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CHARACTERIZATION OF VIRULENCE FACTORS IN AN M50 GROUP A STREPTOCOCCUS STRAIN VIRULENT FOR MICE

by

DER-LI YUNG

A DISSERTATION

Submitted to the graduate faculty of The University of Alabama at Birmingham, in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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ABSTRACT OF DISSERTATION
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Title Characterization of Virulence Factors in an M50 Group A Streptococcus Strain Virulent for Mice

An M serotype 50 group A streptococcal strain, B514, caused several outbreaks of natural infections in mice. Because of its natural pathogenicity in mice, this strain was of interest in developing murine models to study various group A streptococcal virulence factors. The *emm* gene cluster of B514-Sm was first examined by allele-specific PCR and was found to composed of three tandem *emm*-family genes, a pattern which is consistent with the proposed evolution of *emm* locus by gene duplication. SF4 (*mrp50*), SF2 (*emmL50*), and SF3 (*enn50*) genes were cloned individually and the entire gene cluster was sequenced. The gene cluster showed overall greater than 97% DNA identity to that of a human M2 strain T2/44/RB4 except for two small divergent regions present immediately after the signal peptide in both *emm* and *enn* genes. Expression of cloned genes in *Escherichia coli* showed that Mrp50 and Emm50 bind IgG and that Enn50 binds IgA. The transcript level of each gene was then investigated by quantitative Northern hybridization was found to be over 30-fold attenuated relative to strain T2/44/RB4. The M-family proteins were barely detectable even from B514-Sm isolates recently passaged in mice.

A capsule-negative B514-Sm mutant (B514.039) was constructed by inserting a nonreplicative plasmid into the *has*A gene encoding hyaluronate synthase. B514-Sm, B514.039, and two other isogenic strains constructed by collaborators were tested in a pneumonia model and a throat colonization model. Of the additional isogenic strains, one lacked all M protein genes and the other lacked only the gene encoding C5a peptidase. When B514.039 was placed in the intranasal passage of mice, all bacteria recovered from the throats of the mice were encapsulated 1 day after inoculation. Encapsulated revertants

from the mice were analyzed by PCR and, in all cases, the *has*A gene had lost the plasmid insertion and reverted to the normal size. From the pneumonia model, following intratracheal inoculation with B514.039, the incidence of pneumonia within 72 h was significantly reduced from that of the parent strain. The additional isogenic strains had no significant difference from the wild type either in throat colonization or pneumonia models.

Complementation of *mga*-containing plasmids to B514-Sm successfully reversed the attenuated expression of M family proteins. The increase in expression was seen in the *emm* transcript and in the quantity of CnBr-extracted proteins. The DNA sequence of *mga50* was determined; *mga50* had four amino acids substitutions relative to *mga4* and *virR49*. One or more of these amino acid substitutions in Mga50 may be responsible for the attenuated expression of M family proteins in strain B514-Sm. The transcript level of *mga50* was similar to that of *mga2*. Antiphagocytic assays showed that either the capsule or the M-family proteins in strain B514-Sm could provide protection from phagocytosis in human blood.

DEDICATION

To my dear husband, Cameron, my sons, Howard and Darius, and my parents, brother, and sisters for their everlasting love and caring.

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LIST OF ABBREVIATIONS

bp	base pairs
C5a	complement fragment 5a
CFU	colony forming unit
GAS	group A streptococcus
Ig	immunoglobulin
mAb	monoclonal antibody
OF	opacity factor
PCR	polymerase chain reactions
SF	subfamily

INTRODUCTION

Bacterial pathogenicity is related to the ability of a bacterium to cause disease. Several aspects associated with bacterial pathogenesis are the abilities of bacteria to 1) infect mucous surfaces, 2) enter the host through mucous surfaces, 3) multiply in the environment of the host, 4) interfere with host defences, and 5) damage the host. Each of these steps involves a variety of processes to accomplish the task. The availability of good animal models can provide vital information concerning the virulence determinants (factors) to fulfill the five requirements for pathogenicity. *Streptococcus pyogenes* is one of the best-adapted pathogenic microorganisms that causes human infections. The bacteria display limited, but focused, strategies that exploit weaknesses of the host defenses. The subject of this dissertation was to investigate the molecular characteristics of virulence factors of the serotype M50 *S. pyogenes* strain B514 that is virulent for mice. This strain is important for its capability to naturally cause infections in mice. Because of the characteristic of virulence for mice, the animal models representing *S. pyogenes* infections would be available by infecting mice with B514. The mouse models then can be utilized to obtain information about the virulence factors related to the above five requirements for pathogenicity of *S. pyogenes*. To accomplish this purpose, it was necessary to first characterize the molecular basis for the virulence factors of this strain and then to combine those with the study of its pathogenic effect in vivo. This thesis is divided into three major sections. The initial section starts with the molecule characterization of certain virulence factors, the M-family proteins of this strain. The second section investigates the requirement for several virulence factors of strain B514 in mouse models. The requirement for M-family proteins, streptococcal C5a peptidase, and hyaluronic acid capsule for pathogenicity was studied in two mouse

models: a long-term colonization model following intranasal inoculation and a pneumonia model following intratracheal inoculation. This study was done as collaborative work with Dr. Linda Husmann of Emory University. The third section involves further investigation about the *mga* gene of this strain. Mga is a positive transcription activator for the expression of the M-family proteins, C5a peptidase, and some other virulence genes in group A streptococci.

***Streptococcus pyogenes* and its infections.** Gram-positive streptococci are classified into Lancefield groups according to the biochemical and antigenic properties of the carbohydrates of the cell wall (55). One of these groups, the group A streptococci (GAS) or *S. pyogenes*, is a major and exclusively human pathogen. The usual presentations of group A streptococcal infections are pharyngitis or tonsillitis, which occur most often in children of school age or among adults in contact with them. GAS also cause skin infections such as impetigo and scarlet fever (98).

Most GAS infections can be cured by a appropriate treatment with antibiotics (penicillin) treatment. In the absence of antibiotics, two serious complications, rheumatic fever or glomerulonephritis, are the aseptical sequelae related to GAS suppurative infections. In recent years, more virulent and complicated diseases caused by GAS have been reported, and more attention has been directed towards this pathogen. Streptococcal toxic shock-like syndrome is characterized by shock, multiorgan system failure, and destructive soft tissue infection (99). Necrotizing fasciitis is a severe invasive GAS infection occurring in the subcutaneous tissue, in which the fascia and fat are progressively destroyed (98). Streptococcal myositis and bacteremia are also life-threatening aspects of invasive streptococcal disease that have been reported recently (98).

Virulence factors of *Streptococcus pyogenes*. The outcome of an infection depends on the interaction between the virulence factors of the bacterium and the host immune system. *S. pyogenes* has evolved an impressive range of strategies to evade the nonspecific and the specific immune defense systems of humans.

One of the most important virulence factors of *S. pyogenes* is the antiphagocytic M protein, which is an alpha helical coiled-coil structure protein with its carboxyl terminus attached to the cell wall and its amino terminus extending away from the cell (Fig. 1). In the early twentieth century, Lancefield differentiated the GAS strains into more than 70 serotypes on the basis of whether the testing strain showed a precipitating reaction with a member of a panel of antisera to M proteins (56). She also recognized that M protein-rich strains were resistant to phagocytic killing in nonimmune human blood and elucidated an important fact that only type-specific antibodies can opsonize the specific strain and target it for killing by polymorphonuclear leukocytes (PMN) circulating in the blood (57). The epitopes of some opsonic antibodies have been mapped to near the end of the amino terminal portion of M proteins on several different M serotype strains (22, 49). Under the condition that there has been no induction of serotype-specific antibodies to M proteins, the GAS strain can not be killed by PMN and will continue to grow in human blood.

The mechanism by which M proteins confer resistance to phagocytes is complex and may be different among M proteins. Some M proteins bind to the complement factor H (40), which prevents C3 activation by rendering C3b susceptible to inactivation by factor I and restricts the conversion of C3b,Bb (C3 convertase) by competing for factor B (74), thereby resulting in the reduced deposition of C3b on the streptococcal surface (23, 40). M proteins also bind to fibrinogen, an abundant protein in plasma, and the binding contributes to the antiopsonic activity by interfering with C3b deposition on a nonimmune host surface (41, 113, 114). Besides antiphagocytic

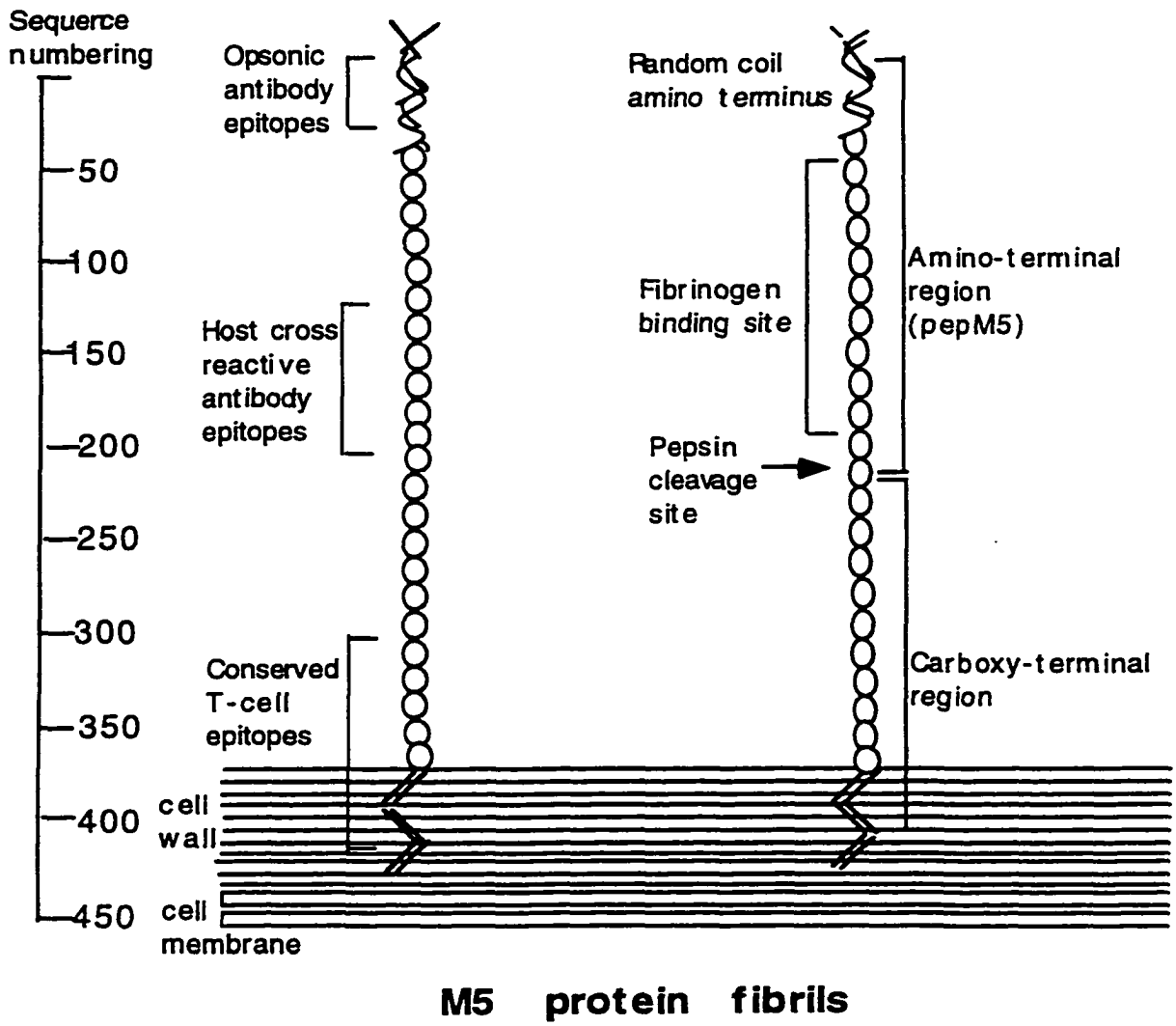

FIG 1. A schematic drawing of M proteins on the streptococcal surface (89). The *emm5* gene cloned from *S. pyogenes* codes for a protein of 450 amino acids. The M protein coiled-coil dimers are approximately 50 nm long and project from the streptococcal surface. Fibrinogen was shown to interact with the amino-terminal half of M proteins (90) and prevents the binding of antibodies to epitopes located away from the amino terminus (22); antibodies directed to the extreme amino-terminal ends of M proteins opsonize streptococci effectively in the presence of bound fibrinogen (90). Opsonic antibody epitopes have been identified within the amino-terminal ~10 amino acids in all M types studied (87). Many M proteins elicit antibodies that crossreact with mammalian tissues, such as myosin and tropomyosin of human heart tissue (19, 22, 27). The majority of crossreactive antibodies from mice and humans recognize the epitopes loocated in the amino-terminal half; those of opsonic antibodies from the extreme amino terminus are not included (89). Major histocompatibility complex class II-restricted T-cell epitopes were found to be distributed through the variable amino-terminal and conserved carboxy-terminal regions of M5 (88).

activity, M proteins also mediate the binding of the bacterium to keratinocytes of the epidermis (70, 71).

Besides the antiphagocytic M proteins, there are M-like proteins on the surface of the cells, and they have DNA sequences similar to those of M proteins. Most M-like proteins bind the plasma proteins, but their roles in virulence are less clear. M-like proteins include immunoglobulin-binding proteins, such as Arp (IgA receptor protein) (58, 68, 96), Mrp (M protein-related protein) (or FcRA) (33, 58, 68, 96), Sir (streptococcal immunoglobulin receptor) (97), and Enn (6) proteins, which bind to Ig via the Fc fragment (nonimmune fashion). Raeder and Boyle (84, 85) found a correlation between the expression level of Ig-binding proteins and the organism's ability to establish invasive skin infections. Certain Mrp proteins of GAS strains contribute resistance to phagocytic killing by human granulocytes besides the M proteins (83). Some M-like proteins bind to the human complement regulator C4BP through the variable N-terminal region and confer antiopsonization activity to the strain (48, 76). A plasminogen-binding protein, PAM protein (4), and protein H, an albumin- and IgG-binding protein (25, 29), are also included in the category of M-like proteins. Not every GAS strain has M-like proteins. The *emm* (encode M proteins) and *emm*-like (encoding M-like proteins) genes have been shown to lie in tandem and are called the *emm* gene cluster. The cluster is located downstream from the *mga* gene and upstream from the *scpA* gene (38).

Streptococcal C5a peptidase is another virulence factor. It specifically cleaves the human serum chemotaxin C5a at the PMN binding site and retards the influx of inflammatory cells and clearance of streptococci at the site of infection (46, 67, 110). From the nucleotide sequences and deduced amino acids determined by Chen (17), streptococcal C5a peptidase is a serine protease which possesses a typical bacterial signal peptide, a catalytic center, and cell-wall spanning and membrane-anchor regions.

The gene encoding streptococcal C5a peptidase, *scpA* loci, is located at the down-stream end of the *emm* gene cluster.

Some strains of GAS grow as "glossy" or mucoid colonies. This is due to the production of a hyaluronic acid capsule (117). The capsule is composed of a high molecular weight glucosaminoglycan made of repetitive units of ß 1-4-linked disaccharide of glucuronic acid and N-acetylglucosamine. The capsule composition is chemically indistinguishable from hyaluronate of human connective tissue (52). In the mid-1980s, the number of clinical encapsulated *S. pyogenes* isolates increased (48). Focal resurgences of rheumatic fever related to the encapsulated GAS strains, the serotypes M-1, -3, -5, -6, and -18, have been reported from different areas in the United States (95). Wessels et al. (108) demonstrated that acapsular mutants of a serotype M18 GAS strain showed a loss of survival in human blood and lowered virulence in mice. They also demonstrated that hyaluronic acid capsule was required by serotype M24 GAS in order to colonize and induce infection in mice pharynx (107). These studies provided evidence that the hyaluronic acid capsule functions to protect GAS from ingestion and killing by phagocytic leukocytes. Besides the antiphagocytic activity, the hyaluronic acid capsule was reported to act as the ligand for attachment of GAS on human keratinocytes (92).

Several surface proteins of *S. pyogenes* can bind to the components of extracellular matrix and basement membrane, such as protein F, a fibronectin-binding protein (102) and GAPDH (glyceraldehyde-3-phosphate dehydrogenase)-like protein (73). The adhesion mediated between the extracellular matrix components and these bacterial adhesins may constitute an important step in colonization of host tissues and the subsequent infection (2).

Several toxins are secreted by *S. pyogenes*, especially the invasive strains (98). Pyrogenic exotoxins (Spe) A, B, and C are the most virulent toxins. SpeA and C can act as "superantigens" by interacting with MHC class II molecules and the T cell

receptor, thereby inducing a T cell response, with the subsequent production of cytokines capable of mediating shock and tissue injury (64). Streptolysins O and S are thiol-activated cytolytic toxins which damage the cell membrane by forming pores and lysing the cells (9).

Unlike the cell-bound streptococcal C5a peptidase, several enzymatic proteins of *S. pyogenes* are secreted out of the cells, such as the cysteine protease (12, 104), hyaluronidase (44), streptokinase (15), and DNAase, amylase, and esterase (20). These enzymes are related to the destruction of the biological barriers of the eukaryotic cells and facilitate the invasion of *S. pyogenes* to deeper tissue sites within the host.

Structural heterogeneity of the *emm* gene cluster. More than 80 serotypes of *S. pyogenes* strains have been found based on the serological typing scheme used by Lancefield (55). Basically, type-specific antibodies are prepared by taking sera from patients or rabbits recovered from streptococcal infection and absorbing the sera with heterologous strains until only type-specific antibodies remain. The antisera contain antibodies specific for surface proteins of *S. pyogenes*, most of which are the M proteins. The DNA sequence of an M protein from an M serotype 6 strain--D471-- was the first to be determined (36), and many more *emm* genes have been sequenced since that time. When the *emm* gene sequences are aligned, the sequences of different M serotype strains show similarity over a large extent of the molecules (Fig. 2). The overall structure of the molecule from the amino-terminal end to the carboxy-terminal end is composed of a highly conserved signal sequence, a variable amino-terminal region, the conserved repeats (A, B, C repeats) with the heptad periodicity typical for coiled-coil proteins (24), then the proline-glycine rich domain residing in the peptidoglycan of the cell wall to stabilize the protein. A segment of LPXTGX motif on the carboxyl end are expected to be cleaved, then the rest of the molecule is crosslinked to the cell wall components (91). Most M proteins contain C

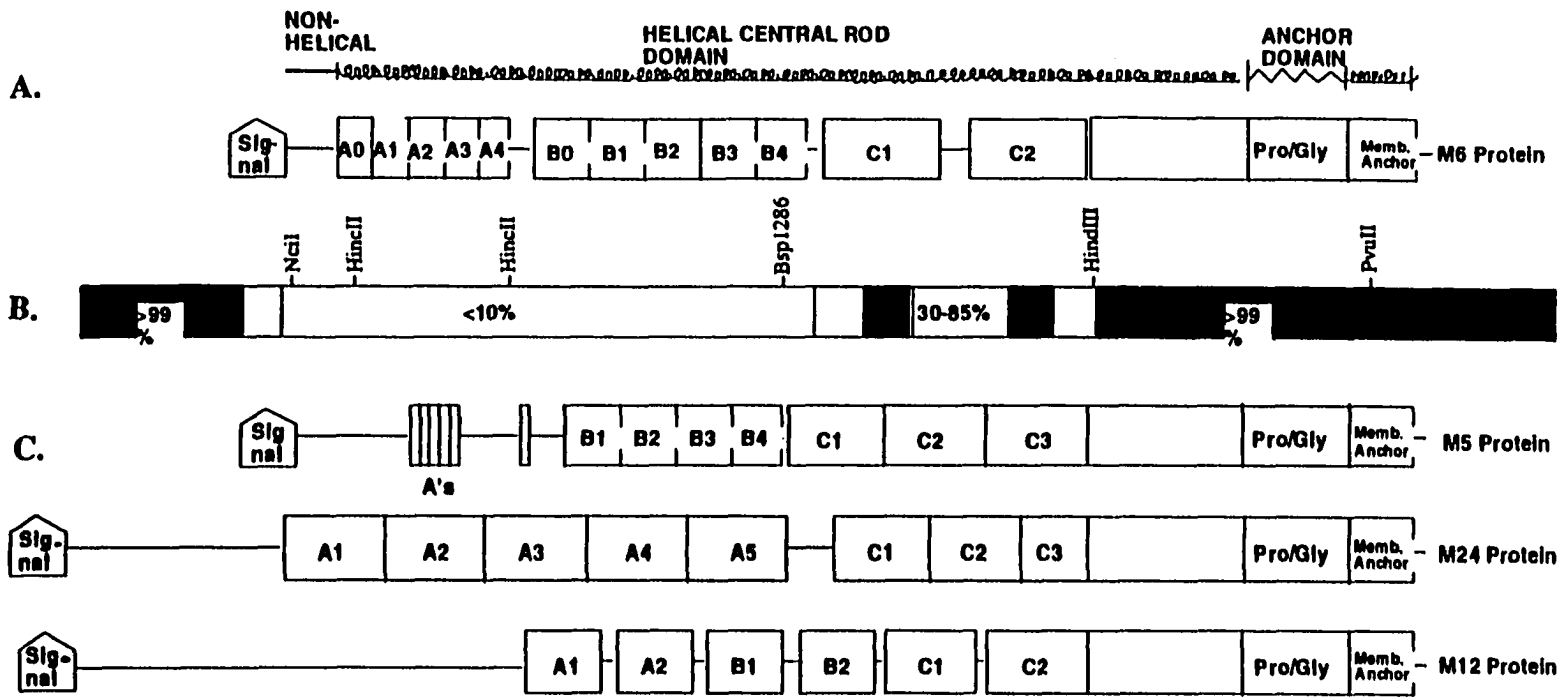

FIG. 2. A. A diagram of the *emm6* gene showing the gene features based on the DNA and amino acid sequences determination and biochemical analysis (24, 36). A GAS M protein, before processing, contains a signal peptide, a nonhelical structure at the extreme amino-terminus, followed by the helical central rod domain, then the proline-glycine (Pro/Gly)-rich wall-anchor domain, and a short positive-charged residues at the carboxy-terminal end. B. the intragenic repeats A, B, and C, three sequences regions present in the gene and M6 protein. C. the similar features of three other *emm* genes--*emm5* (63), *emm24* (66) and *emm12* (87)--and the comparison of DNA sequence homology of these *emm* sequences. The degrees of homology in various regions of *emm* sequences are shown in B.

repeats, but not all have A or B repeats. The location of serotype specific antibodies reacting with M proteins is in the highly variable region of the amino-terminus (22, 49)

Apart from the classical M proteins, the M-like proteins are homologous with the M proteins and share the same overall structure (50). Some phenotypes of M-like proteins were described in the previous section. Together, the M proteins and the M-like proteins are called M-family proteins, the genes are called *emm*-family genes. Over 100 M-family proteins and their nucleotide sequences and deduced amino acids have been studied. Some *emm*-family genes have "class A molecules" repeats instead of C repeats on their carboxyl end (68). When the DNA sequences of *emm*-family genes were aligned, the homology of DNA sequences increases toward the carboxy-terminal end, but decreases toward the amino-terminal end of the DNA sequences (29, 37, 97).

By aligning the conserved carboxy-terminal ends of M-family proteins with the methods of phylogenetic analysis, all known members of this family could be differentiated into four evolutionary distinct *emm* subfamilies -SF1 to SF4 (35, 38). Table 1 lists the *emm* genes of these four subfamilies and some characteristics within the SF genes. Each subfamily has its peculiar subsequence in the conserved proline-glycine rich region which can be used as a marker to ascribe any M-family protein to one of the subfamilies. By these SF-specific subsequences as a primer paired with a second primer to a conserved location for polymerase chain reaction of the chromosomal DNA, each allele of *emm*-family genes can be mapped in any GAS strain. It has been found that one to three tandemly arranged copies of *emm*-family genes are mapped in this cluster, but the alleles within the cluster vary among different strains (38). Nine distinct chromosomal patterns of the genes in the *emm* gene cluster were found by analyzing 44 GAS strains representing 32 different M serotypes (35) (Fig. 3). These nine chromosomal patterns strongly support a model for the generation of the four *emm*-subfamily genes in which the genes are the products of evolutionary events in which the gene duplication is followed by the sequence divergence (35).

TABLE 1. Summary of *emm* family genes divided by their subfamily and allele

Subfamily 1 SF1 genes	Subfamily 2 SF2 genes	Subfamily 3 SF3 genes	Subfamily 4 SF4 genes
emm (36)	*emm49* (30)	*enn49* (30)	*fcrA76* (33)
emm5 (63)	*emmL2.1* (6)	*emmL2.2* (6)	*mrp4* (68)
emm12 (87)	*arp4* (26)	*enn4* (45)	*fcrA64/14* (11)
emm18 (81)	*arp60* (34)	*enn64/14* (80)	*fcrA49* (77)
emm19 (37)	*emm9* (80)	*enn50* (118)	*mrp22* (97)
emm24 (66)	*emm50* (118)		*mrp50* (118)
emm30 37)	*sir22* (97)		
emm55 (34)			
protH (29)			
emm57 (87)			
emmG1 (18)			
fcrAV (94)			
emm1 (32)			
emmL64/14 (11)			
emm3 (78)			
emm53 (4)			
enn5.8193 (111)			
	Characteristics associated with each mosaic allele		
often antiphagocytic	some antiphagocytic		
found in OF$^-$ strains	found in OF$^+$ strains	found in OF$^+$ or OF$^-$	found in OF$^+$ or OF$^-$
react with mAbs 10B6,10F5 (class I)	don't react with mAbs 10B6,10F5 (class II)	don't react with mAbs 10B6,10F5 (class II)	don't react mAbs 10B6,10F5 (class II)
have C repeats	have C repeats	have C repeats	have A repeats
frequently binds fibrinogen	some bind IgA, IgG3 *--arp4, arp60*	some bind IgA-- *enn2*	some bind fibrinogen
some bind IgG3	some bind IgA, IgG1 *-4--sir22*		many bind IgG1, 2, 4
some bind IgG 1-4		some bind IgG 1-4 *--enn33*	
some bind factor H, HAS (human albumn serum) , plasminogen			
	Proposed individual gene names for each allele		
emm	*arp*	*enn*	*fcr*

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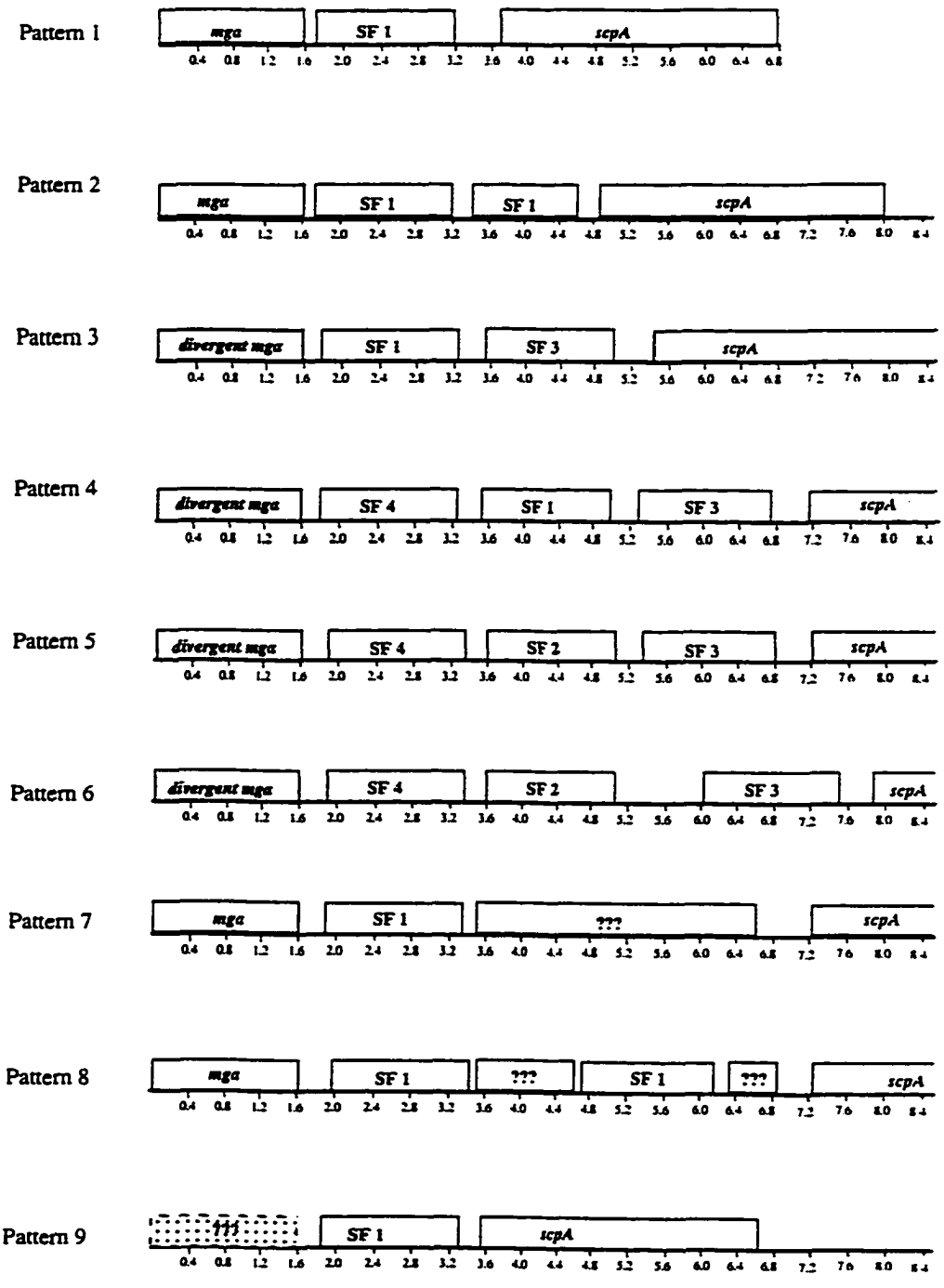

FIG. 3. Proposed maps of nine *emm* chromosomal locus patterns defined by PCR analysis. Numbers below the lines indicate approximate distances in base pairs; boxes above the lines indicate the prescence of genes inferred from the amplification products obtained in PCR reactions (35).

Early studies divide the GAS strains into OF^+ and OF^- groups based on the strain's ability to express serum opacity factor (31, 103, 115). More recently, this division was reported to parallel differences in the reactivities of strains with monoclonal antibodies against the conserved C-repeats in the carboxy-terminal half of the M6 protein, which differentiated GAS strains to class I and II (8). From the evolutionary model of *emm* locus studied by Hollingshead et al. (35), seven out of nine patterns of the GAS strains express a OF^-, class I M protein (all but patterns 5 and 6) which is encoded by a SF-1 gene. Substantial heterogeneity of the *emm* gene clusters containing SF-1 genes was reported. Strains containing an SF-2 allele are nearly all OF^+, class II M protein-expressing strains. This group is more homogeneous and exhibits either pattern 5 or 6.

It is important to consider these features of the evolution of the *emm* gene cluster in order to understand the pathogenesis associated with *emm*-family genes, because particular virulence properties may be associated with specific alleles or suballeles (35). An analysis conducted by Bessen et al. (7) showed that three GAS diseases, uncomplicated pharyngitis, impetigo (skin infection), or acute rheumatic fever, were siginificantly correlated to the *emm* gene cluster chromosomal patterns. The OF^- class I strains are primarily pharyngeal isolates of uncomplicated pharyngitis or isolates associated with acute rheumatic fever. This group is found to have the *emm* gene cluster chromosomal patterns 1 to 3, whereas GAS strains in patterns 4 and 5 are almost all isolates from impetigo. This group included both OF^- class I and OF^+ class II strains. From this study, the association of GAS infection types to the composition of the *emm* gene cluster of the isolates, on the basis of the conserved 3'*emm* genes, could support a notion that M and M-like proteins may contain tissue-specific biologic function (7). It is proposed that if the mosaic structure-like *emm*-family gene products determine, at least in part, the tissue-specificity of infection, then the horizontal transfer

of *emm*-family genes between different GAS strains may play an important role for the emergence of new clones that have unique pathogenic qualities and lead to a change in the principal reservoir for transmission of that clone (5, 7, 111).

A signal response regulator-*mga* gene in a regulon. Upstream of an *emm* gene cluster, there is a gene locus called *mga* (for multigene regulator in GAS). It was found that when transposon Tn916 was inserted about 1.8 kb upstream of the structural gene for M protein of a serotype M6 strain-D471, the amount of M protein-specific mRNA was reduced (14). Later it was detected that the insertion was located 244 bp upstream of an open reading frame, which was later named *mry* (M protein RNA yield). By complementing this insertion mutant with an intact *mry* gene in a replicating plasmid, the expression of M6 protein was restored. This result indicated that Mry can act in *trans* as a positive transcription regulator of the *emm6* gene (75). Other evidence about this positive regulator was that there was a deletion of 400 bases upstream from the transcription start sites of the *emm12* gene, which led to an *emm12*-deleted phase-locked variant--CS64 (87). This gene was also required for the expression of the genes encoding for streptococcal C5a peptidase (16), M-like proteins (e.g., *mrp*, *enn*, *sph*) (79), the opacity factor (*sof*) (62), and a secreted inhibitor of the complement cascade (*sic*) (51). These genes, which are all positively regulated by *mga* (formerly *mry* or *virR*), are now called the *mga* regulon (79, 93). Mga protein is a *trans*-acting positive regulator (75) which binds immediately upstream of and overlaps the -35 region of *emm* and *scpA* promoters with a single 45-bp binding site (60). Sequences homologous to this binding site were found in the other M-family proteins and the C5a peptidase of different M serotypes of GAS strains (60). A consensus Mga binding site has been determined (60). Unlike most DNA binding sites recognized by bacterial regulatory proteins that have a dyad symmetry (72), in this case, the Mga protein binds to a single-half site as a monomer of approximately 30 kDa (60). The

DNA sequences recognized by the Mga protein contain two converging repeat sequences separated from two central adenines by 6 or 7 nucleotides. The unique binding pattern of Mga protein may reflect a novel mechanism for protein-DNA interaction (60).

The Mga protein contains motifs common to the response regulator superfamily of two-component bacterial signal transduction systems. The typical response regulator has been studied more thoroughly and in detail in CheY, which functions in chemotaxis to regulate the flagellar motor (106). CheY is a 14-kDa monomeric protein containing 128 amino acids. When it is phosphorylated by a sensor component, it binds to a flagellum-associated protein, FliM (106). The structure-function relationships of CheY have been described in detail (3, 101, 105). In the case of Mga, the number and location of the predicted interacting domains are unusual (60). Mga is about twice as large as most proteins of this type (62 kDa), it has three possible helix-turn-helix DNA binding domains in the first amino-terminal 130 residues, and there are two putative CheY-like response regulator domains lying in the carboxyl terminus of the molecule (1, 75).

Expression of the *emm* gene of GAS is responsive to environmental changes (13, 61). The responsiveness is regulated by Mga (13, 69, 82), which is also regulated, at least by itself, in response to environmental stimuli, such as the level of CO_2 and the growth phase (59, 69). Regulation of *mga* expression is complex. It is shown that the expression of the *mga6* gene requires the participation of DNA sequences 473 bp upstream of the gene (59, 69), where there is a short segment of sequences conserved in the target sites of many DNA-binding proteins (28). The DNA subsequences are located upstream of the P_1 promoter of the *mga* gene (69). A study using S1 nuclease protection assays showed that *mga* was transcribed from two promoters P_1 and P_2 with different transcription efficiency (69). The *mga* gene was also found to be autoregulated (59, 69), as the Mga binding site can be located in the

upstream region of the gene near the P_2 promoter (79). It is hypothesized that a possible mechanism may involve differential initiation of transcription from these two promoters. In this model, P_1 is responsible for low-level transcription under nonpermissive environmental conditions and P_2 is not active. When encountering a stimulating environment, Mga is activated, possibly by a phosphorylation event with a histidine protein kinase, and regulates its own expression in a feedback loop by activating transcription from P_2 (69). Nevertheless, Bormann and Cleary (10) pointed out that transcription from P_1 is required for sufficient expression of Mga to activate transcription of regulated genes.

All group A streptococci have an *mga* locus in their genomes located upstream of the *emm* gene cluster. DNA sequences of *mga* genes among the GAS strains are heterogeneous (38). It was detected by using three different oligonucleotide probes deduced from a pattern 1 strain D471, and many isolates failed to be hybridized (38). The "divergent" *mga* was found in all strains of *emm* gene cluster patterns 3, 4, and 5, and the *mga* genes that can be hybridized with the oligo probes are those of pattern 1 and 2 strains (36). The diversity of *mga* genes is located mainly in the carboxyl termini (1), which indicates that activation of the response regulators of these two lineages of Mga molecules differs. Within the same pattern strains, *mga* sequences have higher homology, for example, 98% identity between *mga4* and *virR49* (1, 79). Although the two lineages of Mga proteins may differ substantially in their carboxyl termini, they can be complemented (1). For example, Mga4 is able to activate transcription of *emm6* gene in a complementation assay, suggesting that considerable amino acid variation in the carboxy-terminal region of Mga can be tolerated for activation of *emm* genes as long as the critical amino acid residues involved in phosphorylation are intact (1).

A GAS strain B514 virulent for mice--A potential animal model.

The interaction between the virulence factors and the host, and among the virulence factors is complicated for determining the pathogenesis of a disease. The availability of an experimental animal model that is susceptible to infection by the pathogen and develops a disease which mimicks that in human would facilitate studies on the pathogenic mechanism(s) of individual virulence factors. Although at least 95% of GAS infections occur in man, occasionally spontaneous infections can occur in animals. For example, streptococci of M types 50 and 51 have been shown to initiate spontaneous diseases in mice (54). M serotype 51 was discovered to cause acute infections in certain regions of the United States among mice (54). Serotype 50 isolated from Swiss albino and Princeton mice in the USA and in Europe, primarily induced chronic infections (39).

Occasionally, intranasal challenge of mice with type M50 strain can lead to sepsis and death (39). Later, the lethal dosage that killed all of the mice (LD_{100}) of this strain has been determined in the laboratory for protection studies. The LD_{100} in NMRI mice was shown to be 10^5 CFU when administered intranasally and 10^7 CFU when administered intraperitonally (53). Recently, Husmann et al. tried to develop a murine model of GAS infection by inoculating C3HeB/FeJ mice with B514-Sm (a spontaneous streptomycin-resistant B514 isolate) via the intranasal or intratracheal routes (43). No correlation was seen between the development of pneumonia in mice within 1 to 8 days postinoculation and to the doses given to mice via the intranasal route. The number of mice that developed pneumonia after intranasal inoculation was not reproducible and reached a plateau of about 60% regardless of the doses. However, when mice were challenged by the intratracheal route, a relationship was observed between the number of the mice that developed pneumonia and the doses of bacteria administered. The dose at which 50% of the mice exhibited signs of pneumonia within 72 h was 1.0×10^7 CFU (43). All of the mice inoculated with B514-Sm by intranasal

or intratracheal routes eventually became ill, suffering similar lung lesions, with intensive infiltration of inflammatory cells predominantly into the bronchioles and alveoli (43). Other organs such as brain, kidney, spleen, liver, and intestine appeared normal. The majority of mice with pneumonia were also bacteraemic (43).

Husmann et al. (43) successfully developed a pneumonia model by intratracheal inoculation of *S. pyogenes* strain B514-Sm to C3HeB/FeJ mice. To understand the roles of virulence factors of B514-Sm in the development of pneumonia, it is necessary to compare isogenic strains that differ in their expression of specific virulence factors and to test the virulence of parent strain and of isogenic mutant strains in the pneumonia model. It is also relevant to get information on the molecular aspects of individual virulence factor, since several complications raise when interpreting the results from animal models (118). First, GAS are primarily human pathogens, and the capacity of specific strains to colonize and cause disease in rodents determining the suitability of the animal model to reflect the human situation. Second, there is often more than one M-family protein in GAS isolates, rather than a single M protein, so evaluation of virulence contributions from those proteins depends upon understanding the entire *emm* gene cluster. Third, variable gene expression of the different M-family proteins in the same strain has been reported (6, 79), so monitoring expression is important. For these reasons, the most important virulence factor of GAS strain--*emm* genes--was first analyzed in this strain, as the commitment of my graduate research committee. The result about DNA sequences and the expression of *emm* genes of this strain is published as the first paper, "DNA sequencing and gene expression of the *emm* gene cluster in an M50 group A streptococcus strain virulent for mice." The second paper is titled "Role of putative virulence factors of *Streptococcus pyogenes* in mouse models of long-term throat colonization and pneumonia." This was a collaborative study in which I isolated a capsule-negative B514-Sm strain by plasmid insertion mutagenesis, with two other isogenic *emm* gene cluster or *scpA* mutant strains constructed by

Husmann et al. (42). The virulence of the wild-type B514-Sm and three isogenic mutant strains were tested in two mouse models. The third paper is the continued studies on *mga50* gene according to the results from the previous experiments with B514-Sm. It was found that the *mga* gene is defective due to the point mutation(s) in protein sequence which leads to the attenuated expression of downstream *emm* gene cluster. By transforming the *mga* gene cloned in a plasmid from serotype 4 or 6 GAS strains, either plasmid can complement the defective Mga50 and restore the expression of M-family proteins in B514-Sm.

DNA SEQUENCING AND GENE EXPRESSION OF THE *emm* GENE CLUSTER IN AN M50 GROUP A STREPTOCOCCUS STRAIN VIRULENT FOR MICE

by

DER-LI YUNG AND SUSAN K. HOLLINGSHEAD

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ABSTRACT

The strain B514, an M serotype 50 strain, is capable of causing a natural upper respiratory infection leading to death in mice, as reported by Hook et al. in 1960 (E. W. Hook, R. R. Wagner, and R. C. Lancefield, Am. J. Hyg. 72:111-119, 1960). Thus, this strain was of interest for use in developing an animal model for group A streptococcal colonization and disease. The *emm* gene cluster for this strain was examined by PCR mapping and found to contain three *emm* family genes and cluster pattern 5. PCR-generated fragments corresponding to the SF4 (*mrp*50) gene, the SF2 (*emmL*50) gene, and the SF3 (*enn*50) gene were cloned and the entire gene cluster was sequenced. The gene cluster has greater than 97% DNA identity to previously sequenced regions of the gene cluster of the M2 strain T2/44/RB4 if two small divergent regions that encode the mature amino-terminus of the SF-2 and the SF-3 gene products are not included. If expressed, the genes encode proteins which bind human IgG (Mrp50 and EmmL50) or IgA (Enn50). However, in isolates taken directly after passage in mouse, the surface proteins arising from these genes were barely detectable. The transcription of each gene in the B514 strain was investigated by Northern (RNA) hybridization, and mRNA transcripts were detected and quantitated relative to those of the *rec*A gene, a housekeeping gene. Transcription of all three *emm* family genes was found to be over 30-fold attenuated relative to transcription of the same genes in the strain T2/44/RB4. This suggests that the positive regulator, Mga, is either not active in this strain or has a different requirement for activation; it also suggests that capsule may be sufficient to inhibit phagocytosis under this circumstance.

INTRODUCTION

Group A streptococci (GAS) are important human pathogens capable of causing a wide variety of infections, the most common of which are nasopharyngitis and impetigo. Of greater concern are the invasive infections such as necrotizing faciitis,

pyomyositis, and toxic shock syndrome, which appear to have increased in incidence over the past decade. Rheumatic fever and acute glomerulonephritis are also poorly understood sequelae of GAS infection. Understanding the complex pathogenesis of the different group A streptococcal infections is important and will require knowledge of the functions of many related and unrelated virulence factors. Among these factors are a family of proteins which form dimeric fibrils projected from their carboxy termini which are embedded in the wall of the bacterial cell surface (33).

Within this family are the M proteins, defined as such because of their antiphagocytic function and thought to contain epitopes defining the antigenically variable determinants of serotype specificity in their amino termini (24, 25). Recent molecular analyses have revealed over 100 distinct *emm* family genes which encode proteins highly similar to the M protein, most of which do not have antiphagocytic properties but which do have other virulence-related phenotypes (18). For example, the genes *arp*4 (27), *enn*2 (4), *fcr*A76 (13), and *mrp*4 (44) encode immunoglobulin-binding proteins. Additional binding properties associated with members of this family are fibrinogen, plasminogen, factor H, albumin, and C4b binding protein (1, 22, 38, 46, 49).

All of proteins encoded by *emm* family genes have highly similar sites towards their carboxy termini, and by alignment of these carboxy terminal gene regions, four divergent forms within this gene family, called subfamilies (SF1 to SF4), have been identified (16). Individual genes in each subfamily may be mapped on the streptococcal chromosome by PCR amplification with subfamily-specific primers. One to three distinct copies of *emm* family genes are found to make up an *emm* gene cluster in any one GAS strain, but each strain has a different *emm* cluster. The *emm* cluster is located downstream from a transcriptional regulatory gene (*mga*, previously termed *vir* or *mry*) (7, 39) and upstream of the *scp*A gene, which encodes a C5a peptidase (8). Mga is a positive transcription factor for the genes in the *emm* gene cluster and the *scp*A gene (9,

32). At least five different chromosomal patterns of *emm* gene clusters have been found by examination over 300 diverse GAS strains (16, 20), and there are significant associations of these patterns with tissue site of isolation and with streptococcal class (6, 17).

To understand the roles of M and M-like proteins in disease pathogenesis, it is necessary to construct and compare isogenic strains that differ in their expression of specific M and M-like proteins in animal models of disease. There are several complications when interpreting the results from animal models. First, because group A streptococci are primarily human pathogens, the capacity for animal models to reflect the human situation is limited to the capacity of individual strains to colonize and cause disease in rodents. Second, there are often three M or M-like proteins in group A streptococcal isolates from both humans and mice, rather than a single protein, so evaluation of virulence contributions depends upon knowledge of the entire cluster. Third, variable gene expression of the different M and M-like proteins in the same strain has been reported, so monitoring expression is important. For these reasons we investigated the virulence gene characteristics of a natural group A streptococcal strain which is virulent for mice.

The M serotype 50 strain B514 was first identified as the cause of several outbreaks of natural infections in mice (21). This strain was and is highly mucoid on blood agar plates, indicating that it is encapsulated. This strain continues to be more virulent for mice than other GAS strains; when administered intranasally, strain B514 had a 100% lethal dose of 10^5 CFU, as compared with $>10^7$ CFU for five other GAS strains (26), and as little as 20 CFU can give an intense inflammatory response in the mouse lymphoid tissue (50, 51). The reasons for the extreme mouse virulence of the B514 strain are not understood. The B514 strain has also been used to study the protective effects of vaccination (26, 45) and group A streptococcal pneumonia (23). Therefore, this strain is of continuing interest for studies of streptococcal pathogenesis.

To begin examining the role of multiple phenotypes associated with M and M-like proteins in pathogenesis, the *emm* cluster of this strain was mapped by PCR, individual genes of the cluster were cloned, the gene cluster was sequenced and the expression of each gene was examined by quantitative Northern hybridization analysis.

MATERIALS AND METHODS

Bacterial strains and *emm* gene family nomenclature. Group A streptococcal strain B514 is the serotype 50 typing strain, and T2/44/RB4 is the serotype 2 typing strain (40). T2/44/RB4 was received from D. Bessen (Yale University, New Haven, Conn.). A streptomycin-resistant spontaneous variant of B514, recently tested in a mouse model of virulence, was received from M. Caparon (Washington University, St. Louis, Mo.) and is designated herein as B514-Sm1. B514-Sm2 is the same strain after an additional passage in laboratory medium.

The regulatory gene upstream of the *emm* gene cluster was previously called either *mry* or *vir*. It is now called *mga* (43). All homologues of *emm* are referred to as *emm* family genes, but the specific designation *emm* is reserved for genes encoding proteins with known antiphagocytic function. Therefore, the functionally neutral terms *mrp*, *emmL*, and *enn* are used for the SF4, SF2, and SF3 genes, respectively, in this manuscript.

PCR and gene cloning. B514 chromosomal DNA was isolated by a microwave method described previously (20). PCR was used to map the gene cluster with subfamily-specific primers, to generate fragments for the cloning of individual genes, and to generate fragments for gene-specific DNA probes for individual genes. All of the oligonucleotide primers used in this study are listed in Table 1, and an overview map of all the PCR fragments is included in Fig. 1. PCR reactions designed to map the genes in the cluster were carried out as described by Hollingshead et al. (20); a standard

TABLE 1. Oligonucleotides used for PCR mapping, cloning, and probes

Primer	Sequence	Description[a]
For PCR mapping:		
SF3-F	5'-GGTAGAGCTGCTCAAACAGCTACAAGACCT-3'	2409-2438 *emmL2.2* (4)
SF2-F	5'-AACGCTAAAGTAGCCCCACAAAGCTAACCGT-3'	1076-1105 *emmL2.1* (4)
SF4-F	5'-CCAACAAGACCATCACAAAC-3'	1318-1388 *fcrA76* (13)
SF2-LDR	5'-AATCTGCAGTATTCGCTTAGAAAATTAAAA-3'	47-68 *emmL2.1* (4)
SF4-LDR	5'-GAAATCCAAACAAGCACTACCTACTG-3'	24-249 *fcrA76* (13)
DP-2	5'-ATCCCTAATAGTCGCTTTTGAGG-3'	2091-2062 *emm6.1*, 674-645 *scpA* (8, 18)
SF2-R	5'-GTTAGCTTGTGGGGCTACTT-3'	1101-1076 *emmL2.1* (4)
SF3-R	5'-GCTGTTTGAGCAGCTCTACC-3'	2429-2409 *emmL2.2* (4)
CW-1R	5'-GAATGGGTTAGCTGTTTC-3'	Composite from *emm* gene alignment
For cloning and probes:		
UP-8	5'-TGAAAACAGCTCAAAAAAACTGACC-3'	Pair with IG5-R for cloning *mrp50* (SF4)
IG5-R	5'-GGCTAGAAAAGATAGTGTGGGTTG-3'	Pair with UP-8 for cloning *mrp50* (SF4)
SF2-LDR	5'-AATCTGCAGTATTCGCTTAGAAAATTAAAA-3'	Pair with OM2-4 for cloning*emmL50* (SF2) and with OM2-3 for cloning *enn50* (SF3)
LDRALL	5'-GGATCCCCGGGCATCCGTAGCAGTCGCT-3'	Probe of SF4 gene with 2127-R
2127R	5'-TTCTTGGTTGGTTGCTGCTAATT-3'	Probe of SF4 gene with LDRALL
915-R	5'-GTTCTTGATAACGTTTTTCTACTTCTCG-3'	Probe of SF2 gene with SF2-LDR
Enn50-F	5'-TGGAACTTCTGTAAATAATGG-3'	Probe of SF3 gene with Enn50-F
245-R	5'-TTCTTTAGTAGTCTTAGCTAAAGTTGT-3'	Probe of SF3 gene with 245-R

[a] For all PCR mapping primers except CW-1R, the description includes position (in base pairs), gene, and (parenthetically) applicable references(s).

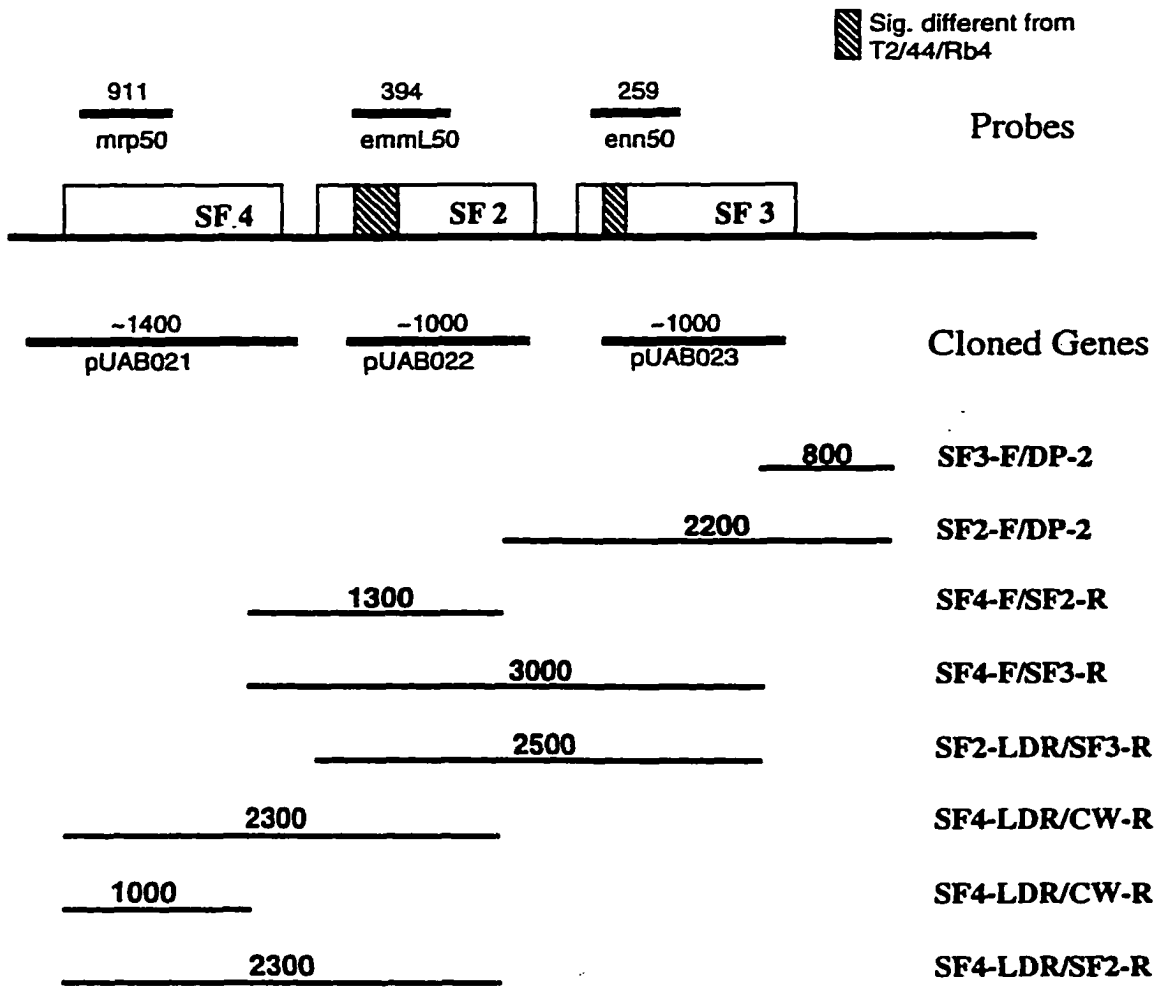


FIG. 1. Chromosomal map of *emm* gene cluster of serotype M50 strain B514. The numbers indicate sizes in base pairs. PCR amplification with the subfamily-specific primers shown in the bottom half of the figure was used to map three genes: an *mrp50* (SF4) gene, an *emmL50* (SF2) gene, and an *enn50* (SF3) gene. Primers for the amplification products are listed to the right of the lines indicating the PCR products. Gene-specific PCR fragments that were cloned for nucleotide sequencing and smaller fragments that were used as DNA probes are indicated below and above the genes, respectively. The two divergent regions in the *emmL50* and *enn50* genes (i.e., regions that are significantly different from T2/44/Rb4 regions) are indicated by the two small hatched boxes.

PCR mixture of 50 μl containing 2.5 mM $MgCl_2$, 200 μM each deoxynucleoside triphosphate, 50 pmole of each primer, and 2.5 U of *Taq* DNA polymerase was used for all other PCRs, with a cycle of 1 min at 95°C, 1 min at 62°C, and 5 min at 72°C, repeated 35 times. For cloning, PCR products were first purified from low-melting-temperature agarose gels (SeaPlaque agarose; FMC) by GeneClean (BIO 101, Inc., La Jolla, Calif.) and ligated to the pCR vector (Invitrogen Co.) before transformation into *Escherichia coli* INVaF'. White colonies were picked from Luria agar plates containing 50 μg of ampicillin per ml, and the plasmid DNA was isolated to screen for plasmids containing the DNA insert of the correct size. The plasmids pUAB021, pUAB022, and pUAB023 contain the SF4, SF2, SF3 genes, respectively, from the *emm* gene cluster of B514 that were cloned in the pCR vectors (Fig. 1).

DNA sequencing. Plasmid DNAs from pUAB021, pUAB022, and pUAB023 were sequenced by the dideoxy chain termination method (41) following procedures described in Sequenase manual (version 1.0; United States Biochemical Corp.) with the primers listed in Table 1. The regions between the cloned genes were sequenced from additional PCR fragments with an Applied Biosciences automated DNA sequencer.

Surface protein analysis and mouse passage. Strain B514 was passaged in mice intraperitoneally by inoculating either 10^6 or 10^4 bacteria. If death due to sepsis had not occurred by 48 h, mice were killed at that time, and individual organs were then dissected and homogenized. The homogenates were plated on sheep blood agar plates, and individual bacterial colonies from each organ were chosen for further analysis. CnBr-extracted surface proteins were analyzed by the method of Raeder et al. (36).

RNA purification. B514 cultures were grown in 37°C standing cultures in Todd-Hewitt yeast broth plus 20 mM glycine in a 5% CO_2--20% O_2 atmosphere. Total cellular RNA was isolated from streptococcal cultures at optical densities at 600 nm between 0.6 and 1.0. Total cellular RNA was purified following centrifugation on CsCl gradients and stored in the presence of vanadyl ribonucleosides as previously described (19).

Northern hybridization. Total cellular RNA (20 μg) from each strain was separated by electrophoresis on formaldehyde-containing agarose gels (28) and transferred to nylon membranes by capillary transfer. The probes used for Northern hybridization were PCR-generated fragments of each of the three genes in the *emm* gene cluster of B514 that include only the gene-specific regions of each gene. These gene-specific probes, shown on Fig. 1, included bp 181 to 1091 of the SF4 gene (*mrpA50*), bp 1643 to 2036 of the SF2 gene (*emmL50*), and bp 3215 to 3472 of the SF3 gene (*enn50*). A *recA* gene probe of 314 bp was previously cloned from GAS strain D471 as described by Dybvig et al. (10). All DNA fragments used for probes were purified from low-gelling-temperature agarose and then radiolabeled with P^{32} by random primer labeling (Boehringer Mannheim Corp.). The probes had specific activities of 2 X 10^6 to 5 X 10^6 cpm/pmole, and they were used at the concentrations of 10 pmol/ml. Northern hybridizations were performed in 6 X SSC (1 X SSC is 0.15 M NaCl plus 0.015 M sodium citrate)--50% formamide at 42°C. Hybridization was visualized by exposure to XAR-1 x-ray film.

Quantitation of transcript levels. Radioactive bands on the blots were quantitated by using a Molecular Dynamics PhosphorImager. All blots that were to be compared were exposed overnight for equivalent time periods on the same screen, and

counts were taken. The internal radiolabeling method used for the probes allowed the detection of small quantities of transcript (100-fold below the detection level for the *recA* transcript). The *recA* gene transcript was used as an internal control in order to compare the relative transcript levels of different genes in the same strain and to roughly compare transcript levels of the same gene in different strains.

First, quantitation controls were performed to examine potential signal variability due to loading volumes, transfer procedures, or stripping of blots for reprobing. There was no loss of signal with stripping of blots, and there was less than a twofold difference in duplicate samples. For experimental runs, three identical blots were first probed with the three *emm*-specific probes; then the blots were stripped and reprobed with the *recA*-specific probe. Ratios of each *emm* gene transcript to the *recA* transcript from the same lane and the same blot were then calculated for reporting. In comparing the PhosphorImager counts of different transcripts, corrections for differing probe length were included. Any differences greater than 10-fold remaining after a correction for probe length were considered significant.

Nucleotide sequence accession number. The DNA sequence of the *emm* gene cluster for serotype M50 has been submitted to GenBank, and the nucleotide accession number is U520008.

RESULTS

PCR mapping of the gene cluster and cloning of individual genes. PCRs were carried out with subfamily-specific primer pairs to examine the structure and order of the *emm* gene cluster of M50 strain B514 as described previously (20). Figure 1 shows the approximate sizes and locations of amplified PCR products of the B514 chromosomal DNA, on the basis of which the strain was shown to have

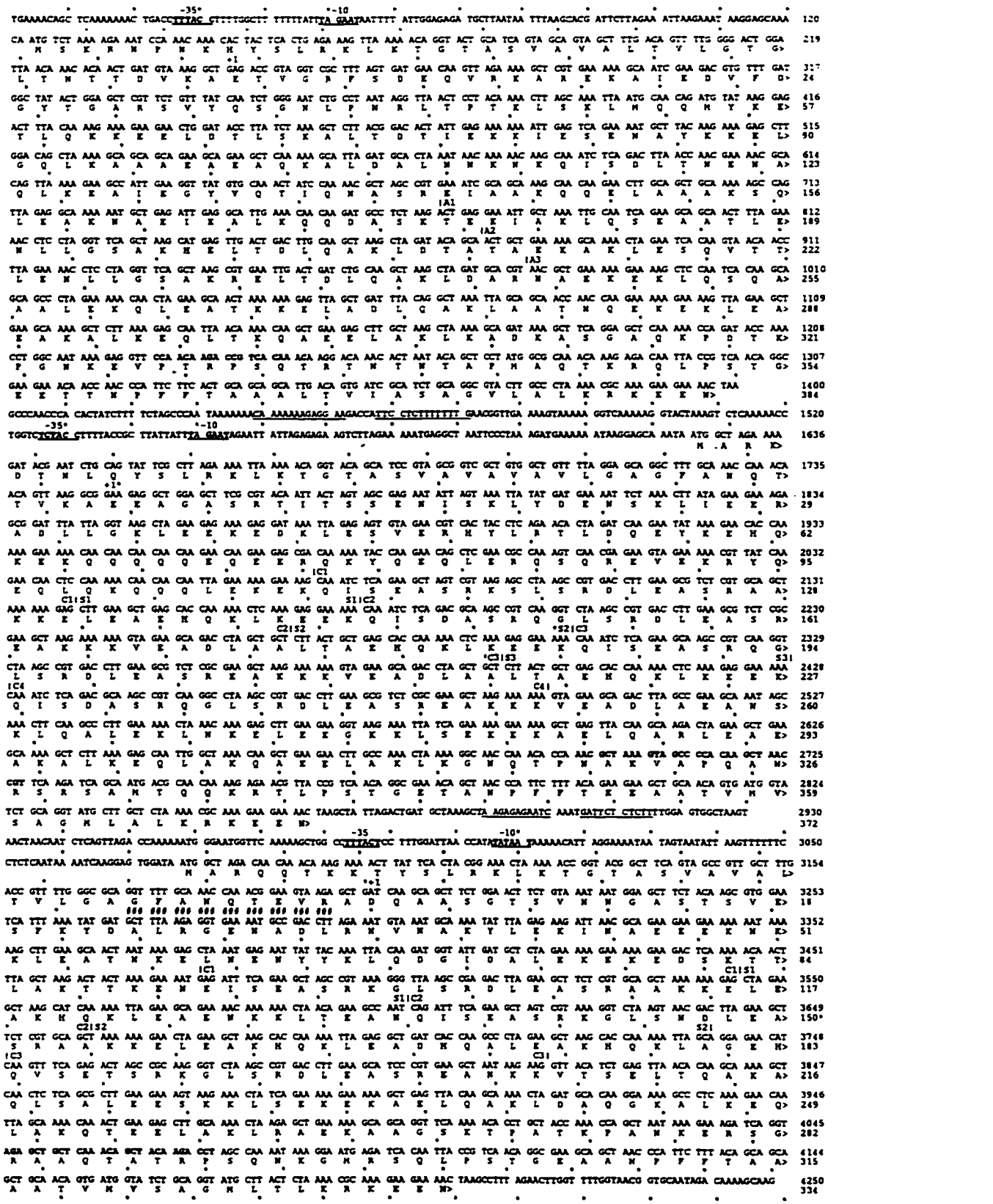
chromosomal pattern 5. The order of genes in B514 is SF4 gene-SF2 gene-SF3 gene, and they are located between the *mga* and *scpA* genes. In order to sequence the individual genes from strain B514, PCR products that encompassed most of each gene were individually cloned in the plasmids pUAB021, pUAB022, and pUAB023 (Fig. 1).

DNA sequence. Figure 2 shows the DNA sequence of the entire *emm* gene cluster of B514. The gene cluster of serotype M50 strain-B514 is over 97% identical to the gene cluster of serotype M2 strain T2/44/RB4 with the exception of two divergent regions characteristic of mosaic genes. The two small divergent regions between the M50 and M2 strains (hatched boxes on the map in Fig. 1) are present near the amino termini, immediately after the signal peptide, in both the SF2 and the SF3 genes. In the M2 strain, both the SF2 gene (*emmL2.1*) and the SF3 gene (*emmL2.2*) were previously characterized for immunoglobulin-binding characteristics and completely sequenced (4) and the SF4 gene was partially sequenced (5).

The SF4 gene (*mrp50*) encodes a protein with 97% identity to FcrA76 which binds the Fc region of human immunoglobulin G1 (IgG1), IgG2, and IgG4 proteins. Like other SF4 genes, instead of having central C repeat regions, the *mrp50* gene has three A repeat regions which have been characterized in *fcrA76* and *mrp4* genes as being the binding site for the IgG Fc (13, 31). Therefore, the *mrp50* gene encodes an IgG-binding protein. Protein expressed from this gene in *E. coli* binds human IgG when expressed and purified in vitro (data not shown).

The SF2 gene, *emmL50*, also encodes a potential IgG-binding protein. Its sequence is highly similar (97% identity) to that of the gene *emmL2.1*, in the entire coding region with the exception of a small region encoding amino acids 1 to 81 of the mature protein (bp 1749 to 1990). The remaining portion of the mature protein

FIG. 2. Nucleotide and deduced amino acid sequences of *emm* gene cluster in the M50 serotype strain B514. DNA sequence was determined by dideoxy sequence analysis with the clones and PCR-generated fragments shown in Fig. 1. Putative promoter sequences (-35 and -10) and ribosomal binding sites are indicated by boldface underlining. The inverted repeats that may serve as potential transcriptional terminator structures are indicated by nonbold underlining. Amino acid residue numbers are given, beginning with the first residue of the processed forms, on the basis of sequence similarity with the leader sequences of other M and M-like protein genes. The *mrp50* (SF4) gene, as indicated here, contains A repeats (A). C repeats (C) are present in *emmL50* and *enn50* genes. The intervening spacers (S) are indicated. The pound signs are directly above the IgA binding site in the *enn50* (SF3).



contains regions homologous to protH (SF1), *emm49* (SF2), and *emmL2.1* (also SF2), all of which bind to the IgG Fc region (4, 11, 12). Mutations in the homologous region in recombinant molecules of protH and *emmL2.1* affect IgG binding (6). A recombinant *emm50* gene product expressed in vitro was also shown to bind human IgG (data not shown).

The 5' end of the SF3 gene of strain B514 contains a domain (amino acids 24 to 32 [ALRGENADLR]) identical to that required for IgA binding in *emmL2.2* (SF3) (2) and similar to that in the genes *arp4* and *arp60* (SF2) (14), which are also human IgA-binding proteins. Again, protein from this gene expressed in vitro was found to bind human IgA (data not shown), although there is a published report that strain B514 does not bind IgA (42).

CnBr-extracted surface proteins. The method of Raeder et al. (36) was used to examine the expression of surface proteins in B514. This procedure preferentially releases the *emm* gene family proteins in all streptococcal strains in which it has been examined (36). CnBr extraction of B514 resulted in very little surface protein relative to the amounts obtained from extraction of other strains even upon concentration (data not shown).

RNA transcripts. Transcription of each of the three genes in the *emm* gene cluster in B514 was analyzed by Northern hybridization gels. PCR fragments from the 5' end region of each gene were used as the *mrp50*, *emmL50*, and *enn50* probes (Fig. 1); limiting probes to the 5' end of each gene makes them gene specific and allows the differentiation of the transcripts of the three separate family members in the *emm* gene cluster. A transcript of the *recA* gene of *Streptococcus pyogenes* was used as the internal marker for standardization of the loading quantities of RNA samples.

Figure 3 displays the autoradiographs of individual gene transcripts of the *emm* gene cluster of B514. Monocistronic RNA transcripts of each of the three genes in the *emm* gene cluster were detected, with sizes appropriate for previously described transcriptional start and stop sites of these genes (15, 34). Duplicate cultures and RNA preparations of the mouse-virulent strain B514 showed the same levels of each relative transcript. The transcripts of the *mrp50* and *emmL50* genes were detected in quantities similar to that of a *rec*A transcript for a housekeeping gene, but the *enn50* gene transcription level was nearly 50-fold lower and barely detectable above the background. The very low level of the *enn50* transcript in B514 might explain the inability of Shalen to detect cell surface binding of human IgA in B514 (42).

In order to quantitate the relative expression levels of these three genes in strains B514 and T2/44/RB4, ratios of counts detected for *emm* transcripts to counts detected for *recA* transcripts on the same gels were calculated (Table 2). Rough estimates of the relative transcript levels for genes in the *emm* cluster of each strain can be made from the values in Table 2. Thus, the ratio of *mrp50* transcripts to *emmL50* transcripts in strain B514 was roughly 1:1, while the ratios of both *mrp50* and *emmL50* transcripts to those of the *enn50* gene in B514 were approximately 35:1. In strain T2/44/RB4, the ratio of transcripts within the gene cluster (*mrp:emmL:enn*) was similar to the internal ratio in B514 (Table 2), but T2/44/RB4 differed from B514 in that it had much more abundant levels of all three *emm* family transcripts in cultures grown under identical laboratory conditions (Fig. 3).

Analysis of *emm* family gene expression after passage in mice. To determine if the very low levels of gene expression were significantly different from the gene expression during passage in mice, individual B514 isolates from dying mice were examined. Bacteria were introduced to mice by the intraperitoneal route, and individual isolates from dessimination sites such as the spleen, lung, and brain were

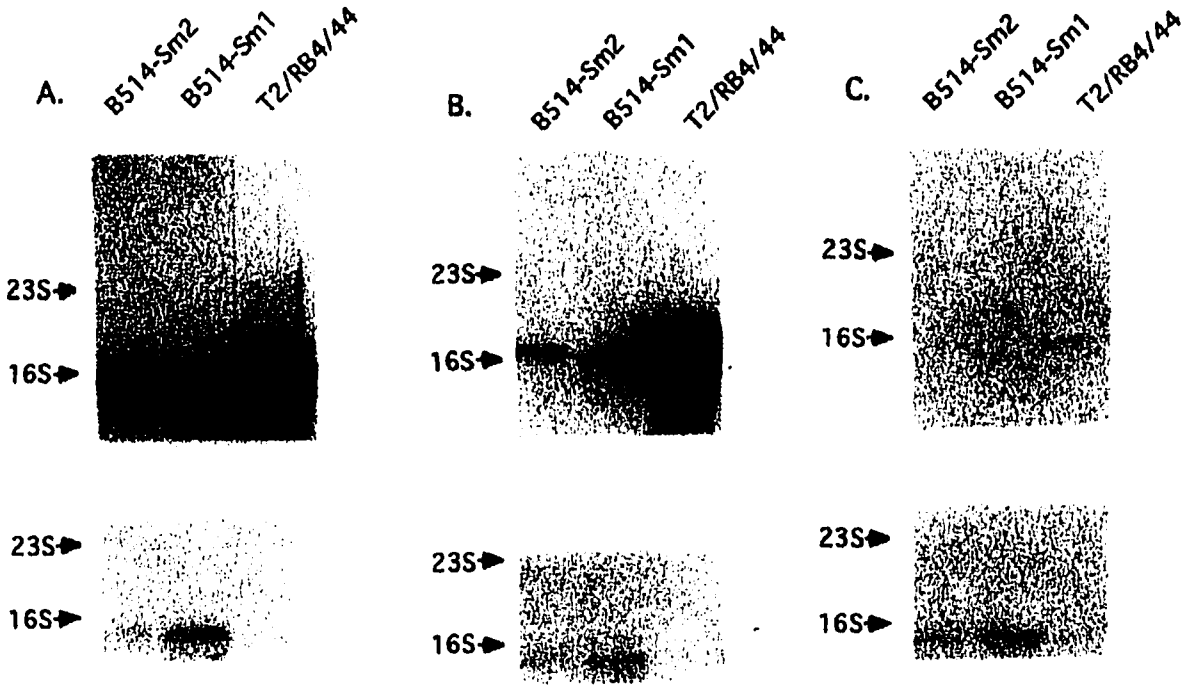


FIG. 3. Northern hybridization to show relative transcript levels in T2/44/RB4 (M2) and two isolates of strain B514 (M50-Sm1 and-Sm2). All strains had been recently passaged in mice, and RNA was extracted from cells grown in vitro under Mga-positive conditions. In this experiment, DNA probes specific for *mrp50*, *emmL50*, and *enn50* genes included only the region that encodes the 5' mature end of each protein (shown in Fig. 1). (A) Transcripts detected with the *mrp50* probe (SF4); (B) transcripts detected with the *emmL50* probe (SF2); (C) transcripts detected with the *enn50* probe (SF3). Each lane contains 20 μg of total cellular RNA from each culture. The exposure time was 4 h (A and B) and 48 h (C). The positions of two ribosomal RNAs are shown on the left. The bottom half of each panel shows the *recA* transcript detected on the same blot, used as the internal standard.

TABLE 2. Ratios of counts of specific *emm* gene transcripts to those of *recA* control transcript[a]

Gene probe	Transcript ratio in strain	
	B514 (M50)	T2/44/RB4(M2)
SF4 gene (*mrp50*)	0.7	32
SF2 gene (*emmL50*)	0.6	59
SF3 gene (*enn50*)	0.02	2

[a] Corrections were made for variations in probe length.

examined for surface proteins by the CnBr extraction method. All of the individual B514 isolates from mice had undetectable surface protein expression compared with that of two other group A strains (Fig. 4). Gene cluster expression in fresh passage isolates was indistinguishable from that in the isolate in which expression was initially quantitated. We infer that expression of this gene cluster may not be critical for virulence in this model system and that phase variation to an "on" state is not the usual outcome of passage of this strain in mice. Additionally, all isolates obtained after passage in mice were highly mucoid on blood agar plates, suggesting high-level expression of the capsule in B514 isolates in vivo as well as in vitro.

DISCUSSION

Strain B514 is important for mouse models of group A streptococcal infection because it causes natural infections in mice. On the basis of the DNA sequence of the *emm* gene cluster in strain B514, it is evident that there are two genes that encode probable IgG- binding proteins and one gene that encodes a probable IgA-binding protein. Binding studies of proteins produced in vitro from cloned genes showed the predicted binding capabilities (data not shown); however, our studies show that these genes are not expressed in the streptococcal host. By PCR mapping, the *emm* gene

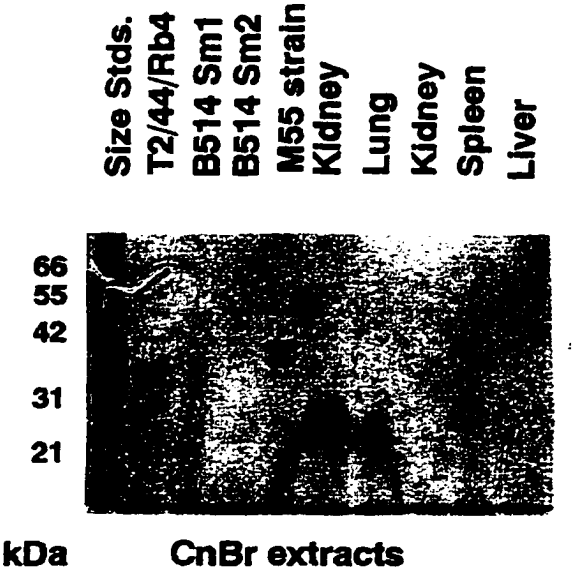


FIG. 4. CnBr extracts of surface proteins from B514-Sm1, from control strains, and from individual isolates obtained immediately after passage in mice. Lane 1, size standards; lanes 2 and 5, protein from an M2 strain and an M55 strain, respectively, isolated from mice (positive controls for strains expressing *emm* loci proteins); lanes 6 to 10, CnBr-extracted proteins from isolated obtained immediately after passage in mice. Stds., standards.

cluster was characterized as having chromosomal pattern 5, which has SF4, SF2, and SF3 genes located between *mga* and *scp*A genes. This pattern is positively associated with the ability to produce a serum opacity factor (SOF) that causes mammalian sera to become opalescent (3, 20). In our collection, 89% of pattern 5 strains are SOF^+, while only 2% of non-pattern 5 strains are SOF^+. B514 is SOF^-, even though it is a pattern 5 strain, and it fails to produce a serum opacity factor.

The sequence of the M50 gene cluster was >97% identical to that of the gene cluster of a serotype M2 strain, T2/44/RB4, except for two small regions near the amino termini of the SF2 gene (81 amino acids) (Fig. 2) and the SF3 gene (23 amino acids) (Fig. 2). One of these two heterogeneous regions may define the M50 type-specific domain of B514, differentiating M50 strains from M2 strains in the Lancefield serotyping system for group A streptococci. Although serotype M2 strains are frequently reported to be associated with human infections, M2 strains are not virulent for mice unless they have been passaged several times through mice to select for virulent variants. In contrast, the serotype M50 strains are not usually found among human isolates, and are among the very few group A streptococci originating from multiple mouse colony outbreaks (21).

Low transcript levels for entire cluster in strain B514. Although transcripts of all three genes in strain B514 could be detected, quantitation studies revealed at least 30-fold less transcript in B514 than in strain T2/44/RB4 (Table 2). The low expression levels were initially surprising because laboratory passage of the strain had been minimized after recent testing for full virulence in mice and care was taken to use growth conditions under which maximum expression of *emm* genes has been obtained. Low levels of expression were consistently found for multiple RNA preparations made from the same strains. The low transcript levels did seem to explain

our inability to detect surface proteins in B514 by a cyanogen bromide extraction procedure (Fig. 4). In most streptococcal strains, two or more proteins are extracted by this procedure, but with B514 very little, if any, protein is extracted.

The very low level of transcription of all three genes in the M50 cluster may represent the uninduced levels of transcription for these genes. These genes are positively regulated by the protein Mga (previously called Mry or Vir) (7), and the regulation involves binding of Mga protein to conserved sites present near the -35 region of the promoters of these genes (29, 34). All of the sequences of the promoter regions are identical in strains B514 and T2/44/RB4 (Fig. 2), so regulation site differences are unlikely to explain the difference in expression between these two strains. Thus, the extremely low level of expression in B514 may indicate that the *mga* gene is either defective or only active under conditions that differ from those required by most streptococcal strains. Footprinting experiments have identified an Mga-binding site for the *emm6* gene and the *scpA* gene located near the -35 region of only one of the two promoters for these genes identified by primer extension experiments (37). It is possible that the basal level of transcription found in the B514 strain results in transcripts originating from promoters that are not under the control of the Mga gene product. A defect in Mga expression in this strain may also explain why the strain is SOF^- even though it is a pattern 5 strain. DNA sequence of the *sor* gene locus revealed a promoter sequence that matches the consensus for Mga binding (29, 37).

The SF3 gene is poorly expressed relative to other genes. As has been previously reported for the genes in this regulon (4, 34), there was a large difference in the relative transcript levels for different genes in the *emm* gene cluster of a specific strain. For example, the SF2 transcript was 30-fold more abundant than the SF3 gene transcript in both the T2/44/RB4 strain and B514 strain (Table 2). This quantitative estimate of difference in transcription levels is consistent with the over 32-

fold difference seen by Bessen and Fischetti (4) and the 16-fold difference for analogous transcripts in strain CS101 seen by Podbielski et al. (34) as well as with additional reports of low-level expression of a gene in this position (48). This could be the result of differential regulation of individual promoters or of differences in promoter strength.

A recent publication highlighted a role for group A streptococcal capsule in pharyngeal colonization of mice (47). The B514 strain is highly mucoid and encapsulated. If the roles in pathogenesis for the capsule and the *emm* gene cluster are overlapping, encapsulation may be important for the virulence of strain B514 for mice in light of the poor expression of *emm* genes in this strain. Whether these roles are equivalent to the roles in human infections is very difficult to address, but strains of certain M serotypes are frequently heavily encapsulated.

Switching from high to low expression of M proteins is referred to as phase variation, and the molecular mechanism by which phase variation occurs is unknown (39). To further test whether in vivo expression of the gene cluster was likely to be much higher in strain B514, passages in mice were performed and expression of this gene cluster in multiple isolates sampled from body sites of dissemination was examined. The low-level-expression or "off " state of this gene cluster in the mouse isolate appeared to be the usual state (Fig. 4).

The *emmL50* gene was found to have a mosaic structure. The heterogeneous 81-bp that was different from *emmL2.1* sequence was instead very similar (>80% identity) to the published sequence of *enn5.6183.2*, which is from a serotype 5 strain (48). The *enn-5.6183.2* gene is in a subfamily distinct from *emmL2.1* or *emmL50*; genes in different subfamilies show an average divergence of 17-27% while genes in the same subfamily show an average divergence of 3%. The M5 strain from which the *enn5.6183.2* gene was isolated has *emm* gene cluster pattern 2, containing two SF1 genes and none from the other subfamilies. The cluster patterns are also thought to

have resulted from evolution of this multigene regulon. The mosaicism of the *emmL50* gene could be explained if a recombination event occurred between an M2-like strain and a strain carrying an *enn5.6183.2*-like gene. This could result in most of the *emm50* gene being almost identical to an *emm2.1* gene while another part of the gene is almost identical to the *enn5*-like gene. The difference in chromosomal patterns (or gene context) suggests that past recombination events involved horizontal transfer between strains that were genetically quite distant from each other, at least with respect to this particular segment of the chromosome. Evidence for the contribution of horizontal transfer events in the evolution of the *emm* gene family has recently been discussed (5, 17, 30, 35, 48).

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ROLE OF PUTATIVE VIRULENCE FACTORS OF *STREPTOCOCCUS PYOGENES* IN MOUSE MODELS OF LONG-TERM THROAT COLONIZATION AND PNEUMONIA

by

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ABSTRACT

To investigate the role of putative virulence factors of *Streptococcus pyogenes* (group A streptococcus, GAS) in causing disease, we introduced specific mutations in GAS strain B514, a natural mouse pathogen, and tested the mutant strains in two models of infection. To study late stages of disease, we used our previously described mouse model (C3HeB/FeJ mice) in which pneumonia and systemic spread of the streptococcus follow intratracheal inoculation. To study the early stages of disease, we report here a model of long-term (at least 21 days) throat colonization following intranasal inoculation of C57BL/10SnJ mice. When the three *emm* family genes of GAS strain B514-Sm were deleted, the mutant showed no significant difference from the wild type in induction of long-term throat colonization or pneumonia. We inactivated the *scpA* gene, which encodes a complement C5a peptidase, by insertion of a nonreplicative plasmid and found no significant difference from the wild type in the incidence of throat colonization. However, there was a small but statistically significant decrease in the incidence of pneumonia caused by the *scpA* mutant. Finally, we demonstrated a very important effect of the hyaluronic acid capsule in both models. Following intranasal inoculation of mice with a mutant in which a nonreplicative plasmid was inserted into the *hasA* gene, which encodes hyaluronate synthase, we found that all bacteria recovered from the throats of the mice were encapsulated revertents. Following intratracheal inoculation with the *hasA* mutant, the incidence of pneumonia within 72 h was significantly reduced from that of the control strain ($P = 0.006$). These results indicate that the hyaluronic acid capsule of *S. pyogenes* B514 confers an important selective advantage for survival of the bacteria in the upper respiratory tract and is also an important determinant in induction of pneumonia in our model system.

INTRODUCTION

The group A streptococcus (GAS, *Streptococcus pyogenes*) is a serious human pathogen, both because of its prevalence and because some of the diseases it causes are severe and even fatal. While uncomplicated pharyngitis is the disease syndrome most commonly associated with this organism, the GAS is also capable of causing a variety of invasive diseases, including necrotizing fasciitis, myositis, bacteremia, streptococcal toxic shock syndrome, and pneumonia. In addition, the delayed sequelae of rheumatic fever and acute glomerulonephritis may follow some types of GAS infection in some people.

The goal of the present study was to examine the importance of several putative virulence factors in the early and late stages of GAS respiratory disease in mouse model systems. For these studies, we used strain B514-Sm, a spontaneous streptomycin-resistant derivative (19) of GAS B514 (type M50), which was originally isolated from Swiss Webster mice and is a natural cause of respiratory disease in these animals (18).

The primary virulence factor of GAS is considered to be the surface-located M protein (23). Although all M proteins have a dimeric coiled-coil structure similar to that of tropomyosin and are attached to the GAS surface by their carboxyl-terminal regions, the serological type of M protein differs from strain to strain because the sizes and sequences of these proteins are highly variable. M proteins are defined by their ability to protect the GAS from phagocytosis by polymorphonuclear leukocytes, a function that has been directly demonstrated by the use of isogenic strain pairs for M6 (28, 36) and M24 (9). The antiphagocytic role of other serological types of M protein has been demonstrated in other strains by showing that anti-M antibodies inhibit the function of M protein and allow phagocytosis of the bacteria.

A protein with sequence similarity to M proteins is termed M related if the GAS strain expressing it has not yet been tested in a phagocytosis assay. Adjacent to the gene encoding the M protein (*emm*), many GAS strains have one or two genes for M-

related proteins with various degrees of structural relatedness to the M protein. They are classified into subfamilies 1 to 4 (SF1 to SF4) on the basis of sequence identities in the conserved carboxyl termini (143 amino acids) required for attachment to the streptococcal surface (17). Often these predicted proteins also include the conserved C repeat region and sometimes the heptad amino acid pattern responsible for the coiled-coil structure of M proteins.

The protection from phagocytosis afforded by M proteins appears to be caused by interference with the alternative complement pathway (4, 30) and may involve the binding of fibrinogen (44, 45). Although the only region whose amino acid sequence is conserved among all M proteins is the C repeat region located near the carboxyl termini of the molecules (37), this region is not required for fibrinogen binding (1) or for resistance to phagocytosis (29). An additional potential virulence role for M family proteins has been suggested on the basis of another function shared by many of them: the ability to bind one or more types of immunoglobulin (Ig). Ig binding by the GAS might confer a selective advantage for invaseness (32) or be important for survival of the bacteria during certain types of disease syndromes (3).

Yung and Hollingshead recently showed that GAS strain B514 has three *emm* family genes (SF4 or *mrp50*, SF3 or *enn50*, and SF2 or *emmL50*) arranged in cluster pattern 5 (48). Recently, several serologically different SF4 M-related proteins (Mrps) in different GAS strains have been implicated in inhibiting GAS binding to phagocytes and in survival of some strains of GAS in human blood (31). Although the *emm*-related genes of B514 are expressed little if at all when this strain is grown under laboratory conditions (48), they may be expressed in vivo, and if so, possibly the SF2 protein (encoded by *emmL50*) and also the SF4 protein (encoded by *mrp50*) would be expected to play an antiphagocytic role (31). In addition, Mrp50 and EmmL50 have been shown to bind human IgG and Enn50 to bind IgA (48).

Another surface protein of GAS considered to be a potential virulence factor is the C5a peptidase, which cleaves the C5a component of complement both in humans (43) and in mice (20). Because C5a may be involved in attracting phagocytic cells that might assist in clearing the infecting GAS, cleavage of C5a is thought to protect the bacteria from immune detection and may be important in the early stages of infection (20).

The surface-located hyaluronic acid capsule of the GAS has also received considerable attention as a possible virulence factor (10, 40, 41, 42, 46). Many of the GAS isolates associated with the resurgence of rheumatic fever during the mid-1980s were mucoid (21), which suggests that the capsule may be important in certain types of GAS disease. Strain B514 has a very mucoid phenotype on agar medium, indicating that it is encapsulated. Viscosity measurements and microscopic examination confirm this conclusion (our unpublished data).

To study the role of these potential GAS virulence factors in invasive disease, we used our recently developed model of pneumonia and systemic spread following intratracheal inoculation of strain B514-Sm in C3HeB/FeJ mice (19). To investigate the earlier stages of disease, we used an inbred mouse strain reported to be less sensitive to GAS (22), strain C57BL/10SnJ. Following intranasal inoculation, we find long-term throat colonization of these mice without significant systemic infection. Thus, we were able to evaluate the role of each of these potential virulence factors in the establishment of GAS respiratory infections and in the late stages of GAS disease by using these mouse models.

MATERIALS AND METHODS

Animals. Virus-antibody-free, 4- to 5-week-old female C3HeB/FeJ and 5- to 7- week-old male C57BL/10SnJ mice were obtained from Jackson Laboratories (Bar Harbor, Maine). Mice were housed on hardwood chip bedding in microisolator cages

in a room kept at 23 $\pm$ 2°C with 50 to 60% relative humidity and a 12-h light-dark cycle. Mice were given tap water and commercial rodent chow (Purina Rodent Chow 2001, Ralston Purina, St. Louis, Mo. U.S.A.) ad libitum. The animals were housed five per cage, were randomly assigned to treatment groups, and were acclimated to the laboratory environment a minimum of 6 days before inoculation.

All protocols involving animals were approved by the Institutional Animal Care and Use Committee.

Bacterial strains. *Escherichia coli* DH5a (14) was used for molecular cloning experiments. *S. pyogenes* B514-Sm (type M50) (19) is a spontaneous streptomycin-resistant derivative of strain B514/33, a natural mouse pathogen (18). *S. pyogenes* JRS4 is a spontaneous streptomycin-resistant derivative of a clinical M6 isolate (strain D471) from the Rockefeller University collection (12). *S. pyogenes* JRS145 is a derivative of strain JRS4 in which the chloramphenicol acetyltransferase gene from *Bacillus pumilus* is fused to the *emm6* promoter in place of the *emm6.1* gene (6).

Media. *E. coli* was grown in Luria broth (35). *S. pyogenes* was cultured in brain heart infusion broth (Remel, Lenexa, Kans.) made up at twice the recommended concentration (2X) and containing supplement B (Difco Laboratories, Detroit, Mich.) (19) or on Todd-Hewitt agar (THA). Kanamycin was used at 300 or 500 μg/ml for *S. pyogenes* and at 40 μg/ml for *E. coli*. Spectinomycin was used at 100 μg/ml for both species.

Plasmid and strain constructions. The *scpA* gene of strain B514-Sm was disrupted by insertion of a nonreplicative plasmid as follows. (Because *scpA* produces a monocistronic message, no downstream polar effects of the insertion should occur.) An internal fragment of *scpA* (7) was amplified from B514-Sm by PCR with the oligonucleotides GGAATTCCCTCAAAAGCGACTATTAGGGAT (scpAfor) and GGGGCCCCGTCTTTTCGACTGATAAAG (scpA10) (Fig. 1). We found that this amplified fragment contained an *Eco*RI site approximately 100 bp from the 3' terminus. Therefore, we digested this fragment with *Eco*RI and inserted it into the unique (polylinker) *Eco*RI site of pCIV2 to create pJRS4012. Plasmid pCIV2 is a pUC18 derivative which has the omega Km-2 interposon (*aphA3* kanamycin resistance gene flanked by transcriptional and translational terminators) (27) in place of the beta-lactamase gene (25). Plasmid pJRS4012 was electroporated into GAS strain B514-Sm, and kanamycin-resistant colonies were selected at 37°C. One clone, called JRS4012, was chosen for further study. Chromosomal DNA from this clone was used as template for PCR (Fig. 1) to confirm that pJRS4012 had inserted into the *scpA* gene of this strain. Primer scpa1, which binds in the *scpA* gene immediately upstream of the *scpA* for binding site, was used with M13rev, which binds to the *lacZ* promoter region in pJRS4012, to amplify a 1.1-kb product in strain JRS4012. Furthermore, this primer pair did not amplify strain B514-Sm or plasmid pJRS4012, verifying insertion of pJRS4012 into the *scpA* gene.

Simultaneous inactivation of *mrp50*, *emmL50*, and *enn50* was accomplished as follows. First, plasmid pUCSpec was constructed for use as a suicide vector in the GAS. Plasmid pUCSpec is a derivative of pUC18 which has the spectinomycin adenyltransferase AAD(9) (24) determinant in place of the beta-lactamase gene. This substitution was accomplished by replacement of the 692 *ScaI*/*NdeI* fragment of pUC18 with the 1.1-kb *NdeI*/*Hind*III fragment (blunted with Klenow fragment of DNA polymerase I) from pDL269 (24). Then, a 0.9-kb fragment internal to the *mrp50*

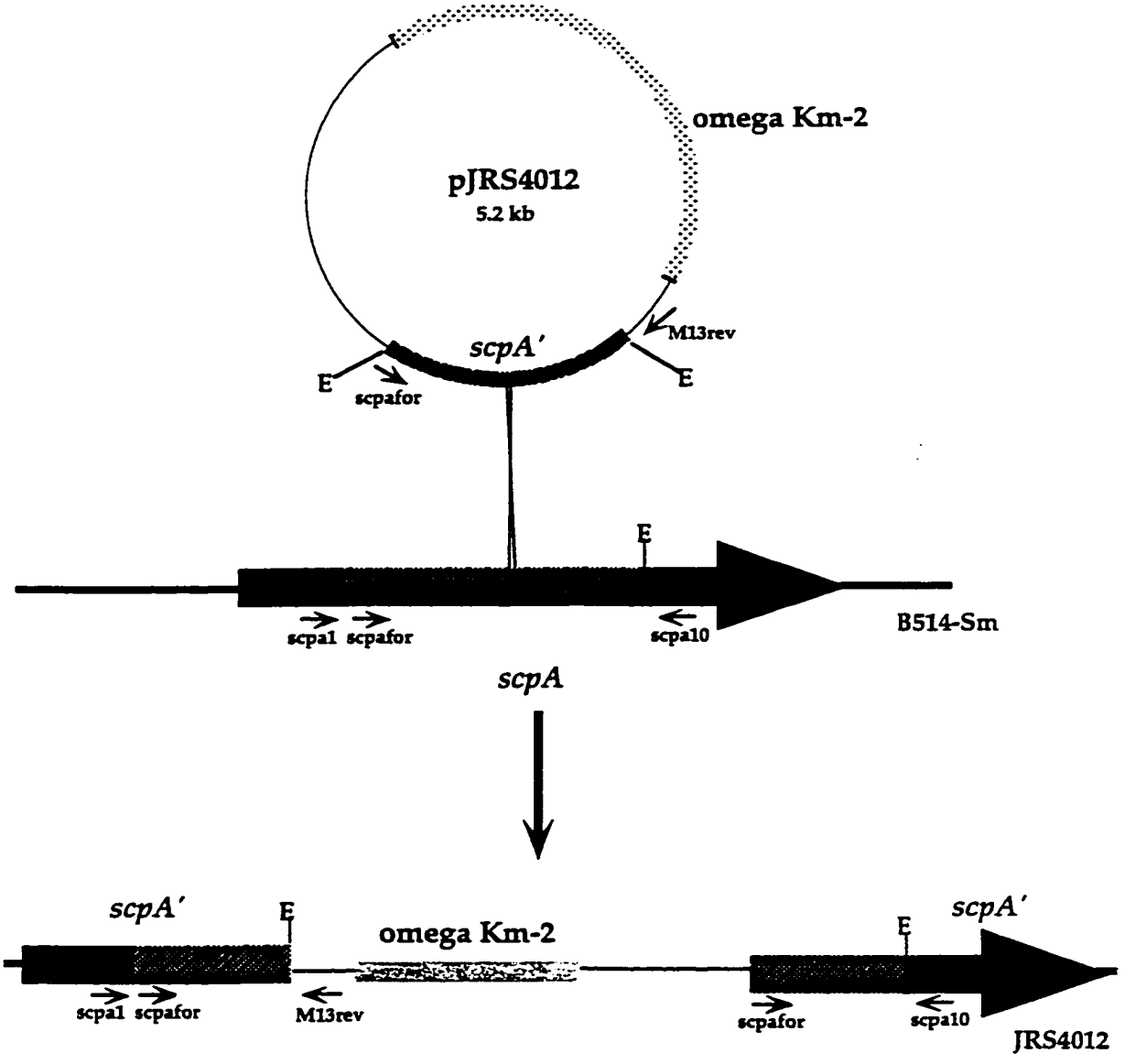

FIG. 1. Construction of insertion mutant JRS4012. The circle represents plasmid pJRS4012, which is based on pCIV2 (pUC orgin) (25) and which contains an internal fragment of the *scpA* gene from GAS strain B514-Sm (striped region) and the omega Km-2 interposon (*aphA3* kanamycin resistance gene flanked by transcriptional and translational terminators; stippled region) (27). The wild-type *scpA* gene is shown on the middle diagram. Homologous recombination between identical *scpA* sequences (striped regions) present on both pJRS4012 and the chromosome led to insertion of pJRS4012 within the *scpA* gene (bottom diagram). The PCR primers used for cloning and for confirming the construction are indicated by small arrows. *Eco*RI restriction sites (E) are indicated. The maps are not drawn to scale.

gene of strain B514-Sm (48) was amplified by PCR with the primer pair CGTGT-TTGATGGCTATACTG (SF4int1)/ GGTTTTTAGCTCCTGAAGCTTTAT (SF4int3; Fig. 2). This fragment was treated with the Klenow fragment of DNA polymerase I and inserted into the unique (polylinker) *Hinc*II site of pUCSpec, creating pJRS4014. The 2.25-kb *Bam*HI fragment of pUC4omegaKm-2 carrying the omega Km-2 interposon (27) was then inserted into the unique *Bam*HI site of pJRS4014, located in the polylinker downstream of the *mrp50* insert, to make pJRS4015. A 0.5-kb fragment internal to the *enn50* gene of B514-Sm (48) was then amplified by PCR with the primer pair GGGGCCGAGGTGAAAATGCCGACCTTA (SF3-1)/ GGAATCGGGATGC-TTCAAGGTCACGGC (SF3-2). This fragment was treated with the Klenow fragment and inserted into the unique (polylinker) *Ecl*136II site of pCIV2 (25) to create pJRS4023. The *enn50* insert of pJRS4023 was then removed by digestion with *Eco*RI and *Hinc*II and cloned into pJRS4015 at its *Xma*I (blunted with the Klenow fragment) and *Eco*RI sites to create pJRS4016. Plasmid pJRS4016 was linearized at a unique *Eco*RI site downstream of the *enn50* DNA insert prior to its electroporation into B514-Sm. Transformants were selected on kanamycin at 37°C and screened for sensitivity to spectinomycin. One transformant, designated JRS4016, was chosen for further study. Chromosomal DNA was subjected to PCR using several primers, as shown in Fig. 2. PCR with primer pair 4032R/OM23R gave a 3.9-kb product from B514-Sm and a 4.1-kb product from JRS4016, as expected. 4032R/omegaI gave the expected 1.0-kb product from JRS4016 and did not amplify B514-Sm. Primer pair omegaII/OM23R gave the expected 1.3-kb product with JRS4016 and did not amplify B514-Sm. These results are consistent with replacement of the three *emm*-related gene sequences with the omega Km-2 cassette as shown in Fig. 2. With SF2-L/SF2-R, a 1.5-kb fragment was amplified in B514-Sm but not in JRS4016, confirming the absence of these sequences in the mutant strain.

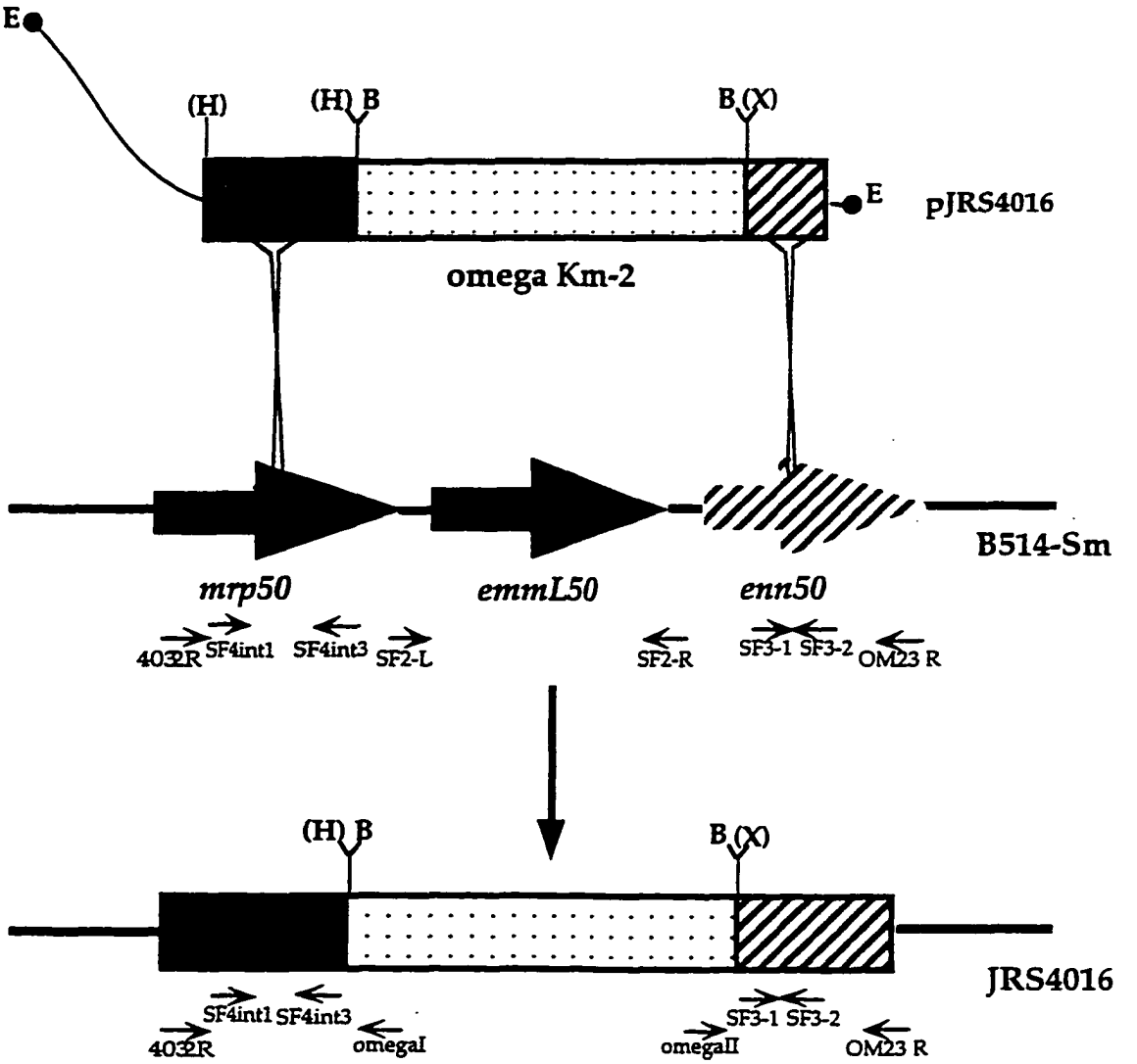

FIG. 2. Construction of insertion-deletion mutant JRS4016. The top diagram represents the chimeric plasmid pJRS4016, shwon linearized at its unique *Eco*RI site (E). Plasmid pJRS4016 contains an internal fragment of the *mrp50* gene (black region), the omega Km-2 interposon (stippled region), and an internal fragment of the *enn50* gene (striped region) cloned into pUCSpec, which has a pUC18 origin and the spectinomycin adenylyltransferase AAD (9) determinant (24). The middle diagram shows the wild-type *emm*-related genes (large arrows) present in GAS strain B514-Sm. Homologous recombination between DNA sequences present on both the plasmid and the chromosome leads to allelic replacement of all three *emm*-related genes with the omega Km-2 interposon to create JRS4016 (bottom diagram). The PCR primers used for cloning and for verifying the construction are indicated by small arrows. Restriction enzyme sites shown are H (*Hinc*II), B (*Bam*HI), X (*Xma*I), and E (*Eco*RI). Parentheses indicate a site destroyed in cloning.

The *hasA* gene, which encodes hyaluronate synthase, was disrupted in strain B514-Sm by insertion of a nonreplicative plasmid as follows. An internal fragment of *hasA* (11) was amplified from strain B514-Sm by PCR using the oligonucleotides ACGTTATCGTTCACCGTTCCC (hasA-F) and AGTGACCTTTTTACGTGTTCCCC (hasA-R). This amplified fragment was cloned into pCRII (Invitrogen, Inc.) to create pUAB031. A 0.5-kb *Ssp*I fragment internal to the *hasA* gene fragment in pUAB031 was isolated and inserted into the *Sma*I site of plasmid pSF151 (39) to create pUAB039 (Fig. 3). pSF151 is not able to replicate in *S. pyogenes* and carries the *aphA3* kanamycin-resistance gene, which is expressed in both *E. coli* and *S. pyogenes*. Plasmid pUAB039 was electroporated into strain B514-Sm and kanamycin-resistant transformants were selected at 37°C. Chromosomal DNA was then was prepared from a nonmucoid, kanamycin-resistant colony, B514.039, and used as a template in PCR with the primers hasA-F and hasA-R. A DNA product of 4.8 kb was amplified, verifying insertion of the entire pUAB039 plasmid into the *hasA* gene (Fig. 3).

Electroporation of DNA into *E. coli* and *S. pyogenes* was performed as previously described (13, 26). Restriction endonucleases, T4 DNA ligase, and the Klenow fragment were used according to the manufacturer's instructions.

Animal inoculations. The mice were anesthetized with ketamine-xylazine and inoculated either intranasally or intratracheally as previously described (19).

Documentation of infection. The animals were observed daily for signs of illness. Throat cultures were obtained as previously described (19). The throat culture swabs were rotated along the surface of a THA plate containing 1 mg/ml streptomycin sulfate and then cultured directly in Todd Hewitt broth containing 0.2% yeast extract (THYB) and 1 mg of streptomycin sulfate per ml for 48 h at 37°C. If at least one

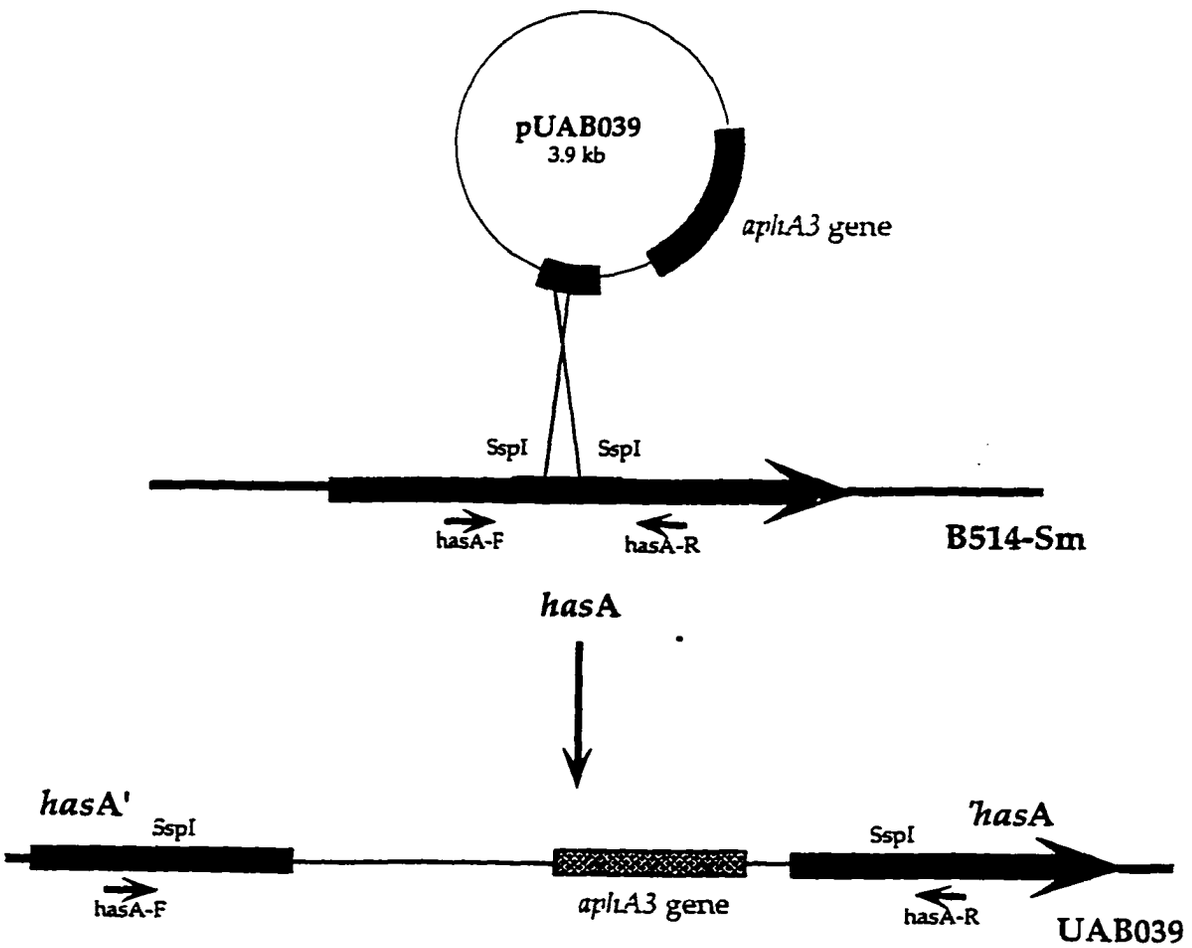

FIG. 3. Construction of insertion mutant UAB039. Plasmid pUAB039 was used to transform B514-Sm to kanamycin resistance. Plasmid pUAB039 contains an internal fragment of the *hasA* gene from B514-Sm (grey region) cloned into pSF151, which is nonreplicative in streptococci and contains the *aphA3* kanamycin resistance gene. Homologous recombination between *hasA* sequences on both the plasmid and the chromosome led to insertion of pUAB039 into the *hasA* gene (bottom line). Some of the PCR primers usesd to verify this construction are shown (small arrows). *Ssp*I restriction enzyme sites are indicated.

colony was seen on agar medium or several cocci in chains were seen in one field from the broth culture, the throat culture was scored as positive.

Blood, trachea, lung, spleen, nasopharyngeal, and meningeal cultures were obtained as previously described (19).

Phagocytosis assay. The ability of strain B514-Sm to resist phagocytosis was assessed as previously described (26) except that 2X brain heart infusion broth with 2% supplement B (19) was used as the growth medium instead of THYB.

Data analysis. Fifty percent lethal dose values were determined by probit analysis using the method of Batson (2). Fischer's exact test was performed by using the InStat program (GraphPad Software, San Diego, CA).

RESULTS

Role of the C5a peptidase in promoting throat colonization. To examine the role of the antichemotactic C5a peptidase in colonization of the throat, groups of five C57BL/10SnJ mice were inoculated intranasally with 10^7 CFU of GAS strain JRS4012 (*scpA*) or B514-Sm (wild type) or with saline solution (Table 1, experiment 1). A dose of 10^7 CFU was used because this was the lowest dose necessary for induction of throat colonization in ca. 90% of the animals. No significant difference was found between the rate of colonization by the mutant and the wild type after 3 weeks.

Since the *scpA* mutation in strain JRS4012 is due to an insertion of a nonreplicating plasmid into the chromosome, it seemed possible that the inserted plasmid could excise, leading to reversion. In control experiments, no such plasmid loss was ever detected during growth in vitro. To check for reversion after growth of

strain JRS4012 in the throats of mice, several colonies from each of the throat swabs were tested for kanamycin resistance (from day 2 postinoculation and then at weekly intervals). All colonies were kanamycin resistant, indicating that reversion had not occurred.

TABLE 1. Effect of mutations in putative virulence genes following intranasal inoculation of *S. pyogenes* in C57BL/10SnJ mice

Expt no.	Strain (relevant genotype) or control	No. with inoculated in throat culture at week 3/total no. of survivors	No. with pneumonia/ total no.	No. with meningeal infection/ total no.
1[a]	JRS4012(*scpA*)	8/10	0/10	0/10
	B514-Sm	9/10	1/11	0/11
	Saline	0/5	0/5	0/5
2[b]	JRS4016(*mrp50 emmL50 enn50*)	8/9	0/10	1/10[c]
	B514-Sm	7/8	1/10[d]	2/10[d]
	Saline	0/5	0/5	0/5
3[b]	514.039(*hasA*)	0/9[e]	0/10	1/10
	JRS4016(*mrp50 emmL50 enn50*)	5/9	1/10	0/10
	Saline	0/4	0/4	0/4

[a] 1 X 10^7 CFU inoculum.

[b] 2 X 10^7 CFU inoculum.

[c] One animal had difficulty maintaining balance and was seen to tilt its head to one side, but the meningeal culture was negative.

[d] One animal had lung lesions and a positive meningeal culture.

[e] Revertant bacteria were cultured from 7 of 9 mice.

Role of the M-like proteins in promoting throat colonization. A deletion-substitution mutation which replaced the three *emm*-like genes (*mrp50*, *emmL50*, and *enn50*) of strain B514-Sm with a kanamycin resistance cassette was used to test the role of the M-related proteins they encode in the throat colonization model.

The frequency of surviving mice with a positive throat culture at week 3 following intranasal inoculation with 2 X 10^7 CFU of the mutant was similar to that for the wild type (Table 1, experiment 2).

Role of the hyaluronic acid capsule in promoting throat colonization. To test the role of the hyaluronic acid capsule in throat colonization, we constructed a mutation in GAS strain B514-Sm in which a nonreplicating plasmid is inserted into the *hasA* gene in the chromosome. Because this plasmid might be able to excise from the chromosome to generate encapsulated revertents, the mutant (strain B514.039) was grown in medium containing kanamycin (500 μg/ml) prior to use in animals. At the time of inoculation, the B514.039 culture contained 0.6% (3 of 516) encapsulated organisms. GAS strain JRS4016 (*mrp50 emmL50 enn50*) was used as the control strain in this experiment since it carries the same kanamycin resistance marker as the *hasA* mutant and since it colonized C57BL/10SnJ mice at the same frequency as the wild-type strain (Table 1, experiment 2).

The frequency of surviving animals that had streptococci in their throats at week 3 following intranasal inoculation was essentially the same for the *hasA* mutant as for the control (Table 1, experiment 3). However, 100% of the bacteria recovered at day 4 from the throats of the animals inoculated with the *hasA* mutant were mucoid. To determine whether incubation of the throat swabs in broth prior to plating altered the fraction of mucoid colonies, a reconstruction experiment was performed in which strain B514.039 was passed successively three times in THYB containing 1 mg of streptomycin per ml and the number of revertents was determined. Following the third passage, there were 0.23% (8 of 3,523) mucoid colonies, indicating that prior incubation of the throat swab in broth does not significantly alter the fraction of mucoid colonies observed.

Table 2 shows the frequency of reversion in the throat cultures at different times after intranasal inoculation with B514.039. On the day following inoculation, significant numbers of encapsulated bacteria were observed in approximately half of the throat cultures that yielded bacteria. By the second day, this proportion had risen to two-thirds, although the initial inoculum contained a maximum of 0.6% mucoid CFU.

Molecular examination of the revertant mucoid colonies. Mucoid colonies were of two phenotypes, Km^r and Km^s. To determine whether the mucoid colonies recovered from mice result from excision of the plasmid from the *hasA* gene or from mutations at another (hypothetical) locus, PCR analysis was performed on five strains (three Km^r, two Km^s) obtained from the throats of five different mice. The primer pair hasA-F/hasA-R (see Materials and Methods and Fig. 3) was used to determine whether the insertion was present in the *hasA* gene in the mucoid revertants. We found that in both the Km^s and Km^r mucoid colonies, the *hasA* gene had been restored to the size present in the wild-type strain, indicating that the plasmid had excised in both types of revertant.

The presence of the Km^r determinant in the resistant revertants and its absence from the sensitive revertants was confirmed by PCR with the primers aphA3-F and aphA3-R. When DNA from the Km^r revertants was used, these primers generated a band identical in size to that obtained from pUAB039, the plasmid containing *aphA3*. However, other primer pairs from within pUAB039 did not generate a PCR product from these Km^r revertants, indicating that only part of the plasmid remains in these strains. The location of the Km^r gene was not further examined in these revertants since it appears that restoration of the *hasA* gene occurred.

TABLE 2. Reversion to mucoidy of *S. pyogenes* B514.039 following intranasal inoculation of C57BL/10SnJ mice with 2 X 10^7CFU

Mouse no.	% Revertants (no. mucoid colonies/total no.)[a] at:					
	Day 1	Day 2	Day 4	Day 7	Day 14	Day 21
1	14 (13/931)[b]	<4.5 (0/22)	100 (369/369)[b]	neg.	neg.	neg.
2	<7.7 (0/13)	<3.6 (0/28)	100 (3/3)	100 (TNTC)	100 (20/20)	100 (96/96)
3	69 (80/116)	71 (10/14)	100 (TNTC)	100 (TNTC)	neg.	neg.
4	neg.	100 (115/115)	100 (TNTC)	100 (TNTC)	100 (72/72)	100 (44/44)
5	100 (16/16)	100 (356/356)	neg	100 (351/351)	100 (TNTC)	100 (4/4)
6	100 (23/23)	100 (95/95)	100 (TNTC)	100 (TNTC)	100 (139/139)	100 (6/6)
7	0.64 (1/156)	88 (29/33)	100 (TNTC)	100 (298/298)	100 (TNTC)	100 (5/5)
8	50 (10/20)	94 (215/228)	100 (TNTC)	100 (TNTC)	100 (TNTC)	100 (14/14)
9	neg.	neg.	dead	dead	dead	dead
10	neg.	<12 (0/8)	neg.	neg.	100 (8/8)	100 (6/6)

[a] Throat swabs were cultured directly on THA and scored after 48 h at 37°C. neg., throat culture was negative; TNTC, too numerous to count (>500 CFU).

[b] Initial plate culture was negative. Results were obtained by plating the broth culture onto THA (see Materials and methods).

Illness observed following intranasal inoculation. Of the 61 animals that were inoculated intranasally, only 6 developed systemic infection (pneumonia or meningeal infection or both). One mouse developed meningeal infection following inoculation with the *hasA* mutant, and 99% (106 of 107) of the bacteria recovered from the meninges, 100% (70 of 70) of the bacteria recovered from the nasopharyngeal washes, and 58% (284 of 488) of the bacteria recovered from the blood culture contained mucoid revertants. This supports the suggestion that the capsule is important for establishment of infection by GAS in this model.

As expected, no streptomycin-resistant bacteria were recovered from the throats of any of the mice inoculated with saline, and these animals showed no signs of illness and remained healthy. In total, the frequency of illness following intranasal inoculation was very low and was not significantly different following inoculation with the wild type or one of the mutants.

Role of the C5a-peptidase in causing pneumonia. To examine the role of these putative virulence factors in the later stages of infection, we used our recently developed model of pneumonia and systemic spread following intratracheal inoculation of C3HeB/FeJ mice (19). The importance of the C5a peptidase was tested by generating a dose-response curve for strain JRS4012 (*scpA*) (under the conditions used previously for wild-type strain B514-Sm (19) (Fig. 4). The curve suggests that the *scpA* mutant may be somewhat less virulent than the wild type in this model. Probit analysis (2) of the results indicates that a dose of 3.3×10^7 CFU would cause 50% of the animals inoculated with JRS4012 to develop pneumonia within 72 h (95% confidence interval, 1.9×10^7 CFU to 5.9×10^7 CFU), which is a small but statistically significant difference from that obtained previously for wild-type strain

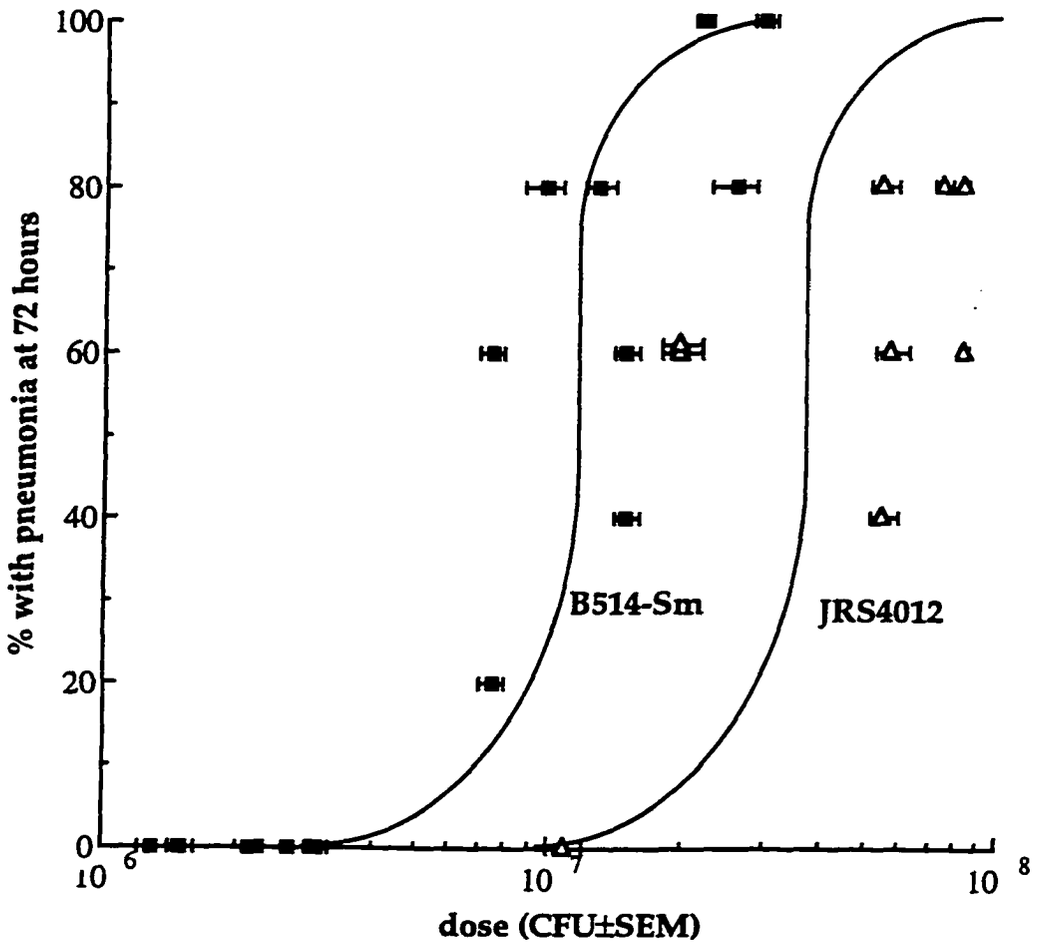

FIG. 4. Dose response of pneumonia in C3HeB/FeJ mice following intratracheal inoculation of *S. pyogenes* strain JRS4012 (*scpA* null) (Δ). The results obtained previously for wild-type strain B514-Sm are included for comparison (■). Each data point represents five mice.

B514-Sm: 1 X 10^7 CFU (95% confidence interval, 8.1 X 10^6 to 1.3 X 10^7 CFU) (19).

To check for reversion of strain JRS4012 following intratracheal inoculation of mice, a minimum of 100 colonies (from the spleen, lung, or tracheal culture) from each of 13 pneumonic animals were scored for kanamycin resistance. All colonies examined from 12 of the 13 animals were kanamycin resistant. Some kanamycin-sensitive colonies (122 of 160) were obtained from the lung of one mouse that had been inoculated with a dose of 2.0 X 10^7 CFU. This indicates that although reversion can occur, it happens only rarely in vivo.

Since we observed a small difference in the degree of pneumonia caused by strains JRS4012, the *scpA* mutant, and B514-Sm, a different approach that might be more sensitive to small differences was tried. Because the C5a peptidase is located on the surface of GAS and is not secreted, a competition experiment should show whether the wild-type GAS is better able to establish infection than the *scpA* mutant. For this experiment, the *scpA* mutant (JRS4012) and the wild type (B514-Sm) were coinoculated intratracheally (Table 3). In three separate experiments, the ratio of wild type to mutant recovered from the lung following the onset of pneumonia did not differ significantly from the input ratio in most mice. Therefore, in this model, it appears that the mutant strain is as effective as the wild type in terms of survival in the mouse lower respiratory tract and establishment of an infection that is characterized by infiltration primarily of polymorphonuclear leukocytes. The large variability in the output ratio among different mice is discussed below.

Role of the M-like proteins in pneumonia. The role of the M-like proteins in promoting pneumonia in C3HeB/FeJ mice was examined following intratracheal inoculation with two different doses of GAS. When mice were inoculated with either GAS

strain JRS4016 (*mrp50 emmL50 enn50*) or the wild type (B514-Sm), the incidences of pneumonia within 72 h were similar (Table 4, experiments 1 and 2). Thus, the degree of infectivity observed for strain JRS4016 was not significantly different from that observed for wild-type strain B514-Sm.

TABLE 3. Effect of intratracheal inoculation of a mixture of *S. pyogenes* strains in C3HeB/FeJ mice

Expt no.	Mutant strain (relevant genotype)	Dose (CFU)	% Wild type in inoculum	Mouse no.	% Wild type in lung culture[a]
1	JRS4012(*scpA*)	5×10^7	13 (31/232)	1	15 (30/196)
				2	8.2 (8/98)
				3	24 (12/49)
				4	<0.51 (0/196)
2	JRS4012(*scpA*)	2×10^7	12 (25/217)	1	14 (41/294)
				2	18 (18/98)
				3	14 (28/194)
				4	8.4 (13/154)
				5	21 (31/146)
3	JRS4012(*scpA*)	7×10^7	47 (183/392)	1	38 (37/98)
				2	46 (77/167)
				3	45 (66/148)
				4	59 (116/196)
				5	53 (80/151)
				6	66 (165/251)
4	JRS4016 (*mrp50 emmL50 enn50*)	2×10^7	23 (109/466)	1	73 (120/164)
				2	13 (19/150)
				3	24 (35/146)
				4	9.5 (18/189)
				5	33 (49/150)
				6	<0.67 (0/150)
				7	22 (32/147)
				8	14 (21/151)

[a] % Wild type determined by scoring the number of Km^S colonies and dividing by the total number of colonies. The wild-type strain was B514-Sm.

To improve the sensitivity of detection of differences, a competition experiment was performed in which strains JRS4016 and B514-Sm were coinoculated intratracheally (Table 3). In this experiment, no consistent increase in the fraction of bacteria

that were wild type was seen, indicating that the wild type had no advantage over the mutant in establishment and growth in the lungs. Thus, the M-like proteins do not seem to have an important role in causing infection in this model.

TABLE 4. Pneumonia in C3HeB/FeJ mice following intratracheal inoculation of *S. pyogenes*

Expt no.	Strain (relevant genotype)	% with pneumonia (no. ill within 72h/total no.)
1[a]	JRS4016 (*mrp50 emmL50 enn50*)	53 (8/15)
	B514-Sm (wild type)	54 (7/13)
2[b]	JRS4016 (*mrp50 emmL50 enn50*)	60 (9/15)
	B514-Sm (wild type)	73 (11/15)
3[a]	B14.039 (*hasA*)	0 (0/14)[c]
	JRS4016 (*mrp50 emmL50 enn50*)	50 (5/10)

[a] Dose was 2 X 10^7 CFU.

[b] Dose was 1 X 10^7 CFU.

[c] By using Fischer's exact test, it was found that P=0.006 when this result was compared to that for JRS4016 in this experiment.

Role of the hyaluronic acid capsule in pneumonia. Since previous experiments demonstrated that the *hasA* mutant strain B514.039 can revert, this strain was again grown in the presence of kanamycin to minimize the number of revertents in the inoculum. Plating experiments showed that the B514.039 inoculum for this experiment contained 1.2% (9 of 773) encapsulated organisms. None of the 14 animals inoculated intratracheally with the *hasA* mutant developed pneumonia within 72 h compared to 5 of 10 for strain JRS4016 (*mrp50 emml50 enn50*) (Table 4 experiment 3). Strain JRS4016 was used as the control in this experiment since it carries the same kanamycin resistance marker as the *hasA* mutant and it induced pneumonia at a

frequency which was not significantly different from that of the wild type (Table 4, experiments 1 and 2) .

At later times (days 5 and 6), two animals inoculated with the *hasA* mutant developed pneumonia. Cultures of the nasopharyngeal washings, throat, trachea, lung, and blood from both of these mice were positive, and all bacteria recovered were encapsulated. In one of these mice, the spleen culture was also positive and yielded exclusively mucoid streptococci, indicating that pneumonia in these animals was caused by an encapsulated revertant.

Role of capsule in protection from phagocytosis. It has been suggested that in some strains of GAS, the capsule has a major role in protection of the bacterium from phagocytosis (10, 40-42, 46). Because encapsulation is important for infection in both of our animal models, we tested whether the highly encapsulated strain B514-Sm can survive in whole human blood. As expected, the positive control (the M6 wild-type strain JRS4) grew in both whole blood and in plasma (Table 5) and the negative control (JRS145, a derivative of JRS4 which lacks the M protein gene) grew in plasma but not in whole blood, presumably due to phagocytosis. Strain B514-Sm also grew in plasma, but not in whole blood, indicating that this strain is sensitive to phagocytosis in spite of the presence of its hyaluronic acid capsule.

DISCUSSION

The GAS (*S. pyogenes*) is a serious human pathogen capable of causing a wide variety of suppurative and invasive infections. While several putative virulence factors have been identified for this organism, very little is known about the role of these factors in vivo in the different stages of disease. To be able to test the role of potential virulence factors, we developed two different models of GAS respiratory infection in mice. To

examine the early stages of disease, we developed a model of long-term (at least 21 days) throat colonization in C57BL/10SnJ mice which we report here. In addition, we previously reported a model of GAS pneumonia in C3HeB/FeJ mice (19) which we used here to examine the role of putative virulence factors in the late stages of disease.

TABLE 5. Sensitivity of *S. pyogenes* strain B514-Sm to phagocytosis in human blood

Donor no.	Strain	Bacterial concn (CFU/ml) in		
		Input	Plasma	Whole blood
1	JRS4	6.0×10^2	3.7×10^4	4.8×10^4
	JRS145	5.1×10^2	2.0×10^4	2.0×10^1
	B514-Sm	2.3×10^2	3.3×10^3	9.0×10^1
2[a]	JRS4	4.2×10^2	1.6×10^4	1.2×10^4
	JRS145	3.2×10^2	1.3×10^4	4.0×10^1
	B514-Sm	1.8×10^2	8.4×10^3	$<1.0 \times 10^1$

[a] This donor had never worked with GAS strain B514 or its relatives.

One surface-located putative virulence factor of the GAS is the C5a peptidase, which in vitro inactivates the complement component C5a (43), an important chemoattractant of polymorphonuclear leukocytes. On the basis of a comparison of the wild-type GAS and mutants with an inactivated *scpA* gene, Ji et al. suggested that the C5a peptidase also inhibits clearance of the GAS by granulocytes in vivo (20). Our model of invasive respiratory infection following intratracheal inoculation results in pneumonia characterized by an influx primarily of polymorphonuclear leukocytes into the alveoli and bronchioles. Thus, we expected the complement cascade to play a role and anticipated that inactivating the *scpA* gene of the infecting bacteria might decrease virulence. We found that the 50% lethal dose for the *scpA* mutant was 3.3×10^7 CFU, while that observed

previously for the wild-type strain was 1.0 X 10^7 CFU (19). This difference, while not large, is statistically significant, so the C5a peptidase may have a small role in invasive infection following both intradermal (20) and respiratory inoculation in mice. However, in a competition experiment in which the wild type and *scpA* mutant were coinoculated intratracheally, we expected selection for the wild type to result in a decrease in the percentage of mutant bacteria in tissues. Because the mutant strain survived as well as the parent, it appears that C5a peptidase has little effect on production of pneumonia in this model.

We found no detectable role for the C5a peptidase during the early stages of disease either, since the incidence of throat colonization following inoculation of the *scpA* mutant was not significantly different from that observed for the wild-type strain. Furthermore, the mice showed no obvious signs of illness (runny eyes, lethargy, ruffled fur), and weight loss was seen only during the first week, even though throat colonization was observed for at least 3 weeks. In all of the mice inoculated intranasally, we saw only one case of cervical lymphadenitis, suggesting that the migration of inflammatory cells to the upper respiratory tract following intranasal inoculation of these mice is minimal.

The M proteins are considered the primary virulence factor of the GAS. These surface-located proteins are thought to protect the bacteria from phagocytic clearance (23), possibly by interfering with complement-mediated uptake (4, 30). While the B514 strain we used does not encode an M protein, it does have genes for M-related proteins and one group of M-related proteins (Mrp proteins) has been shown to promote resistance to phagocytosis in some strains of GAS (31).

A second function of the M-related proteins that has attracted attention as a possible virulence mechanism in some types of GAS disease is their ability to bind immunoglobulins in a nonimmune fashion (5). Among the GAS strains studied by Bessen and Fischetti, nearly all those from cases of impetigo bind IgG, suggesting that

IgG binding activity may be required for survival on the skin (3). Furthermore, a comparison of intraperitoneal versus intradermal inoculation suggested that GAS strains that express an elevated level of Ig binding proteins may have a selective advantage for invasion (32). Bacteria isolated from the spleen exhibited a higher level of Ig binding proteins following intradermal inoculation than following intraperitoneal inoculation.

In contrast, in our model of invasive disease following intratracheal inoculation, we found no detectable role for the M-like proteins; the incidence of pneumonia for the *mrp50 emmL50 enn50* mutant was not significantly different from that observed for the wild type. Furthermore, when the mutant and wild type were coinoculated intratracheally, the mutant appeared to survive as well as the parent. These data indicate that in our model persistence in the lower respiratory tract is not dependent on the M-like proteins and suggest that other factors in strain B514 promote survival at this tissue site. In the coinoculation experiment (Table 3), the great variability in the output ratio of wild type to mutant from mouse to mouse suggests that surviving bacteria may represent an expansion of a limited population that initiated the infection. A precedent for initiation of infection by a small fraction of the inoculum was reported for *Neisseria gonorrhoeae* in human volunteers in which the recovered bacteria were of a specific lipooligosaccharide type that constituted only ca. 0.1% of the inoculum (34).

Since in previous studies expression of Ig binding proteins appeared to promote invasiveness following intradermal inoculation (32), we considered the possibility that the M-like proteins in our model might be important for dissemination of the bacteria from the lung rather than for establishment of the bacteria in the lung itself. However, we observed that following intratracheal inoculation of the *mrp50 emmL50 enn50* mutant, the incidence of positive blood or spleen cultures was not significantly different from that of the wild type (data not shown). We also found no detectable role for the M-like proteins during the early stages of disease. The incidence of throat colonization

for the *mrp50 emmL50 enn50* mutant was very similar to that observed for the wild-type strain.

Although for the strain we studied in these mouse models, the C5a peptidase and M-like proteins do not play a major role in infectivity, our results agree with studies of other model systems that show that the capsule is important. Using a mutant with a defined insertion in *hasA*, which encodes hyaluronate synthase, we found that the capsule was essential for pharyngeal colonization in our model of inbred mice. On the day following intranasal inoculation with the *hasA* mutant, approximately one-half of the throat cultures yielded predominantly encapsulated revertents, even though the inoculum contained only 0.6% revertants. By day 4, all of the bacteria recovered from the throat swabs were mucoid, indicating a strong selection for capsule production for survival of the GAS in the upper respiratory tract of these mice.

Our results are similar to those of Wessels et al., who used a type 24 strain in outbred mice and found that the capsule was essential for pharyngeal colonization (40). Two mutant strains were used in their study. When a mutant containing a transposon insertion in the *hasA* gene was used, all bacteria recovered from the pharynges of mice were revertents. When a mutant with an insertion in *hasA* and a deletion of surrounding DNA was used, the incidence of throat colonization was less than that for the wild type. However, because additional genes were deleted in this mutant. the observed effects cannot be attributed solely to the *hasA* mutation.

In the outbred mouse model using an M type 24 GAS strain (40), systemic infection and death were often secondary to pharyngeal colonization. In contrast, in our model with the M type 50 strain in inbred mice, systemic infection was rarely observed. Therefore, to investigate the late stages of disease, we used our model of pneumonia following intratracheal inoculation. We found the capsule to be critical for induction of this illness. The incidence of pneumonia following inoculation with the *hasA* mutant was significantly reduced compared to that following inoculation with the

wild type, and all surviving bacteria in the lung and blood were encapsulated. This indicates that the capsule is essential for survival in the lower respiratory tract and possibly for the ability of the GAS to cross tissue barriers.

Like the competition study between the wild type and the *mrp50 emmL50 enn50* deletion mutant, the results of the *hasA* study suggest that only a small fraction of the administered bacteria initiate pneumonia and throat colonization in our models. Capsule production was necessary for survival of the bacteria in the upper and lower respiratory tracts, and although the *hasA* mutant inocula used for intranasal and intratracheal inoculation contained only 0.6% and 1.2% (of 10^7 total CFU) encapsulated bacteria, respectively, throat colonization occurred in the majority of animals and pneumonia developed in two mice. This means that throat colonization and pneumonia resulted from a maximum of 10^5 bacteria (those that had the capsules).

The relative contributions of different surface factors to virulence in GAS appear to be strain dependent (10). In some strains, The C5a peptidase (20) and M-like proteins (31) may play a more important role in virulence, and in strains where their roles are not important, the genes for these proteins may not be expressed. In strain B514, we found the capsule to be essential for virulence in our models. The hyaluronic acid capsules on some other strain function like M and M-related proteins to protect the bacteria from phagocytosis in whole human blood (10, 40-42, 46), and with some strains there is a correlation between phagocytosis resistance in human blood and mouse virulence (40, 41, 42). Although we found that strain B514 does not survive in whole human blood, suggesting that neither the capsule nor the M-related proteins protect the GAS in this system, a role for the capsule in phagocytosis resistance in the mouse remains possible since, in vitro, mouse blood has less bactericidal activity than human blood against encapsulated GAS strains (38, 47).

The GAS capsule may have several different functions in promoting virulence in mice. In addition to the possibility that the capsule may protect some GAS strains from

phagocytosis in vivo, it has been suggested that hyaluronic acid might influence the host's cellular immune response (16). In addition, it seems possible that the capsule could be involved in adherence, since capsular polysaccharide on other species of pathogenic bacteria is believed to play this role (8, 15, 33). Although further studies are needed to investigate the precise role that the GAS capsule plays in virulence, our studies have substantiated its critical importance in the models we studied.

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COMPLEMENTATION OF A DEFECTIVE *mga* GENE CORRECTS THE ATTENUATED EXPRESSION OF THE *mga* VIRULENCE REGULON IN AN M50 GROUP A STREPTOCOCCUS STRAIN VIRULENT FOR MICE

by

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ABSTRACT

A group A streptococcal strain that is naturally pathogenic in mice was previously postulated to have a defect in its multigene activator, Mga, that led to an attenuated expression of gene products normally stimulated by Mga (Infection and Immunity, 1996, 64:2193-2200). Mga is a multigene activator with homology to response regulators in two-component bacterial signalling pathways. It activates transcription of a number of monocistronic virulence loci, including a cluster of M and M-like proteins (*emm, mrp, enn*), C5a peptidase (*scpA*), a secreted inhibitor of complement (*sic*), and a serum opacity factor (*sof*). The DNA sequence of *mga50* revealed three amino acid substitutions which might have the potential to affect function. One substitution occurs in a helix-turn-helix domain that could be involved in DNA binding for gene activation, and two others fall within a region where the Mga protein might receive a signal for activation from an unidentified environmental sensor protein. The defect in *mga50* could be complemented for its activation of *emm* family genes by providing either the closely similar complete *mga4* allele or a more divergent *mga6* allele in *trans*. During complementation, the level of *emm50* transcript was restored to quantities that equal those found in most human isolates, and expressions of the M and M-like proteins were also increased. The complementation also allowed resistance to phagocytosis as judged in bactericidal assays. Resistance was mediated by M protein because it could be achieved with complementation in the absence of capsule. The capsule alone could also mediate resistance because encapsulated strains that were not complemented could also resist phagocytosis.

INTRODUCTION

Streptococcus pyogenes (group A streptococci, GAS) is an important human pathogen which commonly causes nasopharyngitis (strep throat), impetigo, and tonsillitis. Severe infections caused by this pathogen include necrotizing fasciitis,

pyomyositis, bacterial sepsis and toxic shock-like syndrome (42). Two non-suppurative sequelae, rheumatic fever and acute glomerulonephritis, also occur as a consequence of some streptococcal infections, and the factors participating these sequelae are poorly understood. To investigate some of the pathogenetic factors contributing to disease, the serotype M50 strain B514 has recently been utilized (18, 20). B514 was the natural cause of multiple outbreaks of respiratory disease in mouse colonies (17). Because the interpretation of data from animal model systems is frequently confounded by the fact that GAS are limited to infection in human hosts, this strain provided the opportunity to utilize model systems in which the natural infection and disease in mice paralleled aspects of natural streptococcal disease in humans. A spontaneous streptomycin-resistant derivative of the B514 strain, B514-Sm, has proven to be useful in a model of long-term throat colonization following intranasal infection that depicts early stages of respiratory disease in C57BL/10SnJ mice, and in a later stage model of pneumonia and systemic spread of streptococci following intratracheal infection in C3HeB/FeJ mice (20).

One important prerequisite for pathogenicity or virulence at any body site is resistance to opsonophagocytosis because resistance permits the spread of the bacterium within the host. This property is elaborated by both M proteins and the hyaluronic acid capsule (2, 29, 33, 39, 46, 47). All strains of GAS share the same chemical composition for their capsular material. The capsule is a hyaluronate, formed of an alternating polymer of ß 1-4-linked disaccharide of glucuronic acid and N-acetylglucosamine. Strains may and frequently do vary in the amount of capsular material, and both rheumatic fever and invasive disease have shown epidemiological associations with strains that are heavily encapsulated (41). In contrast, different strains of group A streptococci frequently have distinct and serologically varied M proteins, the protein mediators of resistance to phagocytosis. The mechanism by which M proteins confer resistance to phagocytes is complex and may not be the same for all

M proteins. Many M proteins bind fibrinogen and, for those that do, resistance may be mediated through this capability (5). Some M proteins bind factor H, and others bind C4BP, both of which interfere with differing aspects of the complement cascade and may influence opsonization (10, 22, 34). Many M proteins bind IgG or IgA in a nonimmune fashion, but this capability has not been well correlated with the antiphagocytic property (3, 13, 25). Natural isolates of GAS also may vary in the amount of M protein present on the surface.

When an unstable acapsular isogenic variant of B514-Sm was utilized in mouse models, a requirement for capsule in both colonization and invasive disease was supported (20). The natural selective pressure within the nasopharynx resulted in the reversion of the capsule mutation in each mouse within 24 h, confirming a previous, similar report (45). A stable mutation which deleted the *emm* gene cluster was not found to differ from its parent strain in the above model systems. This was surprising because M protein is an important virulence factor in human infections, and previous studies of isogenic strains in mouse (21) or rat (16) colonization models had demonstrated a role for M protein. The failure to demonstrate the roles of M-family proteins in B514-Sm may reflect the low expression status of Mga-regulated gene products that was a property of B514-Sm (49).

Mga is a multiple gene regulator of the GAS. Mga belongs to the response regulator family of two-component bacterial signalling molecules, and it communicates information about the external growth environment of the streptococci to the internal gene expression environment within the bacterial cell. Mga activates several clustered virulence genes including the M and M-like proteins (*emm, mrp, fcr, enn, sph*) (36), C5a peptidase (*scpA)* (23) and secreted inhibitor of complement (*sic*) (23). It may also act at a distance on the serum opacity factor gene (*sof*) (28) and has been reported to act, perhaps in an indirect fashion, upon expression of other group A streptococcal loci (*speB, scnA, sls*) (37). Expression of Mga is growth-phase dependent; *mga* is

transcribed throughout exponential phase growth but is shut off during stationary-phase growth (26). It is also stimulated during growth in CO_2 and can vary with osmolarity, temperature, and iron limitations (27, 30).

Most response regulators are transcriptional factors which receive a signal from a sensor component, usually by a phosphorylation, and effect control by binding to sequences upstream of target genes (44). Among the group of general response regulators, the number and location of the response regulator domains and the effector domains of Mga are unusual (26). Mga is about twice as large as most proteins of this type (62 kDa), with three possible helix-turn-helix DNA binding motifs in the amino-terminus and two putative response regulator domains lying in the carboxy-terminal region (1, 32). The expression of *mga* requires DNA sequences extending 473 bp upstream region of the *mga* coding region, and transcription of *mga* is initiated from two separate promoters located within this extended regulatory region (30).

To test the hypothesis that the *mga50* gene of the strain B514 that was naturally virulent for mice was defective, plasmids with complete *mga* genes were electroporated into B514-Sm and its capsule-negative derivative, B514.039. The complementation tests showed that the previously attenuated expression of *mga*-controlled gene products in this strain could be overcome. Transcription of both *mga50* and *emm50* was increased in strains which were complemented with a *mga4* gene, but not with the plasmid vector alone. The *mga50* gene was sequenced and analyzed for potential substitutions that would explain the presumed defective function of Mga50.

MATERIALS AND METHODS

Bacterial strains, media, and plasmids. The strains and plasmids used in the experiments are listed in Table 1. GAS strain B514-Sm is an M serotype 50 strain (17), and T2/44/RB4 is an M serotype 2 strain (40). B514.039 is a derivative of B514-Sm in which the *hasA* locus was insertionally inactivated, leading to the loss of

TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant properties	References
S. pyogenes strains		
B514-Sm	Str^r	(18)
B514.039	Str^rKm^r*hasA*$^-$	(20)
T2/44/RB4		(3, 40)
D471.Rot	Str^r*emm6*	
UAB150	Str^rKm^r*emm6*$^-$	
Plasmids		
pLZ12-Spc	Spc^r	
pMga4	Spc^r*mga4*	(1)
pMga6	Spc^r*mga6*	(1)
pϕMga6	Spc^rpϕSKIIG-*mga6*	(26)

capsule (20). UAB150 is an M protein-negative mutant strain of an M serotype 6 strain, D471.Rot, in which the *emm6* gene locus was replaced with a kanamycin-resistance gene cassette by allelic exchange. Andersson et al. (1) constructed pMga4, in which the blunted *Eco*RI-*Hind*III fragment of of pMGA4-3 containing *mga4* with its predicted promoters P_1 and P_2 was inserted into the blunted *Bam*HI site of plasmid pLZ12-Spc; pLZ12-Spc was constructed by Husmann et al. (19) and is derived from pLZ12 (6) by ligating the blunted 1.7-kb *Pvu*II-*Nde*I fragment of plasmid pDL269 (8), containing the spectinomycin-resistance gene, to the blunted 2.2-kb *Stu*I-*Sal*I fragment of pLZ12. pLZ12-Spc is a shuttle vector capable of replication in both *Escherichia coli* and *S. pyogenes*. pMga6 was also constructed by Andersson et al. (1) through a similar strategy as that used in constructing pMga4. McIver et al. (26) constructed pϕMga6, in which the promoter region was placed by the heterologous lactococcal phage promoter PϕSKIIG (35). *E. coli* was grown in Luria broth (LB broth), and *S. pyogenes* was cultured in brain heart infusion broth (Remel, Lenex, Kans.) made up at

twice the recommended concentration (2X) and adding supplement B (Difco Lab., Detroit, Mich.) to 2% (20). Antibiotics were used at the following concentrations: spectinomycin (Spc), 100 μg/ml for *E. coli* and 500 μg/ml for *S. pyogenes*; kanamycin (Km), 500 μg/ml for *S. pyogenes*, and 200 μg/ml each for spectinomycin and kanamycin when added together.

DNA electroporation and plasmid isolation. Plasmid DNA was isolated from *E. coli* by using a QIAGEN plasmid maxi kit (QIAGEN Inc.). The method for isolation of plasmid DNA from *S. pyogenes* was adapted from that used to isolate the plasmid from *Lactococcus lactis* (19). Electroporation of plasmid DNA to strains B514-Sm and B514.039 was performed according to the protocol as described in Simon and Ferretti (9) with the modification of treating competent B514-Sm cells with hyaluronidase (2.5 μg/ml culture, Sigma Chemical Company) on ice for 15 min after harvesting the cells. About 0.5 μg of each plasmid DNA was electroporated to cells at an electric pulse of 2.5 or 2.0 kV, 25 μFD, and 200 Ω. After delivering the electric pulse, the cells were immediately diluted with 1 ml of chilled culture medium then added to 9 ml of medium. After a 3-h incubation at 37°C, the cells were spread on sheep blood agar plates containing the appropriate antibiotics.

Cell-surface protein extraction. Overnight culture (0.5 ml) of of each strain was inoculated in 10 ml (1 to 20 dilution) of 2X BHI plus 2% supplement B with the optimal antibiotic concentration, and incubated in 5% CO_2 at 37°C. The optical density was measured at 600 nm, and samples of each culture were taken when OD_{600} was between 0.8 and 1.0. The samples were normalized to have similar cell densities. Surface proteins were extracted by CnBr following the method of Raeder et al. (38). Extracted surface protein (10 μl, about one-tenth of volume) of each sample was

analyzed on a polyacrylamide gel containing 12.5% sodium dodecyl sulfate, and then the protein bands were visualized by Coomassie blue staining.

DNA sequencing of *mga50*. B514-Sm chromosomal DNA was isolated following the protocol of Wizard DNA purification kit (Promega Inc.). The oligo-nucleotide primers used for amplifying and sequencing the *mga* locus of strain B514-Sm are listed in Table 2. PCR reactions were performed as described in Hollingshead et al. (15), with the annealing temperature at 58°C for amplifying segments of the *mga50* gene. The amplified DNA product was cleaned with a GENECLEAN kit (BIO 101, Inc). DNA sequencing was performed with an automated sequencer (ABI prism, 377 DNA sequencer) at the DNA Sequencing Facility at the University of Alabama at Birmingham, and the DNA sequences were analyzed by Sequencher 3.0 software and MacVector 5.0.

RNA purification and RNA-slot blot hybridization. Strain B514-Sm and the plasmid-transformed strains were cultured and harvested as previously described (49), except with the media described above. Total cellular RNA was purified following centrifugation on CsCl gradients and stored in the presence of vanadyl ribonucleosides (14). Total cellular RNA of each strain was diluted to certain concentrations and reacted with formaldehyde at 60°C following the protocol described in the "GeneScreen & GeneScreen Plus Hybridization Transfer Membranes, Transfer and Detection" manual (DuPont Inc.). Then the denatured RNA was loaded on nylon hybridization membrane (DuPont Inc.). The DNA probes used to detect transcripts *mga*, *emm*, and *recA* were the PCR products amplified from B514-Sm chromosomal DNA by primers listed in Table 2. The amplified fragments were cleaned with a GENECLEAN kit (BIO 101, Inc). The products were radiolabeled with [α-^{32}P] dATP

with a randomly primed DNA labeling kit (Boehringer Mannheim Corp.). The specific activities of the probes were 2 X 10^6 to 5 X 10^6 cpm/pmol, and they were used at concentrations of 10 pmol/ml. Prehybridization and the following hybridization were according to the protocol described in the manual; the hybridizations were performed in 5 X SSC (1 X SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 50 % formamide at 42°C. Hybridized transcripts were visualized by exposure to XAR-1 X-ray film. The images of transcripts in RNA-slot blots were scanned by a PhosphorImager STORM860 (Molecular Dynamics), and the quantitation of the radioactivity of individual transcript was analyzed with ImageQuant software. Normalized units were defined as the band intensity for any experimental probe at a particular RNA concentration divided by that of a *recA* probe at the same RNA concentration (26).

TABLE 2. Oligonucleotides used for sequencing and probes

Primer	Sequence	Description[a]
For DNA sequencing		
ory14	5'-AATCTGCGAGATTAGAGTAAT-3'	(personal communication with J. R. Scott)
mga-1F	5'-GTGAATGACATAAATGTCGC-3'	445-465
mga-1R	5'-GTAAGTGTTGGTTTTTTTGATGAGG-3'	1088-1064
mga-2F	5'-GAAATTGGCTGTGCTTTTGAG-3'	1521-1541
mga-2R	5'-TCACTAGGAGTCGTACTTTTTCATC-3'	1928-1904
mga-3F	5'-ATCGTGACAAGTCCAGTGCAGG-3'	1112-1133
mga-3R	5'-AACACCGAAGCGTTATGGAGGG-3'	1659-1680
mga-4F	5'-ACAAAAACCAGGCAAGCCAG-3'	2245-2264
virR49-F	5'-TTATCTACCCTCAAACGCCTCATC-3'	1046-1069
virR49-R	5'-AAAAGCCAAAAGGTAAAGGTCAGT-3'	2379-2402
For probes		
Relf1	5'-AATCTGCAGTATTCGCTTAGAAAATT AAAA-3'	7-68 *emmL2.1*(3)
915-R	5'-GTTCTTGATAACGTTTTTCTACTTCTC G-3	428-455 *emmL2.1* (3)
recA-F	5'-ACGAACGTCGAAAGCCCTTG-3'	88-107 *recA* (24)
recA-R	5'-CGGTTTCTTCTGATGCTACTGCC-3'	1104-1126 *recA* (24)

[a] Primers used for amplification as the probe of *mga* gene, the description includes position (in base pairs), gene, and (parenthetically) applicable reference(s).

Phagocytosis assay. The protocol for testing the antiphagocytic abilities of strain B514-Sm and its transformed cells was followed (31) except for the medium used and the inoculum of some strains. All of the strains used in the assay were grown in 2X BHI plus 2% supplement B with appropriate antibiotics and with the addition of 10% heat-inactivated horse serum (Colorado Serum Company). A logarithmic-phase culture of the strains was used for the test. Freshly drawn human blood was mixed with heparin (10 units per ml of blood), and the plasma was obtained by centrifugation of the blood at 5000 X *g* for 10 min at 10°C. Growing cultures of the tested strains were diluted to 200-600 CFU per ml. A mixture of 0.1 ml of culture medium, 0.1 ml aliquots of the bacterial dilutions, and 0.4 ml of either human blood or plasma (control for anti-M antibodies) was incubated at 37°C for 3 h with rotation. The cultures were then plated in 5% sheep blood agar plates plus the appropriate antibiotics and incubated at 37°C.

RESULTS

Restoration of wild-type levels of M-family proteins in strain B514-Sm by complementation with *mga*-containing plasmids. In previous experiments, the expression of the *emm* gene cluster in B514-Sm virulent for mice was found to be attenuated relative to the expression of the same gene cluster in a human serotype M2 strain, T2/44/RB4. A complementation test was performed to see if the *trans*-acting Mga proteins from other GAS strains could complement the putative regulatory defect in strain B514-Sm which led to the attenuated expression of all M-family proteins. Plasmids containing a *mga* gene cloned from either a serotype 4 (AP4) or a serotype 6 (D471) GAS strain were used (Table 1). All plasmids were based on the shuttle vector pLZ12-Spc, and each contained a *mga* gene expressed either under its own native promoter or under a promoter derived from the *Lactococcus lactis* phage

SKIIG (Table 1). Plasmid pMga6 contains *mga6* with its promoter region in a shuttle vector, pLZ12-Spc; pϕMga6 has the same *mga* gene except that the promoter region was replaced by the lactococcal phage promoter; pMga4 contains the *mga4* gene under its native promoter in the same shuttle vector (26). These plasmids were individually transformed into B514-Sm by electroporation. After transformation with the different *mga*-containing plasmids, surface proteins of individual transformants were extracted by CnBr with a standard volume of cells grown to OD 0.8 at 600 nm (38). The major surface proteins extracted by this procedure are the M-family proteins (38). Equivalent quantities of protein from each isolate were then visualized by SDS-PAGE gel resolution and Coomassie blue staining (Fig. 1). Protein levels were compared to those in the parent B514-Sm and also in the strain T2/44/RB4, whose Mga regulon is closely related to that in B514-Sm and which was previously shown to exhibit wild-type levels of M-family proteins (48). M-family proteins were equivalent to those in T2/44/RB4 in transformants containing the plasmid pMga4 (lane 4). Fewer surface proteins were detected in B514-Sm transformed with *mga6*-containing plasmids either in its own promoter or in the phage promoter (lanes 5 and 6). As was seen previously, barely detectable surface proteins were observed in the parent strain--B514-Sm, or in the plasmid vector-transformed B514-Sm strains (lanes 2 and 3). Furthermore, from this analysis, Mga4 protein was found to be more effective than Mga6 protein in complementing the expression of the M-family proteins in strain B514-Sm.

Analysis of *mga50* RNA transcript. The attenuated expression of the *emm* gene cluster in strain B514-Sm was previously found to occur at or prior to the transcription of *emm*-family genes because transcript levels correlated with observed protein levels (49). This had implicated the Mga regulator as being potentially responsible for attenuated expression of the *emm50* gene cluster. To assess whether

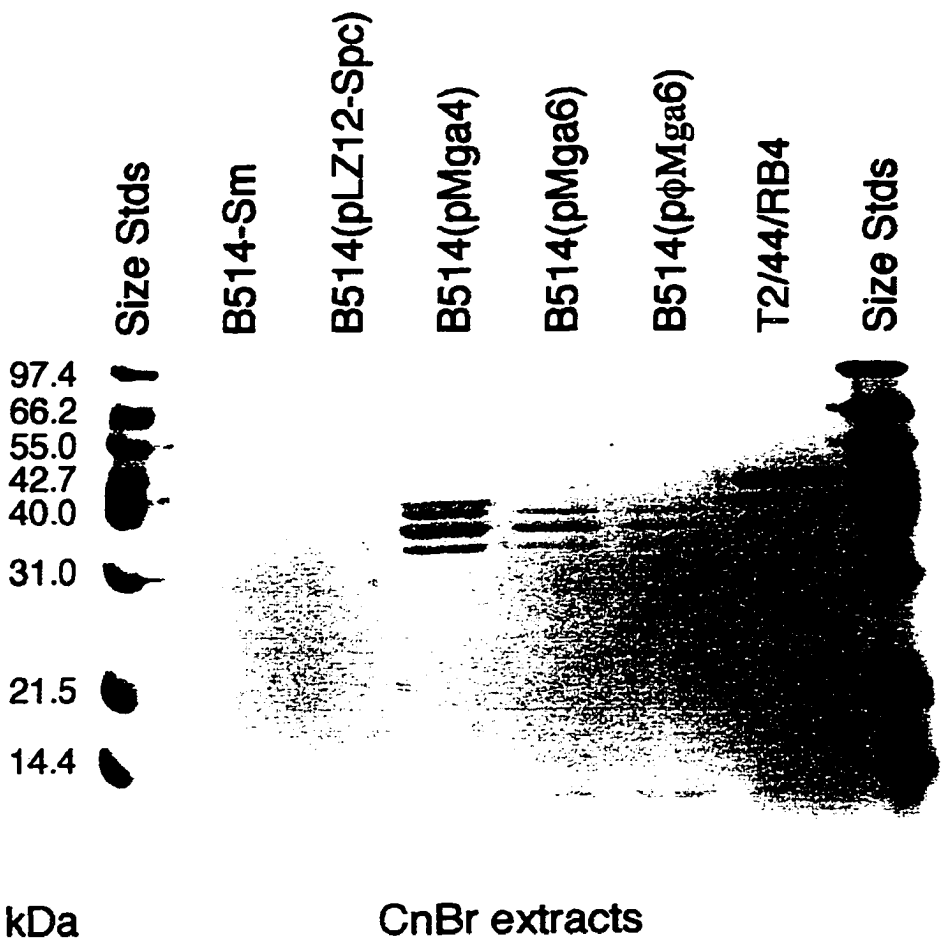


FIG. 1. CnBr extracts of surface proteins from B514-Sm, T2/44/RB4, and *mga* plasmid-transformed B514-Sm cells. Lanes 1 and 8, size standards; lane 2, B514-Sm; lane 3, B514(pLZ12-Spc); lane 4, B514(pMga4); lane 5, B514(pMga6); lane 6, B514-(pφMga6); lane 7, T2/44/RB4. Stds., standards.

the complementation of Mga restored wild-type transcription levels, quantitative RNA-slot blots were performed to detect transcripts of both *emm50* and *mga50*. The *recA* transcript was utilized as a factor for normalizing loading amounts because it has previously been shown to be constitutively expressed (26). Transcript levels were analyzed in strain B514-Sm, with T2/44/RB4 as a positive control, and in B514-Sm transformed with pMga4 or B514-Sm transformed with only the vector itself, pLZ12-Spc. The autoradiographs of the transcripts for *mga50* and *emm50* and the quantitation of these transcripts relative to the housekeeping gene *recA* in different strains are shown in Fig. 2. The transcript of the *mga50* gene was detected in strain B514-Sm, and it was slightly lower than the *mga2* transcript in strain T2/44/RB4 (Fig. 2A, B), with a calculated ratio of about 1:1.5 after normalization with the *recA* gene. The *emm50* transcript in strain B514-Sm was further reduced as compared to the equivalent *emm2* transcript in T2/44/RB4 (Fig. 2A, B), with a calculated ratio of about 1:4. In pMga4-transformed B514-Sm, the level of *emm50* transcript was increased to become approximately equal with the level of transcript for the same gene in T2/44/RB4. In B514-Sm cells transformed with vector alone, the levels of *mga50* and *emm50* transcripts were indistinguishable from those in the parent strain.

Sequencing and analysis of the *mga50* gene. The complete nucleotide sequence of the *mga50* structural gene, including 608 bases upstream from the translation start codon, was completed to allow examination for potential base and amino acid substitutions that might have altered Mga activity in this strain (Fig. 3). The nucleotide sequence contains an open reading frame of 1599 nucleotides which encodes 533 amino acids; the molecular weight of this protein is about 62 kDa. When the upstream 608 bp were compared to the same regions of *mga4* (1) and *virR49* (36), 24 polymorphic sites were found. There were 5 insertions or deletions in either *mga4* or *virR49* relative to *mga50*, and there were 19 substitutions among the three alleles of

FIG. 2. Expression of *mga* gene in strain B514-Sm. (A) Specific RNA transcripts to each gene (*mga*, *emm*, and *recA*) were detected in strains 1, T2/44/RB4; 2, B514-Sm; 3, B514 (pMga4) and 4, B514 (pLZ12-Spc). 2 μg and 0.4 μg of RNA isolated from different strains at similar OD_{600} growing phase were loaded per blot in duplicate. The blot was reacted with DNA probes which were specific to each gene (see Table 2). The blot would be stripped for hybridization with the other probes. The washing condition of the blot for each probe was the same. (B) Quantitation of the *mga* transcript level in strain B514-Sm from hybridization analyses. Data are represented as normalized units defined as the band intensity for any experimental probe at a particular RNA concentration divided by the band intensity of a *recA* internal control probe at the same RNA concentration (26).

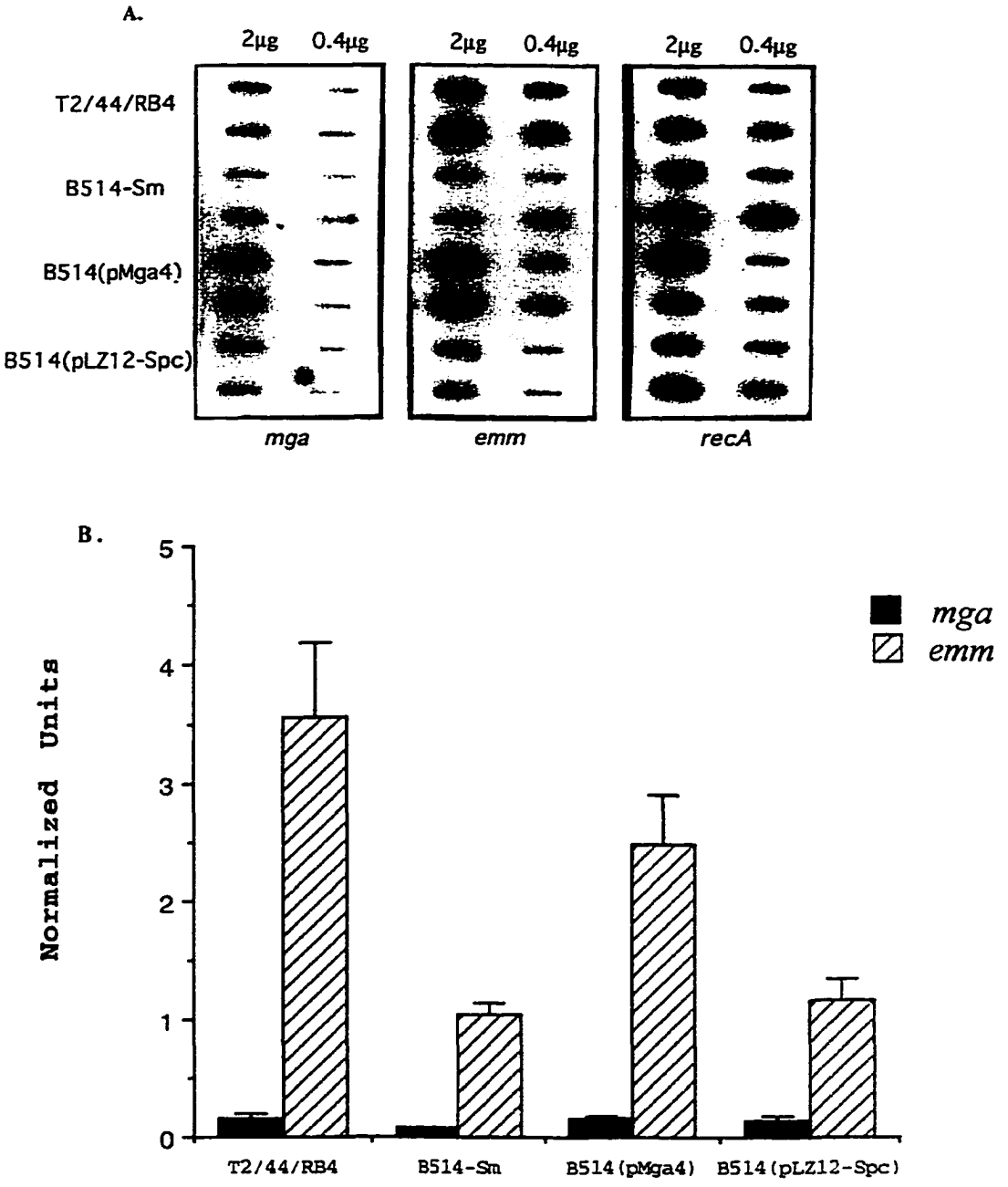
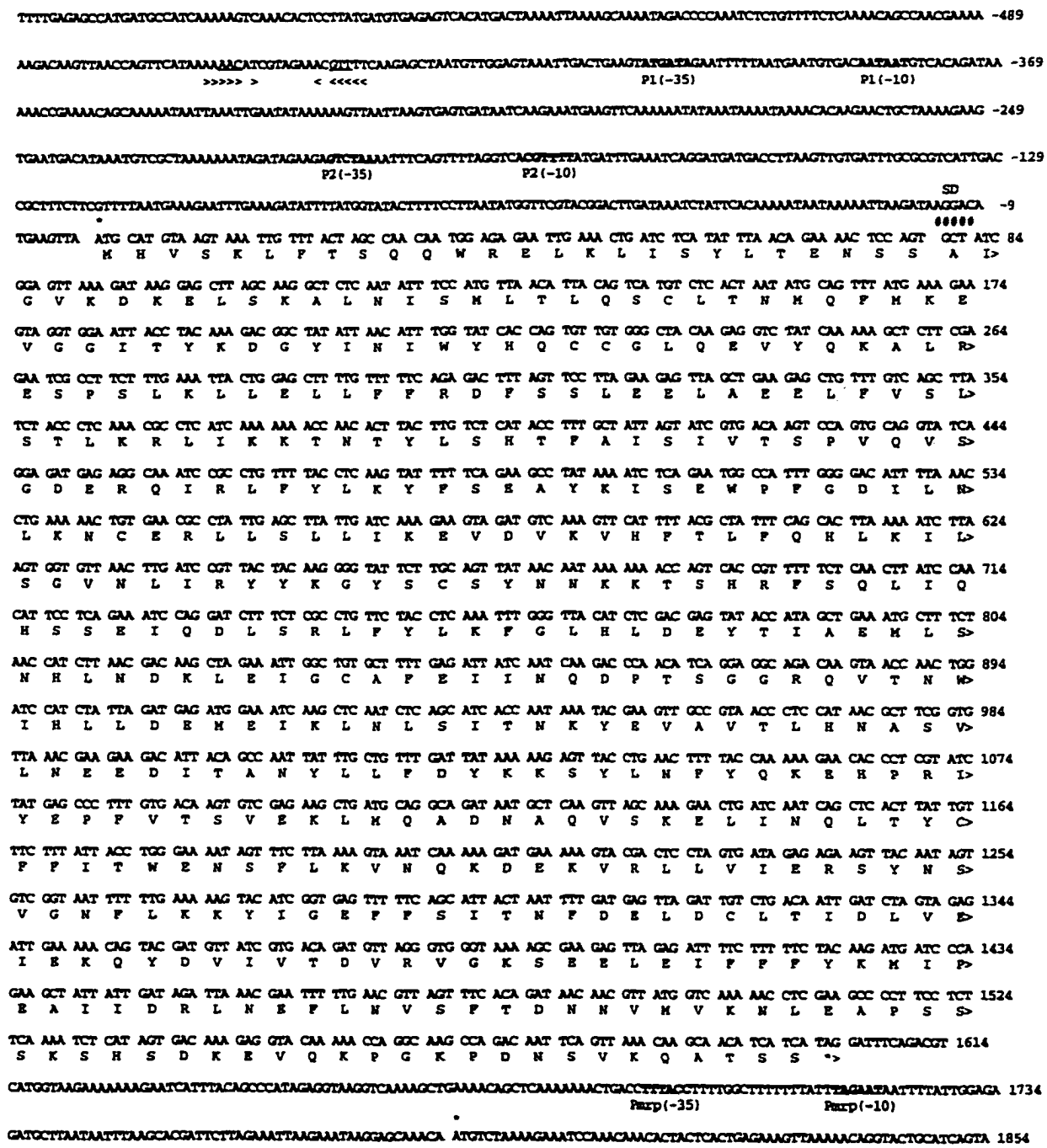


FIG. 3. Sequence of the 2562-bp DNA fragment encoding Mga and adjacent regions. The first base in the ATG codon as the start of translation of Mga is designated as *. The predicted ribosome binding sequences (SD) is indicated as #. Two promoters P1 and P2 required for transcription as designated in *virR49* (34) are found in the upstream region, as indicated by boldface underlining. A region of dyad symmetry in front of P1 promoter is indicated as ><, and there is a core sequence underlined which is conserved in many DNA-binding regulatory proteins (10, 29). No obvious ρ-independent termination signal was found in the downstream of the coding region as in *mga6* (1). The promoter (P_{mrp}) for transcription of *mrp* gene is shown in the downstream region of the *mga* gene. The first coding base of *mrp* is indicated as *.



mga in this region (data not shown). Within the coding region, there were 8 polymorphic amino acid sites among these three *mga* genes (Fig. 4). For four of these sites, *mga50* was synonymous with either the *mga4* or the *virR49* allele. For the other four sites, the amino acids in *mga50* are nonsynonymous with either *mga 4* or *virR49*. One nonsynonymous amino acid is residue 26, which is serine in *mga50* but is asparagine in both *mga4* and *virR49*. This amino acid is in one of the helix-turn-helix effector domains for DNA binding (7). A second nonsynonymous change is residue 361, which is changed to proline in *mga50* and to alanine in the other *mga* genes. Two additional nonsynonymous amino acid residues, 461 and 521, fall within a region where sensor protein(s) might recognize and pass the signal to these response-regulator domains (32). In addition to the single amino acid substitution sites, there is one frameshift of five amino acids which occurs in *virR49* near the carboxyl terminus (Fig. 4). Mga50 matches Mga4 at this location. The frameshift in the Vir49 allele suggests the extreme carboxyl terminus of the protein is not required for Mga regulation. Overall, *mga50* showed 98% homology to *mga4* and *virR49* from the available sequence data. *mga50* differs more substantially from the *mga* gene of an M serotype 6 strain (32); there is only 86% identity, and the majority of variation in this case is found towards the carboxyl terminus of both proteins (1).

Bactericidal test. The antiphagocytic M protein of GAS is considered a critical virulence factor because it enables resistance to killing by polymorphonuclear leukocytes. The capsule of GAS has also been reported to provide some resistance to phagocytosis, especially when it is in a hyper-expressed state and produces mucoid colonies on agar plates (5, 45, 47, 48). In the B514-Sm parental strain, the capsule is overexpressed and the strain is highly mucoid. The strain has given variable results when used in standard bactericidal assays, and we previously reported that it cannot survive in human blood (20). The previous susceptibility to phagocytosis was

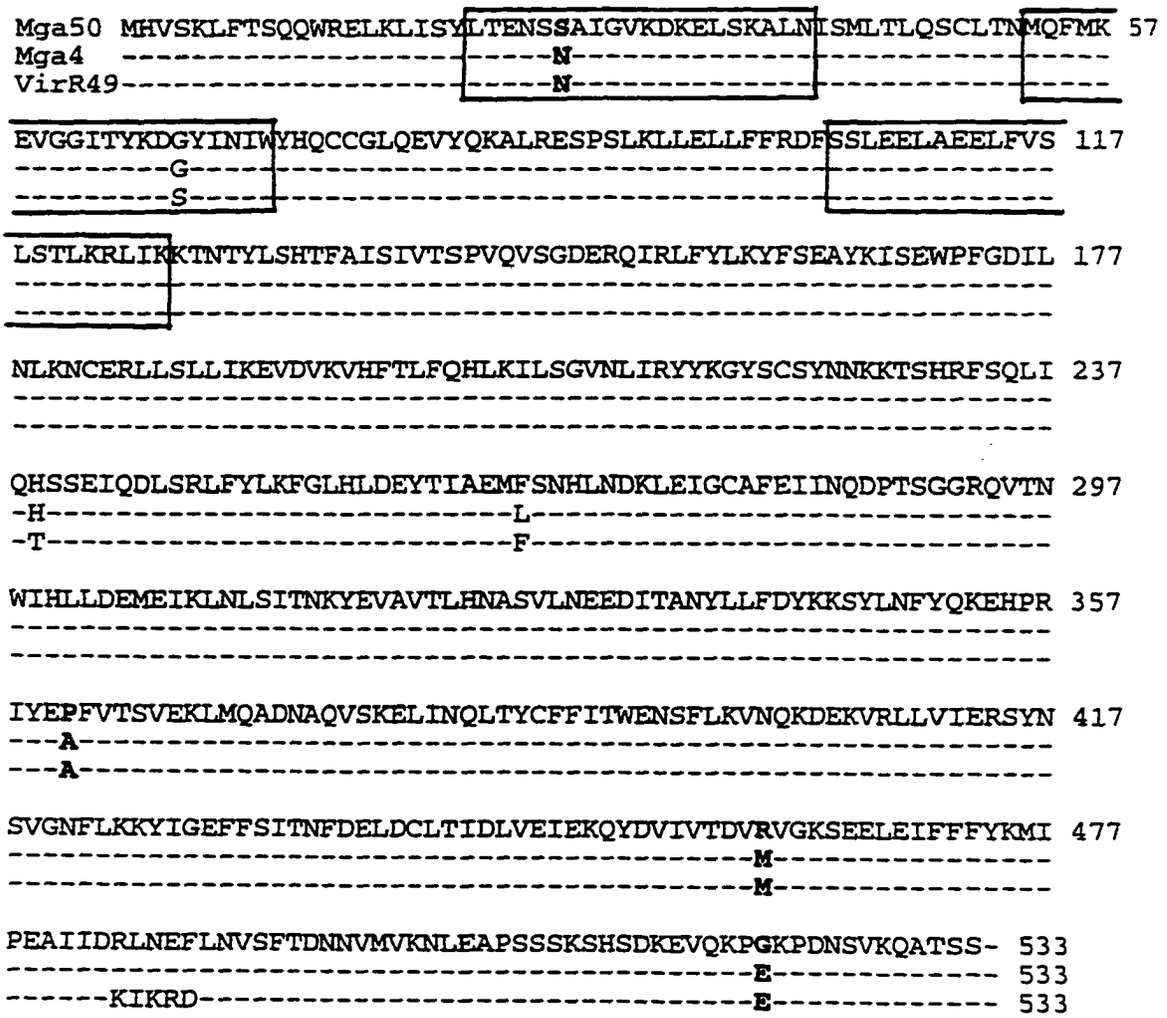


FIG. 4. Comparison of amino acid sequences from different GAS *mga* gene sequences. These sequences are all *emm* gene cluster chromosomal pattern 5 strains, *mga4* (1), *virR49* (36) and *mga50* (this study). Positions of identical residues are marked by -. Three potential helix-turn-helix motifs at the amino termini are marked as boxes. Two possible domains for sensor proteins to recognize are located at the carboxyl terminus, which start at residues 172 to 300, and residue 404 to the end of the protein, as described in (31).

attributed in part to the attenuated expression of the M-family proteins in this strain virulent for mice.

To clearly test whether complementation of Mga50 could restore biological function and result in protection from phagocytic killing, we utilized a version of B514-Sm in which capsule production had been abolished by the insertional inactivation of the *hasA* locus (20). This capsule-negative strain is called B514.039. Each of the pMga plasmids was transformed into B514.039 to exclude the effect of the capsule on the bactericidal assay. B514.039 clones transformed by pMga4 or pLZ12-Spc plasmid were analyzed for production and quantity of surface M-family proteins. As had been observed for the parental strain, complementation of *mga50* was achieved, and higher quantities of protein were produced with the strain B514.039 (pMga4) but not with B514.039 (pLZ12-Spc) (data not shown). All strains were tested for antiphagocytic activity in a direct bactericidal test. The relative survival rates of different GAS strains in human blood are shown in Table 3. It can be observed that the strains that can grow in human blood as well as in plasma include the strains with normally expressed M proteins (strain D471.Rot) and the unencapsulated strain in which M protein expression has been restored by plasmid complementation (strain B514.039 (pMga4)). Strains that failed to grow in human blood include an isogenic version of D471.Rot strain in which the *emm* gene was inactivated (strain UAB150) and the unencapsulated B514.039 strains in which the M protein is still attenuated (B514.039 and B514.039 (pLZ12-Spc)). In these cases, the streptococci cannot survive in human blood but do grow in plasma. From these results, it was clear that the increased expression of the *emm* gene cluster in the absence of any capsule in strain B514.039 (pMga4) was sufficient to restore growth in human blood, a biological property of M proteins.

In the current studies, the heavily encapsulated strain B514-Sm did survive in human blood as well as in plasma. This result differs from our previous observation concerning this strain (20). Because the experiments were assayed with blood from

different donors, and possibly the batch of anticoagulant-heparin used was different, both factors may affect the bactericidal activity of individual human blood for heavily encapsulated streptococci. Nevertheless, compared with the capsule-negative isogenic mutant strain B514.039, the encapsulation of B514-Sm has a protective role against phagocytosis in this test.

TABLE 3. Resistance of *mga*-transformed capsule-negative B514-Sm strains to phagocytosis in human blood

Strain	Total bacterial CFU in:		
	Input	Plasma	Whole blood
D471.Rot	2.4×10^2	5.5×10^3	6.3×10^3
UAB150	5.8×10^2[a]	1.8×10^4	6.3×10^2
B514-Sm	5.1×10^2[a]	1.7×10^4	1.8×10^4
B514.039	5.7×10^2[a]	2.5×10^3	1.2×10^2
B514.039 (pMga4)	1.7×10^2	5.0×10^2	1.5×10^3
B514.039 (pLZ12-Spc)	5.0×10^2[a]	1.4×10^3	1.6×10^2

[a] The number of inoculum is >300 CFU.

DISCUSSION

An M serotype 50 strain B514-Sm is unique among GAS strains in that it showed a natural pathogenicity for mice and originated from a mouse outbreak of respiratory disease. Although isolates of the M50 serotype were found in more than one outbreak in mouse colonies, this serotype has almost never been seen in human isolates, although serotype M62 appears nearly identical to M50 and is found among human isolates with rare occurrence (Beall, personal communication). Because of its mouse virulence, this strain is important for the development of murine models with

accessible procedures to study group A streptococcal pathogenesis (20). B514-Sm is highly encapsulated, but surprisingly, the expression of the most important virulence factors in GAS strains, the *emm* gene cluster, is rather low compared to the M2 strain T2/44/RB4, which has a similar *emm* gene cluster (49). Even though the *emm* gene cluster of B514-Sm is composed of three genes, in order, *mrp* (SF4), *emm* (SF2) and *enn* (SF3) (49), these genes were not associated with bacterial virulence either in the colonization stage or in the later invasive stage in mice infected with B514-Sm (20). This result may be because the expression of these genes is low and results in high pressure selection of the capsule production (48).

In the plasmid complementation expirment, all *mga*-containing plasmids successfully reverted the attenuated expression of the *emm* gene cluster in B514-Sm (Fig. 1). pMga4 under its own promoter was the most efficient. pMga6, either under its own promoter or a constitutive phage promoter, was less efficient. This difference can be explained by the fact that the *mga50* gene is more similar to the *mga4* gene than to the *mga6* gene. Both *mga50* and *mga4* genes belong to the strains that have the same *emm* gene chromosomal pattern 5, which are more divergent from the *mga6* gene that is in the strain has *emm* gene chromosomal pattern 1 (15). It is not known why Mga proteins differentiate into at least two types. In this study, the Mga6 protein can complement the activity of the inactive Mga50, but not as well as the Mga4 protein, which was more homogenous to Mga50. At least three parts of these *mga* genes may have caused the different efficiency in complementation. One was the upstream regions, for example, the promoters. This hypothesis was proved, that when the plasmids with different promoter sequences--pMga4, pMga6, and the pϕMga6--were used to transform into the strain B514.039, these plasmids had different efficiency to complement the defective *mga50* (Fig. 1). The second part was on the carboxyl terminal region, which the sensor components of B514-Sm may not recognize well in Mga6. The third part was the helix-turn-helix DNA-binding motifs, in which Mga6,

after receiving a signal from the sensor proteins, may not interact well enough to activate the *emm* gene cluster of B514-Sm. Since the attenuated expression of the *emm* gene cluster of B514-Sm can be reverted by complementing with *mga*-containing plasmids, it was thought that the *mga* gene of B514-Sm was inactive. Mga protein is a positive transcriptional activator of the *emm* gene cluster, the *scpA* gene, and some other genes. It acts as a two-component bacterial signaling system, in which Mga protein is a response regulator; after receiving a signal from the sensor protein (usually a histidine kinase which transfers a phosphoryl group); the activated response regulator then binds to the regulated genes (43). In the DNA and deduced amino acid sequences of *mga50*, there are four amino acid differences from both *mga4* and *virR49* genes. One is located in the helix-turn-helix DNA-binding motif--Ser^{26} (Asn^{26} in both *mga4* and *virR49* genes); two different residues are in the carboxyl terminus containing the predicted response regulator domains (32)--Arg^{461} and Gly^{521} (Met^{461} and Glu^{521} in both *mga4* and *virR49* genes). The residue Met^{461} is highly conserved in all published Mga protein sequences (1, 4, 12, 32, 36) and is very close to the proposed phosphorylation site, the Asp^{459}. Residue 521 is rather diverse among the Mga proteins (1, 4, 12, 32, 36). Another difference is residue 361, which is alanine in both *mga4* and *virR49* but proline in *mga50*, and the change may affect the protein structure. This Ala^{361} residue is also conserved in the Mga proteins. It is not known whether all four amino acid mutations, or just one or two, cause Mga50 to be inactive. Even though the variability of amino acids in the carboxyl termini of Mga proteins can be tolerated (1), the non-synonymous change of residue 461, which is very close to Asp^{459}, may affect the following activation. The other possibility for inactive Mga50 is the expression of this gene. Okada et al. found that the 473 bp upstream of the *mga* coding region are required for expression of the *mga* gene (30). Even though there were about 20 polymorphisms in this region of *mga50* compared to that of *mga4* or

virR49, the two promoters (P_1 and P_2) found in the *virR49* gene (36) can be located in the upstream region of *mga50* (data not shown). Furthermore, the transcript levels of *mga* in B514-Sm and T2/44/RB4 were nearly similar, which were about 22-fold less than those of the *emm* gene in T2/44/RB4 and 12-fold less than those of the *emm* gene in B514-Sm. From these results, it appeared that the expression of *mga50* was not defective, but that the mutations causing the amino acid changes affected the activity of Mga50.

The expression level of the *emm* gene was about fourfold attenuated in B514-Sm compared to that in T2/44/RB4, according to the RNA-slot blot assays, and is less attenuated than levels we observed previously (49). This difference in quantity of the *emm* transcripts may have resulted from the culture media and the techniques used, both of which could influence the data concerning the extent of attenuation of *emm50* gene cluster of strain B514. Further studies are needed to prove this influence, even though, without any doubt, the expression of M-family proteins in B514-Sm was attenuated when cultured in both media. From the experiments of RNA-slot blot assay, the reverted M-family proteins expressing strain B514 (pMga4) are shown to have the increased transcript level of *emm* gene, which is correlated to the increased transcript level of *mga* gene (Fig. 2).

The group A streptococcal capsule is a virulence factor (5, 45, 47, 48). In our previous study, two mouse models were used to evaluate the role of some virulence factors by delivering B514-Sm or isogenic mutant strains intranasally or intratracheally. It was found that the hyaluronic acid capsule, not the M-family proteins or streptococcal C5a peptidase, was required for pharyngeal colonization and the induction of pneumonia (20). The hyaluronate capsule and M proteins were found to be variably important in the resistance of different GAS to opsonization and phagocytic killing (5). Dale et al. (5) found that type 24 streptococci bound fibrinogen avidly, and encapsulated organisms were completely resistant to opsonization only in the presence

of fibrinogen; and type 18 streptococci bound less fibrinogen than type 24 streptococci and were resistant to phagocytosis only with encapsulation. Assays of complement deposition and C3 binding showed that encapsulated type 24 streptococci were fully resistant to opsonization by C3 only in the presence of plasma. Encapsulated and unencapsulated type 18 streptococci were equally opsonized by C3 in either plasma or serum, but only the encapsulated strains can resist phagocytic killing in blood; this result indicated that opsonization by C3 did not necessarily lead to phagocytic ingestion (5). In this experiment, it was explained that the different amount of fibrinogen binding of type 24 and 18 streptococci was possibly due to the number of B repeats of the M proteins, but the quantities of the M proteins expressed in these two strains may be one of the reasons that there were differential binding of fibrinogen and the necessity of encapsulation in these two types of streptococci. In our bactericidal assay, B514-Sm could survive in human blood, but the capsule-negative mutant strain B514.039 could not; this result indicated that the capsule of B514-Sm does play a role in anti-phagocytosis, contrary to the results that we previously published: that B514-Sm cannot survive in human blood (20). The main purpose of performing bactericidal tests was to determine if the increased expression of M-family proteins in the *mga* plasmid-transformed B514-Sm in the absence of capsule correlates to the anti-phagocytic virulence function of M proteins. The result showed that the increased expression of M-family proteins by complementing with *mga4* gene in the capsule-negative strain B514.039 endowed the strain with more resistance to phagocytic killing.

It is necessary to revert the attenuated expression of *emm* gene cluster in this strain virulent for mice before testing the virulence mechanisms of the products, Mrp, Emm, and Enn proteins, in the murine models. Correcting the inactive form of Mga50 is one way of achieving this. The hyaluronate capsule of this strain plays an important role in mouse group A streptococcal infections because of the low expression of the *emm* gene cluster.

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CONCLUSIONS

DNA sequencing and gene expression of the *emm* gene cluster in an M50 group A streptococcus strain virulent for mice. A serotype M50 strain, B514, can be used for developing murine models of group A streptococcal infections because it causes natural infections in mice. Even though there are data to address the pathological effect from B514-infected mice (43, 116), and the protection studies of inducing systemic or mucosal immunity in the mice challenged by B514 (53, 100), information about the characteristics of individual virulence factors of this strain at the molecular level is scarce. For the purpose of understanding more about the role of individual virulence factors in the development of pathogenicity in animal models, we first characterized the antiphagocytic M protein of this strain. The *emm* gene locus of this strain was examined by allele-specific PCR mapping technique and was found to contain three *emm*-family genes--SF4 (*mrp50*), SF2 (*emmL50*), and SF3 (*enn50*)--and was assigned the *emm* gene cluster of pattern 5. PCR-generated fragments corresponding to the individual genes were cloned, and the entire gene cluster was sequenced. The gene cluster has greater than 97% DNA identity to that of the M2 strain--T2/44/RB4, except that there are two small divergent regions immediately after the signal peptides of the SF2 and SF3 genes. These two heterogeneous regions may define the M50 serotype specificity of B514-Sm, differentiating M50 strains from M2 strains in the Lancefield serotyping system. Even though B514-Sm and T2/44/RB4 strains have similar M-family proteins, serotype M2 strains are not virulent for mice unless they have been passaged several times through mice to select for virulent variants. Likewise, M50 strains are not usually found among human isolates.

On the basis of the DNA sequence of the *emm* gene cluster in strain B514-Sm, it was found that *mrp50* and *emm50* genes encode probable IgG-binding proteins and that the *enn50* gene encodes a probable IgA-binding protein. Binding studies of these three proteins expressed in vitro showed the predicted binding capabilities. However, when the expression of these three genes in B514-Sm was analyzed by quantitative Northern (RNA) hybridization, the transcript levels of all three *emm* -family genes was found to be over 30-fold attenuated relative to those of the same genes in strain T2/44/RB4. The attenuation state of M-family proteins in B514-Sm was not changed even from B514-Sm isolates recently passaged in mice in an attempt to increase M protein expression.

Because all the sequences of the promoter regions are identical in strains B514-Sm and T2/44/RB4, variation in the regulation sites is unlikely to explain the difference in expression between these two strains (personal communication with S. K. Hollingshead). Thus, the low level of expression in B514-Sm may indicate that the *mga* gene, the positive transcription regulator of the *emm* gene cluster, is either defective or only active under conditions that differ from those required by most streptococcal strains. An Mga-binding site for the *emm6* and *scpA* genes located near the -35 region has been identified in one of the two promoters for these genes. It is possible that the basal level of transcription found in the B514-Sm may originate from promoters that are not under the control of the Mga. Another possibility is that B514-Sm is highly mucoid and encapsulated, and the capsule may be important for the virulence of strain B514-Sm for mice because the hyaluronate capsule as well as the M protein have been proved to have antiphagocytic ability (21, 107, 108, 109). The redundancy of these two virulence factors in B514-Sm may lead to the attenuated expression of *emm* genes in this strain.

Role of putative virulence factors of *Streptococcus pyogenes* in mouse models of long-term throat colonization and pneumonia. This has been a collaborative project with Dr. Linda Husmann and colleagues at Emory University, and the purpose has been to investigate the role of putative virulence factors of GAS in two animal models. For this dissertation, the virulence factors tested in these studies were *emm* family proteins, C5a peptidase, and hyaluronate capsule (42). The isogenic mutant strains for individual virulence factors in B514-Sm were constructed by Husmann and coworkers (JRS4016, JRS4012) and us (B514.039) either by deleting the whole *emm* gene cluster or by inactivating *scpA* and *hasA* genes (which encode C5a peptidase and hyaluronate synthease, respectively) by insertion of a nonreplicative plasmid in the genes. These mutant strains were tested in two animal models of infection. The C3HeB/FeJ mouse model in which intratracheal inoculation results in pneumonia and systemic spread of the streptococcus was used to study the late stages of diseases (43). A model of long-term (at least 21 days) throat colonization following intranasal inoculation of C57BL/10SnJ mice was used to study the early stages of diseases. When a whole *emm* family genes-deleted mutant was tested in these models, no significant difference was seen in the induction of long-term throat colonization or pneumonia compared to the wild-type strain. The incidence of throat colonization by the inactivated *scpA* gene mutant and the wild-type strain was not significantly different. However, a small but statistically significant decrease was shown in the incidence of pneumonia caused by the *scpA* mutant. Finally, the hyaluronic acid capsule was demonstrated to be very important for developing disease in both models. Following intranasal inoculation of mice with the *hasA*-inactivated mutant, almost all bacteria recovered from the throats of the mice one day after inoculation were encapsulated revertants with the loss of the inserted plasmid. Following intratracheal inoculation with the *hasA* mutant, the incidence of pneumonia within 72 h was significantly reduced from that of the wild type strain ($p = 0.006$).

These results indicate that the hyaluronic acid capsule of B514-Sm confers an important selective advantage for survival of the bacteria in the upper respiratory tract and is also an important determinant in the induction of pneumonia in these model systems. The minimal effect of M-family proteins and C5a peptidase in strain B514-Sm for establishing disease can be explained by the fact described in the previous study that the expression of M-family proteins (and possibly the C5a peptidase) was attenuated (118). From the results of these experiments, the attenuated expression of *mga*-regulated genes in strain B514-Sm appears to affect its virulence capability, the expression-level of *emm* gene cluster in B514-Sm is not sufficient for their virulent function.

Complementation of a defective *mga* gene corrects the attenuated expression of the *mga* virulence regulon in an M50 group A streptococcus strain virulent for mice. The failure of showing any effect on pathogenicity of *emm* gene cluster and C5a peptidase of strain B514-Sm can be due to their naturally attenuated expression. Therefore, in order to understand the role of these *mga* virulence regulon in the development of group A streptococcal disease in animal models, it was necessary to revert the state of attenuated expression of *mga* regulon to the wild-type level of expression, as in strain T2/44/RB4, whose *mga* regulon is similar to that of B514. One possible cause for the attenuated expression of the *mga* regulon is that the response regulator *mga* gene is defective. To test this hypothesis, the *mga* gene of this strain was further characterized. A complementation test was performed to see if the *trans*-acting Mga proteins from other strains could complement the putative defect of Mga in strain B514-Sm. When B514-Sm was transformed with plasmids containing an intact *mga* gene from either an M serotype 4 or 6 strain, the expression of the *emm* gene cluster was increased. *Mga4* under its own promoter was the most efficient, whereas *mga6*, under either its own promoter or a constitutively expressed phage promoter, was less effective. This indicated that the *mga* gene in strain B514-

Sm was defective and could be complemented by *mga* genes from different M serotypes strains with different effectiveness. To assess whether the defect of *mga50* was due to its expression, and to compare the expression levels of *mga50* and *emm50* in B514-Sm strains transformed with plasmids to those of the wild type strain, quantitative RNA slot blots were performed to detect transcripts of both genes in B514-Sm and its transformants. The level of the constitutively expressed *recA* gene was used for normalizing the loading quantities. The transcript level of the *mga* gene in strain B514-Sm was slightly lower than the *mga2* transcript in strain T2/44/RB4, with a calculated ratio of about 1:1.5. The *emm50* transcript in strain B514-Sm was further reduced as compared to the equivalent *emm2* transcript in T2/44/RB4, with a calculated ratio of about 1:4. An increased transcript level of *emm* gene in strain B514-Sm could be detected when the strain was transformed with a plasmid containing the *mga4* gene. These results indicated that the transcription of *mga50* was not defective, but that the function of the protein as a positive transcription regulator of the *mga* regulon was defective. DNA sequences and deduced amino acid sequences of *mga50* were then determined and compared to the sequences of *mga4* and *virR49*, which occur in strains all having pattern 5 of the *emm* gene cluster. There were, in total, eight amino acid differences from either *mga4* or *virR49*; four out of eight amino acids were different in both *mga* genes. One nonsynonymous substitution was at residue 26, which was located in one of the helix-turn-helix motifs in the effector domain of Mga50. The other substitution was at residue 361, which was an alanine in most *mga* genes but was changed to a proline in *mga50*, and which may cause a structural distortion. The two other substitutions were located in one of the potential CheY-like response regulator domains, arginine at residue 461, instead of methionine in all *mga* genes that have been sequenced so far. This residue is very close to the potential phosphorylation site, aspartic acid at residue 459. This substitution M461R may affect the phosphorylation of D459 and interfere with the subsequent activation. The last substitution is glycine at

residue 521, instead of glutamic acid in both *mga4* and *virR49*. This substitution may also cause a structural distortion. There was also polymorphism found in the upstream region of the coding sequences; nevertheless, the two promoters deduced from *virR49* can be located in this region, and, according to the result of quantitative RNA slot blots, the sequence variation in this region did not affect the expression of this gene. From the results of these experiments characterizing *mga50*, the defect in *mga50* appears to be a point mutation(s) in the encoding gene.

Since the attenuated expression of M-family proteins can be reverted by complementing the strain B514-Sm with intact *mga* plasmids, it was important to know if the increased expression of M-family proteins of this strain could restore biological function and result in protection from phagocytic killing. The wild-type strain B514-Sm was found to be able to survive in human blood in the direct bactericidal assay as a result of the highly expressed hyaluronate capsule. To exclude the factor of capsule, plasmids were transformed into a previously isolated capsule-negative mutant strain--B514.039. With the *mga4* plasmid transformed in B514.039, as for the parental strain, complementation was achieved and a larger quantity of M-family proteins was produced. In the direct bactericidal assay, this transformant was found to survive in human blood to a greater extent than B514.039. It was apparent that the increased expression of M-family proteins in the absence of any capsule in strain B514.039 (pMga4) was sufficient to restore growth in human blood, a biological property of M proteins.

In this dissertation, the *emm* gene cluster of the highly encapsulated strain B514 (M serotype 50) virulent for mice has been characterized. It is proposed to use this strain in an animal model to study the pathogenesis of GAS infections because of its natural ability to infect mice. Even though there are three *emm* gene loci in this strain, which are able to encode three Ig-binding proteins, Mrp, Emm, and Enn, the expression level of these three proteins are attenuated compared to that of a human GAS

isolate--T2/44/RB4 (M serotype 2) strain. This finding is correlated to the results from the in vivo studies which showed that the role of virulence factors, M-family proteins, and C5a peptidase of B514 strain was minimal, and that the hyaluronic acid capsule was required for establishing disease. In this dissertation, it is also shown that the Mga protein, the positive transcription factor of *emm* gene cluster, *scpA*, and some other genes, is defective. Restoration of the wild-type expression level of M-family proteins in strain B514 can be achieved by complementation with the intact *mga* gene from different serotype strains. Certain amino acid changes in Mga protein of strain B514 may result in the defect. The restored expressing M-family proteins of strain B514 was proved to be able to protect the bacteria from phagocytic killing.

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I certify that I have read this document and examined the student regarding its content. In my opinion, this dissertation conforms to acceptable standards of scholarly presentation and is adequate in scope and quality, and the attainments of this student are such that she may be recommended for the degree of Doctor of Philosophy.

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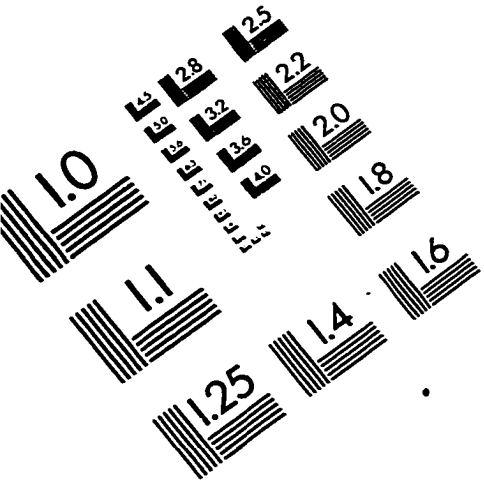

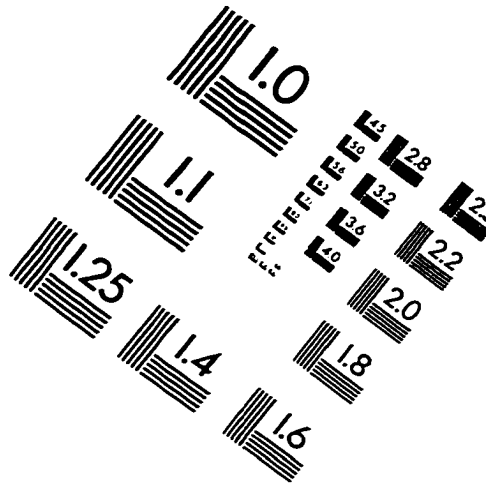

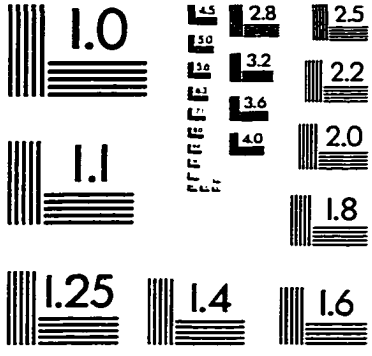

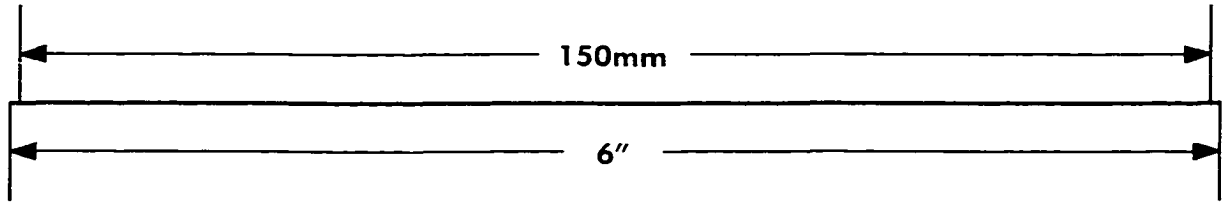

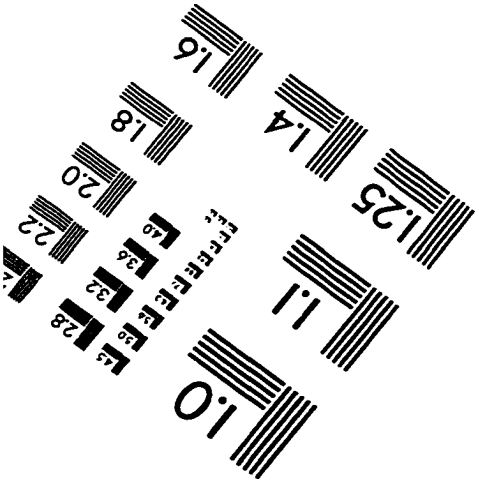

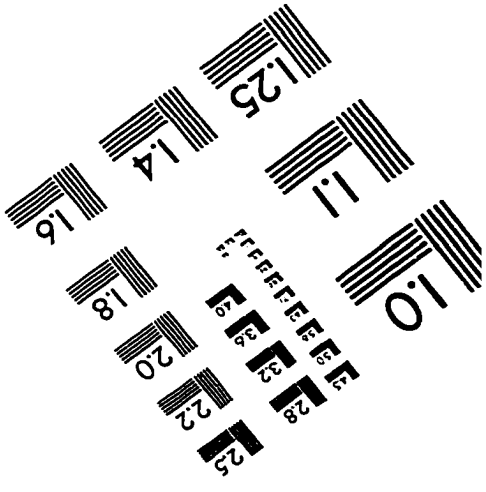

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