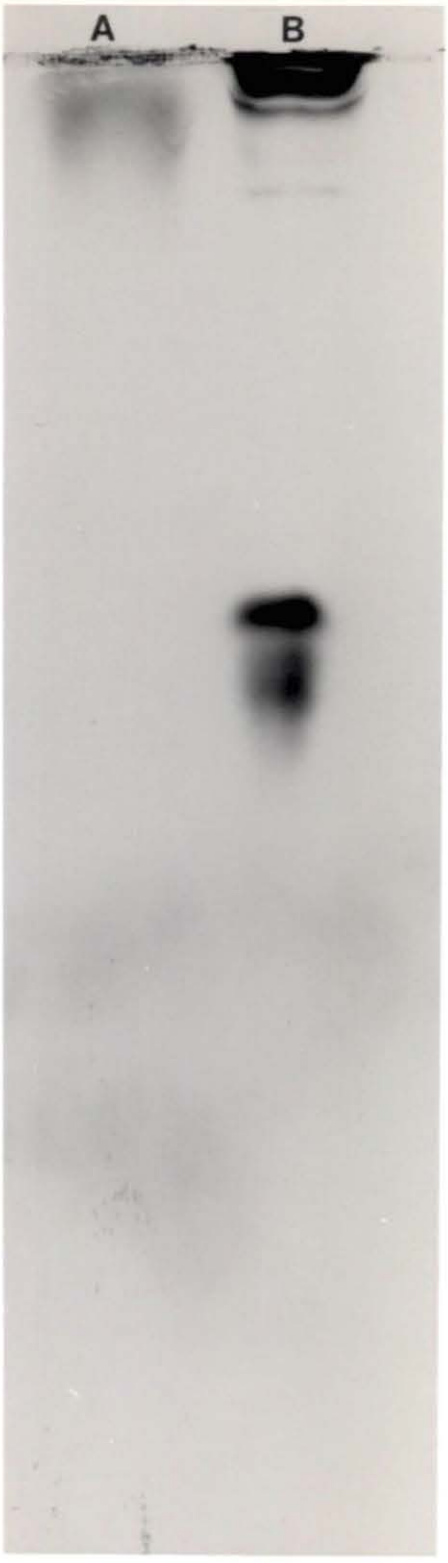

A
B

Figure 4.8: Immunblot analysis of affinity purified sample electrophoresced on a 7.5% SDS-PAGE. Lane A: whole cell lysate reacted with M25.5, lane B: Affinity purified p94 reacted to M25.5, lane C: whole cell lysate reacted with polyclonal antibody and lane D: affinity purified p94 reacted with polyclonal antibody.

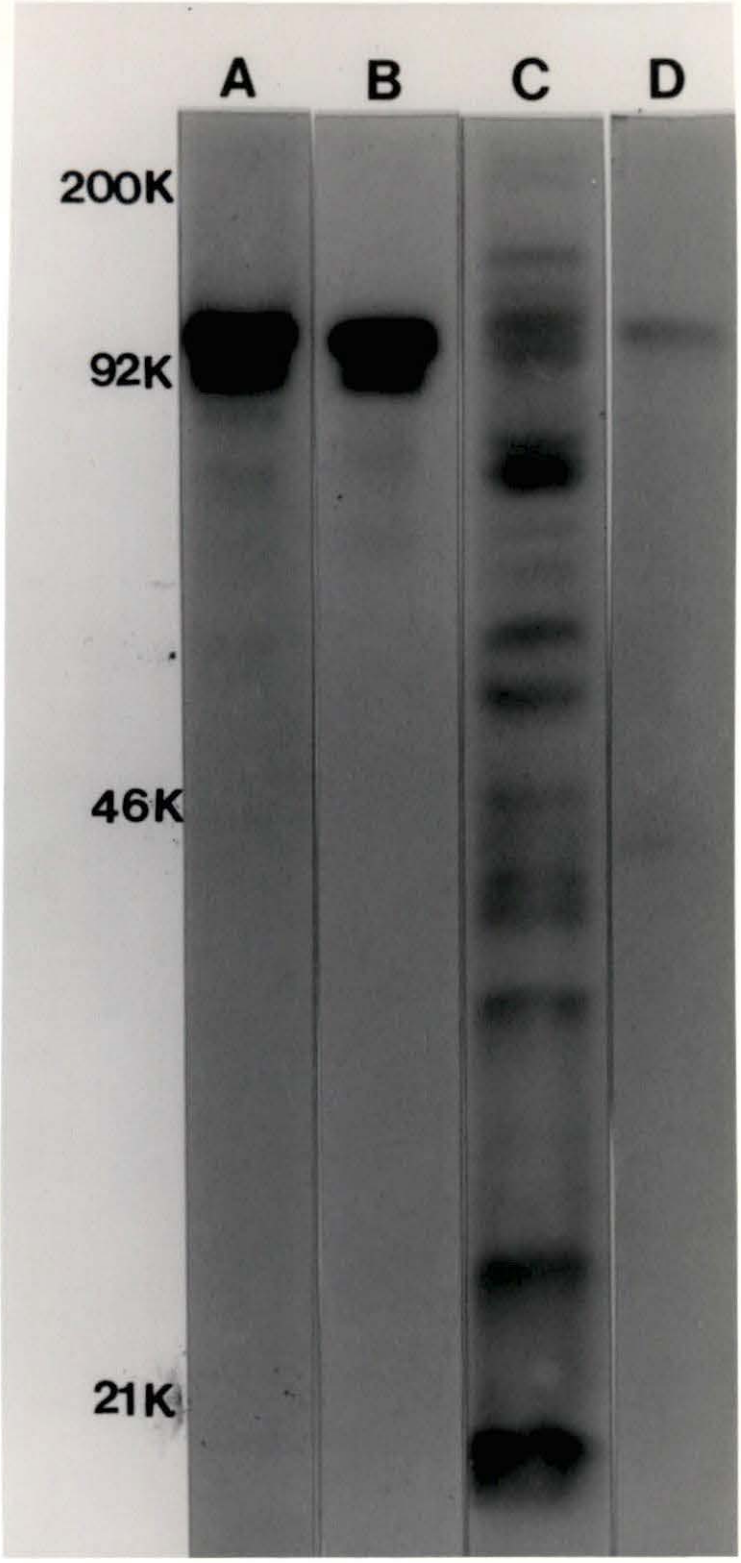
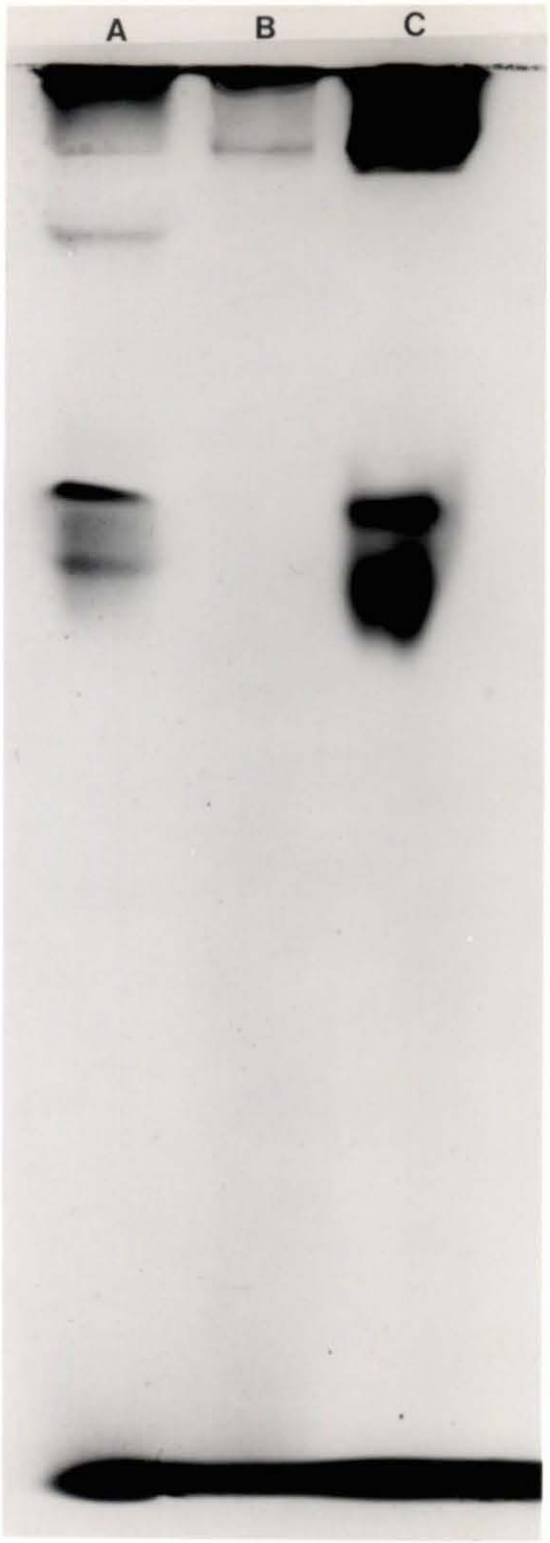

A
B
C
D
200K
92K
46K
21K

Figure 4.9: Native gel for NADH oxidase activity in membrane and supernatant fractions of . Lane A: whole cell lysate, lane B: membrane fraction and lane C: supernatant fraction.


A
B
C

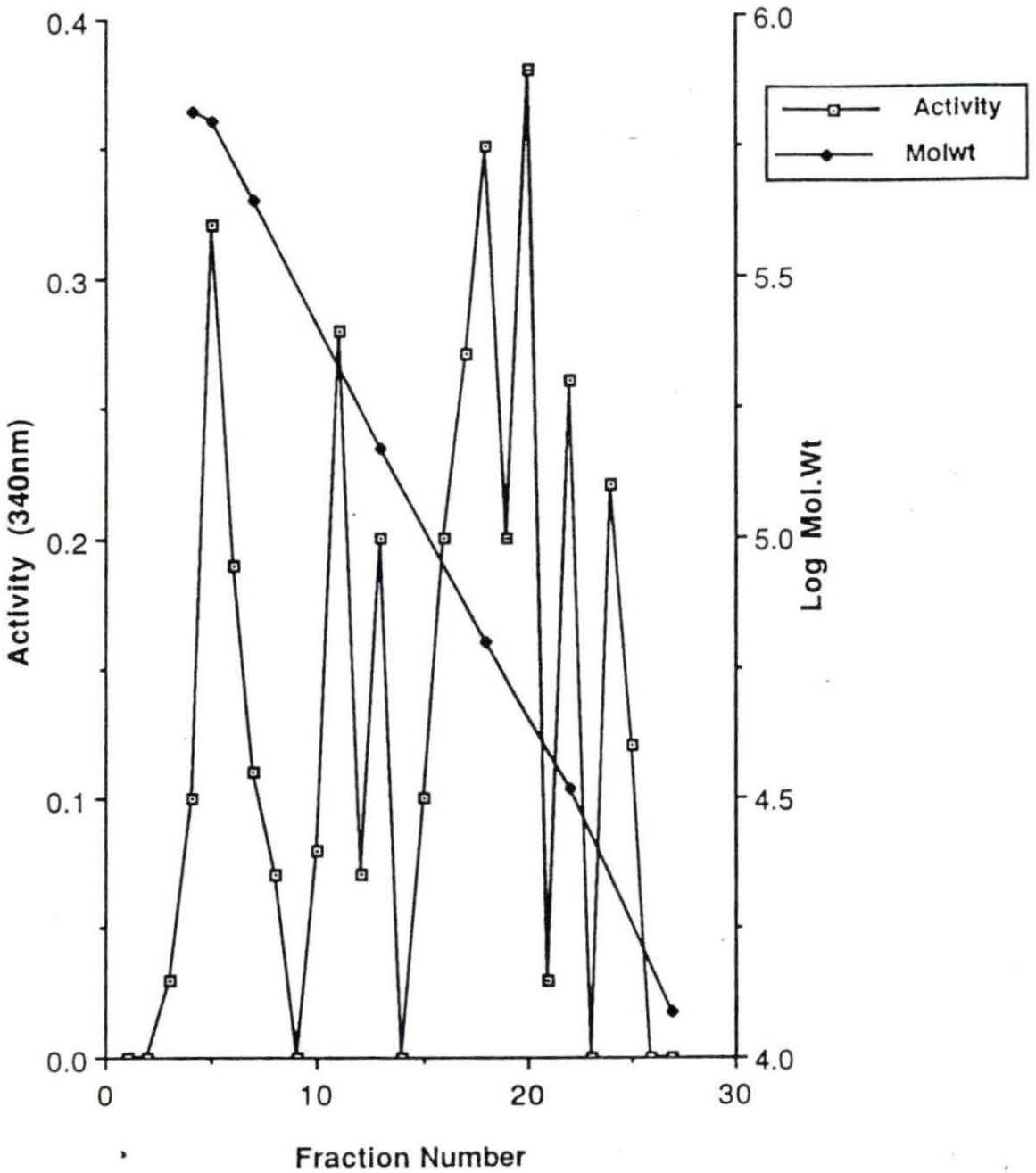


Figure 4.10: Separation of NADH oxidase activity peaks in whole *M. bovoculi* cells solubilized with 0.5% TX-100, by chromatography on Sepharose CL-6B. Activity=NADH oxidase activity, Molwt=standard molecular weight curve.

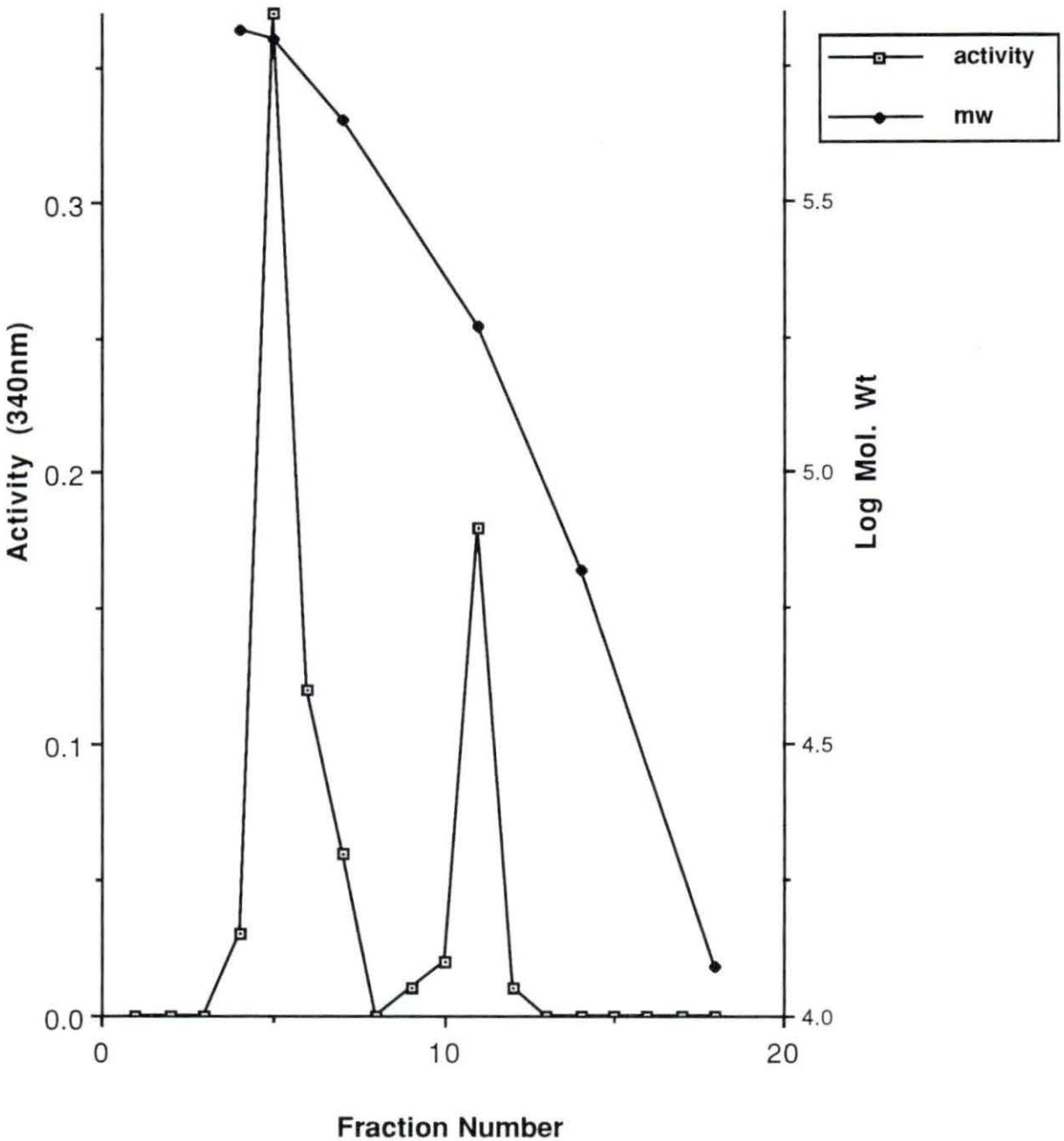


Figure 4.11: Separation of NADH oxidase activity peaks in affinity purified p94, by chromatography on Sepharose CL-6B. Activity=NADH oxidase activity, mw=standard molecular weight curve.

Table 4.6: Molecular weights (kDa) corresponding to NADH oxidase activities in *M. bovoculi*.

Molecular weights of NADH oxidase in kDa		
Gel filtration of whole cell lysate	Native gel of whole cell lysate	Gel filtration of affinity purified p94
630	–	630
187	190	187
148	150	–
63	–	–
56	–	–
33	33	–
19	18	–

Determination of the pI of p94

Although 20 samples with different pH values (Table 4.7.) were collected after isoelectric focusing of detergent solubilized *M. bovoculi* cells, only 16 (samples 3-18) were run on SDS-PAGE gels. The four samples with extreme pH values (pH 3.5 and pH 9.5) were not electrophoresed. The samples separated on gels were transferred to nitrocellulose, reacted with M25.5 and later with radiolabeled (^{125}I) goat anti-mouse IgG. Only samples 3-8 showed bands corresponding to p94 (Fig 4.12). The highest intensity bands were seen from samples 5 and 6. The other samples had bands of decreasing intensity. Therefore, the pI of p94 is between 4.7 and 5.1.

Figure 4.12: Immunoblot analysis of cell lysate subjected to isoelectric focussing, and reacted with M25.5. Lanes 1-8 correspond to samples 3-10 from Table 4.7.

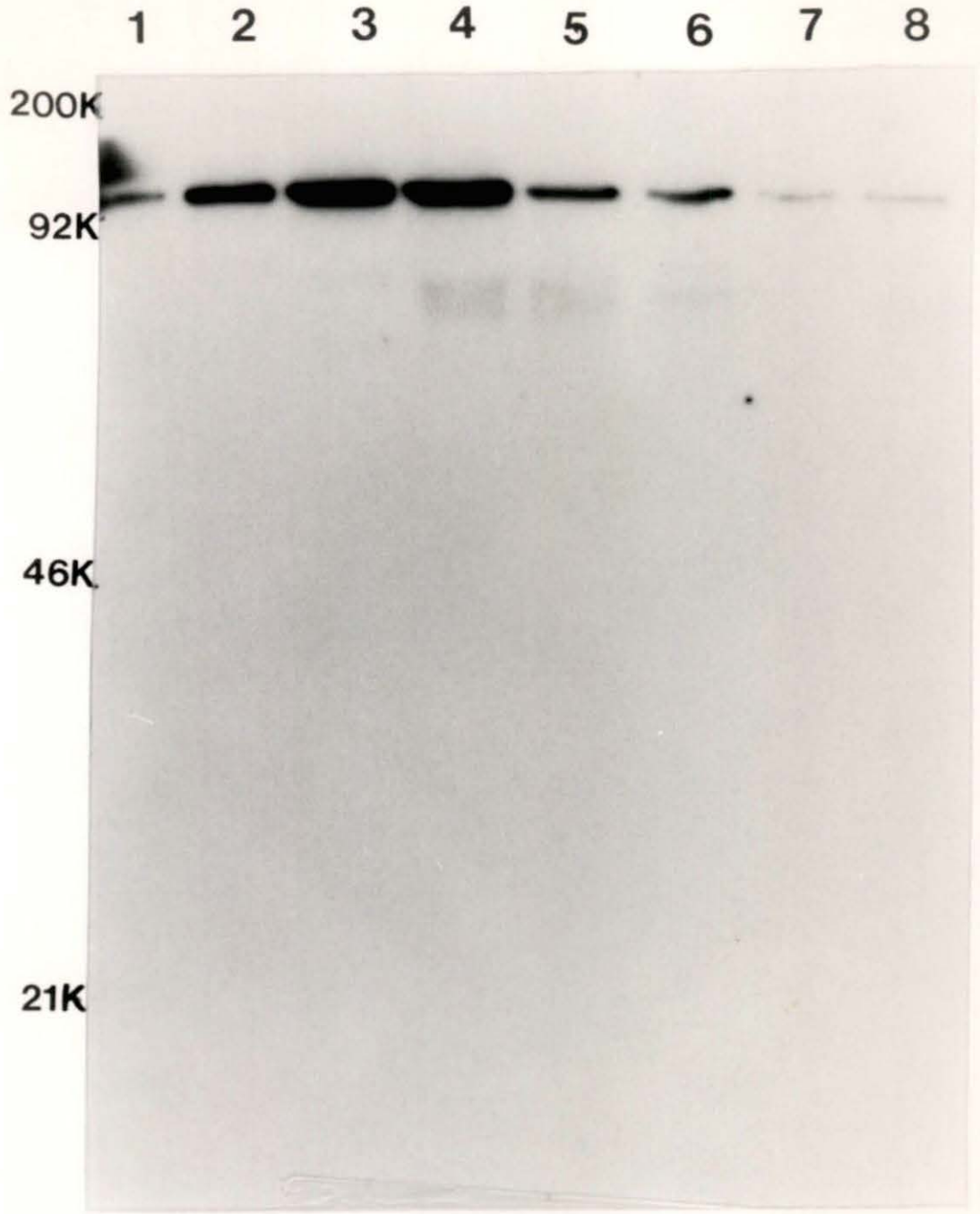

1
2
3
4
5
6
7
8
200K
92K
46K
21K

Table 4.7: The pH values of 20 samples collected after isoelectric focussing of TX-100 solubilized *M. bovoculi* cell proteins.

Sample#	1	2	3	4	5	6	7	8	9	10
pH value	3.7	3.9	4.3	4.45	4.7	5.1	5.6	6.05	6.25	6.5
Sample#	11	12	13	14	15	16	17	18	19	20
pH value	6.8	7.0	7.3	7.5	8.0	8.1	8.3	8.7	9.2	9.4

CHAPTER 5. DISCUSSION

One of the reactive oxygen species produced during incomplete reduction of O_2 to H_2O during respiration, by organisms undergoing aerobic growth, is H_2O_2 [112]. It is produced by a number of mycoplasma species during the metabolism of glucose or glycerol and is characteristic of flavin-terminated electron transport systems [71]. In fact, the virulence of many mycoplasmas has been attributed to reactive oxygen species [110]. Intact cells of *M. bovoculi* release H_2O_2 into the surrounding medium in increasing amounts during the course of time. The production of H_2O_2 was attributed to a 94kDa protein of *M. bovoculi* (p94). This protein was earlier shown to be a trypsin-sensitive, surface located protein [103]. A monoclonal antibody (M25.5), which is specific for p94, completely blocked the production of H_2O_2 by *M. bovoculi* cells. Although, trypsin treatment reduced the production of H_2O_2, trypsin treatment does not seem to eliminate the production of hydrogen peroxide. Trypsin is a mild and selective protease when compared to other proteases such as pronase and is capable of removing high molecular weight proteins from the surface of the membranes. It may be that during the period of time used for H_2O_2 assay, the protein was regenerated by intact cells of *M. bovoculi*[42]. The experiment with trypsin confirms previous observations on the proteinaceuos nature and membrane location of p94, and extends these properties to the principle involved in extracellular H_2O_2 production.

Superoxide dismutases (SOD) are the key defense against superoxide radicals [69]. The enzyme reduces superoxide radicals to H_2O_2 and O_2. Both procaryotes and eukaryotes possess this enzyme. However, the presence or absence of superoxide dismutase in mollicutes has not been confirmed. Assays for activity were conducted both spectrophotometrically and on native polyacrylamide gels using batches of samples prepared by several methods. No evidence for SOD activity was found in *M. bovoculi.*

Eucaryotes as well as procaryotes possess other enzymes that are involved in H_2O_2 production, besides SOD. These enzymes are NADH oxidase, NADPH oxidase, glycerophosphate oxidase and other oxidases [9, 71]. Earlier studies with mycoplasmas have shown NADH oxidase and glycerophosphate oxidase to be involved in H_2O_2 production [71]. To determine whether the production of H_2O_2 by *M. bovoculi* cells was a result of an enzymic reaction, spectrophotometric analysis was performed. The results indicated that NADH oxidase activity and ATPase activity was found on whole cells whereas NADPH oxidase was found only in cell lysates of *M. bovoculi.* Also, addition of NADH to whole cells of *M. bovoculi* increased the production of H_2O_2 which suggests that NADH is probably a rate limiting factor for H_2O_2 production. The cells by themselves produce H_2O_2 which could be due to the presence of internal NADH, spontaneous dismutation of superoxide radicals or other enzymes involved in H_2O_2 production.

Since NADH oxidase activity was found on whole cells of *M. bovoculi*, M25.5 was again used to determine whether the activity could be blocked. The NADH oxidase activity of *M. bovoculi* was blocked to approximately 80-90% by M25.5. In contrast, ATPase activity which is also a membrane activity was not affected by the

addition of M25.5 to the reaction mixture. The results suggested that p94 could be an NADH oxidase. This was quite surprising since p94 is a membrane protein and most mycoplasmas are shown to have a cytoplasmic NADH oxidase [81] with certain exceptions [117, 116]. The NADH oxidase activity was not inhibited by SOD which suggests that O_2^- is not an intermediate during NADH oxidation. The electrons are directly passed on to oxygen, resulting in the formation of H_2O_2 and NAD^+. The NAD^+ production is not affected by the action of catalase and is also insensitive to cyanide. Besides O_2 as its natural electron acceptor, the NADH oxidase of *M. bovoculi* can transfer electrons from NADH to ferricyanide, menadione and DCPIP, thus exhibiting dehydrogenase and diaphorase activities. However, it does not transfer electrons from cytochrome C. This is not surprising since most of the mollicutes lack cytochromes or quinones [79].

The primary role of detergents in protein purification is to bring about solubilization [37]. Membrane bound proteins require detergent solubilization before they are subjected to separation procedures. Efficient isolation of functional proteins from membranes requires the use of suitable detergents. Nonionic detergents disrupt the membranes and provide a lipid-like environment. They allow the isolation of proteins and protein aggregates, whereas zwitterionic detergents are used to obtain active, non-denatured proteins that are devoid of artifactual aggregation. A combination of non-ionic and zwitterionic detergents was used initially to determine the detergent that should be used for solubilization procedures. The three different detergents used (CHAPS, TX-100, NP-40) showed no difference in solubilization as far as NADH oxidase activity was concerned. Although 0.5% detergent was sufficient to solubilize the cells, 1% and 3% detergent was more efficient in resolving activities on gels.

The activity gels for NADH oxidase in *M. bovoculi* reveal various bands with activity. One is a slow moving high molecular weight band and two others are fast migrating proteins. Immunoblot analysis of activity gels with M25.5 indicate that the slow moving band reacts strongly with the monoclonal antibody and one of the fast migrating bands reacts slightly with M25.5. When 0.5% and 3% detergent was used to solubilize cells, reactivity with M25.5 on the immunoblot was also seen in a region of the gel with no activity. This could probably be the monomeric p94 that does not exhibit activity. The same reaction was not observed when 1% detergent was used to solubilize *M. bovoculi* cells.

Osmotic lysis is the best procedure for separating membrane and cytoplasmic fractions because of lack of contamination. Alternative procedures such as sonication are also used when osmotic lysis fails or is not efficient enough to lyse the cells. Since there were two separate activities observed on gels, we were interested in finding out whether the activities could be separated into membrane and cytosol fractions. According to results shown, the membrane fraction contains only the slow migrating high molecular weight activity whereas the supernatant fraction contains both slow migrating and fast migrating activity. The ultracentrifugation procedures used to separate the membrane and the supernatant fractions may not be ideal to separate the two fractions completely or it may be that some of the high molecular weight protein became loose during sonication and was separated from the membrane fraction. Hence, the supernatant fraction also had high molecular weight activity bands.

Affinity chromatography is one of the most powerful procedures that can be used for protein purification. It takes advantage of one or more biological properties of the molecule being purified. When p94 was affinity purified using M25.5, the affinity

purified sample had NADH oxidase activity as measured spectrophotometrically and also on a native gel stained for NADH oxidase activity. The activity on the gel was seen as a slow migrating high molecular weight band. Western blot analysis of an SDS-PAGE with affinity purified p94 revealed bands corresponding to 94kDa by M25.5. Polyclonal antibody against whole *M. bovoculi* proteins reacted with two bands from the same sample (94kDa and 39kDa). The 39kDa protein may be a hydrophobic protein that is not washed off the affinity beads and is copurified with p94. The isoelectric point of p94 was determined to be between 4.7 and 5.1, which is compatible with values found for other mycoplasma membrane proteins [6].

To determine the native molecular weight of NADH oxidases, gel filtration chromatography was performed using whole *M. bovoculi* cells as well as affinity purified p94. Many activity peaks were obtained with whole cells. Affinity purified p94 gave only two peaks with activity. The molecular weights of fractions from whole cells with activity were as follows: 630kDa, 187kDa, 148kDa, 63kDa, 56kDa, 33kDa and 19kDa. The affinity purified sample had activities with molecular weights of 630kDa and 187kDa. This data shows similar high moleculer weight proteins with activity from both the samples. The two proteins (630kDa and 187kDa) are probably heptamers and dimers of p94, since TX-100 which was used to solubilize whole cells and to affinity purify p94 is not a detergent capable of separating artifactual aggregates of proteins. The formation of heptamers could be a separation artifact or a true functional multimer. NADH oxidase(s) have been found in various organisms as monomers, dimers or even hexamers but, to our knowledge, no NADH oxidase has been previously found to be a heptamer. Although the determination of molecular weights on non-denaturing gels is not reliable because the proteins are resolved based

on their charge, molecular size and conformation, approximate molecular weights of bands with activity on gels were determined using known molecular weight protein standards. The molecular weights determined corresponded to most of the activity peaks from gel filtration chromatography (190kDa, 150kDa, 33kDa and 18kDa). The 630kDa band was probably beyond the range of what could be resolved on a native gel. The gel filtration chromatography and activity gel data do corroborate the fact that there are multiple molecular forms of NADH oxidase, but the affinity purified sample contains only activities with high molecular weights that are the heptamers and dimers of p94. Besides these high molecular weight enzymes, *M. bovoculi* contains low molecular weight NADH oxidase(s). *M. pneumoniae* was shown to possess two different NADH oxidases. Many bacteria contain NADH oxidases that are of low molecular weight, such as, the 52kDa dimeric enzyme from *Bacillus megaterium*, 25kDa protein from *Thermus thermophilus* HB8 or the 51kDa NADH oxidase from *Streptococcus feacalis*, etc. NADH oxidase purified from *Mycoplasma capricolum* is a cytoplasmic protein of 72.5kDa and is the only one purified, so far, from a mycoplasma. Some of the above enzymes are involved in the production of water and some others produce H_2O_2 during NADH oxidation. Although the nature and role of the membrane NADH oxidase of *M. bovoculi* is here described, studies have not been conducted to characterize the cytosolic NADH oxidases of this mycoplasma.

These studies identify a 94kDa surface protein of *M. bovoculi* as NADH oxidase. The protein exists as a multimer of 94kDa. The monomer does not appear to be a functional enzyme. Also, NADH oxidase activity in *M. bovoculi*is present both on the membrane, as dimers and possibly heptamers, and in the cytoplasm as low molecular weight moieties.

Earlier work has shown that p94 was involved in metabolism inhibition [7]. Many mycoplasmas possess metabolism inhibition proteins on their surface [20, 28, 120] but, the nature of these proteins and the mechanism by which this occurs has not been dealt with previously. During the process of degradation of glucose or other carbohydrates, the coenzymes NAD^+ and $NADP^+$ are reduced to NADH and NADPH. For the pathways to continue to function, synthesis of NAD^+ and $NADP^+$ or reoxidation of NADH and NADPH becomes essential. During this process, protons and electrons are transported from one membrane component to another. The electrons are further processed by the electron transport system and ATP is synthesized. However, significant electron carriers have not been found in mollicutes. These microorganisms pass electrons directly from NADH to oxygen via NADH oxidase. It is not known whether this oxidation of NADH is coupled to ATP synthesis [79]. NADH oxidase has been implicated as a major factor controlling growth in eukaryotes [72]. In some procaryotes, faster growth rate was correlated with NADH oxidase expression [1]. A surface located p94 or the NADH oxidase may be a major factor for the growth and functioning of *M. bovoculi* cells and the inhibition of this protein is probably the molecular basis for metabolic inhibition in *M. bovoculi.*

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APPENDIX A. CLONING AND SEQUENCING OF THE GENE FOR p94

This appendix summarizes an attempt to clone and sequence the gene for the 94kDa surface protein of *M. bovoculi*. I would like to acknowledge at the outset, Al Campbell, who worked as a technician in our lab previously and initiated this project. The starting material for the project was the N-terminal amino acid sequence obtained from affinity purified p94 (Table A.1.). From the amino acid sequence, a 60bp oligonucleotide sequence was derived using the *E. coli* codon frequency table from the GCG Wisconsin database (Table A.2.). Two non-degenerate 30bp oligonucleotide probes (5' and 3') were then synthesized to probe the genome of *M. bovoculi* (Table A.3.).

Table A.1: The N-terminal amino acid sequence of p94.

Number	1	2	3	4	5	6	7	8	9	10
Amino acid	A	E	I	I	K	Y	K	Y	K	N
Number	11	12	13	14	15	16	17	18	19	20
Amino acid	C	E	F	S	K	I	D	T	P	F

The chromosomal DNA of *M. bovoculi* was obtained by initially lysing the cells with 0.5% SDS. After incubating the lysate with Proteinase K to degrade the pro-

Table A.2: The 60bp nucleotide sequence derived from the N-terminal amino acid sequence of p94.

5'AAA TGG GGT ATC AAT TTT GCT AAA TTC ACA ATT TTT ATA TTT ATA TTT AAT AAT TTC TGC 3'

Table A.3: The 5' and 3' probe oligonucleotide sequence.

Probe	Sequence
5'	GCA GAA ATT ATT AAA TAT AAA TAT AAA AAT
3'	TGT GAA TTT AGC AAA ATT GAT ACC CCA TTT

teins, nucleic acids were extracted with phenol/chloroform and ethanol precipitated. Finally, DNAase free RNAase at 10mg/ml was added to the sample and the DNA was again extracted with phenol/chloroform and ethanol precipitated. The purity of DNA was checked on an agarose gel. The chromosomal DNA thus obtained was digested with various restriction enzymes and electrophoresed on a 0.7% Seakem ME agarose gel for 16-18hrs at 50V constant with 0.5% TBE as running buffer. Fig A. 1. shows the gel with the digested DNA that was stained with 1μg/ml of ethidium bromide for 30min and destained with distilled water for 30min. The DNA was transferred onto a Biodyne nylon membrane (0.45μm pore size) by vaccum transfer. The procedure for transfer was as given by the manufacturer. The probes were labeled with P^{32} by the 5' end labeling method. The labeled probes were then incubated with the membrane and the hybridization was allowed to proceed for 24hrs at 52°C. Later, the membrane was washed three times with 0.5%SDS and 1XSSC, twice at RT and

once at 52^0C (Fig A. 2.). A 11.5Kb EcoRI fragment and a 3.5kb SpeI fragment of the genome hybridized consistently with the 5' and 3' probes.

Smaller DNA fragments are easier to clone than larger DNA fragments. Also, since a protein of 94kDa in molecular size would be coded by a gene of approximately 2.56kb, cloning experiments were started with the 3.5kb SpeI fragment. Phagemid pKS^+ was used as vector and the host strain used to clone the recombinant vector *E. coli* DH5α. *E. coli* DH5α was purchased commercially from Stratagene. The cells were made competent by treating them with 0.1M $CaCl_2$ for 3-4hrs on ice. The cells were transformed with the ligated vector and insert for 10min at 42°C. Blunt end cloning with SmaI was performed since SpeI was found to digest the chromosomal DNA of *M. bovoculi* rather nonspecifically and cohesive ligation was not occuring properly. After several attempts, a single clone containing the 3.5kb fragment was obtained (pKS^+/3.5). To excise the insert, the vector was digested with BamHI and EcoRI which are on either side of the SmaI site in the multiple cloning site of the vector(Fig A.3.). To confirm the presence of the gene for p94 in the 3.5kb fragment, dot blot hybridization with 5' and 3' probes was done. The dot blot consisted of the 6.5kb DNA (3kb vector and 3.5kb insert). *M. bovoculi* chromosomal DNA was used as a positive control and lambda DNA was used as a negative control (Fig A.4.). The probes hybridized with the *M. bovoculi* DNA and the recombinant phagemid DNA but did not hybridize with lambda DNA.

A Maxi prep of pKS^+/3.5 was prepared using the Qiagen Plasmid DNA Kit. The protocol for the preparation of pure DNA was as given in the technical bulletin. The pure DNA at a concentration of 3μg/ml was submitted to the Nucleic Acid Facility for sequencing. The sequencing was performed using the reverse and universal primers

Figure A.1: Ethidium bromide stained gel of chromosomal DNA digested with various restriction enzymes. Lane A: EcoRI, lane B: XbaI, lane C: HpaI, lane D: PstI, lane E: RsaI, lane F: SpeI, lane G: TaqI and lane H: CvnI.

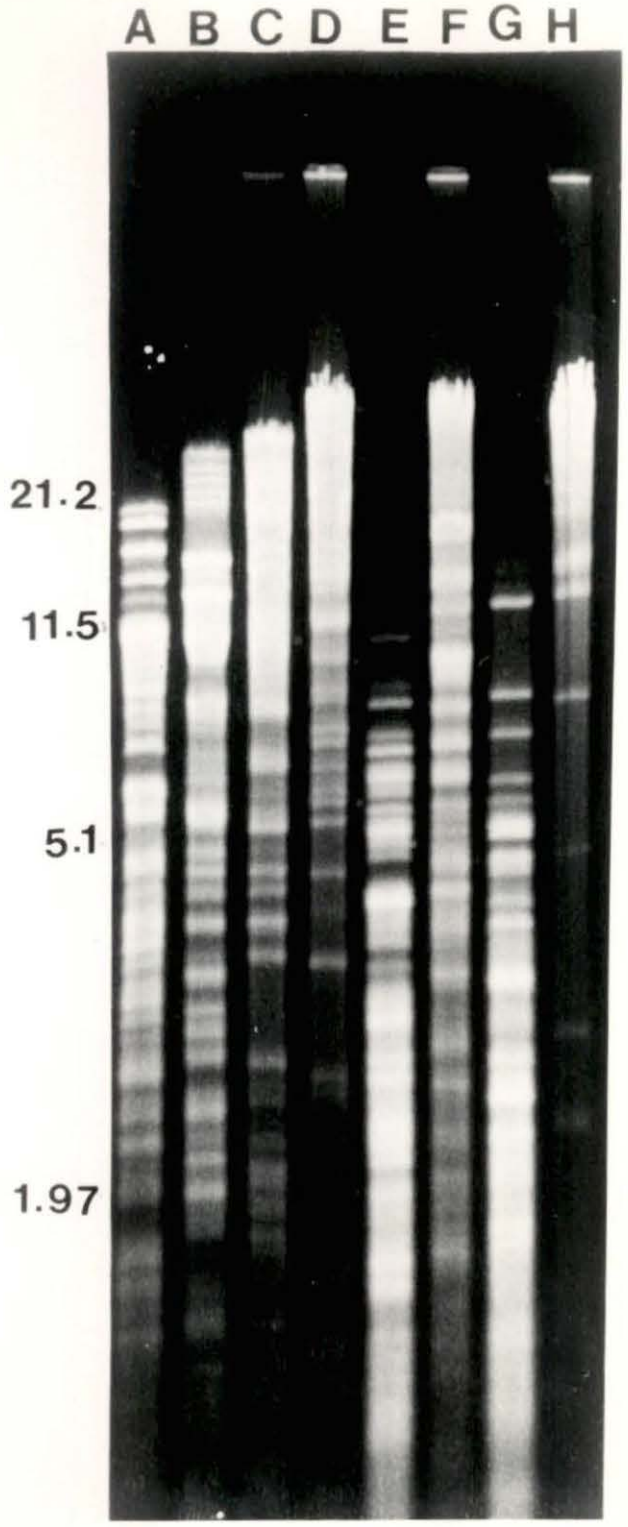

A B C D E F G H
21.2
11.5
5.1
1.97

Figure A.2: Southern blot analysis of DNA digested with various restriction enzymes and probed with 5' probe. Lane 1: EcoRI, lane 2: XbaI, lane 3: HpaI, lane 4: PstI, lane 5: RsaI, lane 6: SpeI, lane 7: TaqI and lane 8: CvnI.

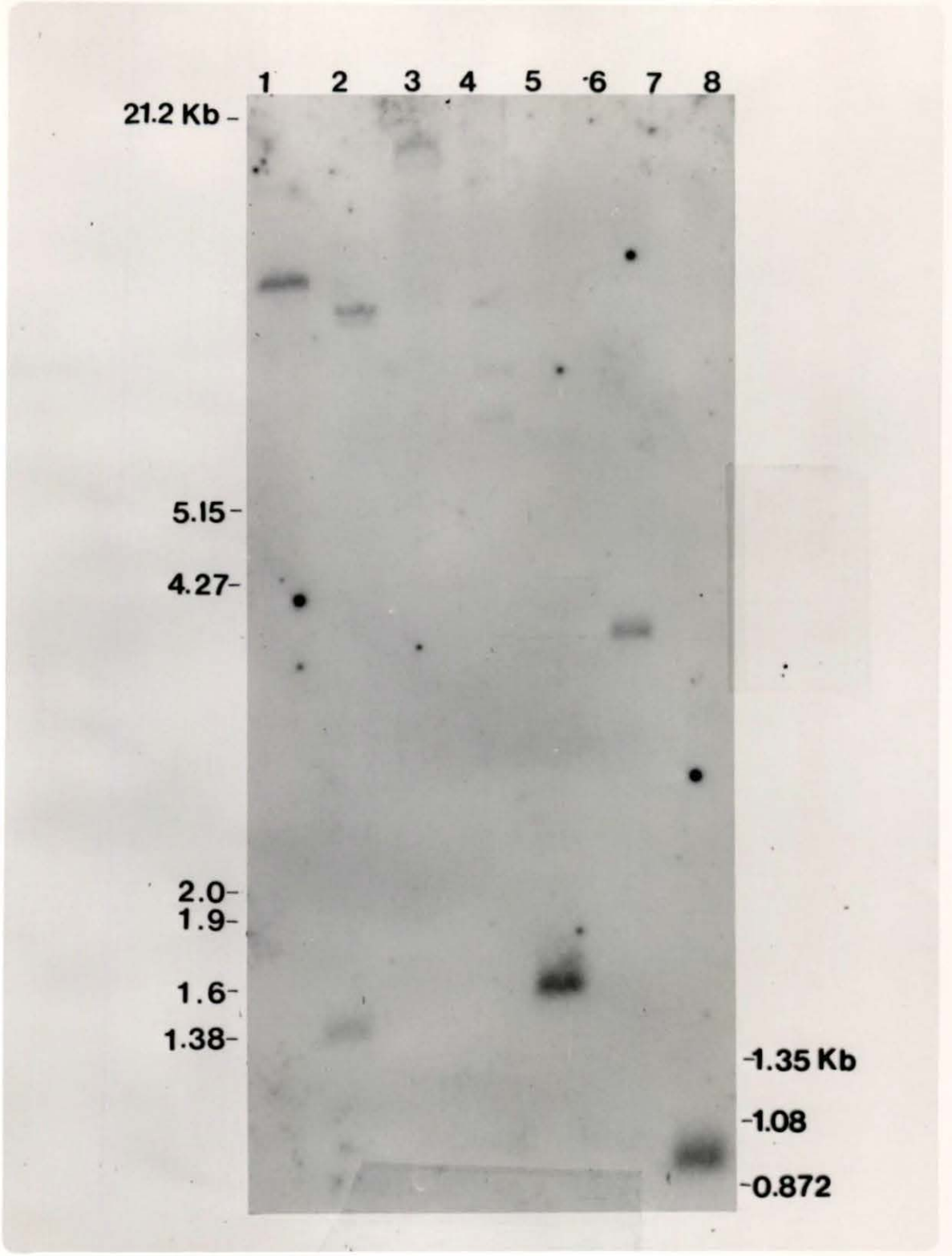

1
2
3
4
5
6
7
8
21.2 Kb
5.15
4.27
2.0
1.9
1.6
1.38
1.35 Kb
1.08
0.872

Figure A.3: Ethidium bromide stained agarose gel of the uncut and BamHI and EcoRI cut pKS$^+$. Lane A: Lambda/EcoRI/HindIII (mol.wt. markers) lanes B and C: cut pKS$^+$/3.5, lane D: uncut pKS$^+$.

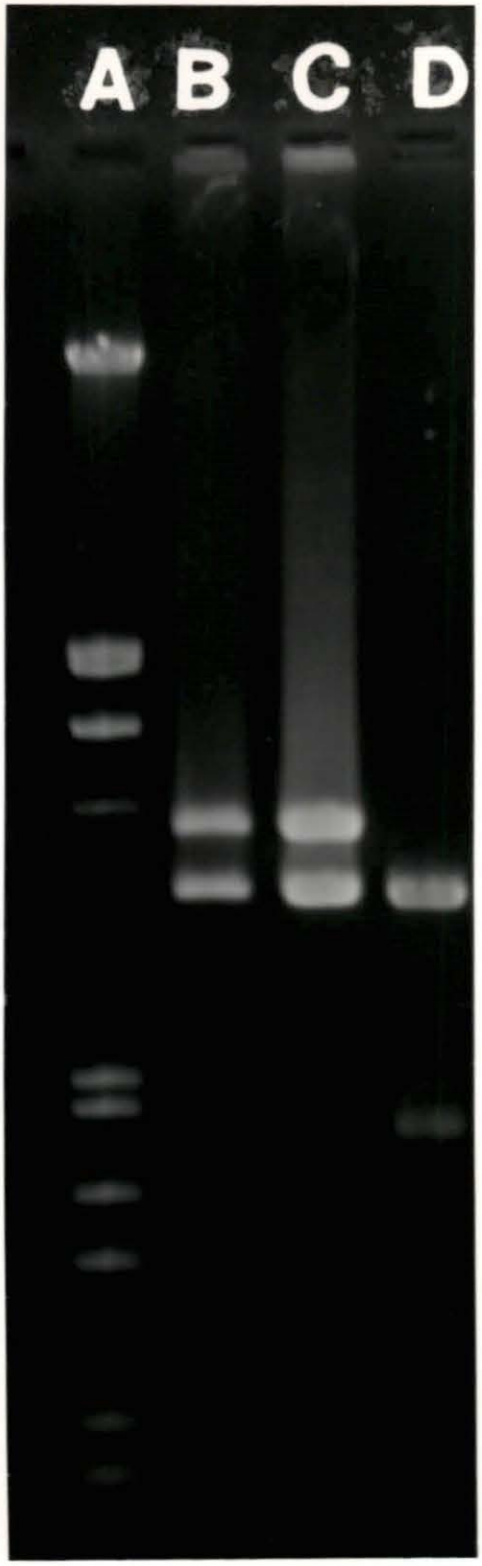
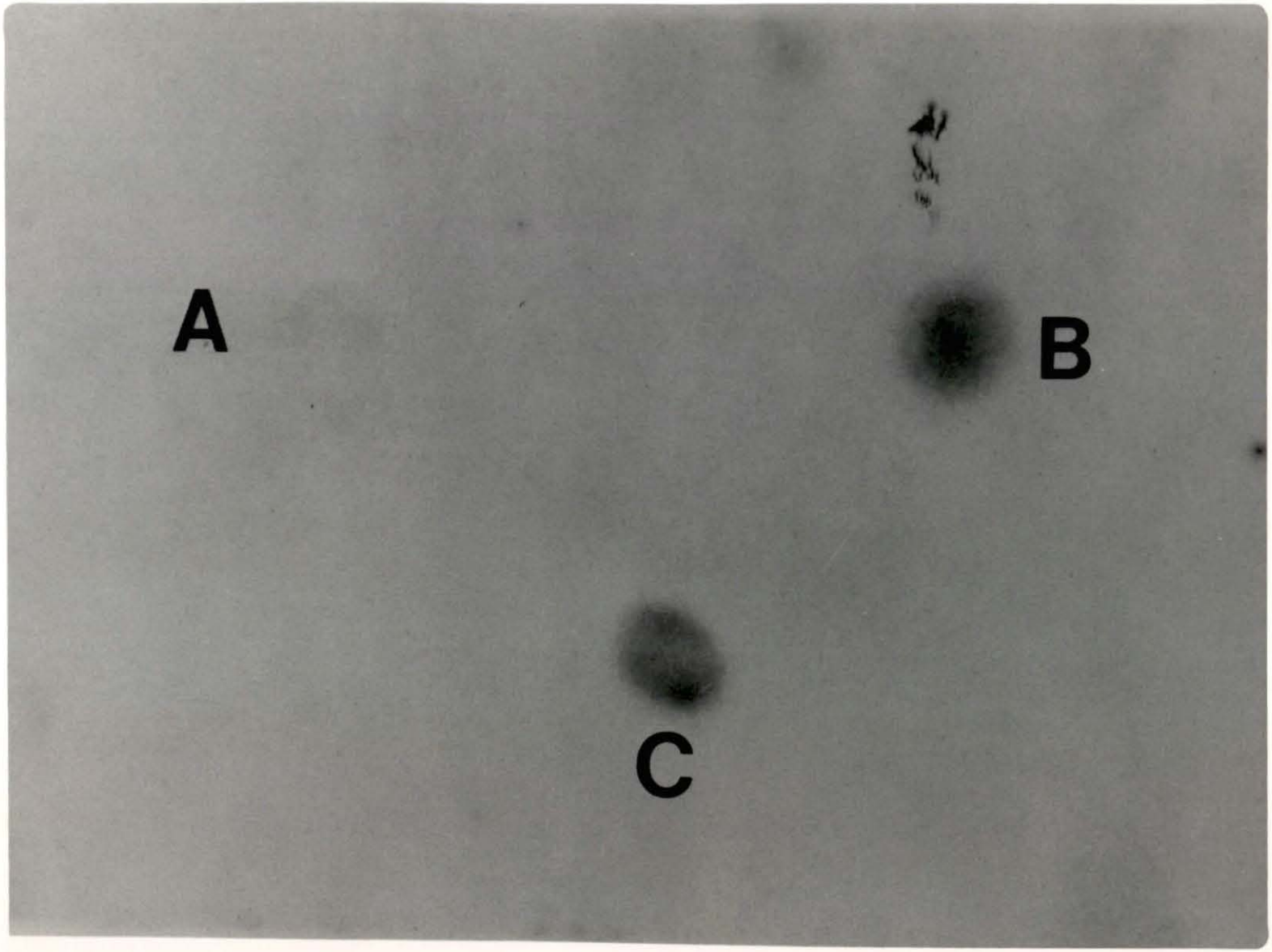

A
B
C
D

Figure A.4: Dot blot analysis of the recombinant phagemid pKS$^+$ DNA. A: lambda DNA, B: pKS$^+$/3.5 and C: DNA.


A
B
C

available in the vector. The sequence obtained is as follows:

The sequence of the 3.5kb fragment obtained using the reverse primer in pKS^+:

CCCCCCCCNNNTTAAAAAATGCCCTTGAACGANTTAGGCCCCACTAAAGGGAACAAAAGCT

GGTACCGGGCCCCCCCTCGAGGTCGACGGTATCGATAAGCTTGATATCGAATTCCTGCAGC

CCCTAGTGGAACAAAAGGGAAAAGAATTAACATCAACATTAACTCCTGTGCAACTTCTCTC

TACGGGTGATAAATATTTTTACTTGAGATTAAGAACATCTAAAGAGGAATATGAGAAAATA

ACCAATGATAGACTTGGATTTACATTAGATAAAGGAAGCCCTGAAACTGATGACTTAGCAT

CTAGTGGCGGAACAAGGCGGCCATACTCCTCATTATCTAAATTATCTAGGTGAAGGTGATA

CTGTCTCATCGGAAATTCCTTCAAACCACAGCTCTGGTGGATGATAATGNAACAGGGAACC

ATATTAAGGCCCAAAAGTTTGGGTTCATCAAANTTATAGCTCTTAACACAAAATTCATTTA

ATTTGGCGGAANGTNTANACAGGTACAANNTTCCNCTNATAANACACTTTATTNCCNGGGN

CCAANTTCCTAGAAANTTTTAANTNCCNTCGGNACTTCANNTGGCTTTTCTTNTNATCCCC

CANCACCNACCGCNACTTGGNAAAGCTNCGGTTNCNCCTTTNGNCNTTTCCNGNAANNCCC

CTTNNCCNATTTTTTTTNTNCCNNCCCCGNCCCACCTTNNAGGTTTNNAGGGCTTTTNCTT

NCCATTGGNAACCNTCCNNCCNTTTCCAACNTNNAGCGGGGGCNTNCGGNCTCGCCNAANT

CCNNNNCTCTTCCCNCTCACCCNCNCTTCCCCNCTTTTTNTGGGGGGTTCGGCCCCANANN

CCNANGCAACTCNNNGGCTCTNCTCNNTCTTNCTCCCCAAAANCANTNGGNAAATGGGNNC

NCCCCNCGNANCNAAACNTCC

The sequence of the 3.5kb fragment obtained using the universal primer in pKS^+:

CCCCCCNTNNNNGAGCCTGAAAANTATAGGGCCANTTGNAGCTCCACCGCGGTGGCGGCCG

CTCTAGAACTAGTGGATCCCCCCTAGTTTCTATATTATACTTGTTGATACAATTGCCAACA

ATTATTGTTTATTTTCCTAGTTCTAAAATTGTAACACTTTTACAAAAGTTCTCTAGCCGCT

TAATTTTGTTTATTTGTGACCTTCTTAGTCTATTATCGCTACTCTTGTTACTTGGTATGTT

CTTCGGCATACACAACAGTTATATCTCTCCTATTCTCATTACTTGTCATATCCTCTCTTTT

AGGATCCGGTACACTCCTTCAGATCTATTCTATATCAAAAATATCGGCCTACTATATTGCA

AAAAGATGATCAGCAAAATGTGCAAAACTGAATTATTGGGCTCAACCAACCGGCTACATCA

ATTCCGGCCTTCCCTGGGCCCCGGCCCACCTTCCCATCCTCTCCCTCATTCTNANCNNANA

NANTCCNGGNTTTATCAANTTANTTAAAACNCCCCTCCAATCGGGTTNCACCCANTNCACT

NATCCTGGCCCCNCTATACCTCNCCNAATNCNGACCNANAAAACGNAANNTNCTTNTTTTT

GGGTTNAAATTNTCATCACACCCAANTTNCNNTGGTNGGNCCCANAANAAAACGCNTTTCN

AAAACCNACTCTANGNGNNNANNCAANCAACNAAACCCCNCANACATNCTNANCCCTCNCC

AACCAGNNGCAAAACCCNNNNTANGGCAANGNCCCNCCGCCCCCGGNGCCTANTTTNAAAA

CACNTNNCNAANANGNCCCNGGCTTCNNAACCCACCGNTGGAAANCAGANAACGGCTCCAC

CGNCTANCCCCCCNNTNCNGGCNNCNNCACCATTNANCANAAAACCNCTTCNCCNCCCGCC

CNANAACTCTCTNNNCACGAACCCAGCCNCACNACGNCACANAANCAANNNC

The sequence was used to make comparisons with the 60bp sequence of p94. However, no matches were found. Sequence comparisons using the GCG Wisconsin Database showed several sequence homologies. Most of the homologies were seen with various regions of mitochondrial genomes from different organisms, such as the ATPases, NADH dehydrogenases, cytochrome oxidase, etc.

Restriction mapping of pKS$^+$/3.5 was done in an attempt to place the gene for p94 within the 3.5kb fragment and subsequently subclone it for sequencing. The restriction map of the 6.5kb plasmid DNA with four different restriction enzymes is given on the next page. The Southern blot analysis of the restriction fragments with 5' and 3' probes did not show any hybridization with either of the probes. The reason for this is not yet clearly understood. It is not known whether this was a technical problem or due to the cloning of a totally incorrect DNA fragment or probably the

restriction fragments that could hybridize with the probes were too small and were electrophoresed off the gel.

The above results show that a 3.5Kb DNA fragment that hybridizes with both the 5' and the 3' probe on a dot blot has been cloned. However, the attempt to clone and sequence p94 was not completely fulfilled. A better attempt at sequencing the gene for p94 would be to use degenerate oligonucleotide probes rather than non-degenerate probes and to identify the cloned DNA fragment on dot blots as well as Southern blots before attempting to subclone and sequence.

The Restriction Map of the 6.5kb pKS$^+$ Circular DNA is shown below: