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Role of Putative Virulence Factors of *Streptococcus pyogenes* in Mouse Models of Long-Term Throat Colonization and Pneumonia

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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 revertants. 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.

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 strain 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 (26, 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 (28). 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 invasiveness (32) or be important for survival of the bacteria during certain types of disease syndromes (3).

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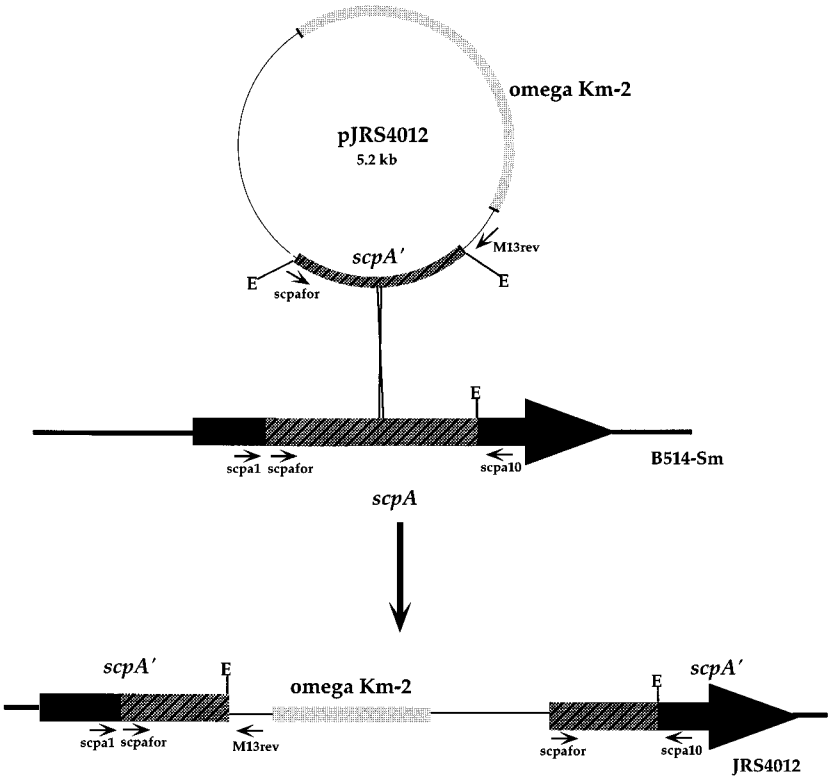

FIG. 1. Construction of insertion mutant JRS4012. The circle represents plasmid pJRS4012, which is based on pCIV2 (pUC origin) (25) and which contains an internal fragment of the *scpA* gene from GAS strain B514-Sm (striped region) and the omega Km-2 interposon (*aphA3* kanamycin resistance gene flanked 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.

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 has been shown 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, 40–42, 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 (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 the 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 mice 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 ± 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.) 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* DH5α (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.*

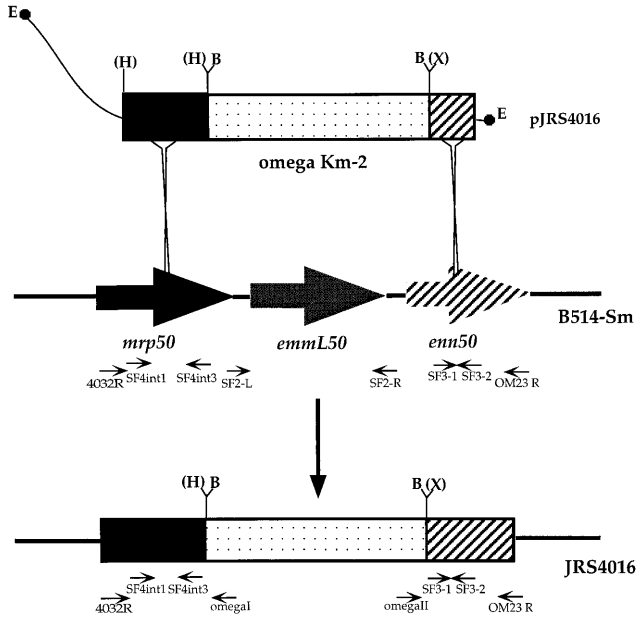

FIG. 2. Construction of insertion-deletion mutant JRS4016. The top diagram represents the chimeric plasmid pJRS4016, shown linearized at its unique *Eco*RI site (E). Plasmid pJRS4016 contains an internal fragment of the *mrp50* gene (black region), the omega Km-2 interposon (stippled region), and an 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 (*Hin*cII), B (*Bam*HI), X (*Xma*I), and E (*Eco*RI). Parentheses indicate a site destroyed in cloning.

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 (2×) 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 GGAATTCCCTCAAAAGCGACTATTAGG GAT (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 scpAfor 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 adenylyltransferase AAD(9) (24) determinant in place of the beta-lactamase gene. This substitution was accomplished by replacement of the 692-bp *Sca*I-*Nde*I fragment of pUC18 with the 1.1-kb *Nde*I-*Hin*dIII fragment (blunted with the Klenow fragment of DNA polymerase I) from pDL269 (24). Then, a 0.9-kb fragment internal to the *mrp50* gene of strain B514-Sm (48) was amplified by PCR with the primer pair CGTGTTTGATGGCTATACTG (SF4int1)/GGTTT TTAGCTCCTGAAGCTTTAT (SF4int3; Fig. 2). This fragment was treated with the Klenow fragment of DNA polymerase I and inserted into the unique

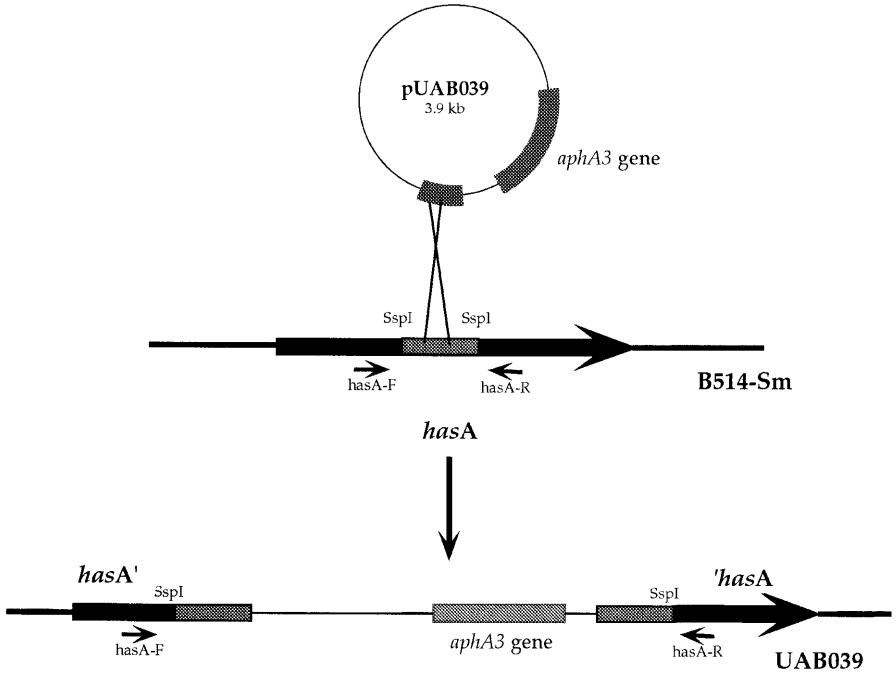

FIG. 3. Construction of insertion mutant UAB039. Plasmid pUAB039 was used to transform B514-Sm to kanamycin resistance. Plasmid pUAB039 contains an internal fragment of the *hasA* gene from B514-Sm (grey region) cloned into pSF151, which is nonreplicative in streptococci and contains the *aphA3* kanamycin 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 used to verify this construction are shown (small arrows). *Ssp*I restriction enzyme sites are indicated.

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 strain 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]	B514.039 (*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] 1×10^7 CFU inoculum.
[b] 2×10^7 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.

(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)/GGAATCGGGA TGCTTCAAGGTCACGGC (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 with 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.

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 with the oligonucleotides ACGTTATCGTTCACCGTTCCC (hasA-F) and AGTGACCTTTT TACGTGTTCCCC (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 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, 29). 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 of streptomycin sulfate per ml 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 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 (29) except that 2× 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, Calif.).

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 the 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 (beginning at day 2 postinoculation and then at weekly intervals). All colonies were kanamycin resistant, indicating that reversion had not occurred.

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 roles 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 2×10^7 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

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

Mouse no.	% Revertants (no. mucoid colonies/total no.)[a] at:					
	Day 1	Day 2	Day 4	Day 7	Day 14	Day 21
1	1.4 (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)[b]	100 (298/298)	100 (TNTC)[b]	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).

revertants, 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 revertants 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 and 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 of the mucoid revertants. We found that in both the Km^s and the 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.

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 solution, 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 C5a peptidase in causing pneumonia. To examine the role of the 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) and comparing the results to those obtained previously (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 to 5.9×10^7 CFU), which is a small but statistically significant difference from that obtained previously for wild-type strain B514-Sm: 1×10^7 CFU (95% confidence interval, 8.1×10^6 to 1.3×10^7 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

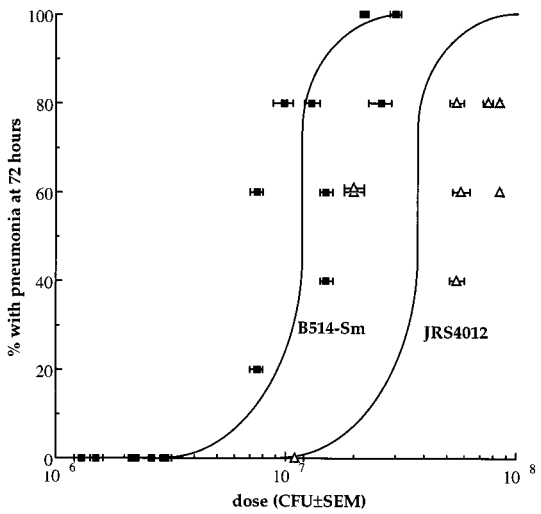

FIG. 4. Dose response of pneumonia in C3HeB/FeJ mice following intratracheal inoculation of *S. pyogenes* JRS4012 (*scpA* null) (△). The results obtained previously for wild-type strain B514-Sm are included for comparison (■). Each data point represents five mice.

from the lung of one mouse that had been inoculated with a dose of 2.0×10^7 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.

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.

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 revertants 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

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[a]	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.

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

Expt no.	Strain (relevant genotype)	% with pneumonia (no. ill within 72 h/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]	B514.039 (*hasA*)	0 (0/14)[c]
	JRS4016 (*mrp50 emmL50 enn50*)	50 (5/10)

[a] Dose was 2×10^7 CFU.
[b] Dose was 1×10^7 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.

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	2×10^1
	B514-Sm	2.3×10^2	3.3×10^3	9×10^1
2[a]	JRS4	4.2×10^2	1.6×10^4	1.2×10^4
	JRS145	3.2×10^2	1.3×10^4	4×10^1
	B514-Sm	1.8×10^2	8.4×10^3	$<1 \times 10^1$

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

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 the 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 roles of putative virulence factors in the late stages of disease.

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 1.0×10^7 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 the 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 inoc-

ulation (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 revertants, 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 tracts 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 revertants. 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 with 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 more important roles 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 strains 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 (40–42). 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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