

## Relative Contributions of Hyaluronic Acid Capsule and M Protein to Virulence in a Mucoid Strain of the Group A *Streptococcus*

ALLON E. MOSES,<sup>1\*</sup> MICHAEL R. WESSELS,<sup>2,3</sup> KARINE ZALCMAN,<sup>4</sup> SEBASTIÁN ALBERTÍ,<sup>2</sup>  
SHIRA NATANSON-YARON,<sup>1</sup> TEHILA MENES,<sup>4</sup> AND EMANUEL HANSKI<sup>4</sup>

*Department of Clinical Microbiology and Infectious Disease, Hadassah University Hospital,<sup>1</sup> and Department of Clinical Microbiology, Hebrew University-Hadassah Medical School,<sup>4</sup> Jerusalem, Israel, and Channing Laboratory, Brigham and Women's Hospital,<sup>2</sup> and Division of Infectious Diseases, Beth Israel Hospital,<sup>3</sup> Harvard Medical School, Boston, Massachusetts*

Received 22 May 1996/Returned for modification 10 July 1996/Accepted 9 October 1996

**The antiphagocytic effect of M protein has been considered a critical element in virulence of the group A streptococcus. The hyaluronic acid capsule also appears to play an important role: studies of an acapsular mutant derived from the mucoid or highly encapsulated M protein type 18 group A streptococcal strain 282 indicated that loss of capsule expression was associated with decreased resistance to phagocytic killing and with reduced virulence in mice. To study directly the relative contributions to virulence of M protein and the hyaluronic acid capsule in strain 282, we inactivated the gene encoding the M protein (*emm18*) both in wild-type strain 282 and in its acapsular mutant, strain TX72. Inactivation of *emm18* was accomplished by integrational plasmid mutagenesis, using the temperature-sensitive shuttle vector pJRS233 harboring a 5' DNA segment of *emm18*. As reported previously, wild-type strain 282 was resistant to phagocytic killing in vitro, both in whole human blood and in 10% serum. The capsule mutant TX72 was highly susceptible to phagocytic killing in 10% serum and moderately sensitive in whole blood. The M protein mutant 282KZ was highly susceptible to phagocytic killing in blood but only moderately sensitive in 10% serum. The double mutant TX74 was sensitive to killing in both conditions. In a mouse infection model, the 50% lethal dose was increased by 60- and 80-fold for the capsule and double mutants, respectively, compared with that of strain 282, but only by 6-fold for the M protein mutant. Integration of the strain 282 capsule genes into the chromosome of a nonmucoid M1 strain resulted in high-level capsule production and rendered the transformed strain resistant to phagocytic killing in 10% serum. These results provide further evidence that the hyaluronic acid capsule confers resistance to phagocytosis and enhances group A streptococcal virulence. The results suggest also that assessment of in vitro resistance to phagocytosis in 10% serum rather than in whole blood may be a more accurate reflection of virulence in vivo of group A streptococci.**

The group A streptococcus (GAS), or *Streptococcus pyogenes*, continues to be a cause of major morbidity and mortality throughout the world. Rheumatic fever, a postinfectious sequela, is the leading cause of acquired heart disease in young people in developing countries. In addition to the resurgence of acute rheumatic fever (36, 37), an increase in the incidence of GAS bacteremia and streptococcal toxic shock syndrome has recently been described (35). Recent publicity generated in response to several cases of necrotizing fasciitis and the fact that the incidence of severe GAS diseases is increasing have suggested an increase in the virulence of GAS (18, 35). Renewed interest in GAS disease has stimulated efforts to define the roles that different virulence factors play in pathogenesis of streptococcal diseases.

M protein is a fibrillar, coiled-coil structure, anchored in the cell wall and extending from the surface of the streptococcus (14, 33). M protein has been considered to be the major virulence factor of GAS (20). M protein-positive strains survive and replicate when incubated in whole blood, whereas M protein-negative strains are readily killed. This property has become a functional definition of an M protein-producing strain. Antibodies directed to the N-terminal portion of the M molecule opsonize M protein-positive strains, rendering them sen-

sitive to phagocytosis. Clinical isolates of GAS almost always produce M protein, and patients recovering from streptococcal infections have anti-M protein antibodies (33). M protein-rich strains often appear mucoid, although Wilson showed that the mucoid colony morphology is a function of capsule production and not of M protein (41). The observation that spontaneous variants of GAS lose M proteins and convert from mucoid to nonmucoid colonies, and that passage through mice restores M protein expression and leads to recovery of mucoid appearance, suggests that these two virulence factors may be jointly regulated by environmental signals. Transcription of *emm* is regulated by the product of the *mga* gene (previously called *mry* or *vir*), but capsule expression appears not to be (5, 7, 24, 29).

Polysaccharide capsules have been shown to facilitate survival of many pathogenic bacteria by interfering with antibodies, complement, and phagocyte-mediated host defense mechanisms (4). Mucoid or highly encapsulated strains of GAS have been implicated as causing unusually severe infections and have been associated with outbreaks of acute rheumatic fever (22). The GAS capsule is composed of hyaluronic acid, a linear polymer of glucuronic acid and *N*-acetylglucosamine repeating units. The genetic locus responsible for hyaluronic acid synthesis (*has*) is composed of at least three genes, *hasA*, *hasB*, and *hasC*, which form an operon. The first gene in the operon, *hasA*, encodes hyaluronate synthase (11, 12); the second gene, *hasB*, encodes UDP-glucose dehydrogenase (13); and the third gene, *hasC*, possesses sequence homology to UDP-glucose py-

\* Corresponding author. Mailing address: Hadassah University Medical Center, P.O. Box 12000, Jerusalem 91120, Israel. Phone: 9722 6777111. Fax: 9722 6434434. E-mail: MOSESA@md2.huji.ac.il.

TABLE 1. Oligonucleotides used for PCR analysis

| Primer <sup>a</sup> | Sequence                              | Location of target region        | Reference |
|---------------------|---------------------------------------|----------------------------------|-----------|
| F1                  | 5'-AAGTTAATGTATGTAAGTA-3'             | 5' end of <i>mga</i>             | 27        |
| F2                  | 5'-GCAAAGAAGAAAATAAGCT-3'             | 3' end of <i>emm</i>             | 28        |
| F3                  | 5'-ATAAGGAGCATAAAAAATGGCT-3'          | 5' end of <i>emm</i>             | 28        |
| F4                  | 5'-AACAGCTATGACCATG-3'                | Reverse of pUC                   |           |
| R1                  | 5'-AGCCAATTTTATGCTCCTTAT-3'           | 5' end of <i>emm</i>             | 28        |
| R2                  | 5'-AGGTCTTGTAGCTGTTTGTAGCAGCTCTACC-3' | PG of <i>emm2.2</i> <sup>b</sup> | 15        |
| R3                  | 5'-AATGGCAAGTTTATCAAATGG-3'           | 5' end of <i>scpA</i>            | 26        |
| R4                  | 5'-AGCTTAGTTTTCTTCTTTGCG-3'           | 3' end of <i>emm</i>             | 28        |
| R5                  | 5'-GTA AAAACGACGCCAGT-3'              | -20 of pUC                       |           |

<sup>a</sup> F, forward; R, reverse.

<sup>b</sup> PG, peptidoglycan-spanning domain.

rophosphorylase (9). Both encapsulated and unencapsulated strains of GAS possess intact *has* loci, and it appears that the expression of the hyaluronic acid capsule is transcriptionally controlled (9).

Previous studies of strain TX72, an unencapsulated Tn916-derived mutant in which *hasA* was interrupted (39), provided evidence that the hyaluronic acid capsule served an important virulence function. TX72 had reduced virulence in mice compared with the wild-type parent strain 282, a mucoid M protein type 18 strain, although TX72 produced a similar amount of M protein as the parent strain did (40). To assess the relative contribution to virulence of M protein and the hyaluronic capsule, we inactivated *emm18* both in strain 282 and in its acapsular mutant TX72. Examination of the mutants in opsonophagocytic assays in vitro suggested that although M protein was the major determinant of growth in whole blood, the capsule was more important in resistance to phagocytosis in 10% serum and in virulence of GAS in a mouse infection model.

#### MATERIALS AND METHODS

**Bacterial strains and media.** The mucoid M protein type 18 GAS strain 87-282 (herein called strain 282) and its Tn916-derived acapsular mutant, TX72, were described previously (39, 40). Inactivation of *emm18* in strains 282 and TX72 (see below) generated the mutants 282KZ and TX74, respectively. M protein type 1 GAS strain 049 (provided by Dennis Stevens) was isolated from a patient with streptococcal toxic shock syndrome. Strain 049(200i) is a mucoid mutant which was derived from strain 049 as described below. *Escherichia coli* DH5 $\alpha$  (32) was used for molecular cloning experiments. GAS strains were grown in Todd-Hewitt broth (Difco, Detroit, Mich.) supplemented with 0.2% yeast extract (THY) or on solid medium containing THY, supplemented with 1.8% Bacto Agar and 5% (vol/vol) defibrinated sheep blood. *E. coli* was grown in Luria-Bertani broth (34). Antibiotics were used at the following concentrations: erythromycin at 750  $\mu$ g ml<sup>-1</sup> and ampicillin at 100  $\mu$ g ml<sup>-1</sup> for *E. coli*; erythromycin at 1  $\mu$ g ml<sup>-1</sup> and tetracycline at 5  $\mu$ g ml<sup>-1</sup> for GAS.

**DNA techniques.** Standard procedures were employed for plasmid cloning experiments (32). Plasmid DNA was purified with Mini or Midi kits from Qiagen (Hilden, Germany). Restriction endonucleases and ligases were used as described in the manufacturers' directions.

Polymerase chain reactions (PCRs) were performed with a thermocycler (M. J. Research) as detailed elsewhere (23). Briefly, for the cloning of *emm18* and analysis of GAS transformants, a simple and a rapid procedure that allows the amplification of specific genes from relatively crude preparations of GAS whole-cell lysates was used (17). *emm18* was amplified from strain 282 by use of all-M forward and reverse primers designed for the amplification of *emm* genes encoding M proteins of various serotypes (28). To facilitate further manipulations, *NotI* sites were incorporated into all-M primers (F3 and R4) (Table 1) and the amplified product was purified with a Wizard kit (Promega, Madison, Wis.). The purified product was digested with *NotI* and cloned into pGEM-5zf (Promega), which was cleaved with the same enzyme. Clones were selected for ampicillin resistance, and one clone containing the appropriate plasmid size (pGEM5-M18) was chosen for further use. PCR analysis of pGEM5-M18 using primers F3 and R4 detected an amplified product of a size identical to that amplified from GAS strain 282 by use of the same primers. The DNA sequence of the 5' end of the insert in pGEM5-M18 was determined by automated DNA sequencing (Applied Biosystems) with primer F3. The sequence obtained for the first 200 nucleotides was 97% identical to that reported by Podbielski et al. for the 5' end of the

*emm18* gene and 99% identical to that reported by Dale et al. (10, 28). For insertional inactivation of *emm18* in GAS strains 282 and TX72, an *EcoRV-HindIII* 1-kb fragment of pGEM5-M18 containing a 5' segment of *emm18* was cloned into the temperature-sensitive *E. coli*-streptococcus shuttle vector pJRS233 (25) to yield the plasmid pM18KZ (Fig. 1A). This plasmid was introduced into strain 282 or TX72 by electroporation, and erythromycin-resistant transformants were isolated at the nonpermissive temperature (37°C) as described previously (25). Growth of transformants at the nonpermissive temperature resulted in the integration of the plasmid through the *emm18* homologous DNA sequences found on both the plasmid and the chromosomal DNA and in the formation of inactive *emm18* alleles (see Results).

To confirm that integration occurred as depicted in Fig. 1A, a PCR analysis using the primers F3 and R4 and primers -20 (R5) and reverse (F4) of pUC (contained in pJRS233) was performed (Table 1; Fig. 1).

For Southern hybridization analysis, chromosomal DNA was prepared from GAS strains following their growth in THY supplemented with 20 mM glycine, as described by Caparon and Scott (6). Probes were labeled by the random-primed DNA method using digoxigenin-11-dUTP (DIG)-tagged oligonucleotides (Boehringer Mannheim). Gel electrophoresis, DNA transfer to positively charged nylon membranes, hybridization, and detection of labeled bands by the DIG LumiPhos detection kit were performed as described in the manufacturer's instructions (Boehringer Mannheim).

The structure of the *mga* locus was determined by PCR analysis using the sets of primers designated in Table 1 and shown in Fig. 2.

Conversion of the nonmucoid M protein type 1 GAS strain 049 to the mucoid derivative 049(200i) was achieved by transforming 049 with the plasmid pSAS200 and then integrating the plasmid into the chromosome. Briefly, this plasmid is a derivative of the plasmid pDY40 comprising a DNA insert containing *hasA*, *hasB*, and a 3-kb upstream segment derived from the strain 282 chromosome, a tetracycline resistance gene plus origin of replication derived from the plasmid pACYC184, and an *EcoRI* fragment of pJRS233 containing the erythromycin resistance gene and the temperature-sensitive origin of replication for gram-positive bacteria (1). Transformation of GAS strain 049 by pSAS200 and selection of erythromycin-resistant chromosomal integrants at the nonpermissive temperature were performed as described above. All resulting transformants were found to be highly mucoid. One such integrant, designated 049(200i), was selected for further study.

**Determination of M protein.** M protein was extracted by HCl treatment of bacterial cells as described by Lancefield (19). Protein extracts were subjected to sodium dodecyl sulfate-11% polyacrylamide gel electrophoresis and transferred to a nitrocellulose filter. M protein was detected by use of purified rabbit anti-synthetic-peptide antibodies directed against the common C-repeat M protein peptide, i.e., anti-SM5(265-291) (kindly provided by David L. Hasty, University of Tennessee).

**Determination of hyaluronic acid capsule production.** To determine the amount of hyaluronic acid capsule produced by each GAS strain, cells from a 10-ml exponential-phase culture were washed twice with water and then suspended in 0.5 ml of water. Capsule was released to the aqueous phase by adding 1 ml of chloroform and then shaking the mixture vigorously. After clarifying the sample by centrifugation, the hyaluronic acid content of the aqueous phase was determined by measuring absorbance at 640 nm after adding to the sample 2 ml of a solution containing 20 mg of 1-ethyl-2-[3-(1-ethylnaphtho-[1,2-d]thiazolin-2-ylidene)-2-methylpropenyl]naphtho-[1,2-d]thiazolium bromide (Stains-all; Sigma) and 60  $\mu$ l of glacial acetic acid in 100 ml of 50% formamide. Absorbance values were compared with a standard curve generated with known concentrations of hyaluronic acid (2, 16).

**Phagocytic assays.** The ability of GAS strains to survive in the blood of a nonimmune individual was tested by the direct bactericidal test of Lancefield (21): 600  $\mu$ l of freshly drawn heparinized human blood was mixed with 300  $\mu$ l of GAS grown to the exponential phase and diluted in THY to a concentration of 300 to 900 CFU ml<sup>-1</sup> so that the final concentration of bacteria in the assay was approximately 100 to 300 CFU ml<sup>-1</sup>. The mixture was incubated at 37°C for 3 h

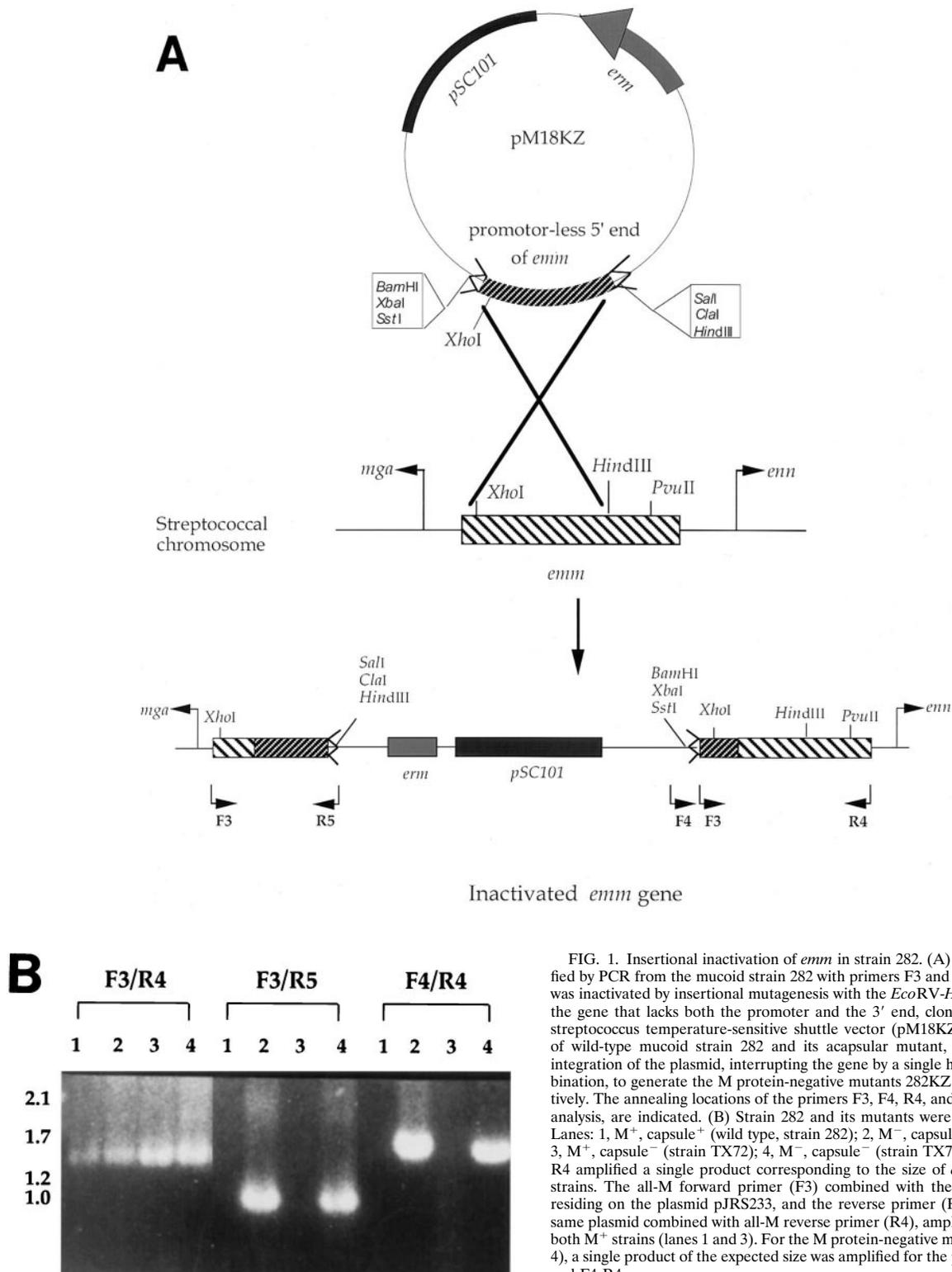


FIG. 1. Insertional inactivation of *emm* in strain 282. (A) *emm18* was amplified by PCR from the mucoid strain 282 with primers F3 and R4. The M18 gene was inactivated by insertional mutagenesis with the *EcoRV-HindIII* fragment of the gene that lacks both the promoter and the 3' end, cloned into an *E. coli*-streptococcus temperature-sensitive shuttle vector (pM18KZ). Transformation of wild-type mucoid strain 282 and its acapsular mutant, TX72, resulted in integration of the plasmid, interrupting the gene by a single homologous recombination, to generate the M protein-negative mutants 282KZ and TX74, respectively. The annealing locations of the primers F3, F4, R4, and R5, used for PCR analysis, are indicated. (B) Strain 282 and its mutants were analyzed by PCR. Lanes: 1, M<sup>+</sup>, capsule<sup>+</sup> (wild type, strain 282); 2, M<sup>-</sup>, capsule<sup>+</sup> (strain 282KZ); 3, M<sup>+</sup>, capsule<sup>-</sup> (strain TX72); 4, M<sup>-</sup>, capsule<sup>-</sup> (strain TX74). Primers F3 and R4 amplified a single product corresponding to the size of *emm18* in the four strains. The all-M forward primer (F3) combined with the -20 primer (R5) residing on the plasmid pJRS233, and the reverse primer (F4) residing on the same plasmid combined with all-M reverse primer (R4), amplified no product in both M<sup>+</sup> strains (lanes 1 and 3). For the M protein-negative mutants (lanes 2 and 4), a single product of the expected size was amplified for the primer pairs F3-R5 and F4-R4.

with end-over-end rotation. Aliquots were plated for quantitative culture at the beginning and end of the 3-h incubation.

Phagocytic killing of GAS in 10% human serum was assayed by the method of Baltimore et al. (1a) with modifications as described previously (40), except that human serum was not absorbed with bacterial cells prior to use. Briefly, approximately  $6 \times 10^6$  bacterial cells from an exponential-phase broth culture were mixed with  $6 \times 10^6$  peripheral blood leukocytes and 10% (vol/vol) human serum,

as a complement source, in 0.5 ml of Dulbecco's modified Eagle's medium. Phagocytic killing was calculated on the basis of quantitative cultures of 25- $\mu$ l aliquots withdrawn from the assay mixture immediately, and after 1 h of end-over-end rotation at 37°C. Mean log kill for each strain was calculated on the basis of log kill values for each experiment.

**Mouse i.p. challenge model.** Mouse lethality studies after intraperitoneal (i.p.) challenge with GAS were performed as described previously (40). Briefly, female

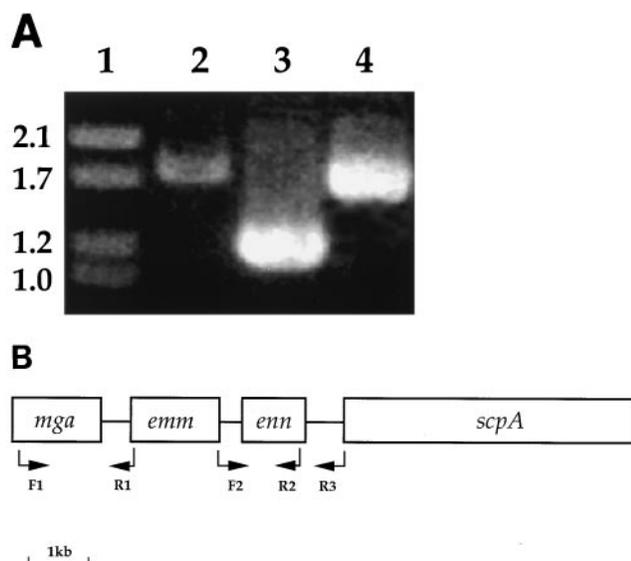


FIG. 2. Gene cluster of *emm18* in strain 282. (A) Lanes: 1, molecular weight standards; 2 to 4, PCR-amplified products obtained with primer pairs F1-R1, F2-R2, and F2-R3, respectively. The numbers on the left are molecular sizes (in kilobases). (B) Schematic map of the gene cluster of *emm18* in strain 282 derived according to data shown in panel A. The *emm18* and *scpA* genes are separated by a 1.7-kb segment, consistent with the presence of *enn18*.

ICR mice (six mice per group) weighing 20 to 30 g were injected i.p. with 10-fold dilutions of exponential-phase organisms. Mortality was determined 3 days after challenge. The 50% lethal dose ( $LD_{50}$ ) was calculated (31) by pooling results of two to five independent experiments.

## RESULTS

**Inactivation of the gene encoding M protein in strains 282 and TX72.** Inactivation of the *emm18* gene in the highly encapsulated strain 282 and its acapsular derivative strain, TX72, was accomplished by integrational plasmid mutagenesis using pM18KZ (Fig. 1A). This plasmid was constructed by cloning a 1-kb *EcoRV-HindIII* DNA segment coding for the 5' portion of *emm18* into the *E. coli-streptococcus* shuttle vector pJRS233, which is temperature sensitive for replication in gram-positive bacteria (25). *emm18* was amplified by PCR using all-M primers (F3 and R4) that were constructed for the amplification of M proteins of various serotypes (28). These primers take advantage of nearly identical sequences of *emm* genes at their 5' ends (consisting of the Shine-Dalgarno sequence and the two first codons of the gene) and at the 3' ends (consisting of the last five codons, the stop codon, and three nucleotides past the stop codon) (28). Thus, the amplified *EcoRV-HindIII* segment lacked DNA sequences 5' to the coding sequence of the gene, including the promoter region and any potential upstream regulatory sequences. Transformation of 282 and TX72 by pM18KZ, followed by propagation of the transformants at the nonpermissive temperature with erythromycin selection, resulted in the integration of the plasmid via homologous recombination through the common *EcoRV-HindIII* segment, present on both the plasmid and the chromosome (Fig. 1A). The integration created two truncated *emm18* genes, one containing an intact upstream region of *emm18* but lacking the 3' portion downstream of the *HindIII* site, and the other containing the complete *emm18* coding sequence but lacking the upstream region including the promoter and any potential regulatory sequences. To confirm that pM18KZ had integrated at the expected site in the 282 and TX72 chromosomes, we per-

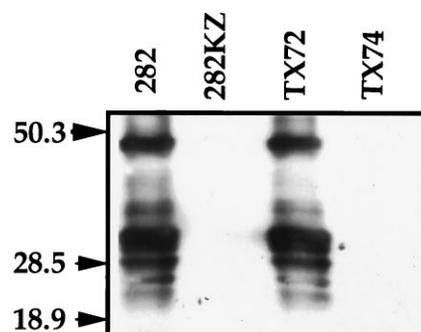


FIG. 3. Western blot of cell extracts of strains 282, 282KZ, TX72, and TX74 probed with purified rabbit antibodies directed against the conserved portion of M protein. Two micrograms of total bacterial protein extract from each strain was loaded onto each lane. The numbers indicated by the arrows represent the sizes of molecular mass markers in kilodaltons.

formed PCR analysis (Fig. 1B) with three sets of primer pairs, F3-R4, F3-R5, and F4-R4 (Table 1). The regions to which each primer anneals are indicated in Fig. 1A. The F3-R4 pair amplified a single product of 1.45 kb in all strains (Fig. 1B) corresponding to the size of *emm18*. As expected for both of the M protein-positive strains, 282 and TX72 (lanes 1 and 3), no amplified bands were visible when the last two pairs of primers were used. In contrast, for the M protein-negative mutants, 282KZ and TX74 (lanes 2 and 4), the last two pairs of primers amplified products of the expected sizes (Fig. 1B). To reconfirm that pM18KZ had integrated at the expected site, we also performed Southern blot analysis. Chromosomal DNA of strains 282, 282KZ, TX72, and TX74 was digested with *HindIII*, *HindIII-XhoI*, and *PvuII-XhoI* and probed with a labeled *EcoRV-HindIII* DNA segment of *emm18*. The analysis detected the expected number of DNA fragments of the appropriate size (data not shown).

To map the *emm* gene cluster in strain 282, we used PCR analysis as illustrated by Fig. 2A. The primer pairs F1-R1 (lane 2), F2-R2 (lane 3), and F2-R3 (lane 4) generated bands with sizes of 1.8, 1.2, and 1.7 kb, respectively. This result is consistent with the presence of an *enn* gene located between *emm* and *scpA* (Fig. 2B). Similar results were demonstrated recently for this strain by Bessen et al. (3). PCR analysis of the mutants 282KZ and TX74 using the primer pairs F2-R2 and F2-R3 produced bands with sizes similar to those observed for strain 282, indicating that the *enn18* gene was not disrupted by the insertional mutagenesis of *emm18* (data not shown).

To demonstrate that strains 282KZ and TX74 lost their ability to express type 18 M protein, we examined Lancefield extracts from these strains by Western blotting using purified rabbit anti-synthetic-peptide antibodies directed against the common C-repeat M protein peptide [anti-SM5(265-291)]. As shown in Fig. 3, the antiserum recognized the same pattern of bands in extracts of strains 282 and TX72. In contrast, no immunoreactive protein bands were observed in the extracts of mutants 282KZ and TX74. Similar results were obtained when we used an antibody raised against whole, killed M protein type 18 bacteria. In an Ouchterlony immunodiffusion gel using serial dilutions of M protein extracts and anti-M18 antiserum, a precipitin line formed between type 18 antiserum and protein extracts of strains 282 and TX72 up to a dilution of 1:8. No precipitin line was visible even for undiluted extracts of mutants 282KZ and TX74 (data not shown). These results indicate that strains 282KZ and TX74 harboring mutated alleles of *emm18* did not express type 18 M protein on their surfaces.

TABLE 2. Phagocytic killing in whole blood

| Strain | CFU/100 $\mu$ l at <sup>a</sup> : |                 |
|--------|-----------------------------------|-----------------|
|        | 0 h                               | 3 h             |
| 282    | 39.7 $\pm$ 13.7                   | 625 $\pm$ 240   |
| 282KZ  | 32.8 $\pm$ 5.9                    | 1.3 $\pm$ 1.9   |
| TX72   | 18.8 $\pm$ 9.8                    | 36.6 $\pm$ 2.0  |
| TX74   | 28.8 $\pm$ 9.9                    | 14.5 $\pm$ 10.2 |

<sup>a</sup> Results are numbers of CFU per duplicate 100- $\mu$ l aliquots at time zero and after 3 h of incubation (mean  $\pm$  standard deviation of three experiments).

Extracts of strains 282 and TX72 yielded similar amounts of M protein, which confirms previous observations that loss of capsule expression in strain 282 by Tn916 insertion does not reduce M protein production (40).

The amounts of cell-associated hyaluronic acid in the mucoid strain 282 and its *emm18*-inactivated derivative, 282KZ, were 52 and 53 fg per CFU, respectively. Strains TX74 and TX72 did not produce detectable quantities of hyaluronate (<0.5 fg per CFU). These results indicate that the inactivation of *emm18* by the procedure described above did not affect the genes involved in the expression of hyaluronic acid capsule. No differences were found among the four strains in expression of group A carbohydrate or hemolysis on blood agar.

**Relative contributions of the capsule and M protein to virulence of strain 282.** The capacity of GAS strains to resist ingestion and killing by phagocytic leukocytes is thought to be an important correlate of virulence. A 3-h incubation of strain 282 in fresh human blood resulted in an average increase of from 39.7 to 625 CFU (Table 2), corresponding to a mean 1.2-log increase (Fig. 4). The M-positive, capsule-negative strain (TX72) increased from an average of 18.8 to 36.6 CFU. In contrast, the M protein-negative mutants 282KZ and TX74 decreased from an average of 32.8 and 28.8 CFU, respectively, to 1.3 and 14.5 CFU, respectively, or a decrease of 1.5 and 0.35 log, respectively (Fig. 4). Poor survival of the M protein-neg-

TABLE 3. Opsonophagocytic assays in 10% serum

| Strain | CFU (10 <sup>6</sup> ) at <sup>a</sup> : |                 |
|--------|--|-----------------|
|        | 0 h                                      | 1 h             |
| 282    | 6.2 $\pm$ 2.2                            | 19.5 $\pm$ 13.4 |
| 282KZ  | 5.1 $\pm$ 1.0                            | 3.9 $\pm$ 2.3   |
| TX72   | 8.9 $\pm$ 2.4                            | 0.37 $\pm$ 0.30 |
| TX74   | 8.3 $\pm$ 3.3                            | 0.28 $\pm$ 0.20 |

<sup>a</sup> Results are CFU (10<sup>6</sup>) at time zero and after 1 h of incubation (mean  $\pm$  standard deviation of four experiments).

ative mutants in human blood could not be ascribed to impaired growth in plasma, since both strains grew during a 3-h incubation in plasma without blood cells, although at slightly lower rates than that of strain 282 (0.82-mean-log increase for 282KZ and 1.2-log increase for TX74 compared with a 1.4-log increase for 282). It is possible that the slightly lower growth rate of 282KZ may account for the somewhat greater killing of this strain compared with that of TX74. In the whole-blood assay, the acapsular strain TX72 increased in number by 0.33 log, a 3-fold increase, compared with the 1.2-log or 16-fold increase for wild-type strain 282. The latter results are similar to those observed previously for strain TX72 (40). Thus, in whole blood, the effect of capsule expression on GAS resistance to phagocytosis was less than that of M protein.

The capacity of the strains to resist killing was also assessed by using isolated human peripheral blood leukocytes and 10% nonimmune human serum as a source of complement rather than whole blood. Under these conditions, after 1 h of incubation, wild-type strain 282 increased in number from an average of  $6.2 \times 10^6$  to  $1.95 \times 10^7$  CFU (Table 3), representing an increase of 0.43 log (Fig. 5). The M protein-negative mutant 282KZ decreased slightly, from an average of  $5.1 \times 10^6$  to  $3.9 \times 10^6$  CFU, or a decrease of 0.23 log. In contrast, acapsular mutants TX72 and TX74 decreased from an average of  $8.9 \times$

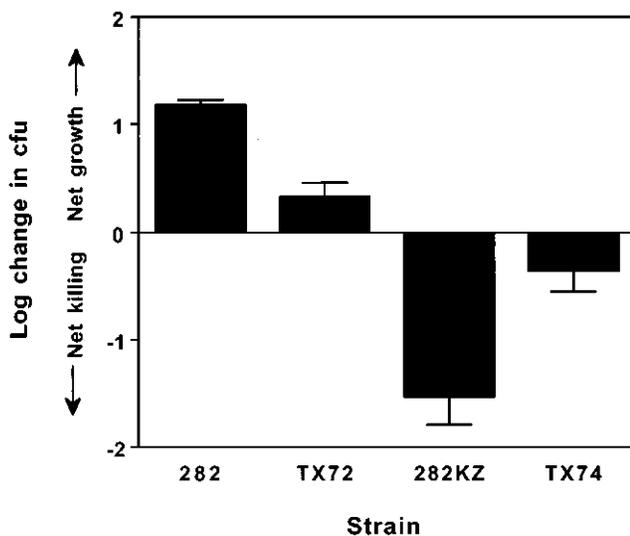


FIG. 4. Resistance to phagocytosis of GAS strains in whole blood. Bars indicate log change in CFU after rotation for 3 h in fresh human blood (bars above the horizontal axis indicate net growth, while bars below the horizontal axis indicate net killing). The results represent the mean  $\pm$  standard error for three independent experiments for wild-type strain 282 (M<sup>+</sup>, capsule<sup>+</sup>), mutant strain TX72 (M<sup>+</sup>, capsule<sup>-</sup>), mutant strain 282KZ (M<sup>-</sup>, capsule<sup>+</sup>), and mutant strain TX74 (M<sup>-</sup>, capsule<sup>-</sup>).

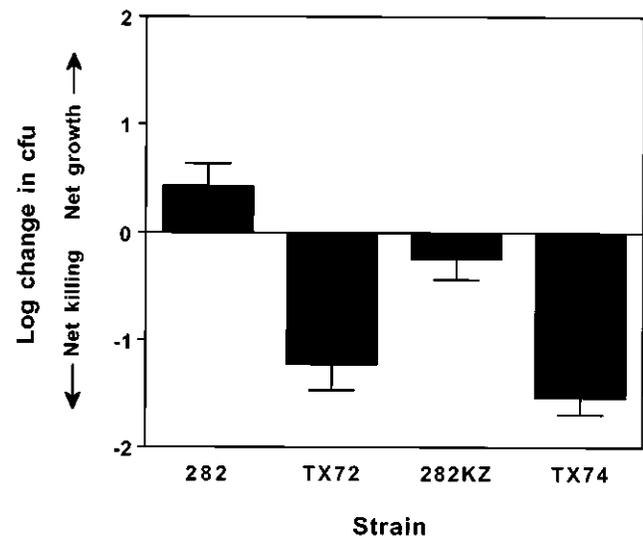


FIG. 5. Resistance to phagocytosis of GAS strains in 10% human serum. Bars indicate log change in CFU after rotation for 1 h in 10% human serum (bars above the horizontal axis indicate net growth, while bars below the horizontal axis indicate net killing). The results represent the mean  $\pm$  standard error for four independent experiments for wild-type strain 282 (M<sup>+</sup>, capsule<sup>+</sup>), mutant strain TX72 (M<sup>+</sup>, capsule<sup>-</sup>), mutant strain 282KZ (M<sup>-</sup>, capsule<sup>+</sup>), and mutant strain TX74 (M<sup>-</sup>, capsule<sup>-</sup>).

TABLE 4. LD<sub>50</sub> of strain 282 and three mutants

| Strain | LD <sub>50</sub><br>(CFU) | No. of<br>mice tested |
|--------|---------------------------|-----------------------|
| 282    | 1 × 10 <sup>7</sup>       | 120                   |
| 282KZ  | 6 × 10 <sup>7</sup>       | 60                    |
| TX72   | 6 × 10 <sup>8</sup>       | 54                    |
| TX74   | 8 × 10 <sup>8</sup>       | 24                    |

10<sup>6</sup> to 3.7 × 10<sup>5</sup> CFU and 8.3 × 10<sup>6</sup> to 2.8 × 10<sup>5</sup> CFU, respectively, representing decreases of 1.2 and 1.5 log, respectively (Fig. 5). Phagocytic killing of the acapsular mutants was markedly reduced when the serum had been inactivated by heating at 56°C, indicating that active serum complement was required for efficient phagocytosis. These results are in contrast to those obtained in the whole-blood assay, in which M protein appeared to be the major determinant of phagocytic resistance; in 10% serum, the hyaluronic acid capsule, and not M protein, appears essential in conferring resistance to phagocytosis.

Previously, we showed that loss of capsule production was associated with an increase in LD<sub>50</sub> of approximately 2 orders of magnitude in mice challenged i.p. (40). Similarly, in the present study, the LD<sub>50</sub> values for acapsular mutants TX72 and TX74 were 60- and 80-fold higher, respectively, than that for wild-type strain 282. By contrast, the LD<sub>50</sub> for M protein mutant strain 282KZ was only sixfold higher than that for 282 (Table 4). These results suggest that the hyaluronic capsule is a major virulence determinant for strain 282 in the mouse model of lethal infection, while M protein appears to have a lesser role.

**Expression of hyaluronic capsule in a nonmucooid GAS strain enhances resistance to phagocytosis.** Results of in vitro assays of phagocytosis using isogenic acapsular mutants provided strong evidence that the hyaluronic acid capsule enhances resistance to phagocytosis. To extend these observations, we asked whether increasing the level of capsule expression in a poorly encapsulated wild-type isolate of GAS would confer resistance to phagocytosis. By integrating into the chromosome a plasmid containing the capsule synthesis genes from strain 282, the nonmucooid M1 strain 049 was converted to a high-level capsule producer. The resulting strain, 049(200i), formed mucooid colonies on blood agar plates and was shown to produce 35 fg of cell-associated hyaluronic acid per CFU, an amount similar to that produced by strain 282, compared with <10 fg per CFU for wild-type strain 049. In 10% human serum, the nonmucooid wild-type strain 049 was readily killed (mean decrease in CFU, 1.4 log), while the encapsulated strain 049(200i) was not (mean increase in CFU, 0.23 log). Thus, increased capsule expression rendered the organisms resistant to phagocytic killing.

## DISCUSSION

Previous studies have provided evidence that both M protein and the hyaluronic acid capsule function to protect GAS from ingestion and killing by phagocytic leukocytes. In general, however, these two major surface molecules of GAS have been studied independently. The question remained unanswered, therefore, whether in an individual strain, M protein, capsule, or both served an antiphagocytic function. Studies of M protein in virulence generally have examined GAS strains rich in M protein but expressing unknown amounts of capsular polysaccharide. Conversely, studies of the hyaluronic acid capsule have used highly encapsulated strains in which the role of M

protein in virulence has not been studied in detail. In the present investigation, we sought to dissect the antiphagocytic function and the role in virulence of these two important surface molecules in the background of a single strain.

Using three isogenic mutants deficient in capsule, M protein, or both, we were able to assess quantitatively the contribution of each molecule to virulence by comparison with the wild type, a highly encapsulated M18 strain. Results of in vitro opsonophagocytic assays indicated that M protein was the major antiphagocytic determinant in whole blood while the capsule was the more important factor in resistance to phagocytosis in 10% serum. The results support the view that both capsule and M protein participate in resistance to phagocytosis for an individual GAS strain and suggest that the relative importance of each may depend on the specific microenvironment within the host. During bacteremia, for example, survival and proliferation of GAS in the bloodstream appear to be highly dependent on M protein expression. At extravascular sites where plasma proteins are present at lower concentrations, such as the skin and soft tissues or on the pharyngeal mucosa, the capsule may play a greater role in resistance to phagocytosis and in virulence. This hypothesis is consistent with the results of previous studies demonstrating that the capsule was critical in persistent GAS colonization of the pharynx after intranasal challenge in mice (38).

A further outcome of these studies was the observation that the behavior of the GAS strains in the whole-blood assay was not entirely predictive of the relative virulence of these strains in an in vivo infection model. The relative virulence of the strains in mice resembled more closely the pattern of resistance to phagocytosis of the strains in 10% serum rather than in whole blood. The whole-blood assay has been very useful as a functional test for M protein expression; however, results of the current studies suggest that measuring in vitro resistance to opsonophagocytosis in 10% serum may be a better method to predict virulence in vivo in a microenvironment where blood is not a major constituent.

The family of M-like proteins has recently been described in detail. These proteins have a role in binding serum immunoglobulins and may have some role in conferring resistance to phagocytosis (8, 30). PCR analysis of strain 282 showed it to contain an M-like gene (*emm18*) located between *emm* and *scpA* (Fig. 2B). The methodology used in the present study to inactivate M protein utilized a DNA fragment encoding the variable portion of the *emm* gene. This approach ensures that even in a strain containing M-like genes, only the M protein gene would be inactivated. Indeed, PCR analysis confirmed that *emm18* was not interrupted in mutants 282KZ and TX74.

Since one of the two truncated *emm18* genes in 282KZ and TX74 contains the promoter but lacks the 3' portion of the gene, it is possible that an amino-terminal fragment of M18 protein is formed but is secreted to the medium rather than anchored to the cell membrane. Such a protein could not be detected by Western blotting or Ouchterlony immunodiffusion of cell extracts. Since disruption in *emm18* resulted in loss of resistance to phagocytic killing in whole blood, we may conclude that if such a protein is produced and secreted, it does not confer resistance to phagocytosis.

The focus of our study on characterizing the function of M protein and capsule in a single strain helps to establish the role of both molecules in virulence; however, it remains uncertain whether results obtained in the background of strain 282 can be generalized to other GAS isolates associated with human disease. It is likely that the biologic activities of M protein and capsule vary somewhat depending on the structure of the particular M protein expressed by a particular strain, the amount

of M protein and capsule elaborated, and the presence or absence of other surface molecules including *emm*-like proteins. The mechanisms underlying the observed differences in relative importance of capsule and M protein in 10% serum versus whole blood are not well understood but may involve interactions of M protein with plasma proteins. M proteins have been shown to bind fibrinogen and other plasma proteins, perhaps with important consequences for resistance to phagocytosis. Binding of fibrinogen to M protein has been correlated with reduced deposition of complement C3b on the bacterial surface. Since C3b is a primary opsonin, fibrinogen binding by M protein has been proposed as an antiphagocytic mechanism. However, type 18 M protein binds fibrinogen poorly, and addition of fibrinogen to serum failed to enhance opsonophagocytosis of type 18 GAS in vitro (10). Whether binding of other plasma proteins to type 18 M protein plays a role in phagocytic resistance in blood is not known.

GAS strains vary widely with respect to capsule production: some express high quantities of the hyaluronic acid capsule and appear mucoid, while others express little or no capsule and appear nonmucoid. Since all isolates tested to date contain a conserved *has* operon (9), it is the degree of capsule expression which determines the particular strain morphology and influences its virulence. In the current investigation, we found that expression of increased amounts of capsule in the background of a poorly encapsulated type M1 strain enhanced resistance to phagocytosis. Together with previous work demonstrating a role for the capsule in pathogenicity of both type 18 and type 24 GAS (10), these findings provide further evidence that the capsule enhances virulence of diverse strains of GAS.

#### ACKNOWLEDGMENTS

We thank Tom DiCesare for expert technical assistance and Carol Berkower and Vered Ozeri for their helpful contribution.

This work was supported in part by the Israel Science Foundation administered by the Israel Academy of Sciences and Humanities (to A.E.M.), by the Ministry of Health Foundation (to E.H.), and by NIH grant AI29952 and an Established Investigatorship from the American Heart Association (both to M.R.W.). S.A. was supported by a fellowship from the Fundación Ramón Areces.

#### REFERENCES

- Ashbaugh, C. D., S. Alberti, and M. R. Wessels. Unpublished data.
- Baltimore, R. S., D. L. Kasper, C. J. Baker, and D. K. Goroff. 1977. Antigenic specificity of opsonophagocytic antibodies in rabbit anti-sera to group B streptococci. *J. Immunol.* **118**:673–678.
- Benchetrit, L. C., L. S. Phuja, E. D. Gray, and R. D. Edstrom. 1977. A sensitive method for the assay of hyaluronidase activity. *Anal. Biochem.* **79**:431–437.
- Bessen, D. E., C. M. Sotir, T. L. Readdy, and S. K. Hollingshead. 1996. Genetic correlates of throat and skin isolates of group A streptococci. *J. Infect. Dis.* **173**:896–900.
- Boulnois, G. J., and I. S. Roberts. 1990. Genetics of capsular polysaccharide production in bacteria. *Curr. Top. Microbiol. Immunol.* **150**:1–18.
- Caparon, M. G., R. T. Geist, J. Perez-Casal, and J. R. Scott. 1992. Environmental regulation of virulence in group A streptococci: transcription of the gene encoding M protein is stimulated by carbon dioxide. *J. Bacteriol.* **174**:5693–5701.
- Caparon, M. G., and J. R. Scott. 1991. Genetic manipulation of pathogenic streptococci. *Methods Enzymol.* **204**:556–586.
- Caparon, M. G., and J. R. Scott. 1987. Identification of a gene that regulates expression of M protein, the major virulence determinant of group A streptococci. *Proc. Natl. Acad. Sci. USA* **84**:8677–8681.
- Cleary, P., and D. Retnoningrum. 1994. Group A streptococcal immunoglobulin-binding proteins: adhesins, molecular mimicry or sensory proteins? *Trends Microbiol.* **2**:131–136.
- Crater, D. L., and I. van de Rijn. 1995. Hyaluronic acid synthesis operon (*has*) expression in group A streptococci. *J. Biol. Chem.* **270**:18452–18458.
- Dale, J. B., R. G. Washburn, M. B. Marques, and M. R. Wessels. 1996. Hyaluronate capsule and surface M protein in resistance to opsonization of group A streptococci. *Infect. Immun.* **64**:1495–1501.
- DeAngelis, P. K., I. Papaconstantinou, and P. H. Weigel. 1993. Isolation of a *Streptococcus pyogenes* gene locus that directs hyaluronan biosynthesis in acapsular mutants and in heterologous bacteria. *J. Biol. Chem.* **268**:14568–14571.
- Dougherty, B. A., and I. van de Rijn. 1994. Molecular characterization of *hasA* from an operon required for hyaluronic acid synthesis in group A streptococci. *J. Biol. Chem.* **269**:169–175.
- Dougherty, B. A., and I. van de Rijn. 1993. Molecular characterization of *hasB* from an operon required for hyaluronic acid synthesis in group A streptococci. *J. Biol. Chem.* **268**:7118–7124.
- Fischetti, V. A. 1989. Streptococcal M protein: molecular design and biological behavior. *Clin. Microbiol. Rev.* **2**:285–314.
- Hollingshead, S. K., T. L. Readdy, D. Li Yung, and D. E. Bessen. 1993. Structural heterogeneity of *emm* gene cluster in group A streptococci. *Mol. Microbiol.* **8**:707–717.
- Hotez, P., M. Caspell, J. Hawdon, C. Beckers, and J. Sakanari. 1994. Hyaluronidases of the gastrointestinal nematodes *Ancylostoma caninum* and *Anisakis simplex*: possible functions in the pathogenesis of human zoonoses. *J. Infect. Dis.* **170**:918–926.
- Hynes, W. L., J. J. Ferretti, M. S. Gilmore, and R. A. Segarra. 1992. PCR amplification of streptococcal DNA using crude cell lysates. *FEMS Microbiol. Lett.* **94**:139–142.
- Kaplan, E. L., R. J. Dwight, and P. Cleary. 1989. Group A streptococcal serotypes isolated from patients and sibling contacts during the resurgence of rheumatic fever in the United States in the mid-1980s. *J. Infect. Dis.* **159**:101–103.
- Lancefield, R. C. 1928. The antigenic complex of *Streptococcus haemolyticus*. I. Demonstration of a type specific substance in extracts of *Streptococcus haemolyticus*. *J. Exp. Med.* **71**:521–537.
- Lancefield, R. C. 1962. Current knowledge of the type specific M antigens of group A streptococci. *J. Immunol.* **89**:307–313.
- Lancefield, R. C. 1957. Differentiation of group A streptococci with a common R antigen into three serological types with special reference to the bactericidal test. *J. Exp. Med.* **107**:525–544.
- Marcon, M. J., M. M. Hrubar, D. M. Hosier, D. A. Powell, M. T. Brady, A. C. Hamoudi, and E. L. Kaplan. 1988. Occurrence of mucoid M-18 *Streptococcus pyogenes* in a central Ohio pediatric population. *J. Clin. Microbiol.* **26**:1539–1542.
- Natanson, S., S. Sela, A. E. Moses, J. M. Musser, M. G. Caparon, and E. Hanski. 1995. Distribution of fibronectin-binding proteins among group A streptococci of different M types. *J. Infect. Dis.* **171**:871–878.
- Perez-Casal, J., M. G. Caparon, and J. R. Scott. 1991. Mry, a *trans*-acting positive regulator of the M protein gene on *Streptococcus pyogenes* with similarity to the receptor proteins of two-component regulatory systems. *J. Bacteriol.* **173**:2617–2624.
- Perez-Casal, J., E. Maguin, and J. R. Scott. 1993. An M protein with a single C repeat prevents phagocytosis of *Streptococcus pyogenes*: use of a temperature sensitive shuttle vector to deliver homologous sequences to the chromosome of *S. pyogenes*. *Mol. Microbiol.* **8**:809–819.
- Podbielski, A. 1993. Three different types of organization of the *vir* regulon in group A streptococci. *Mol. Gen. Genet.* **237**:287–300.
- Podbielski, A., A. Flosdorff, and J. Weber-Heymann. 1995. The group A streptococcal *virR9* gene controls expression of four structural *vir* regulon genes. *Infect. Immun.* **63**:9–20.
- Podbielski, A., B. Melzer, and R. Luttmann. 1991. Application of the polymerase chain reaction to study the M protein(-like) gene family in beta-hemolytic streptococci. *Med. Microbiol. Immunol.* **180**:213–227.
- Podbielski, A., J. A. Peterson, and P. Cleary. 1992. Surface protein-CAT reporter fusions demonstrate differential gene expression in the *vir* regulon of *Streptococcus pyogenes*. *Mol. Microbiol.* **6**:2253–2265.
- Podbielski, A., N. Schnitzler, P. Beyhs, and M. D. P. Boyle. 1996. M-related protein (*Mrp*) contributes to group A streptococcal resistance to phagocytosis by human granulocytes. *Mol. Microbiol.* **19**:429–441.
- Reed, L. J., and H. Muench. 1938. A simple method of estimating fifty per cent endpoints. *Am. J. Hyg.* **27**:493–497.
- Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
- Scott, J. R. 1990. The M protein of group A streptococcus: evolution and regulation, p. 177–203. *In* J. R. Sokatch and N. L. Ornston (ed.), *The bacteria. A treatise on structure and function*. Academic Press, Inc., San Diego, Calif.
- Scott, J. R. 1974. A turbid plaque forming mutant of phage P1 that cannot lysogenize *Escherichia coli*. *Virology* **62**:344–349.
- Stevens, D. L., M. H. Tanner, J. Winship, R. Swartz, K. M. Ries, P. M. Schlievert, and E. Kaplan. 1989. Severe group A streptococcal infections associated with a toxic shock-like syndrome and scarlet fever toxin A. *N. Engl. J. Med.* **321**:1–7.
- Veasy, G. L., S. E. Wiedmeier, G. S. Orsmond, H. D. Ruttenberg, M. M. Boucek, S. J. Roth, V. F. Tait, J. A. Thompson, J. A. Daly, E. L. Kaplan, and H. R. Hill. 1987. Resurgence of acute rheumatic fever in the intermountain area of the United States. *N. Engl. J. Med.* **316**:421–427.

37. **Wald, E. R., B. Dashefsky, C. Feidt, D. Chiponis, and C. Byers.** 1987. Acute rheumatic fever in western Pennsylvania and the tristate area. *Pediatrics* **80**:371–374.
38. **Wessels, M. R., and M. S. Bronze.** 1994. Critical role of the group A streptococcal capsule in pharyngeal colonization and infection in mice. *Proc. Natl. Acad. Sci. USA* **91**:12238–12242.
39. **Wessels, M. R., J. B. Goldberg, A. E. Moses, and T. J. DiCesare.** 1994. Effects on virulence of mutations in a locus essential for hyaluronic acid capsule expression in group A streptococci. *Infect. Immun.* **62**:433–441.
40. **Wessels, M. R., A. E. Moses, J. B. Goldberg, and T. J. DiCesare.** 1991. The hyaluronic acid capsule is a virulence factor for mucoid group A streptococci. *Proc. Natl. Acad. Sci. USA* **88**:8317–8321.
41. **Wilson, A. T.** 1959. The relative importance of the capsule and the M antigen in determining colony form of the group A streptococci. *J. Exp. Med.* **109**:257–270.

---

*Editor:* V. A. Fischetti