Characterization of *Mycobacterium avium* Cytoplasmic Membrane Proteins with an Emphasis on the Major Cytoplasmic Membrane Protein

by

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ABSTRACT

Proteins of the cytoplasmic membrane of Mycobacterium avium were investigated to identify those which were: (1) intrinsic or extrinsic, (2) attached to the cell wall, (3) surface accessible and (4) excreted. In addition , sera containing anti-cytoplasmic membrane proteins were obtained and preliminary purification of the cytoplasmic membrane protein was attempted.

The predominating cytoplasmic membrane protein of 31,000 daltons (MCMP) was found to be intrinsic, attached to the cell wall and possibly surface accessible. The MCMP was not excreted, even in media in which the MCMP is not found in the cytoplasmic membrane.

Other cytoplasmic membrane proteins were also found to be intrinsic; a few were likely to be extrinsic based upon their separation from the membrane in sucrose gradients. Cytoplasmic membrane proteins of 66,000, 115,000 and 129

dalton were surface accessible as judged by I^{125}-Iodobead labeling.

Antisera against the MCMP and other cytoplasmic membrane proteins was obtained and will be useful in further cytoplasmic membrane protein characterization. Acetone precipitation of a cytoplasmic membrane preparation was performed to partially purify the MCMP.

The data from this study can be used for the development of serodiagnostic reagents for detecting mycobacterial infection.

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INTRODUCTION

The genus Mycobacterium is a heterogeneous group of rapid and slow-growing, pathogenic and non-pathogenic bacterial species which share a common characteristic; acid fast-ness. These bacilli are Gram-positive, non-sporeforming and non-motile. The pathogens M. leprae (leprosy) and M. tuberculosis (tuberculosis) are the most studied species of the genus. Recently, there has been an increase in the number of cases of infections caused by members of the Mycobacterium avium-Mycobacterium intracellulare (M. avium complex or MAC) in the Western countries mostly due to the occurrence of infections in patients with acquired immune deficiency syndrome (AIDS) (Cambiaso et al., 1990). M. avium, M. intracellulare and M. scrofulaceum are distinct but closely related species and that M. scrofulaceum is rarely pathogenic (Wolinsky, 1979).

INFECTION IN AIDS

The frequency of M. avium infections among AIDS patients is high, ranging from 25% to 50% (Blaser and Cohen, 1986). Typically, the infection is disseminated and AIDS patients can have as many as 10,000 bacteria per ml in the blood (Wong et al., 1985). Diagnosis by culture can take up

to 4 to 8 weeks. Late diagnosis can delay early antibiotic therapy which is particularly important for AIDS patients. In the developing world, inexpensive acid-fast microscopy is limited by its lack of sensitivity ($>10^4$ organisms/ml specimen) (David, 1973).

EPIDERMIOLOGY OF MAC

Representatives of M. avium, M. intracellulare, and M. scrofulaceum (MAIS) have been isolated from water (Falkinham et al., 1980), aerosol (Wendt et al., 1980) and soil samples (Brook et al., 1984). Since MAIS infections were not transferred person to person (Wolinsky, 1979), environmental sources of MAIS have been postulated (Wolinsky, 1979). Both the frequency of MAIS recovery (Falkinham et al., 1980) or MAIS numbers (Brooks et al., 1984) were higher in southeastern compared to northeastern United States waters and soils. There was also a higher frequency of persons reacting to either PPD-B (Edwards et al., 1969) or PPD-G (Edwards et al., 1970) in the southeastern United States. Thus high frequency of recovery from the environment correlated with higher numbers of MAIS-infected persons. Environmental sources of MAIS infections were also supported by the fact that MAIS isolates from patients with mycobacteriosis and those from natural

aerosols shared common characteristics (Meissner and Falkinham, 1986; Fry et al., 1986).

IMPORTANCE OF CELL SURFACE

The cell surface of MAIS plays an important role in aerosolization, pathogenesis, drug resistance and transport. MAIS have been isolated from aerosols (Wendt et al., 1980) and aerosolization of MAIS from natural waters may possibly be due to the cell surface hydrophobicity and not charge (George et al., 1989). Pathogenesis is influenced by the cell surface as evidenced by the fact that avirulent, opaque colonial variants of M. avium have different surface proteins than virulent transparent colonial parents (Thorel and David, 1984). MAIS resistance to most antituberculous antibiotics could be due to wall architecture (Rastogi, et al., 1981) or permeation barriers (Mizuguchi, et al., 1983). Low rates of transport of various substrates into mycobacterial cells may be responsible for their slow growth. Finally, there exist various cell surface proteins which are antigenic (George and Falkinham, 1989). Therefore, the study of the M. avium cell wall and its components could help in the development of a rapid diagnostic technique by detecting a specific M. avium protein using monoclonal antibodies.

CELL SURFACE LAYER STRUCTURE

M. avium cell wall is composed of a covalent structure comprised of molecules of arabinogalactan-mycolate linked by phosphodiester bonds (Kanetsuna, 1968). The mycolic acids in M. avium are long chain alpha-branched-beta-hydroxyl-acids ranging from 60 to 90 carbon atoms. The thick peptidoglycan layer is composed of chains of polysacharrides formed from alternating units of N-acetylglucosamine and N-glycolylmuramic acid. These units are cross-linked by tetrapeptide side chains containing L-alanine (L-ala), D-glutamic acid (D-glu), D-alanine (D-ala) and meso-diaminopimelic (DAP), thus building a rigid insoluble net surrounding the cytoplasmic membrane. Another major component of the mycobacteria cell wall is a high concentration of lipids. These lipids compose 25% of the cell wall; which results in the acid-fast character of the cells. Inside the cell wall, the next major structure is the cytoplasmic membrane.

MAJOR CYTOPLASMIC MEMBRANE PROTEINS AND THE CYTOPLASMIC MEMBRANE

The M. avium cytoplasmic membrane is the selective permeable barrier surrounding the cytoplasm. The cytoplasmic membrane is commonly composed of diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE) and

phosphatidylinositol (PI) (Minnikin, 1982). These compounds make up the phospholipid bilayer which is both polar and non-polar. The cytoplasmic membrane also contains various proteins (George and Falkinham, 1989) which are thought to perform transport and other functions.

M. avium cytoplasmic membrane proteins are dominated by a 31,000 molecular weight protein called the major cytoplasmic membrane protein (MCMP) which can comprise up to 60% of the total cytoplasmic membrane protein (George and Falkinham, 1989). The MCMP is also present in all MAIS and other mycobacteria (George and Falkinham, 1989). When cells were grown in a low pH Tween 80-containing medium, the MCMP was not found in the cytoplasmic membrane (George and Falkinham, 1989). It has been reported that detergent-grown cells lack the normal MAIS permeability barrier (Mizuguchi et al., 1983), which suggests that the MCMP may function as a porin. Through studying the MCMP and the other cytoplasmic membrane proteins, the function and understanding of these proteins may help in the diagnosis and culturing of M. avium.

DIAGNOSIS

Study of the proteins of the cytoplasmic membrane may help in the development of a rapid diagnostic technique for M. avium complex infections. Since many AIDS patients lack

the ability to produce antibodies (Simpson, et al., 1982), detection of anti-M. avium antibody is not expected to be successful in AIDS patients. Therefore, a modified enzyme-linked immunosorbent assay (ELISA) may be developed in which the patient's serum is immunoassayed using a monoclonal antibody specific for a certain common component of M. avium; the MCMP. This modified ELISA could be a quick, sensitive and inexpensive technique for the diagnosis of M. avium infections.

OBJECTIVES

1. Determine if cytoplasmic membrane proteins are intrinsic or extrinsic.

2. Determine if cytoplasmic membrane proteins are attached to the cell wall.

3. Identify surface accessible cytoplasmic membrane proteins.

4. Determine if the major cytoplasmic membrane protein is excreted into the medium when grown in Tween 80-containing medium.

5. Prepare antiserum for both cytoplasmic membrane and whole cells.

6. Determine what initial steps are best for purifying the MCMP and cytoplasmic membrane proteins.

MATERIALS AND METHODS

BACTERIAL STRAIN

Mycobacterium avium strain 13S (Wendt et. al., 1980) was the principal strain used in this study.

MEDIA

Cells were grown in either Middlebrook 7H9 (M7H9) broth medium (BBL Microbiology Systems, Cockeysville, MD) containing 0.5%(v/v) glycerol (gly) and 10%(v/v) oleic acid-albumin (OAA) enrichment or in a low pH Tween 80-containing minimal medium (George and Falkinham, 1986). The OAA enrichment was prepared by mixing 8.5 g NaCl and 50 g bovine serum albumin (BSA) (Sigma, St. Louis, MO) in 100 ml of distilled water. While mixing, 0.6 ml of oleic acid was added. After all were dissolved, the solution was filtered successively through 5 um, 1.2 um and 0.45 um pore size membrane filters and filter-sterilized by passage through a sterile 0.2 um membrane pore size filter. OAA was added to medium after autoclaving for 15 mins at 15 psi.

The Tween 80-containing minimal medium contained 0.0073 M KH_2PO_4, 0.002 M $MgSO_4$, 0.02 M $(NH_4)_2SO_4$ and 1.0%(v/v) Tween 80 (George and Falkinham, 1986). The pH of this medium was adjusted to 5.5 using 1.0% KOH before autoclaving for 15 minutes at 15 psi.

GROWTH OF CELLS

Stock cultures were grown in 16 x 150 mm screw-capped tubes containing 2.0 ml medium. The 2.0 ml culture was incubated at 37°C for 3 to 4 days (until turbid) on a rotator. The 2.0 ml stock culture was streaked on Plate Count agar medium (Difco Laboratories, Detroit, MI) to check for contamination and Middlebrook 7H10 + OAA + gly to check for purity, by morphology, of the M. avium strain. If free of contaminants, 0.6 ml of stock culture was used to inoculate 6.0 ml of the same medium contained in a 16 x 150 mm screw-capped tube. The 6.0 ml culture was incubated at 37°C on a rotator for 7 days. After incubation, the 6.0 ml culture was streaked as above and if free of contamination was used to inoculate 60 ml of the same medium in a 250 ml screw-capped bottle. The 60 ml culture was incubated at 37°C on a shaker at 100 rpm for 7 days. After incubation, the 60 ml culture was streaked as above and, if uncontaminated, it was used to inoculate 600 ml of the same medium contained in a 1 L screw-capped flask. The 600 ml culture suspension was incubated at 37°C on a shaker at 100 rpm for 7 days (i.e. late log/early stationary phase) and was streaked as above.

ISOLATION OF THE CYTOPLASMIC MEMBRANE

The cytoplasmic membrane fraction was isolated as described by George and Falkinham (1989). Cells were harvested by centrifugation (5,000 x g for 30 min at 4°C). The cells were washed three times in 10 to 20 ml of 40 mM potassium phosphate buffer (pH 6.5) containing 1.0 mM EDTA. The washed cells were finally suspended in 10 ml of the same buffer and the following added: 100 ul of a 30 mM phenylmethylsulfonyl fluoride (PMSF) stock solution, 1 mg RNase A and 1 mg DNase I. The cells were broken by two passages through a cold French pressure cell (Aminco J4-3398A, American Instrument Co., Silver spring, MD) at 20,000 psi. After breakage, $MgCl_2$ was added to a final concentration of 2 mM. The crude extract was centrifuged at 15,000 x g for 15 min at 4°C to remove any unbroken cells. The crude extract was centrifuged at 96,000 x g for 60 min at 4°C. The cytoplasmic membrane pellet (George and Falkinham, 1989) was washed twice with 10 ml of 40 mM potassium phosphate (pH 6.5) and 1 mM EDTA and resuspended in 2.5 ml of the same buffer.

Protein concentration was measured by the method of Lowry, et al. (1951) using bovine serum albumin fraction V (Sigma Chemical Co., St. Louis, MO) as the standard.

SODIUM DODECYL SULFATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Samples were examined for the major cytoplasmic membrane protein in a polyacrylamide slab gel using 0.025 M Tris-HCl and 0.19 M glycine (pH 8.3) containing 0.1% (w/v) sodium dodecyl sulfate as the running buffer. An 11% acrylamide resolving gel was prepared by mixing 6.23 ml of a 30% acrylamide:bis-acrylamide (Bio-Rad, Richmond, CA) solution (28.8 g:1.2 g/ 100 ml. of distilled water, stored at 4^{o}C in darkness), 4.25 ml 1.5 M Tris-HCl (pH 8.6) and distilled water. The solution was degassed for 5 minutes. After degassing, 0.085 ml of 10% (w/v) ammonium persulfate and 0.004 ml of N,N,N',N'-tetramethylethylenediamine (TEMED) (Sigma Chemical Co., St. Louis, MO) was added to the solution. The 4% acrylamide stacking gel was prepared by mixing 0.82 ml of a 30% acrylamide:bis-acrylamide solution, 1.25 ml of 1.5 M Tris-HCl (pH 6.8) and 2.93 ml of distilled water. The solution was degassed for 5 min. After degassing, 0.030 ml of 10% (w/v) ammonium persulfate and 0.005 ml TEMED were added. Gels were poured at room temperature. All samples were prepared using a 4x sample buffer (5.12 ml stacking gel buffer (pH 6.8), 8 ml glycerol, 2 g SDS, 4 ml beta-mercaptoethanol, 3 ml distilled water and a small amount of bromophenol blue) and heated at 100^{o}C for 5 min. After heating, the samples were centrifuged at 16,000 x g

for 1 min at 4°C. All samples were loaded to the wells of the gel and the gel was placed carefully in the electrophoresis chamber containing cold running buffer. Electrophoresis was carried out using a vertical tank apparatus (SE 600 series, Hoefer Scientific Instruments, San Francisco, CA) at 4°C. The protein samples were electrophoresed at 30 ma through the stacking gel. Current was increased to 65 ma upon entry into the resolving gel until the bromophenol blue tracking dye reached the bottom of the gel.

NaOH EXTRACTION OF CYTOPLASMIC MEMBRANE PROTEINS

Cytoplasmic membrane proteins, from cells grown in M7H9 + OAA + gly as described above, were subjected to NaOH extraction as described by Russell and Model(1982). The cytoplasmic membrane fraction was brought to 0.1 N NaOH and incubated at 4°C for 30 min. After incubation, the suspension was centrifuged at 96,000 x g for 60 min at 4°C. The pellet and soluble fraction were separated. The pellet was resuspended in 2 ml of 40 mM potassium phosphate buffer (pH 6.5) containing 2 mM $MgCl_2$, 0.3 mM PMSF and 1 mM EDTA. Proteins in both the supernatant and pellet fractions were separated by SDS-PAGE as described above.

COSEDIMENTATION OF MCMP AND CELL MEMBRANE

A step-gradient of sucrose was prepared as described by Proenca et al. (1987). In a 13.8 ml plastic ultracentrifuge tube, 2 ml 80% (w/v) sucrose solution in potassium phosphate buffer (pH 6.5) containing 2.0 mM $MgCl_2$, 0.3 mM PMSF and 1.0 mM EDTA was added. The solution was frozen at -70°C for 15 to 20 min. Then, a 2 ml 50% (w/v) sucrose solution was added to overlay the frozen 80% (w/v) sucrose layer. The sucrose layers were frozen again at -70°C for 15 to 20 min. After freezing, sucrose was mixed with a 2 ml cytoplasmic membrane fraction to a final concentration of 30% (w/v), then added above the frozen 50% (w/v) sucrose layer and frozen at -70°C for 15 to 20 min. Finally, a 15% (w/v) sucrose solution was added until the tube was full, then frozen at -70°C for 15 to 20 min. A 50% (w/v) sucrose gradient was prepared also. The sucrose gradients were centrifuged at 96,000 x g for 48 hours at 4°C. Following centrifugation, 1.0 ml fractions were removed from the sucrose gradients using a 1 cc syringe. Each fractions were analyzed by SDS-PAGE for the presence of the membrane proteins as describe above.

Each fraction was also assayed for the presence of a cytoplasmic membrane-specific enzyme activity. Measurement of NADH-oxidase (George and Falkinham, 1989) was not possible because of sucrose interference. Another cytoplasmic membrane-specific activity is an NADH-dependent,

nitroblue tetrazolium (NBT)-reducing activity which acts through superoxide (O_2^-) formation. This assay was performed using the following in a 1 ml glass cuvet: 0.795 ml 0.05 M potassium phosphate (pH 7.8), 0.100 ml 1.6 mM NBT in 0.05 M potassium phosphate (pH 7.8), 0.100 ml 4.4 mM NADH in 0.05 M potassium phosphate (pH 7.8) and 0.005 ml of each fraction. The change in absorbance was measured at 560 nm over 5 mins in 1 min intervals on a Spectronic 2000 spectrophotometer (Bausch and Lomb, Rochester, NY).

MAJOR CYTOPLASMIC MEMBRANE PROTEINS ATTACHMENT TO THE CELL WALL

Cells grown in M7H9 + OAA + gly were disrupted by using a Braun Disintegrator (Bronwill Scientific Inc., Rochester, NY). Cells were harvested by centrifugation at 5,000 x g for 15 min at 4°C and suspended in 30 ml 0.04 M potassium phosphate buffer (pH 6.5) containing 2 mM $MgCl_2$, 0.3 mM PMSF and 1 mM EDTA. This cell suspension was placed in a Braun disintegrator bottle and 20 ml of 0.1 mm Ballotini glass beads were added. The bottle was sealed and placed in the Braun disintegrator chamber. The cell suspension with glass beads was chilled by passing CO_2 through the chamber before starting the instrument. The cells were subjected to disruption for 5 min with periodic checks on the temperature of the suspension. After 5 min, the mixture was examined under a phase-contrast microscope to ensure that cells were

broken. The glass beads were removed by passage through coarse grade sintered glass filter (Millipore Co., Bedford, MA) under a vacuum. The broken cell suspension was centrifuged at 30,000 x g for 1 to 1.25 h at 4°C. After centrifugation, the supernatant was saved and the pellet resuspended in 5 ml of the above buffer and the suspended pellet centrifuged at 2,500 x g for 30 min at 4°C to separate whole cells and broken cell walls. Both supernatant and pellet were saved. All samples were run on SDS-PAGE as described above.

IODINATION OF SURFACE PROTEINS

Twenty (20) ml of cells from culture of M. avium strain 13S grown in M7H9 + OAA + gly to a late log/early stationary phase were harvested by centrifugation at 5,000 x g for 30 min at 4°C. The cells were washed twice with 10 ml 40 mM potassium phosphate buffer (pH 6.5) containing 2 mM $MgCl_2$, 0.3 mM PMSF and 1 mM EDTA and centrifuged at 5,000 x g for 30 min at 4°C. The cells were resuspended in 100 ul of the above buffer and transferred in to a 0.5 ml microcentrifuge tube. To radiolabel the surface proteins, 10 uCi NaI^{125} was added to the cell suspension to be labeled and one Iodobead (Pierce Chemical Co., Rockford, IL) and the suspension was incubated at room temperature for 15 minutes with gentle periodic shaking. After incubation, the labeled cell

suspension was separated from the Iodobead. To each labeled and unlabeled cell suspension, 0.15 g of 0.1 mm glass beads were added and the cells broken using a Mini-Beadbeater (Biospec Products, Bartlesville, OK) for 5 mins. After breakage, the suspensions were centrifuged at 4,000 x g for 2 min at 4°C to pellet unbroken cells and glass beads. The supernatant crude extract was recovered and sampled, then the remaining was centrifuged at 4,000 x g for 2 min at 4°C to ensure removal of all cells and glass beads and the cytoplasmic membrane fraction isolated as described by George and Falkinham (1989). The cytoplasmic membrane pellet was recovered and resuspended in 25 ul of the above buffer. Each was subjected to analysis by SDS-PAGE. After electrophoretic separation, the gel was stained as described above. The stained gel was dried and exposed to X-OMAT AR X-ray film (Eastman Kodak Co., Rochester, NY) for 12 to 24 hours.

EXCRETION OF MCMP

To measure possible excretion of MCMP into the medium, cell-free culture medium in which M. avium strain 13S was grown was analyzed by SDS-PAGE after ultrafiltration. Cells were grown in 600 ml of either M7H9 + OAA + gly or Tw80-minimal medium and recovered cell-free culture medium recovered by centrifugation at 5,000 x g for 30 min at 4°C.

After centrifugation, the cell-free supernatant was subjected to ultrafiltration through a PM-10 membrane (Amicon, Danver, MA) overnight under nitrogen pressure at room temperature. The M7H9 + OAA + gly cell-free supernatant was subjected to ultrafiltration to a final volume between 5 to 6 ml. The Tw80-minimal medium cell-free supernatant was subjected to ultrafiltration to a final volume between 15 to 19 ml. The concentrated supernatants were analyzed by SDS-PAGE for the presence of membrane proteins.

ACETONE PRECIPITATION OF CYTOPLASMIC MEMBRANE PROTEINS

Acetone precipitation of cytoplasmic membrane proteins, from cells grown in M7H9 + OAA + gly as described above, proceeded in steps. First, 100 ul of cold acetone was added to 1 ml cytoplasmic membrane preparation in a 15 ml Corex tube (Fisher Scientific, Norcross, GA) and incubated at -20^{o}C for 15 min. After incubation, the suspension was centrifuged at 10,000 x g for 10 min at 4^{o}C and the supernatant transferred to another Corex tube and the pellet dried at room temperature (RT). The pellet was suspended in 50 ul of sample buffer. Using the supernatant, the 20% and 30% cold acetone precipitation process was repeated. The pellets were suspended as above and both pellets and supernatants were analyzed for proteins by SDS-PAGE as described above.

AMMONIUM SULFATE PRECIPATION OF CYTOPLASMIC MEMBRANE PROTEINS

Using cytoplasmic membrane preparation from cells grown in M7H9 + OAA + gly as described above, a 50%(w/v) ammonium sulfate stock solution was prepared by dissolving 5 g $(NH_4)_2SO_4$ in 10 ml distilled water. Five 250 ul aliquots of cytoplasmic membrane preparation were transferred to five separate 1.5 ml microcentrifuge tubes. While vortexing the samples, the appropriate volumes of ammonium sulfate stock solution was added to establish the following percent saturations: 0%, 12.5%, 25%, 37.5% and 50%. Distilled water was added to each sample to compensate for differences in volume of ammonium sulfate stock solution that was added to get different ammonium percent saturation. The suspensions were incubated on ice for 30 min., then centrifuged at 16,000 x g for 5 min. After centrifugation, the supernatants were removed and the pellets were resuspended 100 ul 40 mM potassium phosphate buffer (pH 6.5). Proteins in both supernatants and resuspended pellets were analyzed by SDS-PAGE as described above.

ANTI-MCMP ANTIBODY

Antiserum to M. avium strain 13S whole cells and cytoplasmic membrane preparations from cells grown in M7H9 + OAA + gly were prepared by injecting two rabbits with 10^8

cells/ml for each injection or 20 ug/ml of cytoplasmic membrane preparation for each injection. Each was injected on weekly basis mixed with an equal volume (1.0 ml) of Incomplete Freund's Adjuvant. Injections were performed over a ten week period. Animals were bled 4 times for each antigen (i.e. before injection on the 6^{th}, 8^{th}, and 10^{th} weeks), 3 days after the injection for each vaccine.

Titer of antibody against MCMP for VT40 (whole cells) and VT41 (cytoplasmic membrane preparation) was measured by Western blotting. The cytoplasmic membrane preparation was run on a SDS-PAGE as described above and proteins were transferred to nitrocellulose (Bio-Rad, Richmond, CA.) using a Transphor Electrophoresis Cell (Hoefer Scientific Instrument, San Francisco, CA) using its own power supply. The transfer was carried out overnight at 30 v at $4^{o}C$ using a 25 mM Trizma base (Sigma Chemical Co., St. Louis, MO) buffer containing 0.19 M glycine (Bio-Rad, Richmond, CA), 20% (v/v) methanol (Sigma Chemical Co., St. Louis, MO) and 1% (w/v) SDS. Each strip was soaked in 5% (w/v) Carnation non-fat dry milk in 50 mM Tris-HCl (Bio-Rad, Richmond, CA.) containing 200 mM NaCl (TBS-milk) at pH 7.6 and incubated at room temperature with shaking for 30 min. The antiserum to be assayed for the presence of antibody was diluted with TBS-milk. A strip was incubated with the diluted antiserum 1 hour at room temperature. Each strip was washed three times

for 5 min in TBS-milk. After washing, the strip was incubated with horseradish peroxidase conjugated goat anti-rabbit IgG (Bio-Rad, Richmond, CA) diluted 1:5000 by shaking at room temperature. After incubation with the conjugate, the strip was washed twice in TBS-milk for 5 min and once in PBS buffer (8 g NaCl, 2 g KH_2PO_4, 0.2 g KCl, 1.13 g Na_2HPO_4 in 1 L distilled water). To develop the color, solution A (60 mg 4-chloro-1-napthol in 20 ml. cold methanol) was added to solution B (60 ul 30% (v/v) hydrogen peroxide in 100 ml cold PBS) just before use. The strip was incubated in this solution until color development was seen. The reaction was stopped by washing the strip in distilled water. The strips were stored dry and protected from light.

RESULTS

IDENTIFICATION OF INTRINSIC AND EXTRINSIC CYTOPLASMIC MEMBRANE PROTEINS

NaOH EXTRACTION

Russel and Model (1982) showed that 0.1 N NaOH extraction of a cytoplasmic membrane preparation from Escherichia coli resulted in loss of extrinsic proteins, but not intrinsic proteins. Using this approach, NaOH was added to a cytoplasmic membrane preparation to a final concentration of 0.1 N. The suspension was then incubated 30 min at 4°C followed by centrifugation at 96,000 x g for 60 min at 4°C. After centrifugation, the pellet and supernatant fractions were examined by SDS-PAGE (Figure 1). The treatment indicated that the cytoplasmic membrane proteins, including the major cytoplasmic membrane protein, were all intrinsic proteins except for a 25,100 molecular weight protein which was lost (no longer in pellet or supernatant) during the treatment. The lost of this was discovered by comparing the protein pattern of untreated and treated cytoplasmic membrane preparations (Figure 1). This experiment was performed twice using different culture suspensions with the same results.

COSEDIMENTATION OF MAJOR CYTOPLASMIC MEMBRANE PROTEIN AND CELL MEMBRANE

Cytoplasmic membranes have been isolated and studied using a discontinuous sucrose gradient (Randall, et al., 1987). If the major cytoplasmic membrane protein (MCMP) and other membrane proteins are true intrinsic proteins, they should migrate with the cytoplasmic membrane during centrifugation. Because of the large size of the cytoplasmic membrane, it will sediment faster than proteins which are not intrinsic in the membrane. The cytoplasmic membrane preparations were loaded in a 50% (w/v) (50-30-15) and 80% (w/v) (80-50-30-15) discontinuous sucrose gradient and centrifuged. After centrifugation, fractions were removed in 1.0 ml aliquots from the gradients which were examined by SDS-PAGE and assayed for the presence of the cytoplasmic membrane by NADH-dependent nitroblue tetrazolium (NBT)-reducing activity.

By examination of the 50% (w/v) sucrose gradient fractions by SDS-PAGE, it was found that the MCMP and other cytoplasmic membrane proteins were located in fractions 9 through 12 (Figure 2). The fractions also contained NADH-dependent (NBT) reducing activity. The other fractions did

not contain proteins or NADH-dependent (NBT) reducing activity.

In the 80% (w/v) sucrose gradient fractions, the MCMP was located in fractions 7 through 12; however, the majority of the other cytoplasmic membrane proteins were in fractions 7 through 9 (Figure 3). Fractions 7 through 12 contained NADH-dependent (NBT) reducing activity. Fractions 1 through 5 contained proteins of the following molecular weight: 75,800, 70,800 and 64,600. These proteins were also in fractions 7 through 12 and in the untreated cytoplasmic membrane preparation. Fraction 6 had these three proteins, plus a 55,000 molecule weight protein; however, fractions 1 through 6 had no NADH-dependent (NBT)-reducing activity. This experiment was repeated twice with the same result.

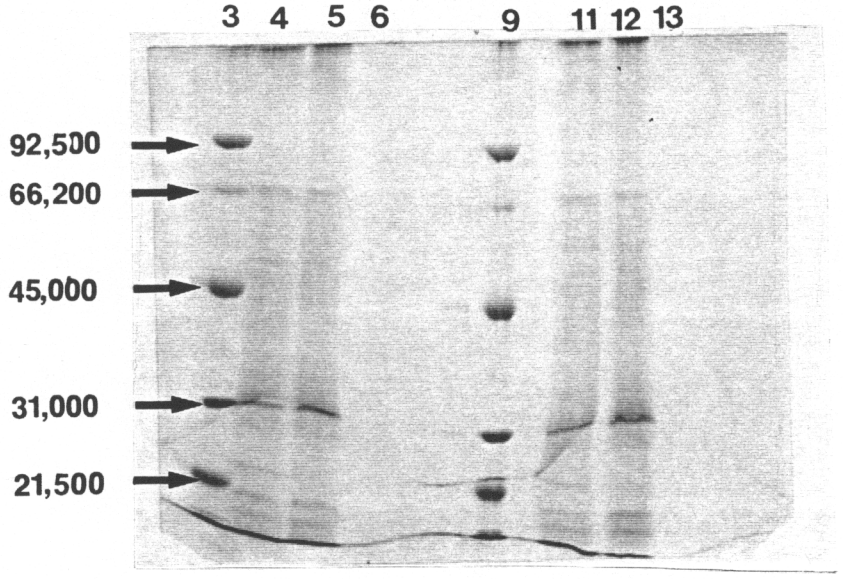

Figure 1. Electrophoretic separation of 0.1 N NaOH treated cytoplasmic membrane preparation loaded on a SDS-PAGE with each lane containing 40 to 45 ug of protein as described under "Materials and Methods". Lanes: (3) (9) protein standards; (4) (11) M. avium strain 13S untreated cytoplasmic membrane preparation; (5) (12) NaOH treated cytoplasmic membrane preparation pellet fraction; (6) (13) NaOH treated cytoplasmic membrane preparation supernatant fraction.

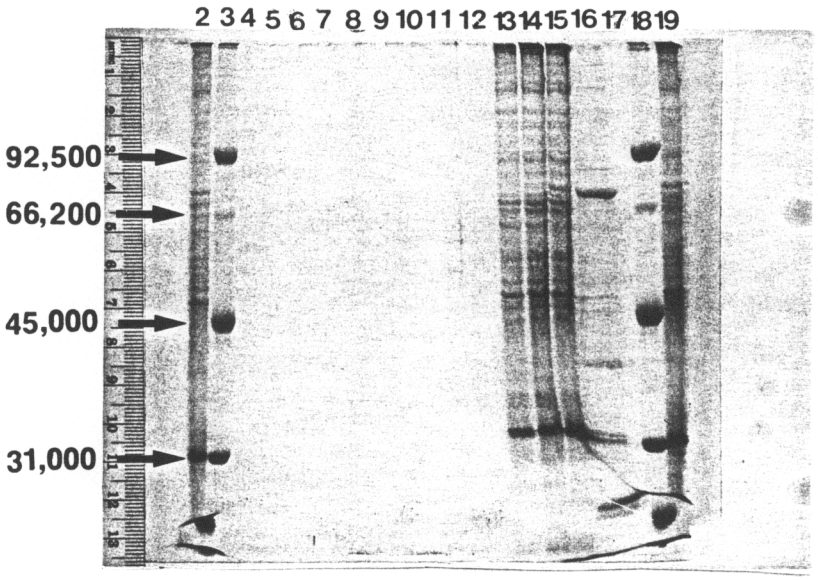

Figure 2. Electrophoretic separation of cytoplasmic membrane proteins from 50% (w/v) sucrose fractions as described under "Materials and Methods". Lanes: (3) (18) protein standards; (2) (19) M. avium strain 13S cytoplasmic membrane preparation; (5-16) fractions from top to bottom of 50% (w/v) sucrose gradient.

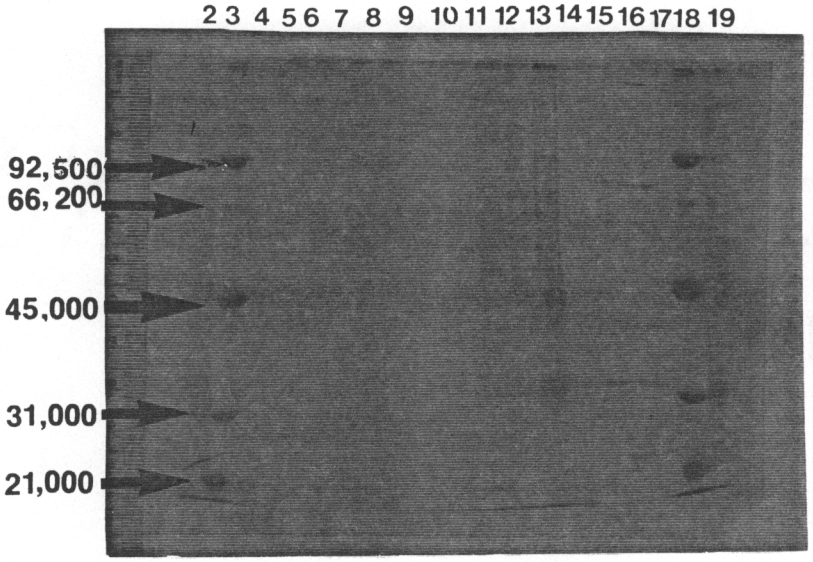

Figure 3. Electrophoretic separation of cytoplasmic membrane proteins from 80% (w/v) sucrose fractions as described under "Materials and Methods". Lanes: (3) (18) protein standards; (2) (19) M. avium strain 13S cytoplasmic membrane preparation; (5-16) fractions from top to bottom of 80% (w/v) sucrose gradient.

MAJOR CYTOPLASMIC MEMBRANE PROTEINS ATTACHMENT TO THE CELL WALL

Cells were broken using a Braun disintegrator with glass beads as described by Work (1971), before isolating the cell wall. M. avium strain 13S cells were broken using this technique. After breakage, differential centrifugation was used to isolate the whole cells, cell wall, and cytoplasmic membrane fractions. The supernatant fraction recovered after disintegration and after cytoplasmic membrane isolation were sampled. All fractions were examined on SDS-PAGE. Assuming that the protein bands with the same molecular weights in one fraction were the same in another fraction, the protein profiles were examined.

The MCMP was located in all fractions; however, it was concentrated in the whole cells, cell wall, and cytoplasmic membrane fractions (Figure 4). The band was barely visible in the soluble supernatant fractions. The cell wall and cytoplasmic membrane fractions had the same protein profiles except that the cytoplasmic membrane fraction contained a 46,800 molecular weight protein which was absent in the cell wall fraction. The supernatant fractions were similar in the lower and mid-molecular weight range of proteins 31,000 through 83,000. In contrast, the supernatant fraction recovered before cell wall isolation contained a 117,500

molecular weight protein which was absent in the supernatant fraction after isolation of the cytoplasmic membrane. The supernatant fraction after cytoplasmic membrane isolation contained 100,000 and 93,300 molecular weight proteins which were absent in the supernatant fraction before cell wall isolation. This experiment was performed twice using different culture suspensions with the same results.

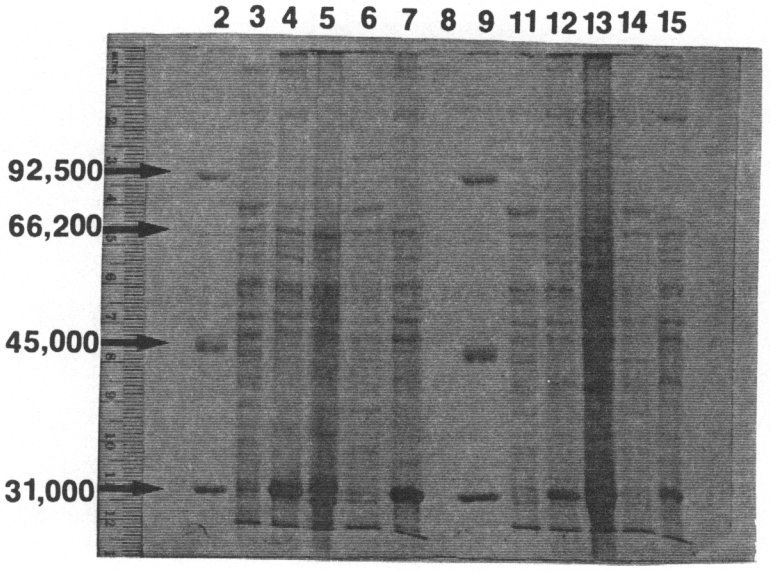

Figure 4. Electrophoretic separation of cell wall isolation fractions as described under "Materials and Methods". Lanes: (2) (9) protein standards; (3) (11) supernatant fraction before cell wall isolation; (4) (11) cell wall fraction; (5) (12) whole cells fraction; (6) (13) supernatant fraction after cytoplasmic membrane isolation; (7) (14) M. avium strain 13S cytoplasmic membrane preparation.

IODINATION OF SURFACE PROTEINS

NaI^{125} in the presence of iodobeads has been used to label tyrosine groups of surface proteins (Fischette, et al., 1985). After exposing the cells to the NaI^{125}, the cells were broken in a Mini-Beadbeater using glass beads. The crude extract was sampled before isolation of the cytoplasmic membrane proteins. The fractions were examined for labelled surface accessible proteins by autoradiography.

The cytoplasmic membrane protein profile was the same with the Mini-Beadbeater breakage procedure as with the French Pressure Cell breakage procedure (Figure 5). The Mini-Beadbeater breakage procedure was easier to perform and less time consuming than the French Pressure Cell breakage procedure.

There were three cytoplasmic membrane proteins which were labeled by I^{125} in the presence of an Iodobead (Figure 6). The MCMP was not labeled using this procedure. The molecular weights of the I^{125} labeled proteins were 129,000, 115,000 and 66,000 (Figure 6). The same three proteins were also labeled in the crude extract fraction after breakage, but there was also a 31,000 molecular weight protein which was labeled. This experiment was performed twice with the same results.

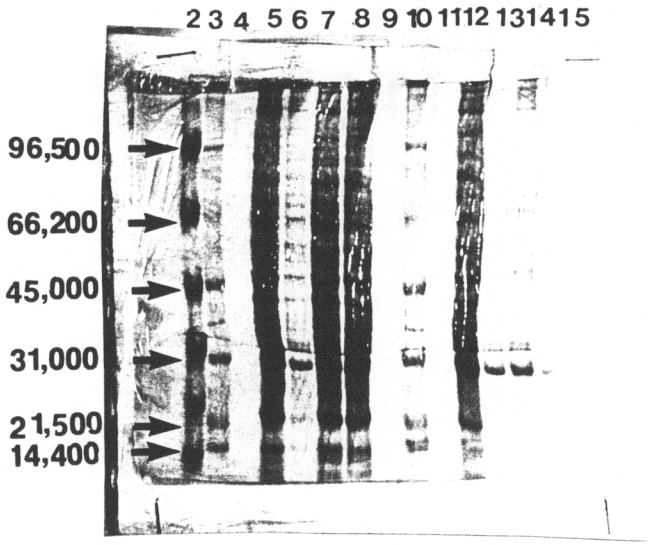

Figure 5. Electrophoretic separation of I^{125} labeled/unlabeled crude extract and cytoplasmic membrane preparation as described under "Materials and Methods". Lanes: (2) prestained protein standards; (3) (10) protein standards; (5) unlabeled crude extract from 10 ml cell suspension; (6) unlabeled cytoplasmic membrane preparation from 10 ml cell suspension; (7) labeled crude extract from 10 ml cell suspension; (8) labeled crude extract from 15 ml cell suspension; (12) unlabeled crude extract from 20 ml cell suspension; (13) labeled cytoplasmic membrane preparation from 10 ml cell suspension; (14) labeled cytoplasmic membrane preparation from 15 ml cell suspension; (15) unlabeled cytoplasmic membrane preparation from 10 ml cell suspension.

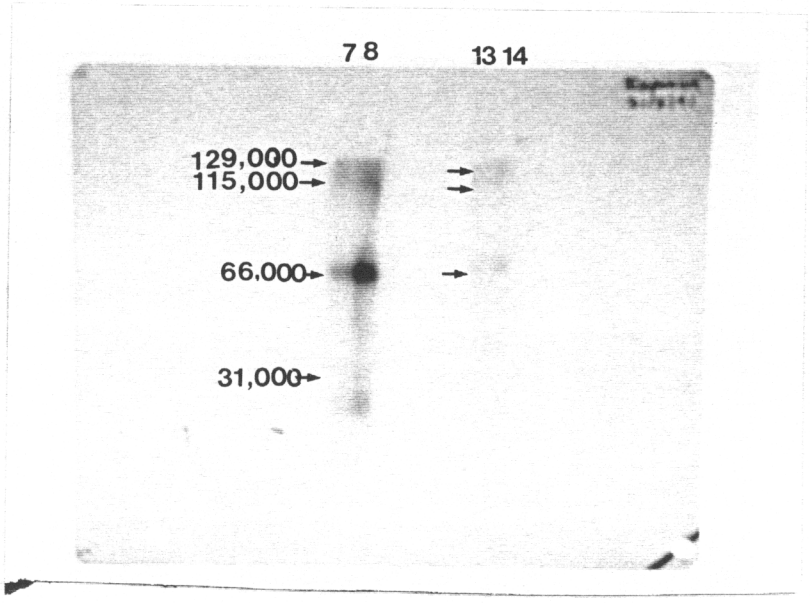

Figure 6. Radioautograph of electrophoretic separation of I^{125} labeled/unlabeled crude extract and cytoplasmic membrane preparation as described under "Materials and Methods". Lanes: (2) prestained protein standards; (3) (10) protein standards; (5) unlabeled crude extract from 10 ml cell suspension; (6) unlabeled cytoplasmic membrane preparation from 10 ml cell suspension; (7) labeled crude extract from 10 ml cell suspension; (8) labeled crude extract from 15 ml cell suspension; (12) unlabeled crude extract from 20 ml cell suspension; (13) labeled cytoplasmic membrane preparation from 10 ml cell suspension; (14) labeled cytoplasmic membrane preparation from 15 ml cell suspension; (15) unlabeled cytoplasmic membrane preparation from 10 ml cell suspension.

EXCRETION OF MCMP

The cytoplasmic membrane fraction of M. avium strain 13S grown in Tween 80-minimal medium does not have the MCMP (George and Falkinham, 1989). This could be due to either the protein not being synthesized and inserted in the membrane or the detergent extracts the protein from the cytoplasmic membrane. If the latter were the case, the MCMP would be found in the cell-free culture medium. Using this rationale ultrafiltration of the media was performed and filtrates were analyzed by SDS-PAGE.

There were no proteins present in the Tween 80-minimal medium filtrate which was concentrated 35 fold. Proteins in the M7H9 + OAA + gly filtrate, which was concentrated 100 fold, were difficult to identify due to interference from the high concentration of bovine serum albumin (Figure 7).

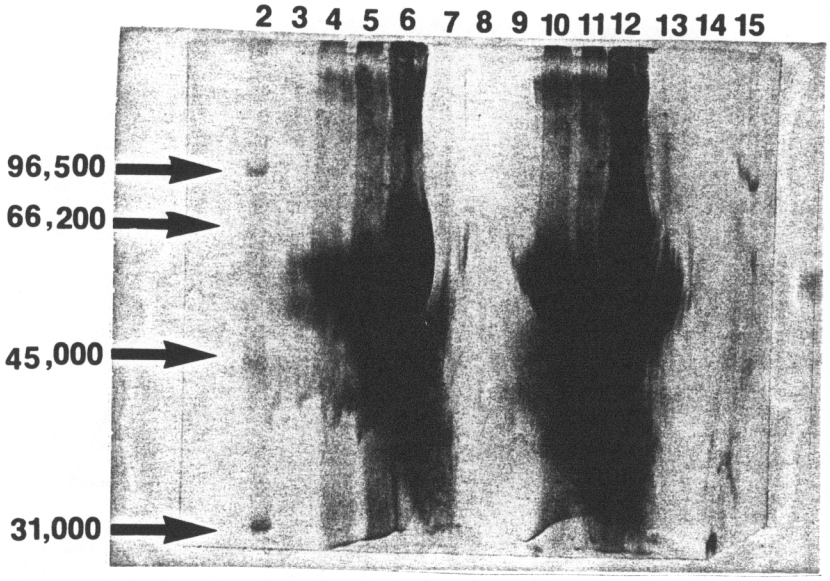

Figure 7. Electrophoretic separation of M7H9 + OAA + gly filtrate and Tween 80-minimal medium filtrate as described under "Materials and Methods". Lanes: (2) (15) protein standards; (4) (5) (10) (11) cytoplasmic membrane preparation from Tween 80-minimal medium grown cells; (6) (12) M7H9 + OAA + gly filtrate; (7) (8) (13) (14) Tween 80-minimal medium filtrate.

INITIAL PURIFICATION STEPS

ACETONE PRECIPITATION

Water-soluble proteins can be precipitated by water-miscible organic solvents such as ethanol and acetone (Robyt and White, 1987). The organic solvents added to the aqueous solution of proteins produce a reduction in the solvating power of water for the charged, hydrophilic protein molecule, leading to aggregation and precipitation of the protein (Robyt and White, 1987). Using this rationale, various concentrations of cold acetone were added to the cytoplasmic membrane preparation. The pellet and soluble fractions after extraction were analyzed by SDS-PAGE.

The majority of the MCMP precipitated at 10% (v/v) (Figure 8). There was a faint MCMP band in the 10% (v/v) supernatant, 20% (v/v) pellet and supernatant and 30% (v/v) pellet and supernatant (Figure 8). The supernatant fractions of the 10% (v/v), 20% (v/v) and 30% (v/v) acetone concentrations have the same protein profile with proteins ranging from 31,000 through 90,000 molecular weight (Figure 8). The pellet fractions of the 20% (v/v) and 30% (v/v) acetone concentration have the same protein profile except for the presence of a 89,000 molecular weight protein located in the 30% (v/v) acetone concentration pellet and absent in the 20% (v/v) acetone concentration pellet. This

experiment was repeated twice using different culture suspensions with the same results.

AMMONIUM SULFATE PRECIPITATION

The salting-out technique can be used to purify proteins based on their hydrophobic character (Robyt and White, 1987). After adding the salt, usually ammonium sulfate, the water solvates the added salt ions resulting in a decrease in the solvation of the protein. This decrease exposes hydrophobic areas of the protein structure, which interact with each other to give an aggregate that precipitates (Robyt and White, 1987).

The following percent saturations of ammonium sulfate were used to precipitate cytoplasmic membrane proteins, focusing on the MCMP: 12.5%, 25%, 37.5% and 50%. The pellet fractions for the different concentrations of ammonium sulfate had the same protein profile containing proteins of the following molecular weights: 97,700, 67,600, 66,200, 61,700, 58,900, 57,500, 51,100 and 31,000 (Figure 9). The protein profile of the pellet fractions were the same as that of the untreated cytoplasmic membrane (Figure 9).

The 12.5% saturation supernatant fraction contained the same proteins as the pellet fraction except for the absence of the 66,200 molecular weight protein (Figure 9). In contrast, the 25% saturation supernatant fraction contained

only the 57,500 molecular weight protein. The 37.5% and 50% saturation supernatant fractions showed no proteins (Figure 9). Overall, the bands in the supernatant were faint. This experiment was repeated three times with the same result.

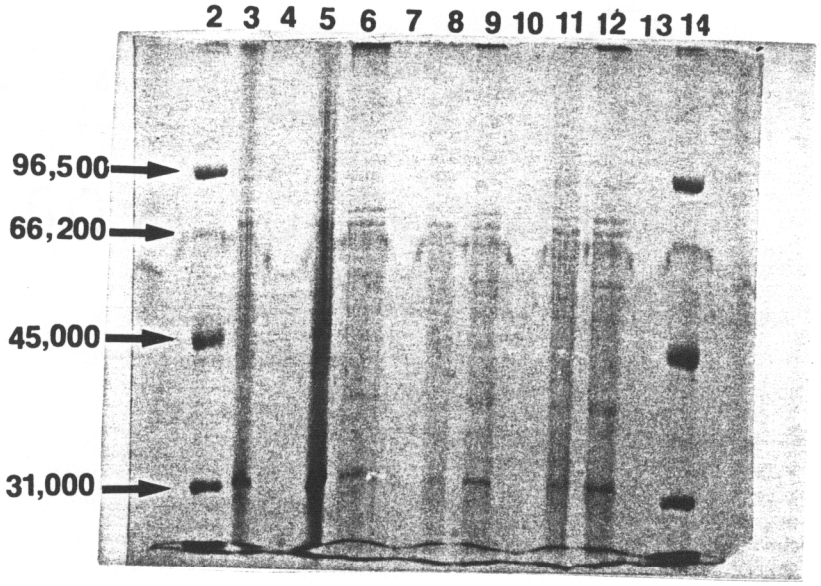

Figure 8. Electrophoretic separation of acetone precipitated cytoplasmic membrane proteins as described under "Materials and Methods". Lanes: (2) (14) protein standards; (3) M. avium strain 13S cytoplasmic membrane preparation without treatment; (5) 10% (v/v) acetone precipitated pellet fraction; (6) 10% (v/v) acetone supernatant fraction; (8) 20% (v/v) acetone precipitated pellet fraction; (9) 20% (v/v) acetone supernatant fraction; (11) 30% (v/v) acetone precipitated pellet fraction; (12) 30% (v/v) acetone supernatant fraction.

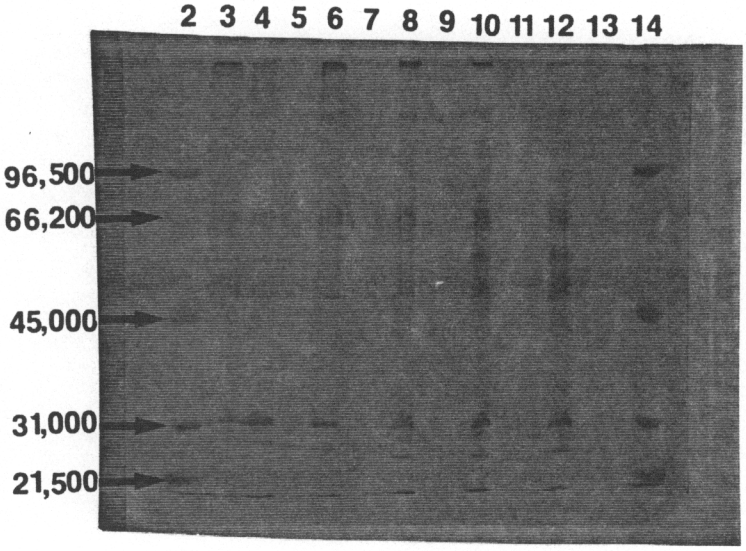

Figure 9. Electrophoretic separation of ammonium sulfate precipitated cytoplasmic membrane proteins as described under "Materials and Methods". Lanes: (2) (14) protein standards; (3) untreated M. avium strain 13S cytoplasmic membrane preparation; (4) 0% precipitated pellet fraction; (5) 0% supernatant fraction; (6) 12.5% precipitated pellet fraction; (7) 12.5% supernatant fraction; (8) 25% precipitated pellet fraction; (9) 25 % supernatant fraction; (10) 37.5% precipitated pellet fraction; (11) 37.5% supernatant fraction; (12) 50% precipitated pellet fraction; (13) 50% supernatant fraction.

ANTI-MCMP ANTIBODY

Two rabbits were injected with either M. avium 13S whole cells or a cytoplasmic membrane preparation from M7H9 + OAA + gly grown cells (one rabbit each). Antibodies to different M. avium proteins were produced. The antisera resulting from the injections were assayed for antibodies specific for cytoplasmic membrane proteins.

An immune response, from both injections, was observed by examining each bleed sample for the presence of antibodies. For both injections, the pre-bleed samples did not contain any antibodies to the antigens; however each sequential bleed sample afterwards did show an immune response. The maximal dilution of the sera permitting recognition of the cytoplasmic membrane proteins was calculated using immunoblots. The VT41 (cytoplasmic membrane preparation) antiserum maximal dilution was 1:6000; whereas, the VT40 (whole cell preparation) was 1:2000.

DISCUSSION

NaOH EXTRACTION OF CYTOPLASMIC MEMBRANE PROTEINS

Russel and Model (1982) used NaOH extraction to determine extrinsic and intrinsic proteins in the outer membrane of Escherichia coli. The NaOH is used to break the covalently bonded extrinsic proteins from the membrane releasing them in to the supernatant. The intrinsic proteins are protected from the effect of NaOH by their embedment in the phospholipid bilayer of the membrane and thus can be recovered in the pelleted cytoplasmic membrane following centrifugation.

Following NaOH extraction, all the proteins, including the MCMP, remained associated with the cytoplasmic membrane except for the 25,100 molecular weight protein (Figure 1). The 25,100 molecular weight protein was lost during the NaOH treatment.

Thus, if NaOH extraction worked in M. avium as E. coli, we would conclude that all proteins of the M. avium cytoplasmic membrane were intrinsic. That conclusion is unlikely, based on the fact that the composition of E. coli outer membrane and M. avium cytoplasmic membrane are different. It is likely that the large amount of lipid in the M. avium membrane would interact with the NaOH (to form soaps with the fatty acids and lipids) and prevent NaOH

extraction of proteins. One way to possibly overcome the neutralization of NaOH by membrane fatty acids would be to use different concentrations of NaOH to identify the effects this would have on the intrinsic proteins of the membrane such as the extraction of some of them.

COSEDIMENTATION OF MCMP AND CELL MEMBRANE

Randall et al. (1987) isolated cytoplasmic membrane proteins by cosedimentation with the cytoplasmic membrane. Using this technique and assuming that intrinsic membrane proteins would migrate with the cytoplasmic membrane and extrinsic proteins would separate because of flotation, the identification of intrinsic proteins was attempted.

In both the 50% (w/v) (50-30-15) and 80% (w/v) (80-50-30-15) sucrose gradients, the majority of the proteins, including the MCMP, were located in fractions which contained cytoplasmic membrane (Figure 2 and 3). This indicated that these proteins were true intrinsic proteins. In the 80% (w/v) sucrose gradient, there were a few proteins in the range between 65,000 and 75,000 molecular weight which were present in fractions that lacked the cytoplasmic membrane (Figure 3). These proteins were also observed in the fractions containing the cytoplasmic membrane as well (Figure 3).

Fractions with the cytoplasmic membrane were located by NADH-dependent NBT-reduction activity. Possibly a portion of this enzyme activity could have been extracted and thus activity might not be associated with the presence of membrane material. Thus, the technique would falsely identify intrinsic membrane proteins.

As was suggested as a problem for NaOH extraction, the presence of high concentration of lipid in the M. avium cytoplasmic membrane might prevent separation of extrinsic proteins. Lipids may so change the mobility of the membrane in the sucrose gradient such that extrinsic proteins will not be separated from the cytoplasmic membrane.

The cytoplasmic membrane was located in different fractions in both the 50% (w/v) and 80% (w/v) sucrose gradient; instead of only one fraction. Perhaps use of more layers of different sucrose concentrations or of a gradient would have resulted in a single narrow band and separation of extrinsic proteins. Finally, the presence of cytoplasmic membrane proteins in a large number of fractions could be due to contamination by material during removal of the 1 ml aliquots from the sucrose gradient with a syringe.

MAJOR CYTOPLASMIC MEMBRANE PROTEINS ATTACHMENT TO THE CELL WALL

Following fragmentation of cells, different fractions were examined for the presence of the MCMP and other proteins by SDS-PAGE. The MCMP was found in all the fractions. However, it was present in highest amounts in the whole cells, cell wall and cytoplasmic membrane fractions (Figure 4).

The identification of the cell wall fraction was based on a method described by Hirschfield et al. (1989). The presence of wall material in that or other fractions could not be confirmed due to the lack of availability of a gas chromatograph to identify common cell wall components.

The MCMP was observed in the cytoplasmic membrane fraction, and also a protein of the same molecular weight as the MCMP was observed in the whole cell fraction (Figure 4). The appearance of this protein could be due to the standard preparation of a sample before being applied to a SDS-gel. During the preparation of a sample, SDS and heat are used to break proteins in to their structural subunits, but this same combination can cause partial hydrolysis of the cell wall of an organism. Due to this partial hydrolysis, various proteins are released from the cell.

Usually in cell wall isolation, the cell wall containing pellet, after breakage, is treated with SDS,

acetone and Tween 80 to remove contaminating membrane and proteins (Hirschfield et al., 1990). Since the cell wall was isolated for the purpose of protein analysis, this harsh treatment was not performed. Such a treatment would denature most proteins allowing for no examinations of the cell wall protein profile. Therefore, it is possible that the MCMP observed in the cell wall fraction is due to residual cytoplasmic membrane bound to the cell wall.

IODINATION OF SURFACE PROTEINS

Surface protein accessibility would be an important characteristic of a protein employed in developing a early diagnostic system for M. avium. Therefore, NaI^{125} in the presence of a Iodobead was used to label the tyrosine groups of any M. avium surface proteins. Both the crude extract after breakage and the cytoplasmic membrane fraction isolated by centrifugation were examined for labeled proteins after being exposed to I^{125}.

There were three cytoplasmic membrane proteins, not including the MCMP, labeled by I^{125} and thus presumably on the surface of M. avium (Figure 6). The same three proteins were also labeled in the crude extract after breakage. Also, a 31,000 molecular weight protein labeled (Figure 6), but only in the crude extract and not in the cytoplasmic membrane fraction. Assuming that the proteins with the same

molecular weights are the same proteins, this protein could possibly be the MCMP.

The labeling of the surface accessible proteins depends on the availability and accessibility of the tyrosine groups. Thus, surface proteins containing tyrosine which are not accessible will not be labeled. This technique was able to identify some proteins which were surface accessible; however, other techniques for identifying surface accessible proteins may identify other surface accessible proteins.

Though a 31,000 molecular weight protein was labeled; as observed in the crude extract but not in the cytoplasmic membrane fraction, it may also not be the MCMP. This could be due to another protein having the same molecular weight as the MCMP. Other techniques should be pursued for identifying surface accessible proteins and their results compared to those presented.

The proteins, which were surface accessible according to I^{125} labeling, are probably good antigens to possible employ in an early diagnostic system for the identification of MAC infections.

EXCRETION OF MCMP

When M. avium strain 13S cells were grown in Tween 80-minimal medium, the MCMP was not found in the cytoplasmic membrane (George and Falkinham, 1989). The possibility that

this results was due to the MCMP in cells grown in Tween 80-minimal medium was examined by ultrafiltration. Using this technique, the cell-free media can be examined by concentrating and analyzing the concentrated media for the presence of the MCMP. As a control, the cell-free M7H9 + OAA + gly medium, in which M. avium strain 13S, was grown also examined for the excretion of MCMP. Such cells contain the MCMP (George and Falkinham, 1989).

The MCMP and other proteins were not observed in the cell-free Tween 80-minimal medium recovered by centrifugation and ultrafiltration. However, the proteins that could be observed in the cell-free M7H9 + OAA + gly medium after ultrafiltration were hard to identify due to the high concentration of bovine serum albumin (Figure 7).

Since the MCMP was not observed in the concentrated cell-free, Tween 80-minimal medium in which M. avium 13S was grown, the synthesis of the MCMP may be repressed when growing in this minimal nutrient medium.

INITIAL PURIFICATION STEPS

If the MCMP were to be used as an antigen to be detected for early diagnosis of MAC infections, purification of the MCMP would be necessary. Therefore , two possible initial steps for purification were performed and compared.

Acetone precipitation of the cytoplasmic membrane preparation resulted in the precipitation of the MCMP at 10% (v/v) of cold acetone (Figure 8). This technique was both quick and relatively easy. It also indicated some partial purification of the MCMP because other proteins were found in other fractions of different acetone concentration (Figure 8).

Ammonium sulfate precipitation of the cytoplasmic membrane preparation was also attempted. All tested percent saturations of ammonium sulfate showed the MCMP being precipitated (Figure 9). Therefore, this technique is not the best initial purification step for the MCMP when compared to the acetone precipitation technique.

A possible reason for the success of the acetone precipitation compared to the ammonium sulfate precipitation could be due to the ability of the organic solvent, such as acetone, to dissolve the membrane. This would release the proteins from the membrane and allow them to precipitate out at certain concentrations of acetone. This event does not occur in the ammonium sulfate precipitation of the cytoplasmic membrane preparation.

ANTI-MCMP ANTIBODY

To examine which M. avium proteins activated an immune response, rabbits were injected with either cytoplasmic

membrane preparation and whole cells from cells grown in enrichment medium. The production of antibodies to these proteins were examined.

The purification of the anti-MCMP antibody was attempted by adsorption using cytoplasmic membrane preparation from cells grown in Tween 80-minimal medium. There was a slight purification that occurred (data not shown); however, the adsorption may work better using whole cells grown in Tween 80-minimal medium.

Once the specific antigen to be used in the early diagnostic system is identified, the antibody to this protein could be purified from the prepared sera and could be used for various studies on the interaction between the antibody and antigen. After these studies, a monoclonal antibody could be prepared.

SUMMARY

The data suggest that major cytoplasmic membrane protein (MCMP) of M. avium 13S is a true intrinsic membrane protein. It can be located in the cell wall of the bacterium and may also be surface accessible. This protein can also be partially purified using 10% (v/v) acetone.

Other cytoplasmic membrane proteins which are true intrinsic proteins also have such characteristics such as attachment to the cell wall and surface accessibility. Some

of these proteins need further study for their possible employment as the antigen to be detected in MAC infections in an early diagnostic system.

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