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Molecular Analysis of the M Protein of *Streptococcus equi* and Cloning and Expression of the M Protein Gene in *Escherichia coli*

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A *Streptococcus equi* gene bank was constructed in the bacteriophage λgt11 cloning vector, and hybrid phage plaques were screened with *S. equi* M protein antiserum. A hybrid phage expressing the *S. equi* M protein (λgt11/SEM7) was identified and lysogenized into *Escherichia coli* Y1089. The cloned M protein appeared in immunoblots as three polypeptides with relative molecular weights of 58,000, 53,000, and 50,000. When reacted with *S. equi* M protein antiserum in an agar double-diffusion assay, the cloned M protein formed a line of identity with a protein in an acid extract of *S. equi*. Furthermore, λgt11/SEM7 protein inhibited opsonization of *S. equi* by antiserum to *S. equi* M protein. In addition, the recombinant protein expressed determinants of the antigen in the immune complexes of purpura hemorrhagica. Native M protein obtained from *S. equi* and recombinant M protein showed very similar molecular weight distributions on immunoblots, appearing as multiple closely spaced bands with molecular weights ranging from 52,000 to 60,000. Antisera prepared separately against each of the acid-extracted polypeptides shown to be important in serum bactericidal responses (molecular weight, 29,000) and nasopharyngeal local antibody responses (molecular weights, 41,000 and 46,000) of the horse each reacted with all three polypeptides in an acid extract. Moreover, antisera against protoplasts and against recombinant M protein of *S. equi* also reacted with these polypeptides. These results suggest that the entire M protein molecule of *S. equi* is present in these preparations and that the fragments in acid extracts carry overlapping segments.

Streptococcus equi causes strangles, a disease of the family Equidae characterized by inflammation of the upper respiratory tract and abscesses of the draining lymph nodes. The disease is found throughout the world and causes heavy economic losses despite the availability of vaccines based on bacterins or M protein-rich extracts. Lack of efficacy of vaccines is probably not due to failure to stimulate serum bactericidal antibodies but to failure to stimulate pharyngeal mucosal antibodies to the *S. equi* M protein (12, 29). Studies in our laboratory have shown that hot acid extracts of *S. equi* contain a series of defined M protein fragments which carry epitopes important in stimulation of bactericidal or mucosal antibodies (12, 31). A 29,000- to 30,000-M_r fragment carries determinants that induce bactericidal antibodies, and the epitopes involved in stimulation of mucosal antibodies are on 41,000- and 46,000-M_r fragments. In addition, recent studies in our laboratory have implicated the M protein of *S. equi* in the pathogenesis of purpura hemorrhagica, an immune-complex-mediated disease of the horse often seen as a sequela of strangles (11). Thus, the M molecule of *S. equi* is of some complexity, with regions that are functionally heterogeneous, analogous to the M molecules of group A streptococci, which have distinct antigenic sites with different immunologic functions (4, 8).

Unlike group A streptococci, for which at least 74 M types have been described (10), precipitin, passive protection, immunoblot, and Southern blot studies suggest that there is only one serotype of *S. equi* M protein (1, 23, 30; J. E. Galán and J. F. Timoney, submitted for publication). *S. equi* is, in fact, unique among the beta-hemolytic streptococci known to carry M proteins in the apparent antigenic stability of its M protein and in the rarity of a carrier state. Clearly, knowledge of the sequence of the M protein gene of *S. equi* and comparisons with the sequences of group A M protein genes such as the M6 gene (16) could provide valuable insight into the molecular basis of antigenic heterogeneity in streptococcal M proteins since DNA homology is known to exist between group A M protein genes and group C DNA (26). Moreover, comparison of the sequence of the M protein gene of *S. equi* with those of its close group C relative *S. zooepidemicus* might help reveal the molecular basis of the ability of the latter to maintain itself as a normal commensal on the pharyngeal mucosa of the horse. Conceivably, since M proteins have been demonstrated in *S. zooepidemicus* (J. F. Timoney and M. Muhktar, unpublished results), a counterpart of the epitope on the 41,000-dalton fragment of the *S. equi* M protein active in mucosal immunity does not exist on the *S. zooepidemicus* M molecules.

As a necessary prelude to the study of these questions, we describe in this paper for the first time both the characterization of the native form of the M protein of *S. equi* and the cloning and expression of its structural gene in *Escherichia coli*. The major immunologically reactive polypeptide fragments in acid extracts were shown to be immunologically cross-reactive with the native and recombinant M molecules and with the M protein antigen found in circulating immune complexes of purpura hemorrhagica in the horse.

MATERIALS AND METHODS

Bacterial strains, bacteriophage, and growth conditions. *S. equi* CF32 has been previously described (12). *E. coli* Y1088, Y1089, and Y1090 (34) were used for growing and screening λgt11 and λgt11 recombinant clones. *E. coli* BHB2688 and BHB2690, used for the preparation of packaging mixes, have been described by Hohn (15). Streptococcal strains were grown on Todd-Hewitt broth. *E. coli* strains were grown on

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L agar or in L broth. When appropriate, ampicillin was added to media at a concentration of 100 μg/ml.

Antigen preparation. (i) Acid extracts. The procedure of Lancefield and Pearlmann (21) was used to prepare acid extracts of *S. equi* CF32.

(ii) Native M protein. Native M protein was obtained as follows. *S. equi* CF32 was grown overnight at 37°C in 200 ml of Todd-Hewitt broth, and the cells were recovered by centrifugation at 7,000 × *g* for 15 min and washed twice with 20 mM phosphate buffer (pH 6). The cells were suspended in 3 ml of 50 mM phosphate buffer (pH 6) containing 10 mg of lysozyme (Sigma Chemical Co., St. Louis, Mo.) per ml, 30 μg of *N*-acetylmuramidase SG (Seikagaku Kogyo Co., Ltd., Tokyo, Japan) per ml, and 0.5 M sucrose, and the mixture was incubated at 37°C for 1 h. Finally, protoplasts were separated by centrifugation at 8,000 × *g*, and the supernatants, containing M protein, were used for subsequent studies.

(iii) *E. coli* lysates. Lysogens were grown at 30°C to an optical density at 600 nm of 0.4 to 0.6 and then incubated at 42°C for 20 min. When required, cultures were induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM and incubated with heavy aeration for an additional 3 to 5 h. Cells were recovered by centrifugation at 5,000 × *g* for 10 min and lysed by freeze-thawing in liquid nitrogen. DNase I (Sigma) was then added to a concentration of 20 μg/ml, and the lysate was incubated at room temperature for 15 min. Cell debris was separated by centrifugation at 10,000 × *g* for 20 min, and the supernatants were saved for further analysis.

Electroelution of polypeptides from SDS-polyacrylamide gel. Acid extracts of *S. equi* CF32 were separated on a sodium dodecyl sulfate (SDS)-polyacrylamide preparative gel (thickness, 1.5 mm). Protein bands corresponding to the 29,000-, 41,000-, and 46,000-M_r polypeptide fragments were located with the aid of a combination of prestained protein standards and Western blots (immunoblots) of a portion of the gel developed with *S. equi* convalescent-phase horse antiserum. The bands were cut out, placed in dialysis sacks, and electroeluted in 20 mM phosphate buffer (pH 6.8) in a TE 50 Transphor apparatus (Hoeffer Scientific Instruments). The eluted protein was concentrated by vacuum dialysis.

Antisera. Lysates from *E. coli* 1089(λgt11) and 1089(λgt11/SEM7) (approximately 500 μg of protein) were homogenized with an equal quantity of complete Freund adjuvant and inoculated subcutaneously into rabbits. Booster doses with incomplete Freund adjuvant were given 3 and 5 weeks later. Serum samples were taken at the first inoculation and a week after the last booster. Antiserum against protoplast M protein was prepared in a similar manner. Electroeluted protein of *S. equi* acid extracts (approximately 20 μg of protein) was homogenized with an equal quantity of complete Freund adjuvant and administered subcutaneously to guinea pigs. A booster dose was given 3 weeks later. Serum samples were obtained at the first inoculation and 2 weeks after the booster. Rabbit antiserum to *S. equi* M protein was prepared as described previously (31). Antiserum against immune complexes isolated from serum obtained from a horse with acute purpura hemorrhagica was produced in guinea pigs as described elsewhere (11). To remove cross-reacting antibodies, all sera were extensively absorbed with a lysate of *E. coli* Y1089(λgt11) prepared as described above and adsorbed onto nitrocellulose filters (Schleicher & Schuell, Inc., Keene, N.H.).

General procedures. Large quantities of λgt11 phage DNA were obtained from *E. coli* Y1088 by the procedures of Maniatis et al. (22) except that cells were grown in super L broth (35 g of tryptone, 20 g of yeast extract, 5 g of NaCl, and 5 ml of 1 N NaOH, each per liter). Recombinant phage DNA was obtained by the method of Ivanov and Gigova (17). Total cell DNA from streptococcal strains was obtained as follows. Cells were grown overnight in 200 ml of Todd-Hewitt broth, recovered by centrifugation at 10,000 × *g*, and washed twice with TE buffer (10 mM Tris [pH7.5], 1 mM EDTA). The cells were suspended in 2 ml of TE buffer containing 20 mg of lysozyme (Sigma) per ml and 50 μg of *N*-acetylmuramidase SG (Seikagaku Kogyo Co.) per ml and incubated at 37°C for 1 h. One milliliter of TE buffer containing 10 mg of preincubated pronase was added, and the mixture was incubated for 30 min at 37°C. The cells were lysed with 1 ml of a 20% SDS solution, adjusted to 50 mM NaCl, phenol extracted three times, chloroform extracted twice, and ether extracted several times. The DNA was finally purified by cesium chloride gradient centrifugation. Packaging mixes of bacteriophage lambda were prepared from *E. coli* BHB2688 and BHB2690 as described by Hohn (15). Restriction endonucleases were purchased from Bethesda Research Laboratories, Inc., Gaithersburg, Md., or Boehringer Mannheim Corp., New York, N.Y., and were used as recommended by the manufacturers.

Construction of phage libraries. *S. equi* CF32 was partially digested with *Eco*RI, and fragments ranging from 1 to 8 kilobase pairs were isolated on a sucrose gradient (22). λgt11 DNA was ligated with T4 DNA ligase (Boehringer Mannheim), digested with *Eco*RI, and treated with calf intestinal alkaline phosphatase (Boehringer Mannheim) before being ligated to the *Eco*RI fragments of *S. equi* DNA. The ligated DNA molecules were packaged into lambda phage particles as described previously (15).

Immunoscreening of recombinant phages. Recombinant phages were plated on *E. coli* Y1090 to give 100 to 300 plaques per plate. After the plates were incubated at 42°C for 4 to 6 h, nitrocellulose filters (82 mm; Schleicher & Schuell) previously impregnated with 10 mM IPTG were overlaid, and the plates were incubated at 37°C for an additional 8 to 12 h. The filters were then removed, washed in NET buffer (50 mM Tris, 150 mM NaCl, 5 mM EDTA, 0.25% [wt/vol] gelatin, 0.05% [wt/vol] Triton X-100 [pH 7.4]) for 1 h, and incubated with rabbit antiserum to *S. equi* M protein for an additional hour. The filters were then washed in NET buffer for 30 min, incubated with affinity-purified peroxidase-labeled goat anti-rabbit serum (Zymed Corp.) for 2 h, and developed with 1-chloro-4-naphthol. Plaques giving a positive signal were picked, suspended in SM medium (50 mM Tris [pH 7.5], 100 mM NaCl, 1 mM $MgSO_4$, 0.2% [wt/vol] gelatin) saturated with chloroform, and rescreened until all the plaques in the filter gave a positive signal. Recombinant phages were then lysogenized into *E. coli* Y1089 as described previously (34).

SDS-PAGE and immunoblotting. SDS-polyacrylamide gel electrophoresis (SDS-PAGE), immunoblotting, and staining with peroxidase-conjugated antibody were performed as described previously (12).

Immunodiffusion. Gel diffusion precipitin analysis was performed in a 1% agarose gel in 60 mM Tris-barbital buffer (pH 8.8). Plates were incubated at room temperature and read after 24 h.

Assay for bactericidal activity. The assay for bactericidal activity was performed with equine leukocytes as described previously (12).

Absorption of test serum. A 100-μl portion of a 1/10 dilution of rabbit antiserum to *S. equi* M protein was

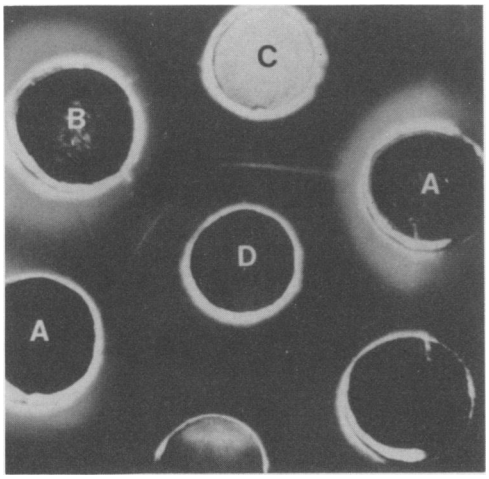

FIG. 1. Immunodiffusion reactions of lysates of *E. coli* 1089(λgt11) (spot A) and 1089(λgt11/SEM7) (spot B) and acid extract of *S. equi* (spot C) with *S. equi* M protein antiserum (D). Lysates of *E. coli* 1089(λgt11/SEM7) containing the M protein of *S. equi* formed a line of identity with the *S. equi* acid extract, whereas lysates of the negative control *E. coli* 1089(λgt11) did not show any reactivity.

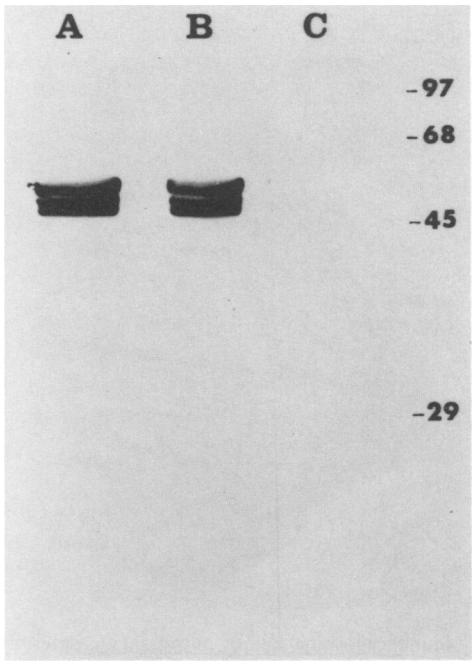

FIG. 2. SDS-PAGE and immunoblot analysis of IPTG-induced (lane A) and noninduced (lane B) *E. coli* 1089(λgt11/SEM7) lysates containing the M protein of *S. equi*. Lane C contained a lysate from the negative control *E. coli* 1089(λgt11). The blot was developed with *S. equi* M protein antiserum and peroxidase-labeled goat anti-rabbit serum. The numbers in the margin are molecular weights in thousands.

incubated with 100 μl of λgt11/SEM7 or λgt11 lysogen lysates (total protein concentration, 5 mg/ml) with slow rotation at 37°C for 1 h. Antigen-antibody complexes were separated by centrifugation at 10,000 × *g* for 20 min, and the supernatants were used in the assay for bactericidal activity.

Southern blotting and DNA hybridization. *Eco*RI-digested chromosomal DNA was transferred to nitrocellulose membranes as described by Southern (27). *S. equi* cloned DNA fragments were isolated from a low-temperature agarose gel (Seaplaque; FMC Corp., Marine Colloids Div., Rockland, Maine) as described by Struhl (28) and labeled with [^{32}P]dCTP by nick translation. Hybridization was performed as described elsewhere (22).

RESULTS

Cloning the M protein gene of *S. equi* in *E. coli*. Fragments (1 to 8 kilobase pairs) of *Eco*RI-digested *S. equi* CF32 total cell DNA were ligated into the *Eco*RI site of λgt11. After in vitro packaging of the DNA molecules, the phage particles were plated on *E. coli* Y1090 and screened for the production of *S. equi* M protein as described above. Approximately 95% of the plaques of the λgt11 library were β-galactosidase negative as determined on X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) plates and were therefore assumed to contain inserts. Approximately 10,000 plaques were screened, and 3 potentially positive clones were found, resuspended in SM medium saturated with chloroform, and rescreened. One was clearly expressing the *S. equi* M protein determinant upon rescreening and was lysogenized into *E. coli* Y1089. This hybrid phage recombinant was termed λgt11/SEM7.

Immunochemical characterization of λgt11/SEM7. λgt11/SEM7 lysates formed a line of identity with *S. equi* acid extract in the immunodiffusion assay when reacted with *S. equi* M protein antiserum (Fig. 1, wells B and C). *E. coli* Y1089(λgt11) lysates, on the other hand, formed no precipitin lines when reacted with anti-*S. equi* serum under the same conditions (Fig. 1, well A). Immunoblot analyses of IPTG-induced and noninduced λgt11/SEM7 lysates are shown in Fig. 2. The cloned M protein appeared as three closely spaced polypeptides with relative molecular weights of 58,000, 53,000, and 50,000 and other lower-molecular-weight bands (Fig. 2, lanes A and B). No difference in the level of expression of induced and noninduced lysogens was detected, indicating that transcription of the protein was independent of the β-galactosidase promoter present in λgt11 (Fig. 2, lanes A and B). *E. coli* Y1089(λgt11) lysates did not react with the *S. equi* M protein antiserum (Fig. 2, lane C). Opsonin absorption studies with λgt11/SEM7 lysates showed that the cloned M protein effectively removed opsonic antibodies from *S. equi* M protein antiserum (Table 1). *E. coli* 1089(λgt11) lysates did not absorb opsonic antibodies from the same antiserum (Table 1).

Purpuragenic determinants in λgt11/SEM7. To determine whether λgt11/SEM7 contained epitopes involved in the pathogenesis of purpura hemorrhagica, lysates were separated in an SDS-polyacrylamide gel, transferred to nitrocellulose sheets, and reacted with an antiserum made against immune complexes obtained from serum from a horse with acute purpura hemorrhagica. The antiserum made against

TABLE 1. Inhibition of opsonic activity of antiserum to *S. equi* M protein by extracts of *E. coli* 1089(λgt11/SEM7)

Treatment	CFU[a]
No absorption	6
E. coli 1089(λgt11)[b]	23
E. coli 1089(λgt11/SEM7)[c]	320
No opsonization	410

[a] CFU in an assay for bactericidal activity with horse neutrophils.
[b] Negative control.
[c] Carries the M protein gene of *S. equi*.

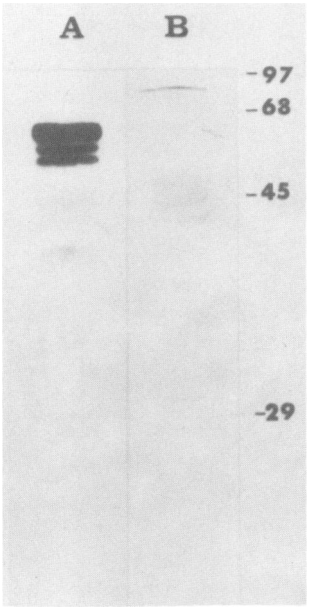

FIG. 3. Immunoblot analysis of purpuragenic determinants expressed by *E. coli* 1089(λgt11/SEM7). Lysates from *E. coli* 1089 (λgt11/SEM7) (lane A) and the negative control *E. coli* 1089(λgt11) (lane B) were separated on an SDS-polyacrylamide gel and then electrophoretically transferred to nitrocellulose. The blot was developed with an antiserum made against immune complexes obtained from a horse with acute purpura hemorrhagica. The numbers in the margin are molecular weights in thousands.

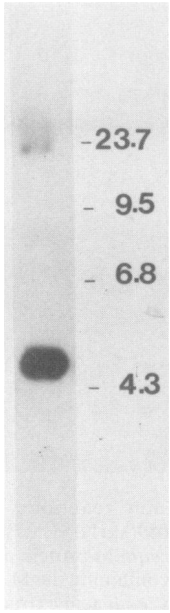

FIG. 4. Southern hybridization analysis of *Eco*RI total cell DNA digest of *S. equi* CF32. The probe was a [^{32}P]dCTP-labeled λgt11/SEM7 insert DNA fragment containing the *S. equi* M protein gene. The numbers in the margin are sizes in kilobase pairs.

immune complexes recognized the same polypeptides as those recognized by the *S. equi* M protein antiserum (Fig. 3, lane A). No reactivities were detected in the *E. coli* Y1089 (λgt11) lysate (Fig. 3, lane B).

Characterization of λgt11/SEM7 insert DNA. λgt11/SEM7 DNA was digested with *Eco*RI and separated on an agarose gel. The size of the insert was estimated as 5.1 kilobase pairs. The insert DNA was isolated from low-temperature agarose gel labeled with [^{32}P]dCTP, and used as a probe in Southern hybridization studies. The probe hybridized with one fragment of 5.1 kilobase pairs in *Eco*RI digests of total cell DNA of *S. equi* under stringent conditions (Fig. 4).

Native M protein. Digestion of the *S. equi* cell wall with lysozyme and *N*-acetylmuramidase SG (mutanolysin) was used to release the M protein. A Western blot of protoplast M protein developed with antiserum to *S. equi* M protein is shown in Fig. 5, lane B. A lysate of *E. coli* Y1089 (λgt11/SEM7) expressing the cloned *S. equi* M protein gene was included for comparison. Multiple bands were observed in the protoplast M protein as well as the recombinant M protein preparations. Most of the reactivity in the protoplast preparation was directed against two polypeptides with approximate relative molecular weights of 60,000 and 58,000. Lower-molecular-weight bands of 53,000 and 49,000 were less prominent. Additional higher-molecular-weight bands were also observed. In the recombinant M protein preparation only three bands, with approximate relative molecular weights of 58,000, 53,000, and 50,000, were visible (Fig. 5, lane A).

Reactivities of antisera against electroeluted polypeptides. Acid-extracted polypeptides of *S. equi* of 29,000, 41,000, and 46,000 M_rs were electroeluted from SDS-polyacrylamide gels, and antisera were prepared. Antiserum against the 29,000-M_r polypeptide recognized several polypeptides in acid extracts of *S. equi*, including the 46,000-, 41,000-, and 29,000-M_r polypeptide fragments (Fig. 6, lane A). Similar results were obtained with the antisera against the 41,000-M_r (lane B) and 46,000-dalton (lane C) polypeptides. In all

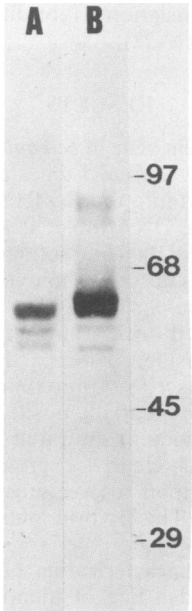

FIG. 5. SDS-PAGE and immunoblot analysis of *E. coli* 1089 (λgt11/SEM7) lysate containing *S. equi* recombinant M protein (lane A) and native M protein (lane B). The blot was developed with *S. equi* M protein antiserum and peroxidase-labeled affinity-purified goat anti-rabbit serum. The numbers in the margin are molecular weights in thousands.

instances, preinoculation sera showed no reactivities in Western blots with acid extracts of *S. equi*.

Reactivities of antisera against *S. equi* protoplast and recombinant M protein. The reactivities of the antisera against protoplast and recombinant M protein of *S. equi* are shown in Fig. 7, lanes A and B, respectively. Both antisera showed similar reactivities and recognized the 46,000-, 41,000-, and 29,000-M_r polypeptide fragments in addition to other bands. Preinoculation sera did not show any reactivities.

DISCUSSION

Although the presence of M-like proteins in *S. equi* has been previously described (2, 3, 6, 23, 31, 33), the information available about this molecule has been derived mostly from studies carried out with acid-extracted M protein and with culture supernatant protein. Thus, little is known about the native or nondenatured form of this molecule. To avoid the degradative effects of acid treatment, we used two muralytic enzymes to extract the M protein of *S. equi*. Most of the reactivity of the monospecific antiserum to *S. equi* M protein was directed against two closely spaced bands with approximate molecular weights of 60,000 and 58,000 (Fig. 5). Additional bands of significantly higher molecular weight were also observed in the protoplast M protein preparation. No clear explanation is available for the multiple banding pattern of the M protein of *S. equi*. Similar multiple banding patterns have been observed for the native M proteins of group A (10) and group G (18) streptococci and the group A M protein produced in *E. coli* (9, 19, 25). A recent study in our laboratory (31) has shown that both high- and low-molecular-weight forms of the M protein are released into the culture medium, the greatest reactivity being with polypeptides of 67,000 and 58,000 M_rs. It is possible that the presence of higher-molecular-weight polypeptides is explained by residual peptidoglycan associated with the M protein. Fischetti et al. (9) have shown that the bands of higher molecular weight on SDS-polyacrylamide gels of the *S. pyogenes* protoplast M protein as compared with those of the M protein produced in *E. coli* are due to the presence of various amounts of residual peptidoglycan derived from the covalent attachment of the M protein to the cell wall. No explanation is available, though, for the multiple banding pattern of *S. pyogenes* M protein produced in *E. coli* or the equivalent lower-molecular-weight bands of native M protein.

We cloned and expressed in *E. coli* the M protein gene of *S. equi*. The recombinant protein showed a precipitin line of identity with a protein in an acid extract of *S. equi* (Fig. 1, spots B and C) after reaction with rabbit antiserum specific for *S. equi* M protein. Furthermore, it absorbed opsonic antibodies from an opsonic serum (Table 1). In Western blot analysis, three closely spaced bands with molecular weights of 58,000, 53,000, and 50,000, along with other lower-molecular-weight bands, were observed (Fig. 2). No clear explanation is available for the multiple molecular weights of the M protein. The top three bands had mobilities similar to those of the native form of the M protein of *S. equi* (Fig. 2 and 5), which strongly suggests that the complete M protein

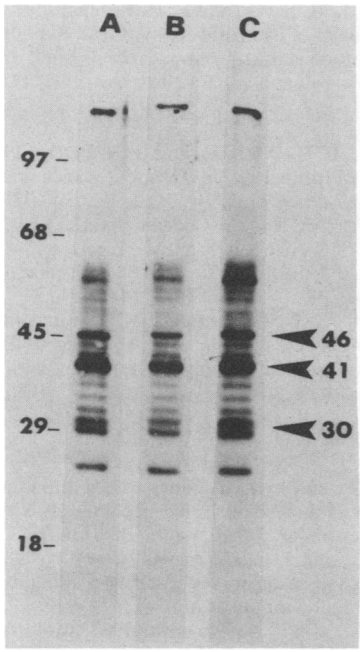

FIG. 6. Immunoblot showing reactivities of antisera made against the electroeluted polypeptides. Acid extracts of *S. equi* were separated on an SDS-polyacrylamide gel, and proteins were electrophoretically transferred to nitrocellulose membranes. Development was with antisera against the 29,000-M_r polypeptide (lane A), the 41,000-M_r polypeptide (lane B), and the 46,000-M_r polypeptide (lane C). The arrowheads indicate the locations of the 30,000-, 41,000-, and 46,000-M_r polypeptides. The numbers on the left (molecular weights in thousands) indicate the positions of the molecular weight standards.

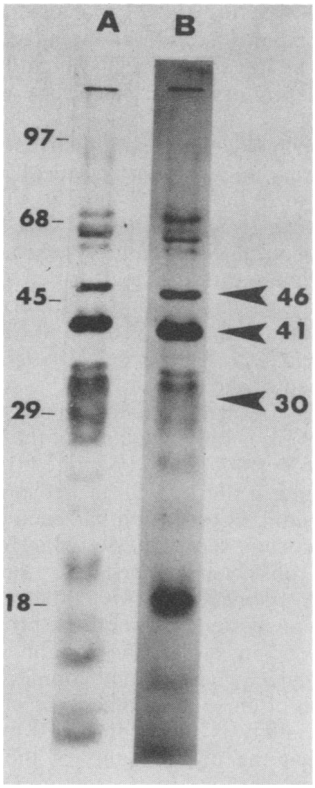

FIG. 7. Immunoblot showing reactivities of antisera made against native (lane A) and recombinant (lane B) M protein. In both lanes the antigen was an acid extract of *S. equi*. The arrowheads indicate the locations of the 30,000-, 41,000-, and 46,000-M_r polypeptides. The numbers on the left (molecular weights in thousands) indicate the positions of the molecular weight standards.

gene was cloned, because the M molecule extracted from protoplasts can be considered to be the intact form of the M protein (9, 32). The lower-molecular-weight bands probably represent degradation products, since they were much less apparent in fresh preparations (data not shown). Interestingly, very similar molecular weight distributions have been observed for the group A streptococcal M protein types 5 and 6 expressed in *E. coli* (9, 19, 24, 25). Furthermore, we have shown that λgt11/SEM7 shares DNA homology with group A streptococcal M protein types 5, 6, and 12 in Southern hybridization studies performed under stringent conditions (13; J. E. Galán, Ph.D. thesis, Cornell University, Ithaca, N.Y., 1986). Similarly, Scott et al. (26) have shown that the M protein type 6 gene of *S. pyogenes* hybridizes with group C streptococcal DNA.

Our Southern hybridization data are consistent with the hypothesis that there is only one copy of the M protein gene in the genome of *S. equi*, since the cloned fragment hybridized with one band in the *Eco*RI chromosomal digest of *S. equi* (Fig. 4). A similar conclusion has been reached for the group A streptococcus serotype M6 (26). However, Kehoe et al. (19) have observed at least two copies of the M5 protein gene in *S. pyogenes*.

We have previously demonstrated the presence of the M protein of *S. equi* in circulating immune complexes of purpura hemorrhagica, an immune-complex-mediated disease of the horse often seen as a sequela of strangles (11). The immunoblot analysis shown in Fig. 3 provides conclusive evidence that the determinants of the M protein involved in this disease are expressed in *E. coli*. Epitope mapping and sequencing of the recombinant M protein will help to better define the determinants involved in purpura hemorrhagica.

Protection against *S. pyogenes* infections in humans is considered to be mediated by serum opsonic antibodies to the M protein (20). In the horse, protection against *S. equi* infections more closely correlates with mucosal nasopharyngeal immunoglobulin A and immunoglobulin G antibodies to specific polypeptide fragments, with molecular weights of 41,000 and 46,000, in acid extracts (12, 14) than with serum bactericidal antibodies (29). Determinants of the M protein able to induce bactericidal antibodies are present on a 29,000- to 30,000-M_r polypeptide fragment in acid extracts of *S. equi*. Antisera against the recombinant and protoplast *S. equi* M proteins recognized several polypeptide fragments in acid extracts of *S. equi*, including those of 46,000, 41,000, and 29,000 M_r (Fig. 7). The similarities in the specificity of these antisera and the antisera made against the different polypeptide fragments eluted from preparative polyacrylamide gels (Fig. 6) suggests that the 46,000-, 41,000-, and 29,000-M_r polypeptides share regions of homology and are simply overlapping fragments of the same molecule. Moreover, these results further indicate that the entire M protein is produced by λgt11/SEM7.

Although the presence of an M-like protein has been well documented (6, 23, 31, 33), its role in the virulence of *S. equi* is less clear. Unlike the situation with group A streptococci, no M protein-negative *S. equi* strain that could be used to study its role in virulence has been isolated. The fact that protection against strangles in the horse correlates with the presence of mucosal nasopharyngeal antibodies to acid-extracted polypeptides with molecular weights of 41,000 and 46,000 (12, 14) and not with serum bactericidal antibodies suggests that determinants on these fragments of the M protein may have an important role in the initial stages of infection when the organism is adhering to or penetrating the nasopharyngeal or buccal mucosa, a phase of pathogenesis that probably does not involve the antiphagocytic properties of the M protein.

Knowledge of the nucleotide sequence of the M protein gene and hence the structure of the M protein will allow prediction of the structures of the epitopes involved in nasopharyngeal antibody responses. It should also provide an insight into why the *S. equi* M protein, unlike its group A (10) and G (18) streptococcal counterparts, is antigenically homogeneous and presents no size variation (Galán and Timoney, submitted). Finally, it will be helpful to determine the precise locations and amino acid sequences of the purpuragenic and opsonic determinants of the M protein.

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