STREPTOCOCCAL M6 PROTEIN EXPRESSED IN *ESCHERICHIA COLI*

Localization, Purification, and Comparison with Streptococcal-derived M Protein

BY V. A. FISCHETTI, K. F. JONES, B. N. MANJULA, AND J. R. SCOTT*

*From the Rockefeller University, New York 10021; and *Department of Microbiology and Immunology, Emory University School of Medicine, Atlanta, Georgia 30322*

M protein, a fibrillar molecule on the surface of the group A streptococcus (1, 2), confers to the organism the ability to resist attack by human phagocytic cells (3), and thus is considered to be one of the major virulence factors for these bacteria. While type-specific opsonic antibodies to the M molecule are able to neutralize its antiphagocytic effect and thus allow ingestion and killing of the streptococcus, the presence in nature of more than 70 distinct M protein serotypes has probably been a major contributing factor in the ability of group A streptococci to survive despite immunological pressures.

M protein has been isolated from streptococci by various methods. These include treatment of the bacteria with acid (4), alkali (5), nonionic detergent (6), pepsin (7, 8), or phage lysin (9). In addition, M protein has been isolated as a product secreted by streptococcal L-forms and protoplasts (10). Since the secreted product is the largest M protein isolated, and is thus most likely to be intact native protein, it would be the preparation of choice for further chemical study. However, the quantities available are too limited to characterize further. M protein isolated following treatment, either with pepsin at suboptimal pH, or with phage lysin, has proved useful for chemical and structural analysis of the M molecules (2, 8, 11–15). Although lysin-extracted M molecule appears to be close in size to the secreted protein (2, 10), its relationship to the secreted molecule, the pepsin-extracted molecule or the native molecule on the surface of the streptococcus, has not yet been determined.

To better understand the complete structure of the M molecule we have used in vitro recombinant DNA technology. We cloned the structural gene for type 6 group A streptococcal M protein into *Escherichia coli* (*E. coli*), where it is expressed (16). Based on electrophoresis in sodium dodecyl sulfate polyacrylamide gels (SDS PAGE),[1] the M protein produced in this new host is similar to

This work was supported in part by National Institutes of Health grant AI11822 (to V. A. Fischetti) and American Heart Association grant 83-1102 (to B. N. Manjula). B.N.M. is an Established Investigator of the American Heart Association.

[1] *Abbreviations used in this paper:* CM, carboxymethyl; ColiM6, Type 6 M protein isolated from *E. coli*; ELISA, enzyme-linked immunosorbent assay; LysM6, Type 6 M protein isolated from streptococci with phage lysin; MD solution, 10 mM $MgCl_2$ and 100 μg DNase; PBS, 0.1 M sodium phosphate-buffered saline, pH 7.0; PepM6, Type 6 M protein isolated with pepsin at pH 5.8; SDS PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TSE buffer, 100 mM Tris, pH 8.0, 20% sucrose, 5 mM EDTA; YT, yeast extract tryptone broth.

that secreted from streptococcal L-forms and protoplasts (10, 16), but larger than M molecules isolated from streptococci by pepsin (8, 11) or phage lysin treatment (2). In addition, the protein prepared from *E. coli* has the same immunodeterminant(s) as M protein isolated from the streptococcus.

In this communication, we describe the localization of the M6 protein in *E. coli* (ColiM6). We also describe the purification and immunological and biological characteristics of the protein prepared from *E. coli*. In addition, we compare its amino terminal sequence with purified M protein isolated from Type 6 streptococci by pepsin extraction (PepM6) and from streptococcal extracts prepared with phage lysin (LysM6).

Materials and Methods

Bacterial Strains and Growth Conditions. The plasmid pJRS42.13 was derived from pJRS42 by digestion with EcoRI and religation. It includes a part of the original vector, pJB8 (17), which is a pBR322-derived replicon, and the structural gene for M6 from *Streptococcus pyogenes* (16). The plasmid is maintained in *E. coli* K12 strain C600NR carrying lambda cI857, a temperature-inducible prophage. All *E. coli* strains were grown aerobically in yeast extract-tryptone broth (YT) (18) at 30°C in the presence of 20 μg/ml ampicillin.

Cell Fractionation. *E. coli* C600NR(pJRS42.13) was grown in YT broth (50 ml) at 30°C to an optical density (OD) of 0.7 at 650 nm. Cells were collected by centrifugation at 7,000 *g* and resuspended in 2.4 ml of ice-cold TSE buffer (100 mM Tris, pH 8.0, containing 20% sucrose and 5 mM EDTA). Lysozyme was added to a final concentration of 0.5 mg/ml and, following gentle mixing, the suspension was placed on ice for 20 min. For the whole cell control, 0.5 ml of this suspension was then removed and processed as described below. $MgCl_2$ was added to the remaining 2.0 ml (50 mM final concentration) to stabilize the resultant spheroplasts, and these were then sedimented at 7,000 *g* for 15 min. The supernatant was filtered through a 0.4-μm Millipore membrane to give the periplasmic fraction, designated SP-1.

The spheroplasts were washed once in 2.0 ml TSE buffer and sedimented at 7,000 *g*. The supernatant was filtered as in SP-1 above and designated SP-2.

To lyse the spheroplasts, 0.5 ml of a solution containing 10 mM $MgCl_2$ and 100 μg/ml DNase (MD solution) was added to the pellet along with 1.5 ml of water. The spheroplasts were then aspirated vigorously several times through a pasteur pipette and frozen (in dry ice/ethanol) and thawed twice. The lysate generated by this treatment was centrifuged at 50,000 rpm for 1 h in a Beckman Type 65 rotor and the supernatant filtered as in SP-1. This cytoplasmic fraction was designated SP-3. The insoluble residue in the pellet was resuspended in 2.0 ml TSE buffer.

Lysis of the whole cell control was accomplished by the addition of 15 μl of Triton-X100 and 100 μl of MD solution to the 0.5-ml sample (see above) and the mixture was frozen and thawed twice and frozen at −70°C.

A sample of each SP fraction (100 μl) was immediately added to an equal amount of SDS loading buffer, boiled for 3 min, and applied to an SDS polyacrylamide gel (see below). The remainder of each SP fraction was frozen rapidly and maintained in aliquots at −70°C.

Analysis of M Protein in E. coli Culture Supernatant. A 50-ml overnight culture of C600NR(pJRS42.13) in YT medium was cleared by centrifugation at 7,000 *g* for 10 min and filtered through a 0.22-μm Millipore membrane (Millipore Corp., Bedford, MA). The medium was then brought to 65% saturation with ammonium sulfate and allowed to remain at 4°C for 18 h. The resulting fine precipitate was collected by centrifugation at 10,000 *g* for 20 min and suspended in 10 ml 50 mM ammonium bicarbonate. After dialysis in this same buffer for 18 h at 4°C, the solution was lyophilized and subjected to SDS PAGE. The presence of M protein was determined by immunoblot analysis (see below).

Immunofluorescence Analysis. An indirect immunofluorescence assay was used to deter-

mine whether a significant amount of M protein was located on the surface of the *E. coli* strain producing the M protein. *E. coli* C600NR carrying plasmid pJRS42.13, C600NR carrying the parent plasmid pJB8, and D471, a type 6 streptococcal strain, were each grown to late log phase and cells were recovered from a 1-ml aliquot by centrifugation at 10,000 *g* for 5 min. The pellet was washed once in cold 1 mM sodium phosphate-buffered saline (PBS), pH 7.0. Antibody (IgG) to type 6 M protein conjugated to fluorescein (19) was added, the mixture placed on ice for 30 min, and the cells washed three times in cold PBS. The cells were mixed with a *p*-phenylenediamine–containing mounting solution to reduce photo bleaching (20) and examined with a Zeiss fluorescence microscope fitted for epifluorescence using a 65× oil immersion lens.

Large-scale Isolation of Crude M Protein from E. coli. 60 l of a YT culture of *E. coli* C600NR(pJRS42.13) was grown to OD 0.7 at 650 nm. The cells were harvested in a Sharples centrifuge (Sharples-Stokes, Warminster, PA) and washed once in 100 mM Tris, pH 8.0. The pellet was suspended in 1 l of ice-cold TSE buffer containing 500 mg of lysozyme and incubated on ice for 20 min. $MgCl_2$ was then added to a final concentration of 50 mM, and the spheroplasts sedimented at 7,000 *g* for 30 min. The supernatant (crude periplasm) was immediately aspirated from the pellet and filtered through a 0.45-μm Millipore membrane. The filtered supernatant was divided into two equal aliquots, one of which was frozen at −70°C and purified separately. The 500-ml supernatant was brought to 65% saturation with ammonium sulfate and placed at 4°C for 18 h. The resulting fine precipitate was sedimented at 7,000 *g*, suspended in 50 ml 5 mM sodium acetate, pH 5.5, dialyzed extensively against this buffer and frozen at −70°C until used for purification.

Purification of M Protein Isolated from E. coli Periplasm. All steps were monitored by SDS PAGE and immunoblot using LysM6 monoclonal antibodies. The crude periplasmic extract (after ammonium sulfate fractionation) was applied to a 1.5 × 12 cm column of CM cellulose (Whatman) equilibrated in 5 mM sodium acetate, pH 5.5. The column was washed with this buffer until the absorption at 280 nm reached baseline. The adsorbed M protein was eluted with 100 mM sodium phosphate, pH 7.0. The eluted protein fractions (containing some contaminating proteins) were pooled and applied directly to a 1.0 × 17 cm column of hydroxylapatite (Bio-Rad Laboratories, Richmond, CA) equilibrated in 25 mM sodium phosphate, pH 7.0. The column was washed with 200 mM sodium phosphate, pH 7.0, until there was no detectable absorbance at 280 nm. The M protein was then eluted with 400 mM sodium phosphate, pH 7.0. Appropriate fractions were pooled, dialyzed extensively against 50 mM ammonium bicarbonate, and stored in the lyophilized state.

Alkaline Phosphatase Assay. The presence of alkaline phosphatase in *E. coli* cell fractions was detected essentially by the method of Brickman and Beckwith (21). Cell fractions SP-1 to SP-3, insoluble residue, and the whole cell lysate were diluted 1:10 in 1.0 M veronal acetate buffer, pH 8.6. A 4% solution of sodium *p*-nitrophenyl phosphate (Sigma) (0.1 ml) was added to each tube and incubated at 37°C for 2 h. The released *p*-nitrophenol color was determined by the absorbance at 420 nm. The concentration of the released *p*-nitrophenol was determined by comparing the absorbance to that of a standard curve of *p*-nitrophenol (Sigma) diluted in 1 M veronal buffer, pH 8.6.

SDS PAGE and Immunoblot Analysis. Proteins were separated on 12% SDS-polyacrylamide gel slabs by the method of Laemmli (22). Molecular weight standards were run concurrently in parallel wells. Gels were either stained with Coomassie Brilliant Blue R in 10% acetic acid–25% *n*-propanol and destained with 10% acetic acid–25% *n*-propanol, or electrophoretically transferred onto nitrocellulose (Schleicher & Schuell, Inc., Keene, NH) (23).

Specific proteins bound to the nitrocellulose membrane were visualized immunologically by the method of Blake et al. (24). After transfer to nitrocellulose, the membrane fraction was reacted either with polyclonal or monoclonal antibodies made to lysin-extracted M6 protein. The bound antibody was detected with alkaline phosphatase-conjugated goat anti-rabbit or goat anti-mouse IgG (Sigma Chemical Co., St. Louis, MO).

The bands were visualized with 5-bromo-4-chloro-3-indolyl phosphate (Sigma) as the alkaline phosphatase substrate and nitroblue tetrazolium (Sigma) as the chromophore.

Bactericidal Assay. The indirect bactericidal assay was carried out as described originally by Lancefield (25) with modifications described by Fischetti et al. (6).

Absorption of Opsonic Antibodies. Purified M6 protein extracted from *E. coli* was lyophilized in two 300-μg aliquots. Rabbit type 6 opsonic antiserum prepared against LysM6 protein (0.5 ml) was added to one, and a similar amount of human serum opsonic for type 6 streptococci was added to the other dried protein sample. The tubes were incubated at 37°C for 1 h and then at 4°C overnight. The precipitate was removed by centrifugation at 20,000 *g* for 15 min and the supernatant was used in a bactericidal assay with type 6 streptococci.

Rabbit Immunization. Antisera to the purified ColiM6 protein was prepared in New Zealand white rabbits. The primary immunization consisted of 100 μg of ColiM6 protein emulsified with complete Freund's adjuvant and given intracutaneously at multiple sites. The animals were boosted after 4 wk with the same dose of the ColiM6 protein in incomplete Freund's adjuvant. Animals were bled 10 d later.

Amino Acid Analysis. Quantitative amino acid analysis was performed on five nmol samples of lyophilized ColiM6, as well as LysM6 (reference 2, manuscript in preparation). Samples were hydrolyzed with 6 N HCl at 110°C for 18 h in evacuated sealed tubes (6).

Amino Terminal Sequence Analysis. Automated Edman degradations were performed with a Beckman Sequencer (updated 890B; Beckman Instruments, Fullerton, CA) as previously described (8) on 30–50 nmol of protein.

Protein Determination. Protein concentration was determined by the method of Lowry et al. (26) using bovine serum albumin as a standard.

Enzyme-linked Immunosorbent Assay (ELISA). The ELISA assay was based on the method of Engvall and Perlman (27) with modifications described by Fischetti (28).

Preparation of Monoclonal Antibodies. BALB/C mice were immunized intraperitoneally with 200 μg of purified LysM6 protein in complete Freund's adjuvant. After 4 wk, mice were bled and tested for antibodies to LysM6 protein by ELISA assay. Mice with high titers of antibodies were given a second dose of antigen (200 μg) intraperitoneally in distilled water. Mouse spleens were excised 3–3.5 d after the booster dose and fusion of spleen cells to P3-NS1/1Ag4-1 (NS-1) myeloma cells was performed essentially by the method of Köhler and Milstein (29) with modifications by Galfre et al. (30). Hybridomas cloned by limiting dilution were grown as ascitic tumors in pristane-primed mice (31) and the ascitic fluid collected and precipitated with 50% ammonium sulfate. Purification of the immunoglobulin was completed by chromatography on protein A Sepharose (32).

Results

Localization of the M Molecule in E. coli. *E. coli* C600NR(pJRS42.13) was converted to spheroplasts and the periplasmic proteins were separated from the spheroplasts by centrifugation (33). The spheroplasts were osmotically and physically lysed, and the membranes along with other macromolecular structures were separated from the soluble cytoplasmic proteins by ultracentrifugation. To separate the proteins present in each fraction, equivalent amounts of each fraction (based on the original cell concentration) were used for SDS PAGE.

Virtually all of the protein reactive with monoclonal antibodies to the LysM6 protein was in the periplasmic fraction of the *E. coli* cells (Fig. 1 *b*, SP-1) with a trace reaction in the cytoplasmic fraction (SP-3). The immunoblot assay of the periplasmic fraction detected only three immunoreactive bands on the nitrocellulose that stained with varying intensity, the center band appearing less concentrated. The apparent molecular weights of the bands were 55,000, 57,000, and 59,000. A faint immunoreactive band, which appears to be a degradation product, was occasionally seen within this fraction at ~35,000 mol wt. An extra

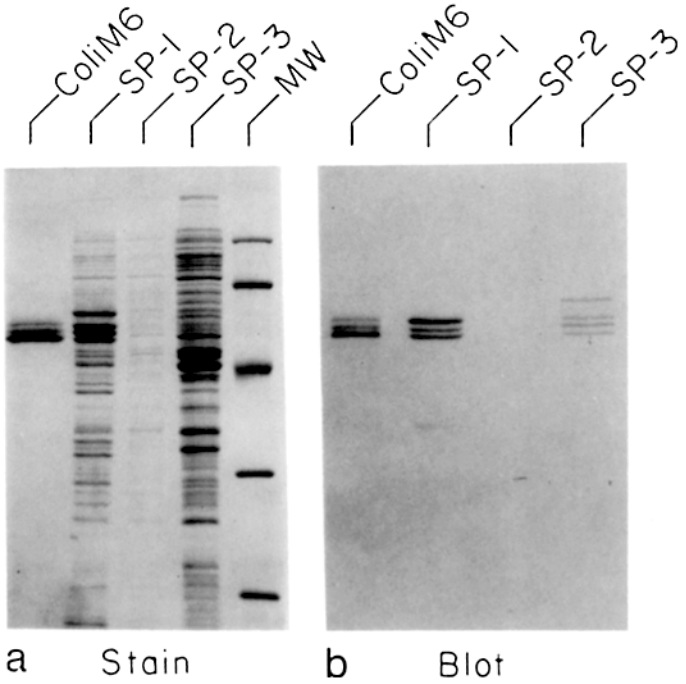

FIGURE 1. (*a*) Coomassie Blue–stained SDS polyacrylamide gel of *E. coli* fractions. *E. coli* strain C600NR(pJRS42.13) was converted to spheroplasts and separated into periplasmic (SP-1), wash (SP-2), and cytoplasmic (SP-3) fractions. Equivalent amounts of each fraction, based on the cell concentration, were applied to a 12% SDS gel along with purified ColiM6 protein. The SDS gel was stained with Coomassie Blue and destained as described in Materials and Methods. Molecular weight standard proteins (*MW*) are phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), and soybean trypsin inhibitor (20,000). (*b*) Immunoblot analysis of *E. coli* fractions. Same protocol as in *a*, except the separated proteins were electrophoretically transferred to nitrocellulose and processed for immunoreactive bands with monoclonal antibody prepared against LysM6 protein as described in Materials and Methods.

weakly reactive band was also observed in the cytoplasmic fraction (SP-3) with an apparent mol wt of 63,000. A stained gel of the same fractions (Fig. 1*a*) revealed that the three immunoreactive bands found in the periplasm were among the prominently staining proteins in this fraction (SP-1). Since alkaline phosphatase is located solely in the *E. coli* periplasm (33), its activity was used as a marker to determine the degree of periplasmic contamination in the nonperiplasmic fractions. Nearly all of the alkaline phosphatase activity was located in the periplasmic fraction, with none seen in the wash (SP-2) or cytoplasm (SP-3) (Table I); the remaining activity was located in the insoluble residue (containing membranes, unlysed cells, and macromolecules). In some experiments a small amount of alkaline phosphatase activity was found in the SP-2 wash fraction (<10%). When this occurred, the immunoblot showed a small amount of immunoreactive protein in this fraction as well.

When the culture supernatant from *E. coli* C600NR(pJRS42.13) was concentrated 100-fold and examined by immunoblot assay with type 6 antiserum, no reactivity was observed. Furthermore, no immunofluorescence due to M6 protein

TABLE I

Presence of Alkaline Phosphatase Activity in E. coli C600NR(pJRS42.13) Cell Fractions

Cell fractions	Percent*
SP-1 (periplasmic)	84
SP-2 (wash)	0
SP-3 (cytoplasmic)	0
Insoluble residue	16

* Expressed as percentage of total activity. Average of two independent determinations.

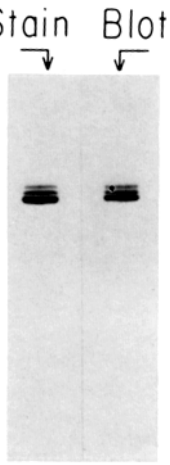

FIGURE 2. SDS PAGE and immunoblot analysis of purified ColiM6 protein. Two samples of purified ColiM6 protein were separated on a 12% SDS polyacrylamide gel in alternate tracks. The gel was cut between the tracks and each processed separately. The proteins in one track (right) were electrophoretically transferred to nitrocellulose and reacted with monoclonal antibodies to LysM6 protein. The bound antibody was visualized as described in the Materials and Methods. The second track (left) was stained with Coomassie Brilliant Blue and destained.

was detectable on the surface of C600NR(pJRS42.13), although the type 6 streptococcal strain D471 exhibited bright fluorescence (data not shown).

Purification of the M Protein Produced by E. coli. Since comparison of the immunoblot with the stained gel revealed that the M6 protein was concentrated within the periplasmic space and was one of the major proteins within it, this fraction served as the starting material for further purification of the ColiM6 molecule. Chromatographic purification of ColiM6 on columns of CM-cellulose and hydroxylapatite (see Materials and Methods) resulted in a preparation that contained three protein bands. These bands could be visualized either by Coomassie Blue staining of the SDS polyacrylamide gel or by immunoblot analysis with monoclonal (Fig. 2) as well as polyclonal antibodies (data not shown) to the LysM6 protein. The mobilities of the protein bands in SDS PAGE of the purified preparation were identical to those of the M protein present in crude lysates of *E. coli* (see Fig. 1 *a*, ColiM6). In contrast to the crude preparation, the lowest of the three bands in the purified preparation was more concentrated in relation

to the other two. The total yield of purified ColiM6 protein from 60 l of culture was 52 mg.

Amino Acid Analysis. The amino acid composition of the purified ColiM6 protein is compared to that of the LysM6 in Table II. Except for the slight differences in the leucine and threonine content, the amino acid compositions of the two molecules are essentially identical.

Sequence Analysis. The amino terminal sequence of the ColiM6 protein through residue 34 is presented in Fig. 3. Despite the three protein bands observed in SDS gels both by Coomassie Blue staining and immunoblot analysis,

TABLE II

Comparison of the Amino Acid Composition of LYSM6 and Colim6 Proteins

Amino acid	Residues/100	
	Lysm6*	Colim6‡
GLU	20.3	20.1
LYS	17.4	16.9
ASP	13.9	14.0
LEU	12.3	11.3
ALA	10.4	9.9
THR	6.7	7.6
GLY	3.4	3.9
ILE	3.0	2.8
ARG	4.2	3.9
SER	3.4	3.2
VAL	3.0	3.3
PRO	0.8	1.4
MET	0.3	0.6
TYR	0.2	0.2
PHE	0.1	0.3
HIS	—§	—
CYS	—	—
TRP	—	—

* Average of two determinations.
‡ Average of three determinations from two different lots.
§ No detectable amounts.

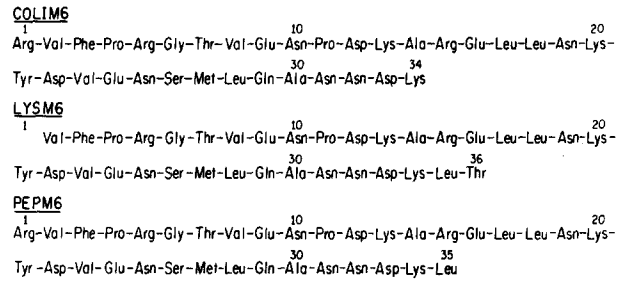

FIGURE 3. Comparison of the amino terminal sequence of three preparations of M6 protein. The amino terminal sequence of the ColiM6 protein is compared with the amino terminal region of purified M6 protein isolated from type 6 streptococci with phage lysin (LysM6) or pepsin (PepM6). The residue numbers in the LysM6 sequence are offset by 1 to correspond with the numbering of the ColiM6 and PepM6 sequences.

only a single residue was observed at each degradation step. The yield of the amino terminal residue at each step was that expected from the amount of protein applied to the sequencer. The amino terminal sequence of the ColiM6 molecule is identical to that of the M6 protein isolated from the streptococci either by limited pepsin digestion (PepM6) or by solubilization of the cell wall by phage lysin (LysM6) (Fig. 3), except for the amino terminal residue in the LysM6 molecule.

Biological Properties of the ColiM6 Molecule. As an indication of the immunogenicity of the purified ColiM6 protein, its ability to induce opsonic antibodies in rabbits was examined. The indirect bactericidal test revealed that after two immunizations, antibodies opsonic for type 6 streptococci are produced (Table III). The ColiM6 protein was also able to effectively remove opsonic antibodies from both human and rabbit opsonic serum (Table IV).

TABLE III

*Production of Rabbit Type 6 Opsonic Antibodies with Colim6 Protein**

	No. of colonies
Inoculum	32
Control‡	2,680
Preimmune serum	2,144
Immune serum	3

* The indirect bactericidal assay was performed with whole heparinized (10 U/ml) human blood (400 μl) as a source of phagocytic cells. A dilution of virulent type 6 streptococci (100 μl) was added in the presence or absence of test serum (100 μl). The tubes were sealed and rotated for 3 h at 37°C and the surviving organisms determined by pour plate method.

‡ The number of colony-forming units after 3 h of rotation with streptococci and blood alone.

TABLE IV

*Removal of Opsonic Antibodies from Rabbit and Human Serum with Colim6 Proteins**

	No. of colonies‡
Rabbit	
Inoculum	20
Control§	790
Rabbit serum:	
Unabsorbed	8
Absorbed with Colim6	620
Human	
Inoculum	7
Control§	1,040
Human serum:	
Unabsorbed	0
Absorbed with Colim6	650

* The indirect bactericidal assay is described in Table III.

‡ Representative results from several experiments.

§ The number of colony-forming units after 3 h of rotation with only blood and streptococci.

Discussion

Nearly all of the M6 protein synthesized by *E. coli* carrying the plasmid pJRS42.13 is accumulated within the periplasmic space. No immunoreactive M protein was found either on the surface of the *E. coli* (by immunofluorescence) or in the culture medium that had been concentrated 100-fold.

Because the ColiM6 protein was localized in the periplasm, which did not contain many other major proteins, purification of the M6 molecule was simplified. The final yield of purified ColiM6 protein was ~1 mg per liter of culture. It is difficult, however, to correlate this quantity with the amount present on the streptococcal surface because of the problems encountered in quantitatively removing M protein from the streptococcal cell wall.

When freshly extracted from the *E. coli* periplasmic space, immunoreactive M6 protein consisted of three closely spaced bands usually appearing with equal intensity on SDS polyacrylamide gels and immunoblot. In some preparations, however, the center band appeared more concentrated, while in others the upper band predominated. A small amount of immunoreactive protein, similar to that seen in the periplasm, was sometimes observed in the cytoplasm. In addition, a small quantity of a larger molecular weight immunoreactive protein, not found in the periplasm, was occasionally present within the cytoplasmic fraction. Whether or not this larger molecular species is a precursor form of the M protein molecules found in the periplasm is currently being investigated.

Since the purified ColiM6 protein was identical in mobility on SDS polyacrylamide gels to that in freshly lysed *E. coli*, it seems likely that little or no major degradation occurred during the purification process (see Fig. 1). It was observed, however, that in the purified ColiM6 preparation, the lowest of the three bands was more concentrated than the other two, while this was not the pattern in the freshly prepared crude periplasm. Whether the increase in concentration observed in the lower band is the result of partial degradation of the upper molecules, charge alterations during purification, or the preferential purification of the lower molecular weight species is not clear at present. The last hypothesis is least likely, since reactive proteins comparable to the two upper bands were not observed by immunoblot analysis of the other fractions during purification.

Multiple closely spaced bands of M protein have previously been observed when M6 was prepared from streptococci with phage lysin (9, 16), while the preparation of M protein obtained by secretion from streptococcal protoplasts or L-forms is a single band on SDS PAGE (10). Because of the differences in appearance on gels of M protein prepared from streptococci by different techniques, we cannot tell whether the banding observed in the ColiM6 preparation is due to modifications peculiar to the *E. coli*. Although the amino terminal region of the ColiM6 appears homogeneous, the occurrence of proteolytic cleavage at the carboxy-terminal region of the larger two proteins resulting in an increase of the lower molecular weight species cannot presently be ruled out.

Muscle tropomyosin, a fibrous molecule that is similar in structure to M proteins (2, 12), displays anomalous behavior on SDS gels. Tropomyosin is composed of two alpha-helical polypeptide chains, termed alpha and beta, each of which are 284 residues long and present at a 3.8:1 alpha/beta ratio in the rabbit skeletal molecule (34). The beta chain differs from the alpha in 39 residues,

most of which are conservative substitutions (35). Despite their identical size, the alpha chain may be separated from the beta chain on SDS gels. It has been suggested that the observed separation is based on the difference between phosphorylated and unphosphorylated species of tropomyosin (36), along with the differences in primary structure (37). In preliminary studies, we did not observe any changes in migration on SDS gels of the three ColiM6 proteins after phosphatase treatment. Thus, further studies will be required along with DNA sequence analysis of the M protein gene to clarify the nature of the multiple banding pattern.

M protein on the surface of the streptococcus is composed of dimers arranged as fibrillar coiled-coil molecules (2, 12). Extraction of the M protein with pepsin at pH 5.8 cleaves the molecule approximately at its center, leaving a portion of the M protein still attached to the cell surface (2). With the exception of the amino terminal residue of the LysM6 molecule, the amino terminal sequences of the ColiM6, LysM6, and PepM6 molecules are identical. This information, along with the knowledge that the PepM6 molecule is a fragment of the M protein derived from the whole cell (2), is evidence that the amino terminus of the M protein is distal to the streptococcal cell wall, and the carboxy-terminal region is involved in its attachment to the cell, as was previously suggested (2).

The fact that the LysM6 protein lacks the amino terminal residue present in the other two molecules suggests that an enzyme with the ability to cleave the amino terminal arginine may be present during the isolation of this molecule. Streptococcal proteinase is a papain-like sulfhydryl-dependent enzyme produced by most group A streptococci. This enzyme has the appropriate specificity to cleave the amino terminal arginine (38) under conditions used for the solubilization of the group A streptococcal cell wall with phage lysin (39).

The similarity in molecular size (16) and near identity of amino acid composition between the LysM6 and ColiM6 molecules indicates that the molecule synthesized in *E. coli* is likely to be the same as the complete M6 protein, as it exists on the streptococcus.

The finding that the amino terminal sequence of the ColiM6 molecule that is exported into the *E. coli* periplasmic space is the same as the M6 molecule isolated from the streptococcus, indicates that the process necessary to remove the amino terminal signal sequence during the export process has the same specificity in both organisms. No immunoreactive M protein could be isolated from the extracellular growth media or observed on the *E. coli* surface. This suggests that the newly synthesized M molecule does not have the structures necessary to penetrate the *E. coli* outer membrane.

We have previously shown by immunodiffusion experiments (16) that the ColiM6 molecule contains antigenic determinants similar to those present on M6 protein isolated from the streptococcus. Further analysis here revealed that the ColiM6 protein is also able to stimulate the production of opsonic antibodies to type 6 streptococci, as well as to remove type 6 opsonic antibodies from both rabbit and human serum. This indicates that the M protein molecule synthesized in *E. coli* and transported to the periplasm has the same immunologically functional determinants as those found on M molecules isolated from the streptococcus (6–8). These results also suggest that the coiled-coil conformation of the M

molecule (2) may be conserved in the *E. coli* preparation, however, physicochemical studies will be necessary to confirm this.

Summary

Type 6 streptococcal M protein produced by *E. coli* bearing plasmid pJRS42.13 (ColiM6) accumulates in the periplasmic space of this new host. No immunoreactive M protein was found either on the surface of the organism or in the culture medium. The ColiM6 protein was purified from the periplasm and the final preparation consisted of three protein bands of apparent molecular weight 55,000, 57,000, and 59,000. These three bands were identical in migration in SDS PAGE to that of the M protein present in freshly prepared crude periplasm. The amino acid composition of the ColiM6 protein was nearly identical to that of M protein isolated from streptococci with phage lysin (LysM6). Furthermore, except for the amino terminal residue of the LysM6 molecule, the amino terminal sequence of the ColiM6 molecule was identical to those of both LysM6 and M protein released from the streptococcus by limited peptic digestion (PepM6). These results reveal that the molecule produced in the *E. coli* and transported into the periplasm may be the complete M protein as it exists on the streptococcus. The results also indicate that the systems that process M protein for transport through the cytoplasmic membrane are similar in the streptococcus and *E. coli.*

The purified ColiM6 protein was able to remove opsonic antibodies from both human and rabbit serum, as well as to stimulate the production of opsonic antibodies in rabbits, indicating that the immunodeterminants on this molecule are the same as those found on streptococcal-derived M molecules.

The authors wish to thank Ms. Victoria Bengyak for the performance of the amino acid analysis and her excellent technical assistance in these studies. We also wish to thank Ms. Sheenah Mische for her expert help with the amino acid sequence analysis and Ms. Clara Eastby for her technical assistance in monoclonal antibody preparation.

Received for publication 29 November 1983.

References

1. Swanson, J., K. C. Hsu, and E. C. Gotschlich. 1969. Electron microscopic studies on streptococci. I. M antigen. *J. Exp. Med.* 130:1063.
2. Phillips, G. N., Jr., P. F. Flicker, C. Cohen, B. N. Manjula, and V. A. Fischetti. 1981. Streptococcal M protein: alpha-helical coiled-coil strecture and arrangement on the cell surface. *Proc. Natl. Acad. Sci. USA.* 78:4689.
3. Lancefield, R. C. 1962. Current knowledge of type-specific antibodies in man follow-inwing infection with group A streptococci. *J. Immunol.* 89:307.
4. Fox, E. N., and M. K. Wittner. 1965. The multiple molecular structure of M proteins of group A streptococci. *Proc. Natl. Acad. Sci. USA.* 54:1118.
5. Fox, E. N., and M. K. Wittner. 1969. New observations on the structure and antigenicity of the M proteins of the group A streptococci. *Immunochemistry.* 6:11.
6. Fischetti, V. A., E. C. Gotschlich, G. Siviglia, and J. B. Zabriskie. 1976. Streptococcal M protein extracted by non-ionic detergent. I. Properties of the antiphagocytic and type specific molecules. *J. Exp. Med.* 144:32.
7. Cunningham, M. W., and E. H. Beachey. 1974. Peptic digestion of streptococcal M

protein. I. Effect of digestion at suboptimal pH upon the biological and immunological properties of purified M protein extracts. *Infect. Immun.* 9:244.

8. Manjula, B. N., and V. A. Fischetti. 1980. Studies on group A streptococcal M proteins: purification of type 5 M protein and comparison of its amino terminal sequence with two immunologically unrelated M protein molecules. *J. Immunol.* 124:261.
9. Fischetti, V. A., J. B. Zabriskie, and E. C. Gotschlich. 1973. Physical, chemical and biological properties of type 6M protein extracted with purified streptococcal phage-associated lysin. *In* Streptococcal Disease and the Community. Michael J. Haverkorn, editor. Excerpta Medica, Amsterdam. p. 26.
10. van de Rijn, I., and V. A. Fischetti. 1981. Immunochemical analysis of intact M protein secreted from cell wall-less streptococci. *Infect. Immun.* 32:86.
11. Beachey, E. H., J. M. Seyer, and A. H. Kang. 1978. Repeating covalent structure of streptococcal M protein. *Proc. Natl. Acad. Sci. USA.* 75:3163.
12. Manjula, B. N., and V. A. Fischetti. 1980. Tropomyosin-like seven residue periodicity in three immunologically distinct streptococcal M proteins and its implications for the antiphagocytic property of the molecule. *J. Exp. Med.* 151:695.
13. Beachey, E. H., J. M. Seyer, and A. H. Kang. 1980. Primary structure of protective antigens of type 24 streptococcal M protein. *J. Biol. Chem.* 255:6284.
14. Manjula, B. N., S. M. Mische, and V. A. Fischetti. 1983. Primary structure of streptococcal pep M 5 Protein: absence of extensive sequence repeats. *Proc. Natl. Acad. Sci. USA.* 80:5475.
15. Manjula, B. N., A. S. Acharya, S. M. Mische, T. Fairwell, and V. A. Fischetti. 1984. Complete amino acid sequence of a biologically active 197 residue fragment of M protein isolated from type 5 streptococci. *J. Biol. Chem.* In press.
16. Scott, J. R., and V. A. Fischetti. 1983. Expression of streptococcal M protein in E. coli. *Science (Wash DC).* 221:758.
17. Ish-Horowicz, D., and S. F. Burke. 1981. Rapid and efficient cosmid cloning. *Nucleic Acid Res.* 9:2989.
18. Maniatis, T., E. F. Fritsch, and J. Sanbrook. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Publications, Cold Spring Harbor, NY. p. 68.
19. van de Rijn, I., J. B. Zabriskie, and M. McCarty. 1977. Group A streptococcal antigen cross reactive with myocardium. *J. Exp. Med.* 146:579.
20. Johnson, G. D., and G. M. del Nogueira Araujo. 1981. A simple method of reducing the fading of immunofluorescence during microscopy. *J. Immunol. Methods.* 43:349.
21. Brickman, E., and J. Beckwith. 1975. Analysis of the regulation of Eschericha coli alkaline phosphatase subunits using deletions and phi-80 transducing phages. *J. Mol. Biol.* 96:307.
22. Laemmli, U. K. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680.
23. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets. *Proc. Natl. Acad. Sci. USA.* 76:4350.
24. Blake, M., K. Johnston, G. Russell-Jones, and E. C. Gotschlich. 1984. A rapid sensitive method for detection of alkaline phosphatase conjugated antibody on western blots. *Anal. Biochem.* 136:175.
25. Lancefield, R. C. 1959. Persistence of type-specific antibodies in man following infection with Group A streptococci. *J. Exp. Med.* 110:271.
26. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Ramdall. 1951. Protein measurement with the folin phenol reagent. *J. Mol. Biol.* 193:265.
27. Engvall, E., and P. Pearlman. 1972. Enzyme-linked immunosorbent assay, ELISA.

III. Quantitation of specific antibodies by enzyme-linked anti-immunoglobulin in antigen coated tubes. *J. Immunol.* 109:129.

28. Fischetti, V. A. 1983. Requirements for the opsonic activity of human IgG directed to type 6 Group A streptococci: net basic charge and intact Fc region. *J. Immunol.* 130:896.
29. Köhler, G., and C. Milstein. 1976. Derivation of specific antibody-producing tissue culture and tumor lines by cell fusion. *Eur. J. Immunol.* 6:511.
30. Galfre, G., S. C. Howe, C. Milstein, G. W. Butcher, and J. C. Howard. 1977. Antibodies to major histocompatibility antigens produced by hybrid cell lines. *Nature (Lond.).* 266:550.
31. Potter, M., J. G. Pumphrey, and J. L. Walters. 1972. Growth of primary plasmacytomas in mineral oil–conditioned peritoneal environment. *J. Natl. Cancer. Inst.* 49:305.
32. Ey, P. L., S. J. Prouse, and C. R. Jenkins. 1978. Isolation of pure IgG1, IgG2a, and IgG2b immunoglobulins from mouse serum using protein A Sepharose. *Immunochemistry* 15:429.
33. Neu, H. C., and L. A. Heppel. 1965. The release of enzymes from Escherichia coli by osmotic shock and during the formation of spheroplasts. *J. Biol. Chem.* 240:3685.
34. Cummins, P., and S. V. Perry. 1973. The subunits and biological activity of polymorphic forms of tropomyosin. *Biochem. J.* 133:765.
35. Mak, A. S., L. B. Smillie, and G. R. Stewart. 1980. A comparison of the amino acid sequence of rabbit skeletal muscle alpha and beta tropomyosins. *J. Biol. Chem.* 255:3647.
36. Montarras, D., M. Y. Fiszman, and F. Gros. 1981. Characterization of the tropomyosin present in various chick embryo muscle types and in muscle cells differentiated in-vitro. *J. Biol. Chem.* 255:3647.
37. Bronson, D. D., and F. H. Schachat. 1982. Heterogeneity of contractile proteins: differences in tropomyosin in fast, mixed, and slow skeletal muscles in the rabbit. *J. Biol. Chem.* 257:3937.
38. Gerwin, B. I., W. H. Stein, and S. Moore. 1955. On the specifity of streptococcal proteinase. *J. Biol. Chem.* 241:3331.
39. Fischetti, V. A., E. C. Gotschlich, and A. W. Bernheimer. 1971. Purification and physical properties of group C streptococcal phage-associated lysin. *J. Exp. Med.* 133:1105.